Advances in Food and Nutrition Research

Volume 47

CONTENTS

Echinacea as a Functional Food Ingredient

Clifford Hall III

Bioactive Peptides and Proteins

Anne Pihlanto and Hannu Korhonen

Plant Products with Hypocholesterolemic Potentials

Pulok K. Mukherjee

CONTRIBUTORS TO VOLUME 47

Numbers in parentheses indicate the page on which the authors' contributions begin.

Jean-Marc Chobert, *Laboratoire d'Etude des Interactions des Molécules Alimentaires, Institut National de la Recherche Agronomique, Rue de la Géraudière, B.P. 71627, 44316 Nantes Cedex 3, France (1)*

Clifford Hall III, *324 IACC, Department of Cereal and Food Sciences, North Dakota State University, Fargo, ND 58105, USA (113)*

Hannu Korhonen, *MTT Agrifood Research Finland, Food Research, 31600 Jokioinen, Finland (175)*

Pulok K. Mukherjee, *Department of Pharmaceutical Technology, Natural Product Studies Laboratory, Jadavpur University, Kolkata 700032, India (277)*

Anne Pihlanto, *MTT Agrifood Research Finland, Food Research, 31600 Jokioinen, Finland (175)*

Gerhard Norbert Schrauzer, *Biological Trace Element Research Institute, 2400 Boswell Rd., Suite 2000 Chula Vista, CA 91914, USA (73)*

MILK PROTEIN MODIFICATION TO IMPROVE FUNCTIONAL AND BIOLOGICAL PROPERTIES

JEAN-MARC CHOBERT

Laboratoire d'Etude des Interactions des Molécules Alimentaires
Institut National de la Recherche Agronomique
Rue de la Géraudière
B.P. 71627, 44316 Nantes Cedex 3
France

I. INTRODUCTION

The actual and potential use of milk proteins as food ingredients has been a popular topic for research over the past 30 years. Milk and dairy products have numerous advantages over competitors when used as ingredients: they are colorless, have a bland taste, are rather stable to processing, are free of

ADVANCES IN FOOD AND NUTRITION RESEARCH VOL 47
ISSN: 1043-4526
DOI: 10.1016/S1043-4526(03)47001-0

toxins and have constituents that can be easily fractionated. As ingredients, dairy products are used mainly for their physico-chemical properties.

The effective utilization of proteins in food systems is dependent on tailoring the protein's functional characteristics to meet the complex needs of the manufactured food products. Many food proteins require modification to improve such functional properties as solubility, foaming and emulsifying activity (EA). Reviews on classical food protein modifications for improved functionality are available in the literature (Means and Feeney, 1971; Feeney and Whitaker, 1977, 1982, 1986).

Functional properties of proteins are closely related to their size, structural conformation, and level and distribution of ionic charges. Chemical treatments, which could cause alteration of these properties, include reactions that either introduce a new functional group to the protein or remove a component part from the protein. Consequently, reactions such as acylation, phosphorylation, esterification, glycation, limited hydrolysis, and deamidation have been used to impart improved functional properties to the dairy proteins.

This review concerns some chemical, enzymatic and genetic methods that modify dairy proteins with an emphasis on current developments.

II. CHEMICAL MODIFICATION OF MILK PROTEINS

Primary structure of proteins can be chemically modified in order to improve their functional properties. This approach has been used with success to study the structure–function relationships (enzymatic function, biological function, physico-chemical and functional properties). Deliberate chemical modification of food proteins can result in alteration of the nutritive value, formation of potentially toxic amino acid derivatives, and contamination by toxic chemicals.

Alteration of amino acid residues can be obtained by heating at acid or alkaline pH. Main classes of reactions used to chemically modify the side-chain of amino acids are acylation, alkylation, oxidation and reduction (Figure 1). Some of them are described in this chapter.

A. PHOSPHORYLATION

1. Reaction conditions

Phosphorylation is an effective way to increase negative charges in a protein molecule and thereby to improve functionality, particularly solubility. Either O- or N-esterification reactions can transfer inorganic phosphate (P_i)

to proteins. In an O-esterification reaction, P_i reacts with the primary or secondary hydroxyl on seryl or threonyl residues, respectively; or with the weakly acidic hydroxyl on tyrosyl residue, forming a –C–O–Pi bond. In N-esterification, P_i combines with the ε-amino group of lysyl residue, the

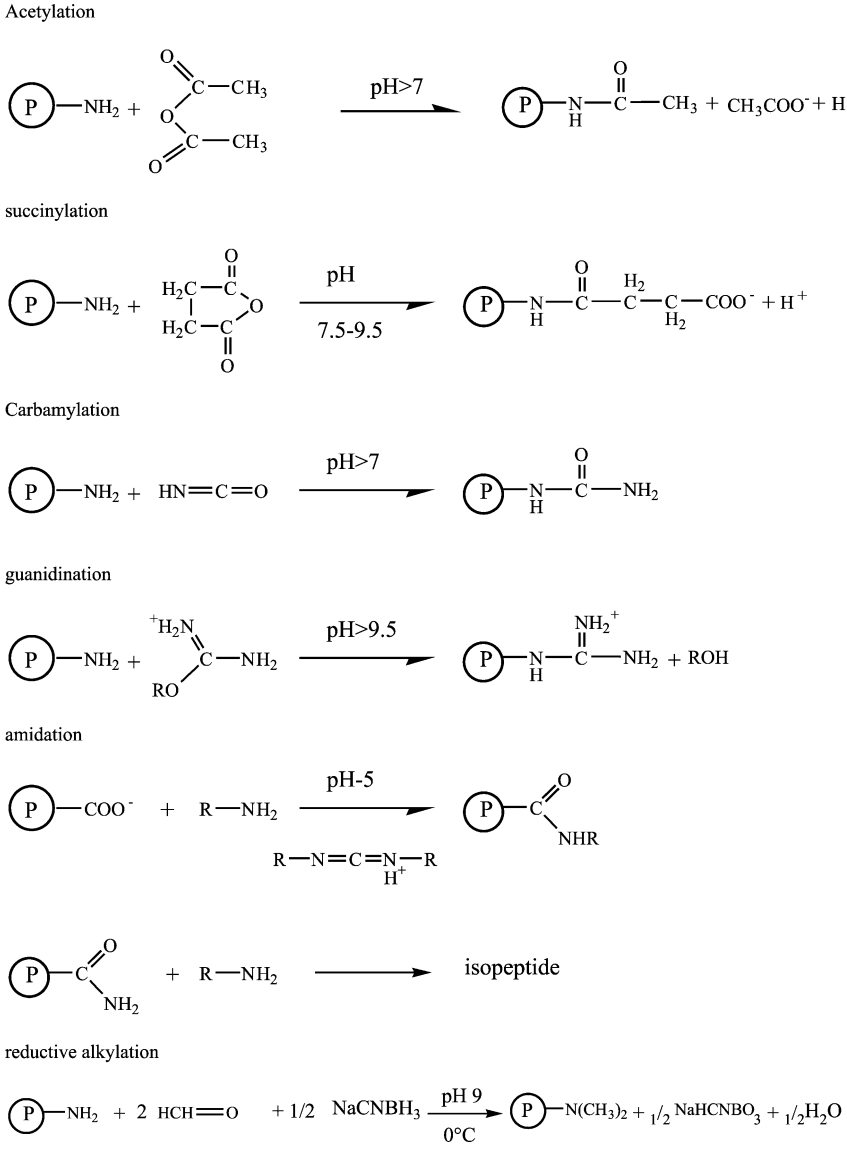
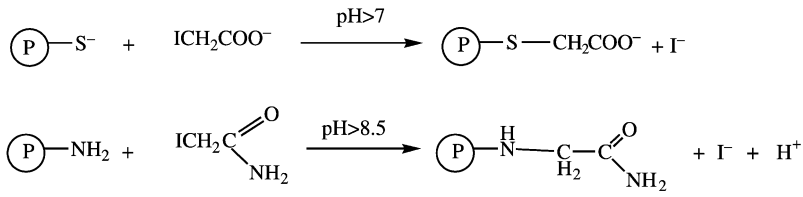
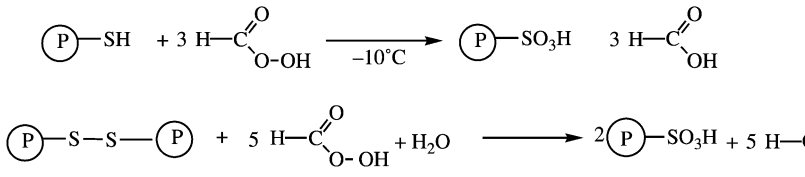


FIG. 1. Main chemical modifications of food proteins.

Carboxymethylation



Oxidation



Esterification

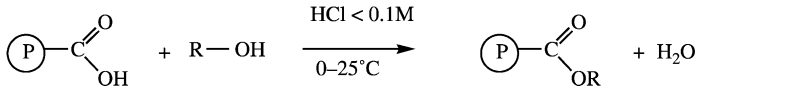


FIG 1. (continued)

imidazole group of histidyl residue, or the guanidino group or arginyl residue, forming a $-C-N-P_i$ bond. The nitrogen-bound phosphates are acid labile and are readily hydrolyzed at pH values at or below 7. Proteins containing oxygen-bound phosphate are acid stable and are the modification of choice for food proteins since the pH of most food systems is 3–7 (Shih, 1992).

Enzymatic phosphorylation by phosphorylases and phosphatases produces phosphoesters such as phosphoserine and phosphothreonine. Chemical phosphorylation of proteins changes their functional properties, improving them sometimes (Yoshikawa *et al.*, 1981; Hirotsuka *et al.*, 1984; Huang and Kinsella, 1986; Chobert *et al.*, 1989; Matheis, 1991). However, the properties of the phosphorylated proteins depend entirely on the degree of denaturation and substitution defined by the reaction conditions and the protein (Medina *et al.*, 1992; Sitohy *et al.*, 1994). Casein was phosphorylated by the commonly used methods, characterized by use of excessive amounts of phosphorus oxychloride and with important additions of concentrated inorganic bases (Matheis *et al.*, 1983; Medina *et al.*, 1992). Thus, obtained phosphorylated caseins were highly cross-linked and partially insoluble and difficult to characterize. Hence, there arose a need to produce monomeric over-phosphorylated caseins more suitable for use and for study of their

functional properties. The monomeric forms are more hydrophilic, and easier to study and used as additives. It was found that the outcome of phosphorylation can be directed by the reaction conditions either towards the formation of polymeric or predominately monomeric phosphoproteins (Sitohy *et al.*, 1994).

Whole casein, which originally contained 6 mol P/mol protein, bound an additional 4, 7 and 11 mol P/mol protein when prepared with 25, 50 and 100 mol $POCl_3$/mol protein in the presence of triethylamine (Sitohy *et al.*, 1995a). α_s-, β- and κ-casein fractions containing 7, 4 and 1 mol P/mol protein, respectively, bound an additional 21, 20 and 9 mol P/mol protein when reacted with 100 mol $POCl_3$/mol protein. The relatively lower extent of phosphorylation achieved for the whole casein, as compared to α_s- or β-casein fractions, might be due to the complexing effect between casein components making some fragments of the protein molecule inaccessible for the reacting reagents. Alternatively, the relatively poor extent of κ-casein phosphorylation might be due to its hydrophobic nature (Kato and Nakai, 1980). Caseins with such properties were obtained not only by use of low molar ratios of $POCl_3$ (25–100 mol $POCl_3$/mol protein) but also due to the presence of triethylamine allowing the reaction to proceed with such low $POCl_3$ molar ratios. The phosphorylation yields obtained in these conditions (low $POCl_3$/protein molar ratios; 25–100 in the presence of triethylamine) were higher than those obtained by Matheis *et al.* (1983) and even higher in some cases than those obtained by Medina *et al.* (1992) who used extremely high $POCl_3$/protein molar ratios (1000–2000) in the presence of an inorganic base.

The SDS-PAGE patterns of the same samples showed small intermolecular associations in the phosphorylated caseins especially when compared with the results obtained by Matheis *et al.* (1983) and Medina *et al.* (1992) whose phosphorylated samples were entirely unable to enter the SDS-PAGE gel due to high cross-linking.

The possibility of using basic amino acids in the form of free bases as the only base of the reaction was studied in order to eliminate the use of tertiary amines, which are nutritionally unacceptable (Sitohy *et al.*, 1995b,c; Haertlé and Chobert, 1999).

The extent of phosphorylation was proportional to the applied $POCl_3$/protein molar ratios. Phosphoamidation was proportional to the basic amino acid/$POCl_3$ molar ratio, which is in agreement with the previously observed importance of triethylamine acting as the proton scavenger (Sitohy *et al.*, 1994). The highest phosphorylation yield was observed for 80 mol $POCl_3$/mol protein and 6 mol lysine or arginine acid/mol $POCl_3$. However, the highest phosphorylation achieved varied according to the (basic) amino acid used. This difference is clearly due to the different nucleophilicity of the three

amino acids used. The p*K* of the lateral groups of arginine, lysine and histidine are 12.48, 10.53 and 6.0, respectively. Hence, the pH of the starting reaction media using L-arginine, L-lysine and L-histidine in the form of free bases were 10.8, 9.7 and 7.6, respectively. It is well known that protonated primary amines are unreactive. Thus, basic pH is essential for the rapid formation of the phosphoamide bond. The obtained phosphorylation yields might seem low but they may be acceptable for food purposes and they are close to the highest phosphorylation (6 mol P/mol casein) reported by Matheis *et al.* (1983) using 2000 mol $POCl_3$/mol protein. The secondary phosphoamide bond formation depends on the initial substitution of protein with $-POCl_2$ and $=POCl$ showing higher yields for arginine than lysine and confirming the role of activated phosphate groups in secondary grafting of the amino acids on β-lactoglobulin. The use of arginine or lysine may be advised as a reaction base for low phosphorylation yield. The secondary grafting of the basic amino acid used for the reaction improved this method of eliminating the unacceptable quaternary amines (triethylamine). When other amino acids such as leucine, methionine, threonine or tryptophan were added (at a molar ratio of 2 mol/mol $POCl_3$) in addition to the basic amino acid (molar ratio of 5 mol/mol $POCl_3$) to the reaction medium (80 mol $POCl_3$/mol protein), the phosphorylation yield was reduced to 2 mol P/mol protein. The arginine and lysine grafting yields were reduced to 2 mol amino acid/mol protein and the added amino acids did not form significant amounts of secondary phosphoamide bonds. This indicates the importance of the appropriate buffering by arginine and lysine conferred by the basicity of their lateral side-chains.

2. *Effect of phosphorylation of casein on its functional properties*

The pH–solubility curves presented in Figure 2 generally show that phosphorylated whole casein has greater solubility than native casein in neutral and basic pHs resulting from the displacement of the isoelectric point towards acidic values. The gradual displacement of the isoelectric points towards the acidic side demonstrates the gradual increase in the negative charges with increasing phosphorylation yield. However, highly phosphorylated proteins are not resolubilized below their isoelectric point indicating some cross-linking. The solubility curves of the phosphorylated casein fractions (α_s, β and κ) show the same dependence of solubility profiles pointing to the monomeric state of the modified proteins since the polymeric form is poorly soluble and shows diffuse changes in the isoelectric points as previously found by Medina *et al.* (1992).

The emulsifying activity index (EAI) curves for phosphorylated whole casein solution versus pH showed shift of their minima towards the acid

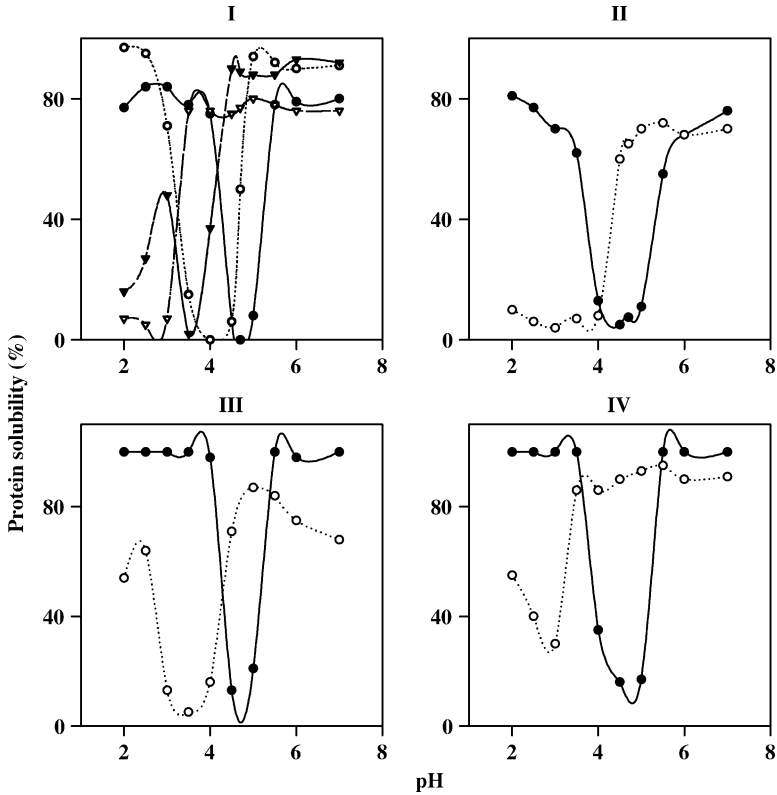


FIG. 2. pH–solubility curves of phosphorylated caseins. I, whole native (•) and phosphorylated caseins [4 (○), 7 (◆) and 11 (▽) mol P/mol protein]. II, III and IV are α_s-, β- and κ-casein in the native (•) and phosphorylated (○) forms. (*Source*: From Haertlé and Chobert (1999), by courtesy of Food & Nutrition Press, Inc.)

values (Figure 3). It was proportional to the extent of phosphorylation and to the shift of isoelectric point. EAI of the phosphorylated whole casein was lower in the pH range 6–7 than that of the native whole casein. This is quite visible in the case of the highly phosphorylated derivatives and was due to significant hydrophilicity increase. Near the isoelectric point of the native whole casein, EAI of the phosphorylated whole casein was higher than that of the native caseins. EAI decreased in the acidic pH range 2–4, which is close to the isoelectric points of the modified proteins. All the phosphorylated whole casein samples showed high stability at the pH range 3–6, and lower stability at the pH range 6–7, when compared to the native sample. Generally, all the phosphorylated caseins behave similarly, with phosphorylated κ-casein showing the biggest improvement of emulsifying properties.

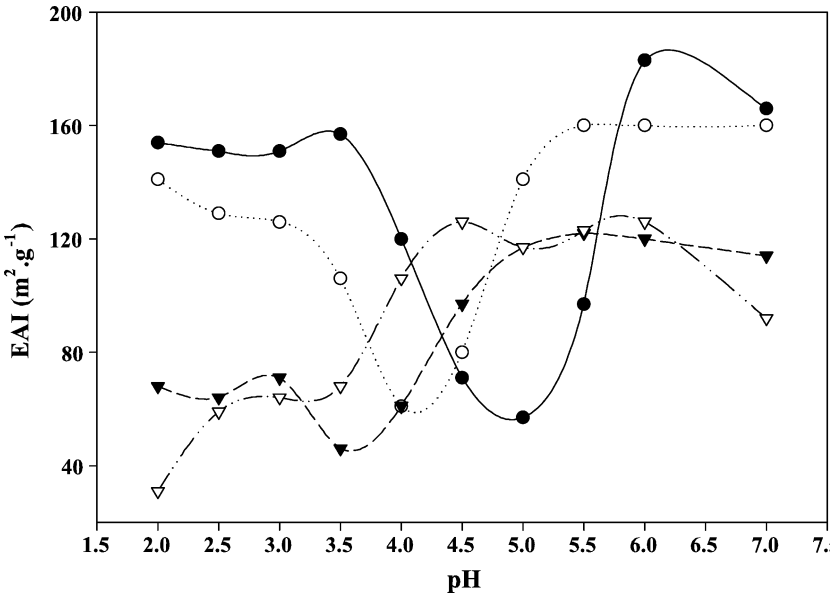


FIG. 3. Emulsifying activity index of native (●) and phosphorylated whole caseins [4 (○); 7 (◆) and 11 (▽) mol P/mol protein]. (*Source*: From Haertlé and Chobert (1999), by courtesy of Food & Nutrition Press, Inc.)

Since the emulsifying properties depend on the balance between hydrophobicity and hydrophilicity at the protein surface, a very high yield of phosphorylation may disrupt this balance. The higher improvement of emulsifying properties of κ-casein was due to its original high hydrophobicity, which diminished after phosphorylation.

Table I shows that the foaming properties of whole casein improved by slight phosphorylation. The lowest phosphorylated form of casein (4 mol P/mol protein) showed higher foam hydration and stability than the native whole casein. However, the highly phosphorylated whole casein (11 mol P/mol protein) showed poor foaming properties. The foam hydration of α_s-casein deteriorated while that of κ-casein improved by phosphorylation. This discrepancy seemed to be caused by a different initial hydrophobic/hydrophilic balance of the proteins in their native states. However, foam stabilities of all casein fractions were reduced by phosphorylation, with κ-casein being only slightly affected.

The highest phosphorylated samples of β-lactoglobulin in the presence of arginine and lysine showed EAI of 254 and 229 m^2/g, respectively, which represented an improvement as compared to EAI of native

TABLE I

FOAMING PROPERTIES OF NATIVE AND PHOSPHORYLATED CASEINS

Protein sample	1[a] TF(s)	2 FV max	3 LV max	4 FV 20 min	5 LV 20 min	6 HL (s)
Native whole casein	201	50	3.7 ± 0.05	30.6 ± 0.08	0.71 ± 0.05	460 ± 15
Phosphocaseins:						
(4 mol P/mol protein)	201	50	4.25 ± 0.04	36.5 ± 0.11	0.78 ± 0.07	471 ± 10
(7 mol P/mol protein)	204	50	4.11 ± 0.05	34.1 ± 0.15	0.71 ± 0.04	460 ± 11
(11 mol P/mol protein)	218	50	2.75 ± 0.02	15 ± 0.09	0.24 ± 0.02	453 ± 12
Native α-casein	206	50	6.75 ± 0.06	41.3 ± 0.09	1.63 ± 0.05	556 ± 14
Phospho α-casein	212	50	5.2 ± 0.05	21.2 ± 0.08	0.79 ± 0.07	421 ± 13
Native β-casein	203	50	6.14 ± 0.09	47 ± 0.21	1.72 ± 0.04	552 ± 11
Phospho β-casein	206	50	6.24 ± 0.16	34.7 ± 0.34	1.1 ± 0.05	476 ± 5
Native κ-casein	206	50	4.42 ± 0.04	48.7 ± 0.15	1.33 ± 0.11	475 ± 16
Phospho κ-casein	205	50	5.65 ± 0.09	45.1 ± 0.13	1.1 ± 0.12	445 ± 14

Source: From Haertlé and Chobert (1999), by courtesy of Food & Nutrition Press, Inc.

[a]1: Time of foaming (s); 2: Maximum foam volume (ml); 3: Maximum liquid volume in foam; 4: Foam volume after 20 min; 5: Liquid volume in foam after 20 min; 6: Half-life of foam (s).

β-lactoglobulin (160 m^2/g). The stability of emulsions prepared with phosphorylated β-lactoglobulin was also higher than the stability of emulsions prepared with native β-lactoglobulin. Emulsion prepared with phosphoarginyl β-lactoglobulin amide exhibited the highest stability. Such improvement in the emulsifying properties of the phosphorylated proteins agrees well with the results reported by Sung *et al.* (1983), Hirotsuka *et al.* (1984), and Huang and Kinsella (1987). According to these authors, the improvement in emulsifying properties could be explained in light of the increased negative charges on the phosphorylated proteins, which tends to cause electrostatic repulsion between the negatively charged emulsion faces, reducing the flocculation and coalescence of the emulsions. Consequently, a significant improvement of emulsifying properties can be achieved with moderate phosphorylation.

B. ESTERIFICATION

The esterification reaction is an important tool for modifying food proteins. Esterification with different alcohols leads to the blocking of free carboxyl groups thus raising the net positive charge, making the modified proteins more basic (Wilcox, 1967; Mattarella *et al.*, 1983; Halpin and Richardson, 1985). The basicity of the modified protein depends on the degree of esterification and on the original content of basic amino acid residues on the protein molecules. This modification has shown its impact on the folding and peptic hydrolysis of β-lactoglobulin (Briand *et al.*, 1995). While β-lactoglobulin is resistant to peptic hydrolysis in aqueous and physiological conditions, an ethylated β-lactoglobulin derivative was highly susceptible to pepsin hydrolysis in aqueous conditions. The esterified β-lactoglobulin exhibited 22 new sites of pepsin cleavage (Briand *et al.*, 1995; Chobert *et al.*, 1995) as compared to the peptic peptides obtained when hydrolysis of native β-lactoglobulin was performed in hydro-ethanolic conditions (Dalgalarrondo *et al.*, 1995). Hence, the reaction might be a good tool for increasing peptic digestibility of food proteins or producing specific new unconventional peptides exhibiting novel activities. Moreover, the high isoelectric points of the esterified proteins might endow them with new physico-chemical and functional properties (Mattarella and Richardson, 1983; Halpin and Richardson, 1985; Chobert *et al.*, 1990).

Since the initial work of Fraenkel-Conrat and Olcott (1945), protein esterification has been described in a number of studies (Mattarella *et al.*, 1983; Chobert *et al.*, 1990, 1995; Bertrand-Harb *et al.*, 1991; Briand *et al.*, 1994, 1995). The conventional procedure involves three steps. The first step is the mixing of reactants (protein, alcohol and acid). The second step is the esterification reaction itself, which generally ranges in length from one to several days, at 4°C. The last step is reaction termination and product recovery.

The length of the reaction, which may reach 10–12 days, could limit the reproducibility and applicability of this reaction. Additionally, the method used to recover the product at the end of the reaction can lead to side-reactions. In some procedures (e.g., Halpin and Richardson, 1985; Bertrand-Harb *et al.*, 1991), the reaction medium at the end of the reaction time was combined with an equal volume of water, then dialyzed against water for several days, which can lead to deesterification (Bertrand-Harb *et al.*, 1991). Another way to recover the reaction product was by drying under vacuum (Briand *et al.*, 1995).

Protons are essential catalysts of the esterification process, which will not proceed to any extent if they are absent in the reaction medium. Consequently, determining the optimum amount of protein catalyst required for the reaction is extremely important (Sitohy *et al.*, 2000).

1. Factors influencing esterification. Study of β-lactoglobulin as a model

a. Influence of time-course of reaction. The reaction time-course (8, 16, 24, 48, 72 and 96 h) was followed in an esterification process using β-lactoglobulin (3% protein concentration) dispersed in 95% ethanol in the presence of 0.7N HCl. The extent of esterification increased gradually with the length of reaction as 11, 14, 23, 28, 32 and 36%, respectively. It is obvious that under the conditions used, the esterification did not reach its maximum even after 96 h of reaction. Consequently, in order to increase the extent of modification after shorter reaction times, conditions should be modified drastically. For example, protein and acid concentrations can be increased. The length of reaction is alcohol-dependent since it was observed that esterification with methanol reached its maximum within 24 h by using less concentrated solutions.

b. Influence of temperature. Comparative experiments (2% protein, 0.7N HCl final concentration) were carried out by esterifying β-lactoglobulin with 99.7% ethanol at three different temperatures (4, 10 and 20°C). The reaction was stopped after 8 h. The extent of esterification increased with the increase of temperature, being 2, 4 and 8% at 4, 10 and 20°C, respectively. However, the products obtained in higher reaction temperatures developed violet coloration after drying showing that high temperatures may induce side-reactions. Wilcox (1967) stated that higher temperatures may give insoluble esterified products. Consequently, although higher temperatures may increase the reaction rates, they should not be used in order to avoid undesired side-reactions.

c. Influence of the presence of water. Water is required for the reaction at proportions depending on the type of alcohol (ethanol, methanol, and *n*-propanol) used. Water is required to dissociate hydrochloric acid supplying

the protons needed for the activation of the carboxyl groups during the esterification reaction. Water in the medium may also be used to form a hydration layer around the protein molecules, which helps us to organize the hydrophobic moieties in the globulin core (Tanford, 1980), thus making the carboxyl groups more accessible for esterification. The importance of water for the esterification reaction was indirectly considered by Halpin and Richardson (1985) who used 95% ethanol to esterify β-lactoglobulin while using anhydrous methanol for the same purpose.

d. Influence of the type of alcohols. The comparison of different alcohols used for esterification showed that methanol is the most reactive and can esterify most of the carboxyl groups of β-lactoglobulin, leading to a maximum esterification extent of 97% when traces or 1% water is present in the starting alcohol. The final amount of water in the reaction medium also depends on its quantity contained in the added acid.

The difference between reactivity of alcohols agrees with the results of Fraenkel-Conrat and Olcott (1945) and Halpin and Richardson (1985) who found that methanol was most reactive as an esterifying agent followed by ethanol and then *n*-butanol. Mattarella *et al.* (1983) found that in methanol the esterification reaction progressed quicker than in ethanol, while there was no evidence for esterification in propanol, butanol or pentanol even after one-week reaction time. The reason for such a difference according to the alcohols used is due to their different polarity and structural hindrance, which is especially evident in isopropanol.

e. Effect of the protein concentration. The effect of the protein concentration was investigated using different conditions. Esterification was performed for 24 h at 4°C in 95% ethanol and 0.7N HCl. β-Lactoglobulin was added at 2, 3, 4 and 5% (Table II). Increase of protein concentration from 2 to 3% slightly enhanced the extent of esterification. However, a further increase till 4 and 5% had an adresse effect on the reaction, resulting in poorer extents of esterification. During esterification, carboxyl groups of the protein should first be activated with protons before reacting with the alcohol. Consequently, the most important factor for the reaction is not the protein concentration *per se*, but the concentration of activated carboxyl groups. Taking into account that acid concentration was equal in all preparations with different protein concentrations, it can be concluded that the ratio H^+/COOH became lower when the protein concentration was increased up to 4 or 5%. Hence, with higher protein concentrations, the reactivity of carboxyl groups was lower and, consequently, the extent of esterification was reduced. In this series of preparations, the initial H^+/COOH ratio was equal to 24 when using 2% protein and 0.7N HCl,

TABLE II

EXTENT OF ESTERIFICATION OF β-LACTOGLOBULIN WITH 95% ETHANOL AS INFLUENCED BY PROTEIN AND HYDROCHLORIC ACID CONCENTRATIONS IN THE REACTION MEDIUM AT 4 °C

Acid normality	0.7N	1.4N		1.75N	
Time (h)	24	14	24	14	24
Protein concentration (%)		Extent of esterification (%)			
2	36 ± 0.71				
3	38 ± 0.87				
4	21 ± 1.04	44 ± 0.46	51 ± 1.10		
5	21 ± 0.75			45 ± 1.01	57 ± 0.80

Source: From Haertlé and Chobert (1999), by courtesy of Food & Nutrition Press, Inc.

decreasing to 12 when switching to 4% protein and 0.7N HCl. In the latter case, the activated carboxyl groups available for the reaction are expected to be less numerous and, consequently, the relatively lower extent of esterification is justified.

In another series of experiments, the H^+/COOH ratio was kept a constant (24 mol H^+/mol COOH group, as in the case of a 2% preparation) during the esterification of 4 and 5% β-lactoglobulin. Results in Table II show that protein concentration in such conditions is an essential factor for the reaction. The extent of β-lactoglobulin esterification in 95% ethanol was remarkably enhanced up to 51% after 24 h reaction when using 4% protein. Increasing the protein concentration up to 5% with the same H^+/COOH molar ratio equal to 24, further increased the extent of esterification up to 57%. Even by decreasing the reaction time to 14 h, protein was more esterified when present at 4 or 5%, compared to that obtained after 24 h with 2% protein. Consequently, protein concentration is an important driving force for the reaction if supplied in the form of activated carboxyl groups. This factor can not only maximize the esterification yield but also shorten the time required to attain this maximum.

2. *Application to α-lactalbumin*

α-Lactalbumin (5%, w/v) was esterified for 6 h at 4°C with $>99.5\%$ alcohols (methanol, ethanol, n-propanol) using 20, 40 and 60 molar ratio (MR, mole acid/mole carboxyl group) (Sitohy *et al.*, 2001a). α-Lactalbumin was more resistant to esterification than β-lactoglobulin with the different alcohols used. Even in the presence of methanol, the extent of esterification did not exceed 52% with the highest concentration of HCl. This may be due to the more compact conformation of α-lactalbumin, which may be better protected than β-lactoglobulin against the nucleophilic attack by the alcohols. α-Lactalbumin may be subject to conformational changes in acidic conditions followed by a strong increase of surface hydrophobicity. Hence, the contact with hydrophilic alcohols should be limited. However, methanol was still more reactive as an esterifying agent than the other two alcohols used with both proteins. In order to achieve higher extents of esterification in the case of α-lactalbumin, either the reaction time should be prolonged or the molar ratio of HCl should be increased.

SDS-PAGE patterns of esterified α-lactalbumin did not differ from those observed for native protein, indicating no sign of hydrolysis or polymerization. Hence, the conditions used for esterification did not affect the molecular size of the modified protein.

3. *Application to β-casein*

Extent of esterification of β-casein increased gradually parallel to the increase in the molar ratio of HCl (Sitohy *et al.*, 2001a). As observed with β-lactoglobulin, methanol achieved the highest rates of esterification. However, propanol was more efficient than ethanol, which was the opposite of the results obtained with β-lactoglobulin. This discrepancy might be due to conformational differences between these two proteins.

The relatively low extents of esterification obtained using ethanol and propanol could be due to the following factors: (1) low polarity of the reaction medium; (2) inadequate protein concentration; (3) insufficient amount of catalyst HCl.

In order to increase the polarity of the medium, ethanol and propanol were used at a concentration of 95% during esterification of 5% β-casein using a molar ratio of 30, 40 and 50. The extent of esterification decreased considerably (about 50% decrease) when compared with the values obtained in the presence of $>99.5\%$ alcohol. Although water might be needed to enhance the polarity of the reaction medium, it should not exceed a certain limit.

The role of protein concentration was examined by esterifying β-casein for 6 h with 99.7% ethanol and 99.5% propanol using a molar ratio of 70 and protein concentrations of 3, 4 and 5%. The respective extents of esterification were 26, 45 and 56% with ethanol and 36, 49 and 54% with propanol indicating that a protein concentration of 5% was the most efficient as was observed with β-lactoglobulin.

In order to examine the effectiveness of the third important factor playing a role in esterification yield, β-casein (5%) was esterified using higher molar ratio (60, 70 and 80). The modified product was recovered either by centrifugation or by filtration under vacuum. The extent of esterification could be increased up to 59 and 56% in the presence of ethanol and propanol, respectively, when using a molar ratio of 80 and when the resulting product was recovered by centrifugation. These values were slightly lower (51 and 53%) when the modified product was recovered by filtration. Since centrifugation is more time consuming than filtration under vacuum, and leaves the reactants in contact for an additional period, this may be one of the causes of a higher esterification yield. However, the use of filtration under vacuum may have the advantage of reducing the time and energy costs associated with freeze-drying (3–4 days).

4. *Factors influencing pepsinolysis of ester derivatives of β-lactoglobulin*

Esterification generally enhances the susceptibility of proteins to peptic action (Briand *et al.*, 1995; Chobert *et al.*, 1995). This was most evident with

β-lactoglobulin methyl ester, which underwent the highest degree of esterification. It was easily hydrolyzed by as low as 0.125% enzyme/substrate ratio (E/S) of pepsin, at a low temperature (4°C) and for short incubation times (15–30 min) reaching a high degree of hydrolysis (DH) independent of the substrate protein concentration. Hence, esterification can be a good method for improving the peptic digestibility of pepsin-resistant proteins, such as β-lactoglobulin and some legume proteins (Sakai *et al.*, 1997; Vieths *et al.*, 1999). Consequently, it can eliminate their allergenicity and enhance their biological value (see Section III.B.). Tricine-SDS PAGE of peptic hydrolysates of esterified β-lactoglobulin were characterized mainly by low molecular mass bands in the case of methyl and ethyl derivatives, while there were additional bands of medium molecular mass in the case of propyl ester derivatives. Hence, both the degree of esterification and the nature of the ester group are responsible for this difference (Sitohy *et al.*, 2001e). A high extent of esterification (100%) increased the amount of peptic cleavage sites throughout the protein molecule, giving rise to low molecular mass peptides of about 3 kDa. In contrast, peptic hydrolysates of propyl ester derivatives of β-lactoglobulin (44% esterified) showed an additional band of a medium molecular mass of 5–6 kDa. Reversed phase-high performance liquid chromatography (RP-HPLC) profiles of peptic hydrolysates of esterified β-lactoglobulin showed a broad population of hydrophilic peptides independent of the nature of the ester group or of the extent of esterification. This was due mainly to the increased availability and to the spread of the peptic cleavage sites throughout the esterified molecules, leading to their lysis into small hydrophilic peptides. RP-HPLC profiles of esterified β-lactoglobulins differed from those of native β-lactoglobulin. This means that esterification gives rise to new peptides. There were only slight differences between the profiles of methyl and ethyl ester hydrolysates. However, RP-HPLC profile of hydrolysate of β-lactoglobulin propyl ester was different from those obtained with methyl and ethyl esters. In order to obtain more hydrophobic peptides by peptic hydrolysis, the esterifying agent should have a relatively long aliphatic chain and the conditions of hydrolysis should be mild.

5. Peptic hydrolysis of ester derivatives of β-casein and α-lactalbumin

In a recent work (Sitohy *et al.*, 2001b) 100% methyl-, 59% ethyl- and 56% propyl-esters of β-casein, and 52% methyl-, 36% ethyl- and 25% propyl-esters of α-lactalbumin were prepared. The degree of pepsinolysis (% DH) was enhanced considerably after esterification. Methyl esters of both proteins yielded the highest levels of DH. Compared to SDS-PAGE of peptic hydrolysates of native proteins, those of esterified β-caseins demonstrated the

disappearance of the bands corresponding to peptides of medium and high molecular masses. SDS-PAGE of peptic hydrolysates of esterified α-lactalbumin showed the disappearance of the bands corresponding to peptides of low molecular masses. Compared with native protein, RP-HPLC profiles of peptic hydrolysates of esterified β-casein showed more hydrophobic peptides. The major changes in RP-HPLC of peptic hydrolysates of esterified α-lactalbumin concerned peptides eluted lately (hydrophobic), while the distribution of peptides eluted early (hydrophilic) remained constant.

6. *Improvement of functional properties of milk proteins by esterification*

Milk proteins have good inherent functionalities as well as high nutritional values. Hence, they are used as ingredients in a wide range of food products (Huffman, 1996; McCrae *et al.*, 1999). Due to its amphiphilic properties, β-casein is one of the most surface active milk proteins (Mitchell *et al.*, 1970; Benjamin *et al.*, 1975). However, because of the acid isoionic points of milk proteins, their functional properties are worst in that range of pH, when compared to the alkaline range. Adsorption of β-lactoglobulin to fat globules increases with increased pH, exhibiting good EA in the alkaline pH range (Yamauchi *et al.*, 1980; Nagasawa *et al.*, 1996; Hattori *et al.*, 1997). Adsorption of β-lactoglobulin on the oil–water interfacial layer is strongly reduced with decreasing pH reaching 61.6 and 12.9% at pH 9 and 3, respectively. This can be attributed to pH-dependent structural changes since β-lactoglobulin molecules are more rigid at acid pH (Shimizu *et al.*, 1981, 1985; McCrae *et al.*, 1999). Recently, it was confirmed that pH influences the interfacial composition and stability of emulsions prepared from egg yolk proteins (Le Denmat *et al.*, 1999). Generally, the most important factors determining the emulsifying properties of whey proteins are protein concentration, pH, ionic strength and history of processing and storage conditions. EA also depends on the flexibility of protein molecules since flexible molecules such as caseins can spread over the oil–water interface in contrast to more rigid proteins such as the whey globulins (Hunt and Dalgleish, 1994). When β-lactoglobulin adsorbs at an oil–water interface, it denatures partially exposing its highly reactive free sulfhydryl groups (Corredig and Dalgleish, 1995), which can then interact with similar groups of neighboring molecules forming polymers. The second major whey protein, α-lactalbumin, was found to inhibit β-lactoglobulin polymerization (Dickinson and Matsumura, 1991; Monahan *et al.*, 1993). Hence, the ability of a protein molecule to form and stabilize oil droplets is closely linked to the structure and conformation of the molecule (Das and Kinsella, 1990).

Since good solubility and emulsification properties at acidic pHs are required for some food applications such as acid soft drinks and acid foods,

modification of proteins improving their functional properties in this pH range may be needed. Esterification of proteins was proved to block the protein negative charges thus increasing the net positive charge and raising the protein isoionic points. This should render the modified proteins more soluble and likely much more tensio-active in the acidic range of pH. The isoionic point of β-lactoglobulin was raised from 5.2 in its native state to 6.2, 8.7 and 9.8 after its esterification with butanol, ethanol and methanol, respectively (Mattarella and Richardson, 1983; Halpin and Richardson, 1985). Similar features were observed during esterification of β-casein (Chobert *et al.*, 1990). The magnitude of change in the isoionic point values depends on the degree of esterification (Chobert *et al.*, 1990; Bertrand-Harb *et al.*, 1991; Sitohy *et al.*, 2000). Alternatively, methyl and ethyl esterification of β-lactoglobulin resulted in randomized protein structures (Mattarella *et al.*, 1983). Generally, partial denaturation of proteins results in increased surface activity compared to the native globular forms (Mitchell *et al.*, 1970). Hence, esterified β-lactoglobulins were more efficient in lowering the interfacial tension at the oil–water interface as a result of increased surface hydrophobicity (Halpin and Richardson, 1985). It was also reported that EA of esterified β-lactoglobulins is worse than that of the native form (Mattarella and Richardson, 1983; Chobert *et al.*, 1990). This discrepancy might be due to differences in pH or any other limiting factors used in each study. In previous studies, the change of EA of esterified proteins was followed by measurement of turbidity according to the method of Pearce and Kinsella (1978). Since the oil droplet size may better reflect EA, Sitohy *et al.* (2001c) measured the change of this parameter in order to elucidate the changes in EA and stability of different esterified milk proteins in the acidic and neutral ranges of pH. This yielded information on the suitability of using esterified proteins as ingredients in acid foods or to fortify acid soft drinks. Moreover, it was reported that oil droplet size influences the organoleptic perception of food emulsions and that the interfacial layer surrounding the oil droplet may be a determinant factor controlling the stability of the emulsion towards flocculation and coalescence (Mine, 1998; Aluko and Mine, 1999; Le Denmat *et al.*, 1999).

Three milk proteins (β-lactoglobulin, α-lactalbumin and β-casein) were esterified to different extents with methanol, ethanol and propanol; then their solubility was studied in the pH range 3–10. Solubility of β-lactoglobulin esters depends on the degree of esterification as well as on the nature of the grafted ester groups. Samples of highly esterified β-lactoglobulin (99%) gave rise to a more homogenous protein population with a minimum of solubility near pH 10, while those with low degrees of esterification gave a heterogeneous population showing two solubility minima (Figure 4). Consequently, two opposite effects of esterification on the solubility of

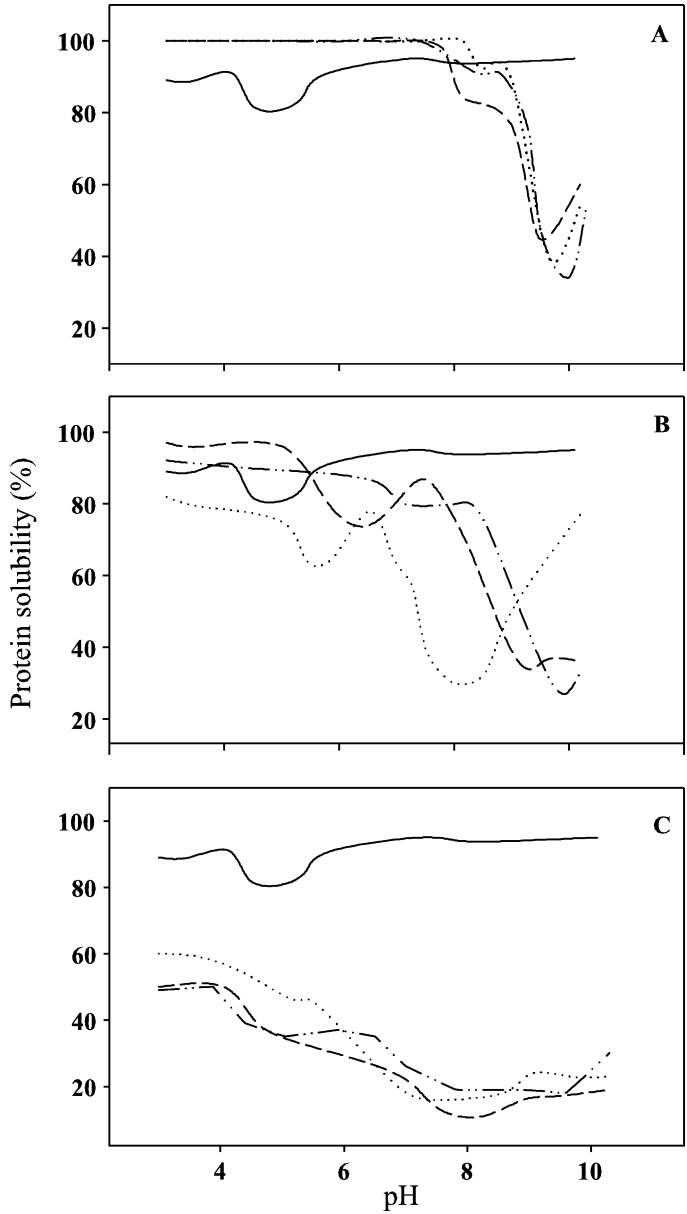


FIG. 4. Solubility profiles of β-lactoglobulin esterified to different extents with [A]: 0% (—), 85% (· · · · · ·), 94% (–··–) and 99% (– –) methanol; [B]: 0% (—), 44% (· · · · · ·), 49% (–··–) and 73% (– –) ethanol; [C]: 0% (—), 34% (· · · · · ·), 39% (–··–) and 49% (– –) propanol. (*Source*: From Sitohy *et al.* (2001c), by courtesy of Wiley-VCH Verlag, GmbH.)

β-lactoglobulin in the acidic pH range could be perceived. The first factor improving solubility results from the isoelectric point shift towards the alkaline pH. The second factor lowering solubility results from replacing hydrophilic carboxylic groups by hydrophobic ester groups. The overall protein solubility is a result of the interplay between these two effects. The first effect is more efficient in the case of the most esterified derivatives and when the grafted ester group is of a less hydrophobic nature (e.g., methyl). The second effect is more prominent in case of derivatives with a low degree of esterification and when the grafted ester groups are more hydrophobic (e.g., propyl). This may explain the reduced solubility reported in case of some esterified proteins at acid pHs (Mattarella and Richardson, 1983; Chobert *et al.*, 1990).

α-Lactalbumin methyl ester was more soluble in the acidic range of pH and less soluble in the alkaline range of pH as compared to unmodified protein (Figure 5). The low methylated sample (38%) showed two regions with a minimum of solubility (pH 5.6 and 6.8) compared to pH 7.2 and 8.8 for the 52% methylated derivative. Similarly, the highly ethylated α-lactalbumin sample (36%) showed improved solubility in acid conditions and decreased solubility in the alkaline range of pH value. The solubility of the low ethylated derivative (11%) was improved whatever the pH. The solubility curves of α-lactalbumin propyl esters were similar to that of ethyl derivatives. However, the reduction of solubility of propyl esterified derivatives was more pronounced when compared to α-lactalbumin ethyl esters showing solubility levels of 47–50% and 67–70%, respectively. As observed with β-lactoglobulin ester derivatives, the nature of the grafted ester group is an important factor for the solubility of the modified α-lactalbumin. Comparison of solubility of α-lactalbumin propyl esters with that of β-lactoglobulin propyl esters shows a different behavior for these two families of esters. While grafted propyl ester groups decrease the solubility of β-lactoglobulin in the acidic pH range, they improve the solubility of α-lactalbumin. Consequently, the conformation of the esterified protein might play an important role on the solubility of the protein. Hence, it is not only the nature of the grafted ester group that determines the solubility of the modified protein but also the nature of the protein itself.

As observed with β-lactoglobulin and α-lactalbumin, the isoelectric points of esterified β-casein shifted towards the alkaline pH range. The magnitude of this shift was proportional to the extent of esterification. β-Casein methyl esters (Figure 6A) being the more esterified samples (60, 88 and 100% esterification) showed higher magnitudes of isoelectric point shift compared with other types of esters. The decrease of solubility in the alkaline pH range (7–9) was more evident for β-casein methyl esters, giving a minimum of solubility as low as 20% for the highly esterified samples (88 and 100% esterified). The minimum

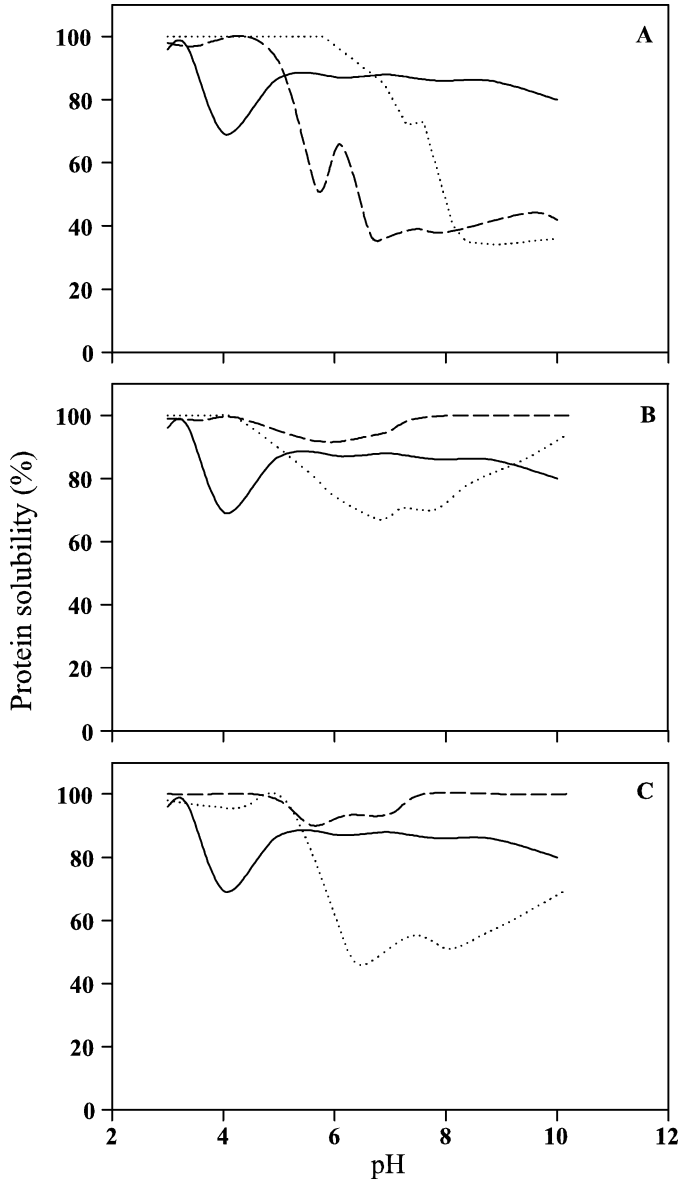


FIG. 5. Solubility profiles of α-lactalbumin esterified to different extents with [A]: 0% (—), 38% (– –) and 52% (· · · · · ·) methanol; [B]: 0% (—), 11% (– –) and 36% (· · · · · ·) ethanol; [C]: 0% (—), 8% (– –) and 24% (· · · · · ·) propanol. (*Source*: From Sitohy *et al.* (2001c), by courtesy of Wiley-VCH Verlag, GmbH.)

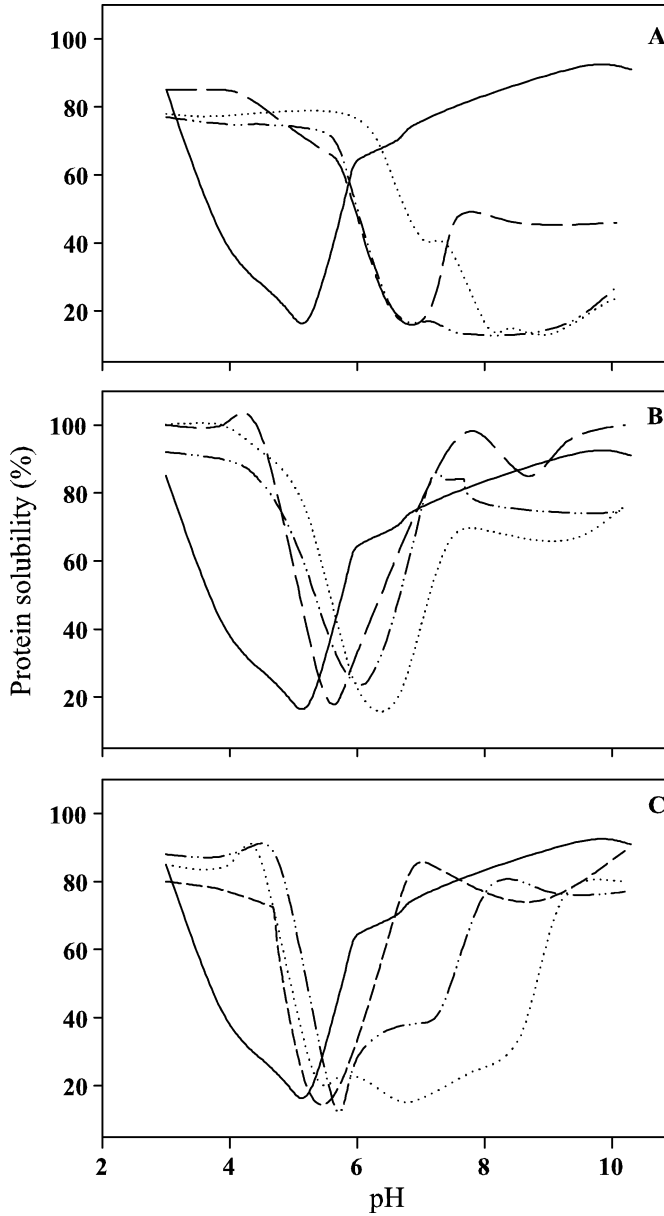


FIG. 6. Solubility profiles of β-casein esterified to different extents with [A]: 0% (—), 66% (– –), 88% (–··–) and 100% (·····) methanol; [B]: 0% (—), 17% (– –), 33% (–··–) and 59% (·····) ethanol; [C]: 0% (—), 26% (– –), 38% (–··–) and 56% (·····) propanol. (*Source*: From Sitohy *et al.* (2001c), by courtesy of Wiley-VCH Verlag, GmbH.)

of solubility of β-casein esters is mainly observed for one pH value or one pH range in contrast to two minima previously observed with β-lactoglobulin and α-lactalbumin. This may be because the flexible structure of β-casein makes the alcoholic groups more accessible to nucleophilic attack, giving rise to more homogeneous esterified populations. The hydrophobic core of β-lactoglobulin is certainly more resistant to the nucleophilic attack of the alcoholic groups at the beginning of the reaction, becoming only accessible with the progress of esterification or at higher protein concentrations giving rise to different molecular populations.

Comparison of methyl and ethyl esters of β-casein shows a more important shift of isoelectric point for the methyl derivative due to the higher extent of modification. Moreover, the solubility of β-casein methyl esters is smaller in the alkaline range of pH (7–9) due also to the high extent of modification. However, although ethyl and propyl esters of β-casein displayed similar extents of substitution (~20 to ~60%) and had a similar shift of isoelectric points, the propyl ester derivatives were less soluble in the alkaline pH range than the ethyl ester derivatives probably because of the more hydrophobic nature of grafted propyl groups. It can be concluded that the solubility was improved only between pH 4 and 5. This property might find some practical application when using esterified proteins as additives for beverages instead of protein hydrolysates that often have a bitter taste (see Section III.C.).

Comparison of the solubility of all three esterified proteins showed that the magnitude of the shift in the isoelectric points decreased in the following order: β-lactoglobulin > α-lactalbumin > β-casein.

Emulsifying properties of esterified milk proteins were also studied in the pH range 3–7 by measuring the oil droplet size using laser light scattering (Table III). EA and stability of esterified milk proteins in the acidic pH range 3–5 were generally higher compared to the corresponding native proteins. This improvement was associated with the degree of solubility, the degree of esterification, the type of ester group grafted and the nature of the used protein. The highest EA improvement was observed for methyl ester derivatives at pH 5 where the native proteins have poor emulsifying properties (Sitohy *et al.*, 2001c).

7. *Change in biological properties*

a. Formation of complexes between DNA and esterified dairy proteins. As with many natural basic proteins such as histones and lysozymes, plasmid DNA can interact with methylated α-lactalbumin and methylated β-lactoglobulin forming complexes (Sitohy *et al.*, 2001d). The stability of the complex was tested at different pH, temperatures and salt concentration, and after enzyme digestion with DNase I and pepsin. Incubation at 37°C for long

TABLE III

EMULSIFYING ACTIVITY AND STABILITY OF NATIVE AND ESTERIFIED MILK PROTEINS MEASURED BY OIL DROPLET DIAMETER, DIRECTLY AFTER EMULSIFICATION OR AFTER A TWO-WEEK STORAGE AT 4 °C USING 4 MG/ML PROTEIN CONTENT AND 15% SUNFLOWER OIL

pH	Emulsifying activity (oil droplet diameter, μm)				Emulsifying stability (oil droplet diameter, μm)			
	Native	Methyl	Ethyl	Propyl	Native	Methyl	Ethyl	Propyl
β-Lactoglobulin								
4	1.37 ± 0.06	1.17 ± 0.04	1.13 ± 0.08	1.13 ± 0.04	1.40 ± 0.03	1.24 ± 0.03	1.40 ± 0.05	1.30 ± 0.03
5	1.64 ± 0.08	1.18 ± 0.06	1.15 ± 0.06	1.32 ± 0.03	1.93 ± 0.05	1.33 ± 0.03	1.34 ± 0.04	1.41 ± 0.04
7	0.97 ± 0.06	1.20 ± 0.04	1.25 ± 0.08	1.46 ± 0.06	1.02 ± 0.02	1.30 ± 0.05	1.48 ± 0.06	1.61 ± 0.05
α-Lactalbumin								
4	0.94 ± 0.02	1.11 ± 0.04	0.95 ± 0.07	1.21 ± 0.03	0.88 ± 0.02	1.07 ± 0.02	0.97 ± 0.02	1.22 ± 0.02
5	1.21 ± 0.03	0.92 ± 0.07	1.03 ± 0.06	1.24 ± 0.04	1.15 ± 0.03	0.93 ± 0.02	0.95 ± 0.02	1.21 ± 0.02
7	0.71 ± 0.02	1.06 ± 0.06	1.64 ± 0.05	1.67 ± 0.06	0.67 ± 0.01	1.07 ± 0.02	1.60 ± 0.04	1.67 ± 0.05
β-Casein								
4	U	1.13 ± 0.06	1.21 ± 0.01	1.35 ± 0.06	U	1.20 ± 0.02	1.29 ± 0.03	1.21 ± 0.02
5	U	1.25 ± 0.05	1.54 ± 0.05	1.85 ± 0.05	U	1.21 ± 0.02	1.89 ± 0.03	1.85 ± 0.11
7	0.96 ± 0.01	U	U	U	0.96 ± 0.01	U	U	U

U: Unmeasurable; *Source*: From Sitohy *et al.* (2001c), by courtesy of Wiley-VCH.

periods (up to 24 h) allowed the interaction of DNA with low concentrations of esterified proteins to take place. High temperature treatment (100°C) for short periods of time enhanced complex formation after 5 min of heating in case of both DNA-methylated α-lactalbumin and DNA-methylated β-lactoglobulin. The DNA-methylated β-lactoglobulin complex was more stable than the DNA-methylated α-lactalbumin complex when the thermal treatment at 100°C was extended to 10 min. Both complexes of DNA with methylated α-lactalbumin and DNA with methylated β-lactoglobulin were formed in large amounts and were more stable at acid pH (3–6). In contrast, their amounts were smaller and they were less stable at basic pH (8–9). Generally, larger amounts of stable DNA-methylated β-lactoglobulin complex were obtained at acid pH when compared with DNA-methylated α-lactalbumin complex. These complexes were still highly stable at very acidic pH (1–2) but not at very basic pH (10–11). Formation and stability of the complexes of DNA studied with esterified protein were generally salt-concentration-dependent. Magnesium chloride had the biggest inhibitory effect on the formation and stability of these complexes while potassium acetate had the smallest. The inhibitory effect of KCl on both the complex formation and stability was observed in the range 0.4–1.0 M. DNA complexed with esterified milk proteins and with lysozyme was more resistant to hydrolysis with DNase I than was unbound DNA. Additionally, DNA complexed with methylated α-lactalbumin was more resistant to DNase I digestion than its complex with methylated β-lactoglobulin or with lysozyme. All the studied proteins complexed with DNA were resistant to pepsin.

b. Inhibition of bacteriophage replication with esterified milk proteins. DNA-binding proteins can have antiviral and tumor suppressor activities. Milk proteins have already been reported to have antiviral effects, which were ascribed to lactoferrin (Hasegawa *et al.*, 1994; Harmsen *et al.*, 1995), a mammalian iron-binding protein of 80 kDa (Baker *et al.*, 1994). Human lactoferrins exhibit potent antiviral activities against cytomegalovirus (Hasegawa *et al.*, 1994; Harmsen *et al.*, 1995), HSV-1 infection (Fujihara and Hayashi, 1995; Marchetti *et al.*, 1996) and inhibit the replication of HIV-1 in a T-cell line if they are added prior to infection or during the virus adsorption step (Puddu *et al.*, 1998).

Lactoferrin-mediated protection of mice from cytomegalovirus infection was reported to be linked with T-cell-dependent increase in natural killer cell activity (Shimizu *et al.*, 1996). Lactoferrin potent antiviral inhibitory effect against HIV-1 in T-cell line was attributed to its action on HIV binding or on the entry of this virus into the cells no matter if it was in the apo or saturated form (Puddu *et al.*, 1998). Antiviral and antitumor activities are correlated to

each other. Factors that enhance protein–DNA cross-linking, e.g., the antibiotic Givocarcin V, show antiviral and antitumor activities (Morimoto *et al.*, 1981; Nakano *et al.*, 1981). RNA– and DNA–protein interactions are essential for some pathogenic viruses. Tat protein is essential for HIV viral replication and activates transcription by binding to the transactivation-responsive (TAR) site located at the 5′-end of the viral transcript. In hepatitis C virus (HCV), a ~350 nucleotide region in the 5′-end of the viral transcript is required for cap-independent translation and is vital for virus replication (Xavier *et al.*, 2000). Hence, the disruption of such interaction with external nucleic acid binding proteins can result in significant reduction of virus replication and infection.

DNA-binding properties of esterified milk proteins have been studied previously. These interactions are the result of increased net positive charge on the modified proteins caused by esterification (Sitohy *et al.*, 2001d). Hence, it was interesting to verify the possible use of esterified milk proteins in inhibiting viral replication. Since esterified milk proteins bind to DNA in a nonspecific way (Sitohy *et al.*, 2001d), this will probably endow them with broad specificity against viruses, whatever their mutated forms could be. This could be of interest since viruses adopt mutation strategies to escape immune control (Alcami and Koszinowski, 2000) and produce viral proteins that are no longer recognized by antibodies (Hill *et al.*, 1996; Zeidler *et al.*, 1997). Bacteriophage M13, which is specific for *Escherichia coli*, is a well-characterized filamentous 6407-nucleotide single-stranded bacteriophage (Van Wezenbeek *et al.*, 1980). Its structure and mechanism of replication are well known (Sambrook *et al.*, 1989; Makowski, 1994; Marvin, 1998). Hence, it can be used as a model phage for investigating the antiviral action of esterified milk proteins.

Esterified proteins showed an antiviral activity against bacteriophage M13 due to their DNA-binding properties (Sitohy, M., Chobert, J.-M., Karwowska, U., Gozdzicka-Jozefiak, A., and Haertlé, T., unpublished data). Methylated β-lactoglobulin, ethylated β-lactoglobulin and methylated α-lactalbumin formed complexes with the M13 DNA with an efficiency depending on their degree of esterification. The more the esterification extent of the protein, the higher the DNA complexing capacity and the higher its antiviral activity. The antiviral effect was associated with a simultaneous inhibitory effect on viral replication, which reinforces the previous conclusion that antiviral activity originates from interactions between esterified proteins and viral DNA. Methylated β-lactoglobulin showed even higher antiviral activity and a stronger inhibitory effect on the replication of bacteriophage M13 than native basic proteins such as calf thymus histone and chick egg lysozyme, probably as a result of the formation of a more stable DNA–protein complex in the case of esterified milk proteins. Complexes of esterified proteins with DNA

were previously found to be resistant to hydrolysis (Sitohy *et al.*, 2001d). Consequently, the formation of complexes with viral DNA will definitively exclude it from the replication system inhibiting the overall process. During replication, some regions of the viral DNA are exposed to interactions with proteins. Esterified proteins present in the viral medium can attack the viral DNA, forming complexes and stopping the replication machinery. The virus life cycle depends on the organization of its structures through the interactions with specific proteins (Johnson and Chiu, 2000). Consequently, the nonspecific interactions with esterified milk proteins may disrupt the virus life cycle through disrupting its structural organization.

C. GLYCATION OF β-LACTOGLOBULIN USING MILD CONDITIONS

The nonenzymatic browning or Maillard reactions are of great importance in food manufacturing. This reaction was first described by the French biochemist Louis Camille Maillard at the beginning of the 20th century (Maillard, 1912). The browning reaction can be defined as the sequence of events that begins with the reaction of the amino group of amino acids, peptides, or proteins with a glycosidic hydroxyl group of sugars; the sequence terminates with the formation of brown nitrogenous polymers or melanoidins (Ellis, 1959; O'Brien and Morrissey, 1989; Ames, 1990; Friedman, 1996; Chuyen, 1998). This is also one of the principal pathways of final degradation of organic matter in nature.

These nonenzymatic reactions are responsible for numerous changes on food properties and may impair food safety. Although these reactions are of great importance in the production of aroma, taste and color, they are often accompanied by a reduction of the nutritive value of different foods and by the formation of toxic compounds harmful for human health (Ledl and Schleicher, 1990). Results of nonenzymatic browning can be either desirable or undesirable. The brown crust formation on bread is desirable; the brown discoloration of evaporated and sterilized milk is undesirable. For products in which the browning reaction is favorable, the resulting color and flavor characteristics are generally experienced as pleasant. In other products, color and flavor may become quite unpleasant.

The reaction velocity and pattern are influenced by the nature of the amino acid or protein and carbohydrate involved in the reaction. Generally, lysyl residue is the most reactive amino acid because of the free ε-amino group. Reaction of the ε-amino group of lysyl residues of proteins with reducing sugar results in the so-called "Amadori product" via the formation of a Schiff's base and the Amadori rearrangement (Ledl and Schleicher, 1990; Friedman, 1996). Through this reaction, the conjugation of sugar to the protein does not require chemical catalysis, but just heating in order to

accelerate the spontaneous reaction. A well-controlled Maillard reaction can thus be a good method for protein processing in the food industry. Since lysine is the limiting essential amino acid in many food proteins, its destruction can reduce the nutritional value of proteins. Foods that are rich in reducing sugars are very reactive and this explains why lysine in milk is destroyed more easily than in other foods (De Man, 1999).

Study and characterization of the Maillard reaction products could allow one to control the formation of advanced Maillard products (AMP), responsible in part for noxious effects in diabetes and in age-related cardiovascular diseases (Al-Abed *et al.*, 1999). This knowledge could lead to a better control of these reactions in order to modify food proteins and their functions. Maillard reactions are one of the simplest ways to modify food proteins because they take place when a protein and a sugar are just heated together (Chuyen, 1998).

β-Lactoglobulin is a major whey protein. It is present in the milk of various ruminant species (Godovac-Zimmermann *et al.*, 1990a,b; Sawyer and Holt, 1993; Ochirkhuyag *et al.*, 1998). This protein constitutes a major waste product of the cheese industry. Only recently, its use increased as a food additive thanks to its good nutritional properties (Smithers *et al.*, 1996). Consequently, the improvement of β-lactoglobulin functional properties may be of considerable interest to industry.

To improve their functional and physico-chemical properties, dairy proteins have been modified by several methods (Haertlé and Chobert, 1999), such as phosphorylation (Sitohy *et al.*, 1995c), esterification (Chobert *et al.*, 1995; Sitohy *et al.*, 2001c), alkylation (Kitabatake *et al.*, 1985), and reductive amidation (Mattarella *et al.*, 1983); (see Section II.A., B.). However, most of these methods, using toxic chemicals, cannot be used in food processing.

β-Lactoglobulin showed better emulsifying properties after glycation with glucose-6-P (Aoki *et al.*, 1997) and better heat stability and solubility after glycation with glucose, mannose or galactose (Nacka *et al.*, 1988). Study of the Maillard reactions between β-lactoglobulin and lactose revealed the presence of α-lactulosyllysine (Jones *et al.*, 1998), a unique lactosylation site in the early step of the reaction (Léonil *et al.*, 1997; Fogliano *et al.*, 1998) and a heterogeneity of protein glycoforms (Morgan *et al.*, 1997).

1. Reaction conditions. Glycation in the presence of different sugars

Protein polymerization and glycation site specificity were investigated according to the nature of sugar used for modification of β-lactoglobulin (Chevalier *et al.*, 2001c). Among the six common sugars used, arabinose and ribose induced the highest degree of modification. Glucose, galactose and

rhamnose were less reactive and lactose generated the lowest degree of modification. Proteins substituted with ribose or arabinose formed polymers stabilized by sugar-induced covalent bonds. When other sugars were used, part of the aggregated proteins were stabilized only by hydrophobic interaction and disulfide bonds. According to mass spectrometry analysis, leucine 1 (N-terminal amino acid), lysine 14 and lysine 47 were modified in the presence of galactose, glucose or lactose. Lysines 69, 75 and 135 were modified only in the case of protein glycated with glucose. Lysine 100 was modified only when protein was glycated with lactose. No glycation site could be detected for proteins glycated with ribose or arabinose due to the higher degree of modification, which inhibited the tryptic hydrolysis used before mass spectrometry analysis.

2. *Change in protein structure*

Heating of β-lactoglobulin for 3 days at 60°C did not induce major conformational changes, as observed by peptic hydrolysis, since only an additional 5% of the protein was hydrolyzed when compared with native β-lactoglobulin. Only minor modifications could be observed in far- and near-UV circular dichroism spectra of these proteins. Nevertheless, some changes in aggregation behavior were observed by SDS-PAGE, showing polymerization of β-lactoglobulin after heating. When β-lactoglobulin was heated in the presence of sugars, larger structural modifications were observed depending on the sugar used (Chevalier *et al.*, 2002). Conformational modification of β-lactoglobulin was related to the degree of glycation. The more reactive the sugar was, the more denatured was the glycated protein. According to peptic hydrolysis data, near- and far-UV circular dichroism spectra and microcalorimetry analysis, proteins modified with ribose or arabinose (the most reactive sugars) showed important conformational changes. In contrast, proteins modified with lactose or rhamnose (the less reactive sugars) had similar three-dimensional structures to native β-lactoglobulin. According to previous work (Chevalier *et al.*, 2001a,c), glycation of β-lactoglobulin induced polymerization of protein monomers. Moreover, as observed by calorimetry analysis, Maillard glycation increased the temperature of denaturation of proteins glycated with galactose, glucose, lactose or rhamnose. These results correlated well with those obtained in a previous study of the functional properties of glycated β-lactoglobulin (Chevalier *et al.*, 2001c; see below), which demonstrated the importance of the sugar used for the improvement of emulsifying and foaming properties of the derivatives.

3. *Change in functional properties*

The whey produced during cheese and casein manufacturing contains approximately 20% of all milk proteins. It represents a rich and varied mixture of secreted proteins with wide-ranging chemical, physical and functional properties (Smithers *et al.*, 1996). Due to their beneficial functional properties, whey proteins are used as ingredients in many industrial food products (Cheftel and Lorient, 1982). According to Kinsella and Whitehead (1989), functional properties of foods can be explained by the relation of the intrinsic properties of the proteins (amino acid composition and disposition, flexibility, net charge, molecular size, conformation, hydrophobicity, etc.), and various extrinsic factors (method of preparation and storage, temperature, pH, modification process, etc.).

Numerous attempts were made to improve the functional properties of whey proteins through physical, chemical and/or enzymatic treatments (Haertlé and Chobert, 1999). Many studies were carried out with β-lactoglobulin, the major whey protein. Focusing on the improvement of solubility, heat stability, foaming properties and emulsifying properties, this protein has been conjugated with ester (Mattarella and Richardson, 1983; Sitohy *et al.*, 2001c), gluconic or melibionic acids (Kitabatake *et al.*, 1985), carbohydrates (Waniska and Kinsella, 1988; Bertrand-Harb *et al.*, 1990) and phosphoric acid (Sitohy *et al.*, 1995a–c).

However, most of these methods utilize toxic chemicals and are not permitted for potential industrial applications. Recently, some attempts were made to improve the functional properties of β-lactoglobulin by conjugation with glucose-6-phosphate (Aoki *et al.*, 1997).

In a recent work (Chevalier *et al.*, 2001d), the functional properties (solubility, heat stability, emulsifying and foaming properties) of β-lactoglobulin after glycation of the protein with several sugars (arabinose, galactose, glucose, lactose, rhamnose or ribose) were studied. Protein samples were heated in the presence or in the absence (heated control) of different sugars for three days at 60°C. Subsequent glycation induced a modification of the solubility profile, shifting the minimum solubility towards more acidic pH. Native β-lactoglobulin was soluble over the whole pH range studied (Figure 7A). After heating, a 50% decrease of solubility was observed in the pH range 4.0–5.5 with a minimum observed at pH 5, which is near the pI of the protein (Figure 7A). After heating β-lactoglobulin in the presence of arabinose or ribose, the resulting glycated derivatives exhibited 35% solubility at pH 4 (Figure 7B), which is lower than the solubility obtained with heated β-lactoglobulin and β-lactoglobulin glycated with the other sugars. Major conformational modification induced by the glycation could explain such a decrease. However, due to the shift of their isoelectric point, these

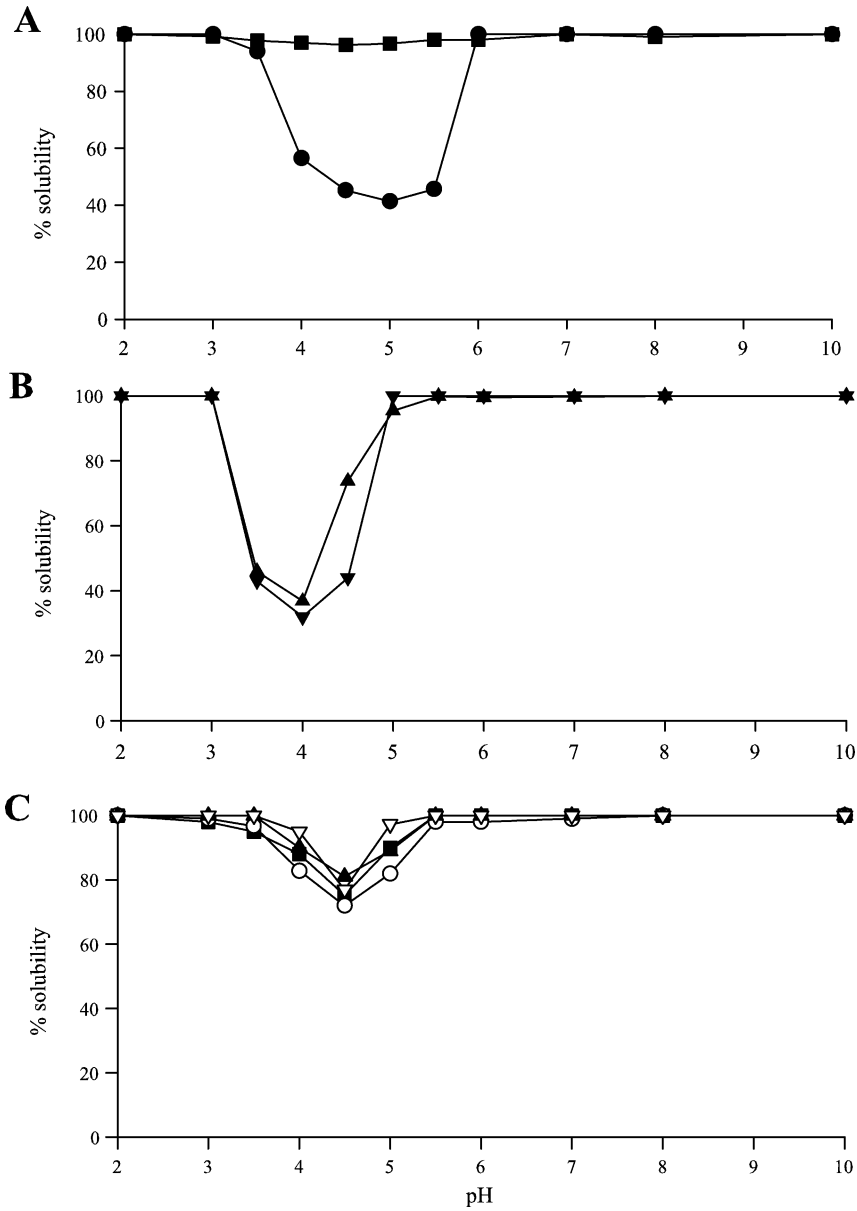


FIG. 7. Solubility of native, heated and glycated β-lactoglobulin (BLG) as a function of pH. The protein concentration was 2 mg/ml. **A**: native (■) and heated (•) BLG; **B**: BLG glycated with arabinose (▲) and ribose (▼); **C**: BLG glycated with galactose (■), glucose (○), lactose (▲) and rhamnose (▽). (Reprinted from Chevalier *et al.* (2001d), Copyright 2001, with permission from Elsevier Science.)

derivatives showed an increased solubility at pH 5 as compared with heated β-lactoglobulin. In the case of proteins modified with galactose, glucose, lactose or rhamnose, 75% solubility was observed at pH 4.5 (Figure 7C), showing a protective effect of glycation by these sugars against the decrease of solubility due to heating.

Glycated proteins exhibited a better thermal stability when heated at pH 5 as compared to native or heated control.

Glycation of β-lactoglobulin with arabinose or ribose (the most reactive sugars) improved its emulsifying properties. Solubility is a determining factor for a protein's ability to form an emulsion (Kinsella and Whitehead, 1989). Despite the fact that native β-lactoglobulin is highly soluble in the pH range 2–10, its EAI was low at acid pHs and increased with increasing pH (Shimizu *et al.*, 1985), suggesting that structural changes occur in β-lactoglobulin at acidic pH that influence its emulsifying properties. Such effects close to the pI of β-lactoglobulin could be at the origin of the decrease of emulsion stability (ES) (Klemaszewski *et al.*, 1992) observed in the $d_{3.2}$ values.

Recent studies have shown that lactosylation of β-lactoglobulin in a dry system did not significantly alter the three-dimensional structure of the protein. In contrast, in an aqueous system, an important structural change was observed at the protein–protein interface (Morgan *et al.*, 1999a–c). This suggests that protein modified with galactose, glucose, lactose or rhamnose could present structural changes as compared with the native protein. Polymerization observed with glycated β-lactoglobulin (Bouhallab *et al.*, 1999; Chevalier *et al.*, 2001a) could be involved in these changes.

Proteins modified with galactose, glucose, lactose or rhamnose were less glycated. Consequently, they may have conserved a globular three-dimensional structure close to the conformation of the native protein, accounting for the increase in $d_{3.2}$ at pH 5.

Foaming properties were better when β-lactoglobulin was glycated with glucose or galactose (moderately reactive sugars). These results suggest that the nature of the sugar is an essential factor for improving the functional properties of glycated proteins by processes of Maillard reaction.

4. Change in biological properties

The protein fraction of milk is known to contain many valuable components and biologically active substances, which confer special properties for the support of infant development and growth (Meisel and Schlimme, 1990). Many milk-born bioactivities are latent, requiring proteolytic release of bioactive peptides from inactive native proteins (Schanbacher *et al.*, 1998). Milk protein-derived bioactive peptides include a variety of substances that are potential modulators of various regulatory processes and reveal

multifunctional bioactivities (Meisel, 1998). Opioid agonist (Meisel, 1986), opioid antagonist (Tani *et al.*, 1990), inhibitor of angiotensin converting enzyme (ACE) (Yamamoto, 1997; Pihlanto-Leppälä *et al.*, 1998), immunomodulator (Brantl *et al.*, 1981), antimicrobial (Zucht *et al.*, 1995; Dionysius and Milne, 1997) and antithrombotic (Chabance *et al.*, 1995) activities have been largely described (Meisel, 1997). β-Lactoglobulin is known to contain an ACE-inhibitory sequence (Mullally *et al.*, 1997) but its biological function in milk is still not well known (Sawyer *et al.*, 1998). Some of the biological properties of milk proteins are discussed in Section III.E.

Since many attempts are made to control food storage and to preserve food from oxidation and microorganism contamination, it is interesting to subject protein to oxidoreductive modification in order to see whether new biological properties could be induced. The Maillard reaction is one of the major reactions modifying proteins in food and in nature. The study of how it influences the biological properties of derived proteins and peptides is of particular interest. The consequences of this reaction on the biological properties of the modified products have been largely studied on model systems, which consist of heating a single amino acid with a reducing sugar. The Maillard reaction induced: (1) antioxidative activity of glucose–glycine (Anese *et al.*, 1994), xylose–lysine (Yen and Hsieh, 1995) and xylose–arginine reacting systems (Beckel and Waller, 1983); (2) antimicrobial activity of xylose–arginine and glucose–histidine systems (Einarsson *et al.*, 1983; Einarsson, 1987a,b) and of glucose–glycine system (Stecchini *et al.*, 1993); (3) cytotoxic activity of glucose–lysine and fructose–lysine systems (Jing and Kitts, 2000); (4) clastogenic activity of ribose–lysine (Vagnarelli *et al.*, 1991) and of glucose–lysine systems (Kitts *et al.*, 1993). Proteins modified by the Maillard reaction can also present some of these properties. For example, lysozyme modified with dextran by glycation revealed a significant antimicrobial activity against both Gram-negative and Gram-positive bacteria (Nakamura *et al.*, 1991); glycation of casein with glucose or lactose resulted in enhancement of antioxidant activity when compared with native casein (McGookin and Augustin, 1991).

As shown in Section II.C.3., functional properties (such as thermal stability, emulsifying and foaming properties) of β-lactoglobulin modified by the Maillard reaction were improved, depending on the sugar used during modification. Glycated β-lactoglobulin used as a food ingredient for its functional properties may also change the oxidative conditions and stress and/or can influence cellular and microorganism growth. A recent study was carried out to determine the extent to which this reaction can convey antioxidant, antimicrobial, mutagenic or cytotoxic activities to β-lactoglobulin, and to its tryptic and peptic hydrolysates (Chevalier *et al.*, 2001b).

Antioxidant properties of β-lactoglobulin modified with six different sugars (see Section II.C.1.) were estimated using a radical scavenging activity test. Glycation induced a radical scavenging activity to β-lactoglobulin, whose intensity depended on the sugar used for modification (Figure 8). Proteins modified with ribose and arabinose showed higher radical scavenging activity, depicted by about 80 and 60% of 2,2-diphenyl-1-picrylhydrazyl (DPPH) absorption decrease at 515 nm.

Einarsson *et al.* (1983) and Einarsson (1987a,b) observed an inhibition of bacterial growth by Maillard reaction products prepared with model systems of arginine–glucose, arginine–xylose and histidine–glucose. Antimicrobial activity has never been observed with glycated proteins except in the case of lysozyme modified with dextran by the Maillard reaction (Kitts *et al.*, 1993). Nonmodified lysozyme exhibits an inhibition of growth only for Gram-positive bacteria. Conjugation of dextran to lysozyme resulted in an extension of the antimicrobial spectrum to Gram-negative bacteria. In the study of Chevalier *et al.* (2001b), antimicrobial properties against different bacterial

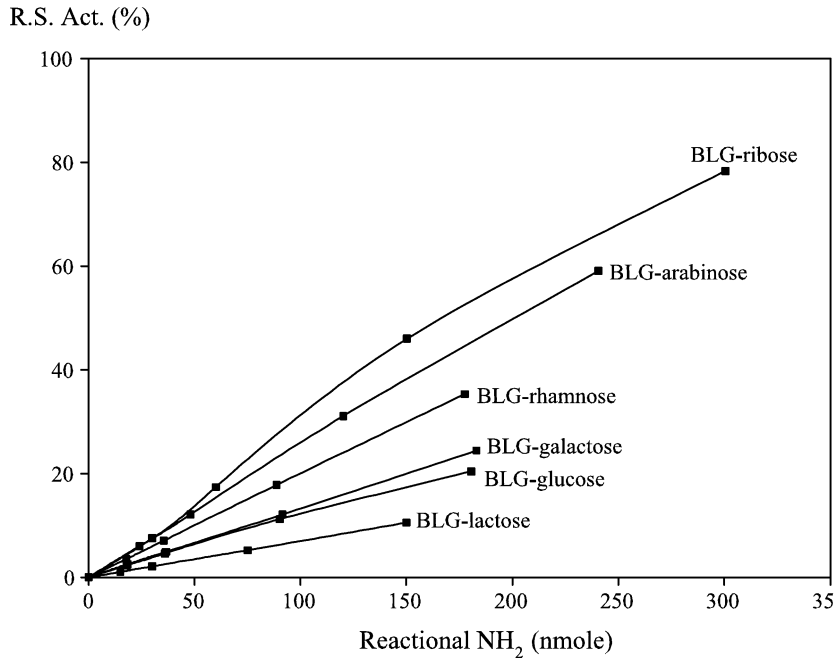


FIG. 8. Radical scavenging activity (R.S. Act.%) of β-lactoglobulin glycated with arabinose, galactose, glucose, lactose, rhamnose or ribose as a function of the number of reactional primary amino groups of β-lactoglobulin. (*Source*: From Chevalier *et al.* (2001b), by courtesy of American Chemical Society.)

strains were studied with a diffusion disk method. No antimicrobial effect of any glycated form of β-lactoglobulin against *E. coli*, *Bacillus subtilis*, *Listeria innocua* and *Streptococcus mutans* was observed. As native β-lactoglobulin did not show any antimicrobial activity, its modification by the Maillard reaction could not induce any extension of the antimicrobial spectrum. Only the appearance of new antimicrobial activity could be expected. Such an activity is certainly more difficult to induce by a nonspecific chemical modification because of the large spectrum of resistance mechanisms in bacteria.

Cytotoxic tests were performed using two cell lines and the MTT [3, (4,5-dimethylthiazoyl-2-yl) 2,5 (diphenyl-tetrazolium bromide] rapid colorimetric assay. Cytotoxicity test with MTT showed no enhancement of cytotoxicity by modified proteins and their peptic and tryptic peptides against COS-7 and HL-60 cells after glycation.

Maillard reactions are known to produce mutagenic, DNA-damaging and cytotoxic substances especially during food processing and cooking (O'Brien and Morrissey, 1989). This certainly concerns AMP. Heterocyclic amines are the most potent mutagens known to be formed during heat processing of food (Friedman, 1996). Such compounds should not be formed (at least in detectable quantity) in the conditions used in the study by Chevalier *et al.* (2001b) because of the mild heat treatment applied (72 h at 60°C). Consequently, in the case of products studied earlier, some innocuousness can be expected.

D. THERMAL MODIFICATIONS OF STRUCTURE AND CO-DENATURATION OF α-LACTALBUMIN AND β-LACTOGLOBULIN

Milk and dairy products are submitted to thermal treatments in order to remove undesirable microorganisms. For example, microbial agents rapidly spoil whey from the cheese industry unless it is treated or used rapidly. Currently, heating is the most commonly used pasteurization/sterilization treatment (heating at 135–150°C during 2 s). The development of new technologies should be able to solve this problem by more selective treatments such as filtration, high pressure, and magnetic or electric fields.

Although less frequently discussed, heat processes often influence the textures and chemistries of the intermediate and end products, and thermal treatments are not without consequences on milk proteins that are denatured. Denaturation of proteins occurs under precise conditions of pH, temperature and ionic strength leading to their unfolding. Denaturation is significantly slower when proteins are near their isoelectric point. Only β-lactoglobulin is irreversibly denatured at pH 7 and 70°C; α-lactalbumin is denatured at pH 6.7 and 65°C. Aggregation of these proteins, besides hydrophobic

interactions, is often stabilized by intra- and intermolecular bonds. This can be due to thiol–disulfide interchange catalyzed by free thiol groups (McKenzie, 1971) and hydrophobic or electrostatic interactions (McSwiney *et al.*, 1994). The capacity and rate by which whey proteins aggregate is controlled by factors such as protein concentration, pH, temperature and presence of other components. According to Aguilera (1995), aggregation can take place in three steps: denaturation, aggregation, and gelification. Aggregation is more rapid near the isoelectric point of proteins. For a review on the effect of thermal treatment on milk protein structure, see Cayot and Lorient (1998).

β-Lactoglobulin is the major whey protein. Hence, its molecular properties may control whey protein aggregation. β-Lactoglobulin resists denaturation at acidic pH but can be easily denatured at alkaline pH (McKenzie and Sawyer, 1967; Waissbluth and Grieger, 1974). It aggregates and is subjected to conformational changes at intermediate pHs. At pH 7.5, a reversible transition (Tanford transition; Tanford *et al.*, 1959) takes place increasing the reactivity of the free thiol group Cys 121. At alkaline pH, denaturation occurs in two steps: unfolding of α-helix and of exposed β-sheet portions followed by unfolding of other β-sheets. Thermal denaturation of β-lactoglobulin occurs in the temperature range 50–90°C with conformation changes similar to those observed during the first step of alkaline denaturation, such as unveiling of the thiol group. β-Lactoglobulin denaturation is reversible as long as its temperature is lower than 65–70°C. At neutral pH, three steps have been described by Roefs and de Kruif (1994): (1) Initiation: reversible reactions, which lead to monomeric β-lactoglobulin; then an irreversible modification of structure unveiling the free thiol group directed towards the exterior of the molecule; (2) Propagation: reactive free thiol group involved in chemical exchange with one of the two disulfide bridges of another nonreactive molecule of β-lactoglobulin. An intermolecular bridge is formed and a new free thiol group becomes reactive on the second molecule and so on; (3) End of reaction: reactive intermediates form a dimer or polymer without reactive free thiol group.

All whey proteins are not able to self-aggregate. α-Lactalbumin, that changes conformation at 64°C, is devoid of free cysteyl residues needed to initiate and propagate the exchange reaction. However, in the presence of proteins with a free thiol group (β-lactoglobulin, bovine serum albumin), α-lactalbumin may take part in aggregation (Gezimati *et al.*, 1997).

Study of heat denaturation of major whey proteins (β-lactoglobulin or α-lactalbumin) either in separated purified forms, or in forms present in fresh industrial whey or in recomposed mixture respecting whey proportions has been recently carried out (Bertrand-Harb *et al.*, 2002), indicating significant differences in their denaturation depending on pH,

temperature of heating, presence or absence of other co-denaturation partner, and existence of a previous thermal pre-treatment (industrial whey). α-Lactalbumin, usually resistant to tryptic hydrolysis, aggregated after heating at 85°C and over. After its denaturation, α-lactalbumin was susceptible to tryptic hydrolysis probably because of exposure of its previously hidden tryptic cleavage sites (Lys–X and Arg–X bonds). Heating over 85°C of β-lactoglobulin increased its aggregation and exposure of its peptic cleavage sites. The co-denaturation of α-lactalbumin with β-lactoglobulin increased their aggregation and resulted in complete exposure of β-lactoglobulin peptic cleavage sites and partial unveiling of α-lactalbumin tryptic cleavage sites. The exposure of α-lactalbumin tryptic cleavage sites was slightly enhanced when the α-lactalbumin/β-lactoglobulin mixture was heated at pH 7.5. Co-denaturation of fresh whey by heating at 95°C and pH 4.5 and above produced aggregates stabilized mostly by covalent disulfide bonds easily reduced by β-mercaptoethanol. The aggregates, stabilized by covalent bonds other than disulfide, arose from a similar thermal treatment but performed at pH 3.5. Thermal treatment of whey at pH 7.5 considerably enhanced the tryptic and peptic hydrolysis of both major proteins.

III. ENZYMATIC PROTEIN PROCESSING

Protein modification, traditionally carried out by direct chemical means, can also be accomplished using enzyme catalysis. Chemical modification is not very desirable for food applications because of the harsh reaction conditions, nonspecific chemical reagents, and the difficulty to remove residual reagents from the final product. Enzymes provide several advantages including fast reaction rates, mild conditions and high specificity. Under optimum conditions, enzyme-catalyzed reactions proceed 10^8–10^{11} times more rapidly than the corresponding nonenzymatic reactions, resulting in reduction of energy costs and increased processing efficiency. Enzymatic methods are consequently more attractive to the food processor. Moreover, most enzymes can be produced in large quantities, each having appropriate physical, chemical and catalytic properties. The cost of enzyme production is reasonable if one uses microbial fermentation or biotechnological processes.

Besides the proteases, which have been investigated extensively and are the only modifying enzymes currently in use commercially, there are transglutaminase, protein kinase, and peptidoglutaminase. These enzymes have only been reported for use in food protein modification on a laboratory scale. Feeney and Whitaker (1977, 1982, 1986) addressed possible

approaches applicable to the modification of proteins and discussed the impact of these potential modifications on the structure and properties of the proteins in great detail.

A. LIMITED PROTEOLYSIS AND FUNCTIONAL PROPERTIES

A large number of papers describes the effect of proteolytic breakdown of food proteins on their functional properties. They comprise animal proteins such as casein (Chobert *et al.*, 1987, 1988a,b; Haque and Mozaffar, 1992; Agboola and Dalgleish, 1996; Slattery and FitzGerald, 1998; Smyth and FitzGerald, 1998), soybean proteins (Puski, 1975; Adler-Nissen and Olsen, 1979; Mohri and Matsushita, 1984; Adler-Nissen, 1986a; Kim *et al.*, 1990b), wheat gluten and gluten fractions (Popineau and Pineau, 1993), among others.

Hydrolysis of peptide bonds causes several changes in proteins: (1) the NH_3^+ and COO^- content of the protein increases, increasing its solubility; (2) the molecular mass of the protein decreases; and (3) the globular structure of the protein is altered, exposing the previously hidden hydrophobic groups.

Proteolytic modification has special importance for the improvement of solubility of proteins. This effect becomes significant even after very limited proteolysis. Hydrolysis of casein to DH of 2 and 6.7% with *Staphylococcus aureus* V8 protease increased the isoelectric solubility to 25 and 50%, respectively (Chobert *et al.*, 1988a). However, it should be noted that the solubility profiles were not identical, due to a shift of the isoelectric point of the modified proteins. Solubility of a protein hydrolysate depends on the enzyme used (Adler-Nissen, 1986a). Protamex™ (a *Bacillus* proteinase complex) hydrolysates of sodium caseinate (DH 9 and 15%) displayed 85–90% solubility between pH 4 and 5 (Slattery and FitzGerald, 1998).

Emulsifying properties of proteins are sensitive to proteolytic modification (Mietsch *et al.*, 1989). Limited hydrolysis (DH 2 and 6.7%) of casein decreased the EA at all pH (Chobert *et al.*, 1987, 1988a), whereas the ES at DH = 2% was higher than that of native casein (Chobert *et al.*, 1987). The EA of casein was reported to decrease with increasing net charge and with decreasing hydrophobicity due to proteolysis (Mahmoud *et al.*, 1992). Since the *S. aureus* V8 protease is highly specific for glutamate residues, and as these residues are uniformly distributed in the sequence of caseins, a poor EA was not expected at these DH values. This could be due to some properties of the obtained peptides, which had the glutamyl residue at the C-terminal end. While using trypsin for hydrolysis of dairy proteins (Chobert *et al.*, 1988b), it was reported that a limited hydrolysis of casein (DH of 4.3, 8.9, and 9.9%) improved its EA, showing the importance of the specificity of the enzyme used. However, ES of such hydrolysates was lower than that of unmodified casein. This could be attributed to the fact that the obtained peptides were not

amphiphilic enough to impart a high stability to the emulsion. Hydrolysis of casein with trypsin, chymotrypsin and Rhozyme-41 resulted in an improvement in EA (Haque and Mozaffar, 1992). Protamex hydrolysates (DH 0.5 and 1.0%) of casein showed higher EA at pH 2 as compared with unmodified casein (Slattery and FitzGerald, 1998). However, at higher DH of 9.0 and 15%, hydrolysates exhibited lower EA compared to caseinate.

Generally, the pH of protein solutions during emulsification affects their emulsifying properties via electrostatic effects. The emulsifying capacity of protein hydrolysates is usually low at the isoelectric point. Addition of salt improves the emulsifying properties at p*I* (Turgeon *et al.*, 1992). Several studies have shown no correlation between emulsifying capacity and high solubility (Chobert *et al.*, 1988a,b; Turgeon *et al.*, 1992).

The effect of limited proteolysis of whey proteins with covalently immobilized trypsin has been investigated. A carefully controlled limited proteolysis yielded a fraction (residues 41–100 with a disulfide bond to residues 149–162) of the core β-barrel of β-lactoglobulin (Chen *et al.*, 1993, 1994; Huang *et al.*, 1994, 1996). The emulsifying properties of these peptides were two to three-fold larger than that of the native protein in the pH range 3–9. Furthermore, the stabilities of emulsions formed with the domain peptide fraction were greater as indicated by the lack of separation of an oil phase after standing at ambient temperature for one week, whereas emulsions formed with the native protein separated (Huang *et al.*, 1996).

Because of the lower structural stability and altered molecular characteristics of the domain peptides, their interactions and thus their gelling properties were significantly different from those of the native protein. Solutions of a limited proteolysate of β-lactoglobulin formed gels at 60°C, whereas the untreated protein required temperatures of 70–80°C for gelation (Chen *et al.*, 1994). The domain peptides, even in the presence of other whey proteins, alter the gelation properties of β-lactoglobulin. Thus, limited proteolysis of whey protein isolate exhibited gelation characteristics that were different from those of untreated whey protein isolate. For example, 10% solutions of enzyme-treated whey protein isolate at pH 7 exhibit a gelation point at 77°C, while untreated whey protein isolate only gelled after holding at 80°C for 1.4 min. The enzyme-treated whey protein isolate solution formed an opaque particulate gel at 80°C whereas the whey protein isolate gel was clear and fine-stranded. Values for hardness, cohesiveness, gumminess and chewiness were significantly greater for enzyme-treated whey protein isolate gels (Huang *et al.*, 1999).

Enzymatic hydrolysis modifies the foaming properties of casein. Protamex hydrolysates of sodium caseinate (DH 0.5 and 1.0%) displayed increased foam expansion at pH 2, 8 and 10 as compared with unhydrolyzed caseinate (Slattery and FitzGerald, 1998). Hydrophobic peptides resulting from

plasmin hydrolysis of β-casein improved foam formation and stabilization at pH 4 (Caessens *et al.*, 1999).

A commercial range of hydrolysates of whey proteins with DH ranging from 8 to 45% was used to make emulsions with soybean oil (Singh and Dalgleish, 1998). As estimated by the particle sizes, the maximum emulsifying capacity was obtained from hydrolysates with a 10 or 20% DH. Higher hydrolysis resulted in peptides that were too short to act as effective emulsifiers, and, at lower proteolysis, the somewhat reduced solubility of the hydrolysates slightly decreased their emulsifying power. All the emulsions were unstable when they were subjected to heat treatment at high temperatures (122°C for 15 min), but emulsions prepared from the less hydrolyzed peptide mixtures were stable to heat treatment at 90°C for 30 min.

One of the applications of enzymes in the preparation of food gels is the production of cheese. During this process, chymosin hydrolyzes a specific bond of κ-casein, resulting in the destabilization of the micelle structure followed by aggregation and formation of an insoluble coagulum.

Enzymatic gelation of partially heat-denatured whey proteins by trypsin, papain, pronase, pepsin, and a preparation of *Streptomyces griseus* has been studied (Sato *et al.*, 1995). Only peptic hydrolysate did not form a gel. The strength of the gel depended on the enzyme used and increased with increasing DH. Hydrolysis of whey protein concentrate with a glutamic acid specific protease from *Bacillus licheniformis* at pH 8 and 8% protein concentration has been shown to produce plastein aggregates (Budtz and Nielsen, 1992). The viscosity of the solution increased dramatically during hydrolysis and reached a maximum at 6% DH. Incubation of sodium caseinate with pepsin or papain resulted in a 55–77% reduction in the apparent viscosity (Hooker *et al.*, 1982).

B. ALLERGENICITY

One of the applications of proteases is to decrease the risk of allergenicity when cow's milk is used as a substitute for human milk. Human and cow's milk differ in their protein composition. β-Lactoglobulin, the major whey protein in cow's milk (9.8% of the total protein content) is absent from human milk. Hydrolyzing the protein can reduce allergenicity. Asselin *et al.* (1989) compared the allergenicity of whey protein hydrolysates produced by pepsin, chymotrypsin, trypsin, pancreatin and combinations of these proteases. DH was not the only important parameter influencing allergenicity. A hydrolysate with 20.5% DH produced with pancreatin had a higher residual content of α-lactalbumin and β-lactoglobulin as compared with a 11.2% DH chymotryptic hydrolysate. Ultrafiltration could be used to reduce the residual antigens in whey protein hydrolysates (Asselin *et al.*, 1989).

Nakamura *et al.* (1993) showed that using a combination of two nonspecific endoproteases and a DH of up to 25%, the antigenicity of whey proteins can be reduced by 1000 times. Antigenicity can be lowered further by including proteases specific for glutamyl, aspartyl, lysyl and arginyl residues, which play important roles in allergen peptides.

The potentiality of a protein to cause an allergic reaction is related to the size of the protein, its primary, secondary and tertiary structures. Antigenicity of casein hydrolysate prepared by using pancreatic proteases was studied by Mahmoud *et al.* (1992).

C. BITTER PEPTIDES

Depending on the nature of the protein and the protease used, progressive proteolysis can liberate bitter peptides from proteins, the bitterness of which is a function of amino acid composition and sequence as well as the peptide chain length (Adler-Nissen, 1986b). An excellent review of the chemistry of bitterness has been published (Roy, 1997) and the reader is directed to this for more details.

Bitterness has long been associated with the formation of hydrophobic peptides (Ney, 1971) and it has been reported (Ney and Rezzlaff, 1986) that peptides having a Q-value (calorific value of the amino acids in a peptide) greater than 1350 kcal/mol are bitter. Bitterness is also related to the DH; bitter peptides contain typically between 3 and 15 amino acids (Pawlett and Bruce, 1996). Treating casein with proteases with differing specificities results in increased bitterness with an increase in the DH. The level of bitterness is dependent on the hydrophobicity of the peptides formed, which in turn, is related to specificity. It is, therefore, possible to influence the level of bitterness by protease selection.

Although hydrolysis can result in improved functional properties and increased digestibility, the formation of bitterness usually has a negative impact on the product acceptability. A logical extension of the use of enzymes for the hydrolysis of food proteins is the use of enzymes for further hydrolysis of the bitter peptides formed. Peptidases are a class of enzymes that cleave single or a pair of amino acids from the ends of peptide chains. Exopeptidases are specific either for the N-terminal (aminopeptidases) or the C-terminal (carboxypeptidases) ends. Key to the use of peptidases in either debittering or bitter prevention restricts the DH. Peptidases have been applied to hydrolysis of α-lactalbumin and casein to produce a nonbitter and highly soluble ingredient for use in nutritional drinks and health-food products.

Among amino acids some L-amino acids are known to be bitter ones. Aromatic amino acids, basic amino acids, and branched-chain amino acids are bitter amino acids. Several bitter amino acids are essential for humans.

Therefore, when an amino acid mixture is administrated orally, the bitterness of these amino acids is crucial. According to Kirimura *et al.* (1969), γ-Glu-Phe is not bitter but sour and astringent. Improvement of the bitter taste of amino acids by γ-glutamylization and an enzymatic method for synthesizing γ-Glu-Phe from Gln and Phe involving bacterial glutamyltranspeptidase have been recently described (Suzuki *et al.*, 2002). Results indicate that γ-glutamylization of Phe can abolish its bitterness and improve the taste. According to several panel members γ-Glu-Phe has a lemon-like refreshing sourness.

Several patent applications pertaining to peptidases have highlighted the growing interest in this type of enzyme. A patent from Gist brocades (1996) demonstrated a process for producing an aminopeptidase preparation by *Aspergillus niger*. Röhm GmbH (1997) and Novo Nordisk (1998) used modern biotechnology to engineer strains of *Aspergillus*, which produce amino- and carboxy-peptidases, respectively. Both of these enzymes were shown to enhance food protein hydrolysis and help prevent the development of bitterness.

D. TRANSGLUTAMINASE AND DAIRY PRODUCTS

In nature, various enzymes are known to cross-link proteins. The physiological function of protein cross-linking is to enhance the strength of the molecular structure of proteins and protein networks. The substrate specificity of cross-linking enzymes is a key aspect in developing protein–protein cross-linking structures. Enzymes like transglutaminase and protein disulfide isomerase have a defined reaction mechanism and are therefore specific, whereas enzymes like peroxidase and lipoxygenase have a more random reaction mechanism resulting in a broad substrate specificity.

Transglutaminase (EC 2.3.2.13) catalyzes an acyl-transfer reaction in which the γ-carboxamide groups of peptide-bound glutaminyl residues are the acyl donors. The enzyme catalyzes *in vitro* cross-linking in whey proteins, soya proteins, wheat proteins, beef myosin, casein and crude actomyosin refined from mechanically deboned poultry meat (Zhu *et al.*, 1995). In recent years, based on the enzyme's reaction to gelatinize various food proteins through the formation of cross-links, this enzyme has been used in attempts to improve the functional properties of foods. Commercial transglutaminase is usually obtained from animal tissues. The complicated separation and purification procedure results in an extremely high price for the enzyme, which hampers a wide application in food processing. Recently, studies on the production of transglutaminase by microorganisms have started. The enzyme obtained from microbial fermentation has been applied in the treatment of food of different origins. Food treated with microbial transglutaminase appeared to have an improved flavor, appearance and texture. In addition,

this enzyme can increase shelf-life and reduce allergenicity of certain foods (Zhu *et al.*, 1995).

α_{s1}-Casein and soybean globulins were polymerized and gelatinized by Ca^{2+}-independent transglutaminase from a variant of *Streptoverticillium mobaraense*, which is totally independent of Ca^{2+}. In this aspect, microbial transglutaminase is quite unique from other mammalian enzymes. Such a property is very useful in the modification of functional properties of food proteins, because many food proteins, such as milk caseins, soybean globulins and myosins, are susceptible to Ca^{2+} (Nonaka *et al.*, 1989). This enzyme polymerized such albumins as bovine serum albumin, human serum albumin and conalbumin in the presence of dithiotreitol. Rabbit myosin was polymerized by the present enzyme but actin was not. An RP-HPLC analysis after enzymatic digestion of the polymerized α_{s1}-casein showed existence of the ϵ-(γ-Glu)Lys bond. Thus, it was confirmed that the polymerization was formed by a catalytic reaction of the transglutaminase.

Several suspensions and emulsions containing commercial sodium caseinate or skim milk were gelatinized by microbial transglutaminase treatment. The characteristics of the gels were largely affected by the enzyme concentrations employed (Nonaka *et al.*, 1992). For caseinate gels, generally the higher enzyme concentration gave steep decreases in breaking strength, strain and cohesiveness of the gels. The creep tests on emulsified gels prepared with two different enzyme concentrations showed that the gel made with a higher enzyme concentration was more viscoelastic. For skim-milk gels, the enzyme caused a substantial increase of the breaking and hardness, while the strain and cohesiveness showed little or no changes.

Microbial transglutaminase is capable of incorporating amino acids or peptides covalently into proteins (Nonaka *et al.*, 1996). This reaction can improve the nutritive values of food or feed proteins, because covalently incorporated amino acids or peptides behave like amino acid residues in a protein.

Many food protein substrates of microbial transglutaminases were gelled upon incubation with it. The characteristics of such a gelation procedure and the gels formed are as follows: proteins that are not gelled by heating can be gelled; gels that normally melt at elevated temperature no longer melt after the microbial transglutaminase gelation; protein in oil-in-water emulsions, even in the presence of sugars and/or sodium chloride, can be gelled; gel firmness increases after heating; the gels can no longer be solubilized by detergents or denaturants. Concerning dairy products, many researchers have shown that milk casein, which has no capability of gel formation even by heating, was a very good substrate for various transglutaminases. It was found that a heat-resistant firm gel was formed from casein in the transglutaminase reaction. An example is the use of microbial transglutaminase in

yoghurt production. Yoghurt is a milk gel formed by acidic fermentation with lactic starter, but it may suffer from problems of serum separation with a change in temperature or physical impacts. Adding microbial transglutaminase can solve this, because microbial transglutaminase improves the water-holding capacity of the gel. The microbial transglutaminase reaction also makes it possible to produce dairy products, such as ice cream and cheese with low-fat contents or a reduced content of nonfat-solids (for a review, see Motoki and Seguro, 1998).

Transglutaminases from different sources exhibit marked differences in substrate specificity. The relatively low substrate specificity of microbial transglutaminase means that this enzyme can cross-link a large variety of proteins and that its application potential is higher than that of transglutaminase from bovine blood plasma and pig erythrocytes. Previous cross-linking of β-casein with microbial transglutaminase increased the gel strength of low pH-gelled β-casein. This increase showed a maximum depending on the incubation time of cross-linking. In addition, cross-linking of β-casein resulted in protein with heat-induced gelling properties, whereas uncross-linked β-casein did not form a gel under these conditions (Wijngaards *et al.*, 2000).

Emulsifying properties of milk proteins were significantly altered after cross-linking with transglutaminase from *Streptoverticillium mobaraense*. With a low degree of cross-linking, the stability of β-lactoglobulin stabilized oil–water emulsions towards strong flocculation or coalescence was reduced after cross-linking (Færgemand *et al.*, 1997). With extensive cross-linking, the stability of milk protein stabilized oil–water emulsions was poorer than that of the native emulsions. The stability towards creaming and the stability in ethanol were generally improved after cross-linking.

Transglutaminase might be used in stabilizing products like yoghurt, fresh cheese, whipping cream and novel milk products. The preparation of cross-linked caseinates as functional ingredients in foods may also be worthwhile (Lorenzen *et al.*, 2000). However, extensive studies are necessary to get a better understanding of cross-linking in dairying. A possible mechanism by which transglutaminase affects heat stability of milk has been recently reported (O'Sullivan *et al.*, 2002). As potential nonfood uses of transglutaminase, the preparation of foils or films, coatings, medical polymers and carriers for immobilizing enzymes should be mentioned (Table IV).

Since application of microbial transglutaminase-catalyzed modification on food proteins is vast, intense attention must be focused on nutritional efficiency of such cross-linked proteins. It has been demonstrated that the glutamine–lysine dipeptide could be metabolized in rats, and that lysine was

TABLE IV
POTENTIAL USE OF TRANSGLUTAMINASE IN PROCESSING OF MILK PROTEINS AND DAIRY PRODUCTS

Caseinates	↗ Gelation, emulsifying properties, viscosity
α_{S1}-Casein	Films, coating, artificial skin, immobilizing enzymes
Whey proteins	Packaging film
Yoghurt, fresh cheese	↗ Gel strength, ↘ syneresis
Ice cream	↗ Water binding, gelation properties
Proteolysates	Covalent incorporation of lysyl peptides

integrated in rat tissues (Iwami and Yasumoto, 1986; Friedman and Finot, 1990). Moreover, rats fed with cross-linked caseins grew normally as compared to rats fed with native caseins (Seguro *et al.*, 1996), suggesting that the cross-linked proteins are cleaved and that the lysine in the moiety is metabolically utilized in the body.

In summary, enzymatic hydrolysis presents numerous possibilities to modify the properties of proteins. Several food-grade enzymes with different specificities are now available. The selection of an enzyme is mainly dictated by its cost, while the cost of an enzyme accounts for only a small percentage of the protein hydrolysate production cost.

E. LIBERATION OF BIOLOGICALLY FUNCTIONAL PEPTIDES

The isolation and characterization of encephalins in 1975 led to the detection in 1979 of the opioid activity of peptides issued from partial proteolysis of dairy proteins (Brantl *et al.*, 1979, 1981, 1982; Henschen *et al.*, 1979; Lottspeich *et al.*, 1980). Peptides that are inactive within the amino acid sequence of a protein may be released by digestive processes *in vivo* and may act as physiological modulators of metabolism. In analogy to the endogenous opioid peptides (endorphins), opioid peptides from food proteins have been called exorphins (Zioudrou *et al.*, 1979; Clare and Swaisgood, 2000). Peptides with antihypertensive, immunomodulating, antithrombotic, and opioid activities have been found in bovine and human milks, and in fermented products and cheese (Smacchi and Gobetti, 2000).

1. Peptides with opioid agonistic and antagonistic activities

Most of the known bioactive peptides derived from milk proteins are opioid peptides (Table V). Those derived from caseins are called casomorphins or casoxins, while those from whey proteins are lactorphins, lactoferroxins or serorphin. Major opioid peptides are fragments of β-casein.

TABLE V
MAIN BIOLOGICAL PROPERTIES OF PEPTIDES ISSUED FROM MILK PROTEINS

No.	Sequence	Fragment	Name	Biological activity			
				Opioid IC50[a]	ACE Inhibition IC50[b]	Immuno-modulatory (% control[c])	Ca^{2+} binding capacity
1	YPFPGPIPNSL	β-Cn (f60-70)	β-Casomorphin-11	10			
2	YPFPGPI	β-Cn (f60-66)	β-Casomorphin-7	14	500	−21/+26	
3	YPFPG	β-Cn (f60-64)	β-Casomorphin-5	1.1	0		
4	RYLGYLE	α_{S1}-Cn (f90-96)	α-Casein exorphin	1.2			
5	RYLGYL	α_{S1}-Cn (f90-95)	α-Casein exorphin	12			
6	YLGYLE	α_{S1}-Cn (f91-96)	α-Casein exorphin	45			
7	YLGF * NH2	α-La (f50-53)	α-Lactorphin	300	733		
8	YLLF * NH2	β-Lg (f102-105)	β-Lactorphin	160	172		
9	YL	β-Lg (f102-103) α_{S1}-Cn (f91-92)			122		
10	LF	β-Lg (f104-105)			349		
11	YGFQNA	SA (f399-404)	Serorphin	85			
12	SRYPSY * OCH3	κ-Cn (f33-38)	Casoxin 6	15			
13	YIPIQYVLSR	κ-Cn (f25-34)	Casoxin C	50			
14	AVPYPQR	β-Cn (f177-183)	β-Casokinin-7		15		
15	FFVAP	α_{S1}-Cn (f23-27)	α-Casokinin-5		6		
16	FPEVFGK	α_{S1}-Cn (f28-34)	α-Casokinin-7		140		
17	TTMPLW	α_{S1}-Cn (f194-199)	α-Casokinin-6		16	+121/+162	
18	YQQPVLGPVR	β-Cn (f193-202)	β-Casokinin-10		300	−28/+14	
19	PGPIPN	β-Cn (f63-68)	Immunopeptide			+122/+139	
20	LLY	β-Cn (f191-193)	Immunopeptide			+148	

TABLE V (*continued*)

MAIN BIOLOGICAL PROPERTIES OF PEPTIDES ISSUED FROM MILK PROTEINS

No.	Sequence	Fragment	Name	Biological activity			
				Opioid IC50[a]	ACE Inhibition IC50[b]	Immuno-modulatory (% control[c])	Ca^{2+} binding capacity
21	YG	α-La(f50-51) α-La(f18-19) κ-Cn (f38-39)		>1000		+101	
22	YGG	α-La (f18-20)				+35	
23	RELEELNVPGEIVES ∗ LS ∗ S ∗ S ∗ EESITR	β-Cn (f1-25)4P	Caseinophospho-peptide				629
24	DIGS ∗ ES ∗ TEDQAMEDIM	α_{S1}-Cn (f43-58)2P	Caseinophospho-peptide				328
25	QMEAES ∗ IS ∗ S ∗ S ∗ EEIVPNS ∗ VEQK	α_{S1}-Cn (f59-79)	Caseinophospho-Peptide				841
26	FKCRRWNRMKKL GAPSITCVRRAF	LF (f17-41)	Lactoferricin	Antimicrobial			
27	MAIPPKKNQDK	κ-Cn (f106-116)	Casoplatelin	Antithrombotic			

S ∗ phosphoseryl; *Source*: adapted from Meisel (1998).

[a]IC_{50} value (μmol/l) given for peptide concentration inhibiting [^{3}H]-naloxone binding, by 50%.

[b]IC_{50} value (μmol/l) given for peptide concentration inhibiting the activity of ACE, by 50%.

[c]Values indicate % stimulation (+) and inhibition (−) in relation to control (= 100).

These β-casomorphins were found in analogous positions in sheep, water buffalo and human β-casein. β-Casomorphins correspond to the fragment 60–70 of the β-casein sequence. These compounds are liberated from casein by enzymatic *in vitro* digestion (Henschen *et al.*, 1979; Teschemacher and Koch, 1990; Meisel and Schlimme, 1996; Meisel, 1998) as well as under *in vivo* conditions. Opioid antagonists have been found in bovine and human κ-casein (casoxins) and in α_{S1}-casein (Meisel and Schlimme, 1996). The opioid antagonist lactoferroxin has been found in human lactoferrin (Meisel, 1998). Various synthetic casoxins were isolated as C-terminally methoxylated peptides (fragments 33–38, 34–38 and 35–38 of κ-casein). They were more active than native fragments. Tryptic fragment 25–34 of bovine κ-casein (casoxin C) showed a relatively high opioid antagonistic activity in comparison to the esterified peptides (Meisel and Schlimme, 1996). Casoxins are opioid receptor ligands of the μ-type with relatively low antagonistic potency as compared with naloxone. Recently, a tryptic peptide with an anxiolytic activity has been isolated from bovine α_{S1}-casein (fragment 91–100, named α-casozepine; Miclo *et al.*, 2001).

2. *ACE-inhibitory activity*

These peptides exert their bioactivity through the inhibition of the ACE. ACE is a multifunctional enzyme (Meisel and Schlimme, 1996) located in different tissues (brain, plasma, lung, kidney, heart, skeletal muscle, pancreas, spleen, placenta, arteries, testes, uterus and brush border; Meisel and Schlimme, 1996), and plays a key physiological role in the regulation of local levels of endogenous bioactive peptides. Exogenous ACE inhibitors having a hypertensive effect *in vivo* were first discovered in snake venom (Ferreira *et al.*, 1970; Kato and Suzuki, 1971). Several food protein sources including fish, maize and milk protein contain sequences that inhibit ACE (Meisel and Schlimme, 1996). Peptides have been found in α_{S1}-, β- and κ-caseins, and in bovine serum albumin (Table V).

ACE-inhibitory peptides are produced during the manufacture of dairy products, e.g., secondary proteolysis during cheese ripening. The ACE-inhibitory activity in cheese was mainly associated with the low molecular mass peptide fraction.

3. *Peptides with immunomodulatory activities*

The bioactivity of immunopeptides has been characterized by different *in vitro* and *in vivo* test systems. Casein-derived immunopeptides including fragments of α_{S1}- and β-casein (Table V) have been shown to stimulate phagocytosis by murine macrophages, and to exert a protective effect against

Klebsiella pneumoniae infection in mice after intravenous treatment (Meisel and Schlimme, 1996). The mechanism by which milk protein derived peptides exert their immunomodulatory effects is not yet defined. However, opioid peptides may affect the immunoreactivity of lymphocytes via the opiate receptor. There is a remarkable relationship between the immune system and opioid peptides, because opioid μ receptors for endorphins are present on T lymphocytes and human phagocytic leukocytes (Meisel, 1998).

4. Peptides with antimicrobial activities

Antimicrobial peptides have been derived from lactoferrin (Meisel, 1998). An antimicrobial peptide fragment was produced *in vitro* upon enzymatic cleavage of lactoferrin with pepsin. The resulting peptide had better bactericidal properties than the undigested lactoferrin, suggesting that its smaller size may facilitate access to target sites on the microbial surface. The antimicrobial activity of lactoferrin seemed to be correlated with the net positive charge on the peptide. An α_{S2}-casein fragment (165–203), named casocidin-I, containing a high proportion of basic amino acids was also found to be an antibacterial agent which can inhibit the growth of *E. coli* and *Staphylococcus carnosus* (Meisel and Schlimme, 1996).

5. Peptides with antithrombotic activities

Casoplatelins are peptides obtained from the caseinomacropeptide (fragment 106–162) of bovine κ-casein (Meisel and Schlimme, 1996). These peptides are inhibitors of the aggregation of platelets as well as of the binding of human fibrinogen γ-chain to a specific receptor site on the platelet surface. The main antithrombotic peptides of κ-casein are the sequences 106–116, 106–112, 112–116 and 113–116. Three amino acid residues (Ile 108, Lys 112, Asp 115) of κ-casein fragment 106–116 are in homologous positions as compared with the γ-chain sequence of human fibrinogen (Meisel and Schlimme, 1996). These residues seem to be important for the inhibitory effect, which is due to the competition between antithrombotic peptides and the γ-chain for the platelet receptors.

6. Peptides with mineral-binding properties

Micelles in bovine milk contain physiologically significant amounts of calcium and phosphorous because of phosphorylated seryl residues in α_{S1}-, α_{S2}- and β-casein. These casein phosphopeptide (CPP) fragments help to create thermodynamically stable casein micelles super-saturated with calcium and phosphate, thus contributing to the stability of milk during

heat processing (Holt, 1994; Holt and Horne, 1996). *In vitro*, cleavage of casein with enzymes such as trypsin and alcalase leads to a number of CPP fragments (Adamson and Reynolds, 1995, 1996). If CPP exerts a function *in vivo*, one may expect that these casein-derived fragments are relatively resistant to proteolytic breakdown in the intestinal tract. Naito and Suzuki (1974) showed that this is indeed the case. In later rat studies, these results were confirmed; furthermore, it was shown that the formation of CPP in the intestine could increase the concentration of soluble calcium (Lee *et al.*, 1980, 1983; Sato *et al.*, 1991; Hirayama *et al.*, 1992). Meisel and Frister (1989) found that CPP fragments were also released in the intestine of mini pigs.

Cell culture and animal and human studies have subsequently addressed the question of whether CPP in the diet improves the absorption of calcium. The animal studies that tried to find positive effects of dietary CPP on intestinal Ca absorption, Ca balance and bone formation showed controversial results (see Steijns, 2001 for a review).

Complexes of Ca, CPP and phosphate have also been shown to reduce caries in a dose-dependent fashion, by increasing the level of calcium phosphate in the plaque, thus influencing the demineralization/remineralization process (Reynolds *et al.*, 1995), and to significantly reduce the adherence of *Streptococci* to tooth enamel (Schuepbach *et al.*, 1996).

Most published research on the anticariogenic properties of milk proteins concentrates on the whole casein, its hydrolysates, isolated fractions from the hydrolysates such as CPP, and some proteose peptone fractions (Scholz-Ahrens and Schrezenmeir, 2000; Grenby *et al.*, 2001; Warner *et al.*, 2001). Whey proteins have also been studied for their anticariogenic properties (Reynolds and Del Rio, 1984; Grobler, 1991; Loimaranta *et al.*, 1999). Therefore, it is possible that whey proteins, as a whole or after hydrolysis, could exhibit some cariostatic effects that might find applications in food and other formulations (Warner *et al.*, 2001). Although whey proteins gave a lower level of protection against caries than CPP, they are readily available and their preparation on a large scale is economically competitive.

IV. GENETIC ENGINEERING OF MILK PROTEINS AND PROTEASES

One of the most important factors that determine whether or not a protein is usable in the fabrication of a food product is its functionality. The functionality results from a combination of physico-chemical properties that define the behavior of the food protein in food systems. It is evident that a detailed understanding of food protein functionality requires an intimate knowledge of the protein structure. The bovine milk protein system has been studied in great detail. Although providing useful structure–function

information on proteins, chemical derivatization often results in polydisperse protein products because of more or less random reactions with protein functional groups of varying reactivity. Thus, specificity of modification is lost, which can hamper interpretation of structure–function information.

Recent developments in recombinant DNA technology can be used to systematically alter single amino acids in the primary sequence of proteins. It is known that single changes in amino acids can markedly alter the functional characteristics of proteins.

In the genetic engineering of enzymes, one probably requires the precision of modification inherent in recombinant DNA techniques to alter the K_m, V_{max}, specificity, etc. of the enzyme. It is now feasible to genetically engineer enzymes to change their thermal inactivation, kinetic characteristics, and specificity to make them more suitable for use in food processing and analysis. Manipulation of the functionality of food proteins for the most part will probably not require such precision and it becomes possible to alter their properties with more generalized changes. This section will discuss the use of some changes in β-lactoglobulin to improve its functional properties and the use of oligodeoxynucleotide directed mutagenesis for specific alterations in the primary structure of trypsin to change its characteristics.

A. THERMOSTABILITY OF β-LACTOGLOBULIN

β-Lactoglobulin serves as an excellent model system because the various requirements to carry out protein engineering have been established, including a high resolution structure and a system for expression of the recombinant protein.

Various preparations of whey proteins have found applications in food formulations for either their nutritional or functional properties. For example, the texture of yoghurt is highly dependent upon the incorporation of β-lactoglobulin in the curd. To achieve this, the milk is heated to 85°C to aggregate β-lactoglobulin onto the casein micelle. On the other hand, the use of whey proteins in beverage formulations is limited where thermal processing is required and a clear, nonturbid solution is desirable. Applications are limited due to its tendency to form aggregates that scatter light upon heating. The free thiol group (see Section II.D) initiates thermal-induced reactions leading to the formation of macromolecular complexes. β-Lactoglobulin cannot form effective gels when reducing conditions are used.

The events leading to the macromolecular associations observed with β-lactoglobulin appear to be mediated by disulfide interactions. The initial solution proposed to reduce thermoinduced aggregation was to remove the Cys 121 and replace it with alanine (Cho *et al.*, 1994). Unfortunately, recombinant protein could not be purified from inclusion bodies. The alternative

for enhancing thermostability was to engage the free thiol in a disulfide bridge, thus introducing a third disulfide linkage into the protein. Due to their close vicinity with Cys 121, Ala 132 and Leu 104 were chosen for replacement with a cysteine. A132C and L104C proteins were created. In contrast to C121A, both of these proteins refolded properly. The conformational stability of the mutant proteins against thermal denaturation was substantially increased (8–10°C) as compared with wild-type β-lactoglobulin. The midpoint of the denaturation profile of A132C β-lactoglobulin is at a guanidine hydrochloride concentration of 4.5 M as compared to 3.6 and 3.4 M for L104C and wild-type β-lactoglobulin, respectively. Neither the A132C nor the L104C polymerized upon heating at 65°C, in contrast to the wild-type protein. Thus, a valuable performance attribute was engineered into the protein by modification of its thermal stability (Cho *et al.*, 1994).

B. GELATION OF β-LACTOGLOBULIN

The curd formed during the manufacture of cheese and other fermented dairy products results in a partial denaturation and aggregation of proteins. Although the curd is primarily composed of caseins, some whey proteins may be entrapped depending on the process and particularly on the temperature to which the milk is heated prior to fermentation. Yoghurt has a significant amount of β-lactoglobulin in its curd due to the high temperature used in the process, which is sufficient to denature this protein. Incorporation of β-lactoglobulin into the curd is desirable for textural and flavor properties of the product.

The design of thermostable variants of β-lactoglobulin coupled with the fact that chemical thiolation could increase the gel strength, suggested a strategy for engineering proteins with enhanced gelation ability (Kim *et al.*, 1990a; Lee *et al.*, 1993). Increasing the free thiol group content might enhance gelation. Two mutations F82C and R40C were engineered and the resulting gel strength measured by a penetrometer that measured the weight necessary for a flat-tipped needle to puncture the gel inside a capillary tube. Wild-type β-lactoglobulin when heated at 90°C for 15 min formed gels with strengths of 14–19 g over a concentration range 9.4–10%, but it did not form a gel below 9%. In contrast, F82C β-lactoglobulin formed a gel at concentrations down to 8% with a gel strength of 23.7 g. Gels of R40C/F82C β-lactoglobulin were formed at concentrations as low as 6.8% and had a gel strength of 16.5 g (Batt, 1997).

The key issue with a number of modifications that have been made to β-lactoglobulin is their performance as food ingredients, especially when added to complex formulations (Lee *et al.*, 1994). As a model system, yoghurt is simple and could be improved by reducing whey syneresis. Since the double

mutant R40C/F28C formed stronger gels at lower temperatures as compared to the wild-type β-lactoglobulin, its functionality as a food additive for yoghurt has been explored. Very small amounts of R40C/F28C β-lactoglobulin reduced the amount of whey formed in yoghurt that was processed at 70°C, a temperature 15°C lower than the standard regimen.

C. DESIGN OF RECOMBINANT ENZYMES USED FOR PROTEIN MODIFICATION

The cost of enzyme purification is a major factor in the expense of most enzyme processes. If a bioreactor is incorporated into the process, then costs associated with bioreactor preparation and regeneration as well as the operational stability of the immobilized enzyme are major factors in the economics of the bioprocess (Swaisgood, 1991).

One of the approaches has been to genetically design fusion proteins with an affinity domain linked to the enzyme. This technology provides for one-step purification as opposed to the multistep processes required for purification of commercial enzymes. Furthermore, immobilization can be achieved at the same time, leading to a minimization of bioreactor preparation costs. Fusion proteins with streptavidin as an affinity domain have been designed (Sano and Cantor, 1991; Walsh and Swaisgood, 1994; Lee and Swaisgood, 1998).

Cross-linking of the polypeptide chains can dramatically alter the functionality of food proteins. This result may be achieved by sulfhydryl–disulfide interchange (see Section II.D.). More extensive cross-linking can be obtained by using transglutaminase to catalyze formation of isopeptide bonds between the γ-carboxamide group of glutaminyl residues and the ε-amino group of lysyl residues (see Section III.D.). An immobilized streptavidin-transglutaminase with a specific activity nearly 50% of that obtained for purified guinea pig liver enzyme has been designed and expressed (Oh *et al.*, 1993; Huang *et al.*, 1995).

The functional behavior of a food protein depends on a number of molecular properties including the chemical characteristics of the surface such as the distribution of the charge and polar and nonpolar residues, and the flexibility of the structure (Huang *et al.*, 1996). These characteristics are modified by limited hydrolysis (see Section III.A.). Size and structure of a polypeptide are important for good functionality. Consequently, DH must be carefully controlled (Huang *et al.*, 1996). Such a control is best achieved by using immobilized enzymes, which in turn avoids the necessity of a downstream enzyme inactivation step that may destroy the structure of the polypeptide, prevents enzyme autolysis, and avoids contamination of the product

with the proteinase (Swaisgood, 1991). An effort to develop enzyme purification and immobilization technology has been done, and the genetic construction and expression of a trypsin–streptavidin has been investigated (Higaki *et al.*, 1989).

One of the major goals in protease engineering is to understand how structure determines function. Site-directed mutagenesis can provide a powerful approach to design enzymes with novel specificities (Perona and Craik, 1995). The aspartyl 189 residue of trypsin (S_1) is crucial for substrate orientation and specific lysis of its Arg–X and Lys–X bonds (P_1). Sequence alignments of other seryl proteases reveal that the vicinal amino acid residue 188 (S_2) is highly conserved and can be either a lysine or an arginine (Perona and Craik, 1995). Consequently, the amino acid in position 188 plays an important role differing, however, from outright definition of protease specificity determination. K188 was replaced with aromatic amino acids (Chobert *et al.*, 1998a) or histidine (Briand *et al.*, 1997) in order to change the interactions of the substrate-binding site and, hence, modulate catalytic properties of this protease. In other series of experiment the highly conserved G187, K188 and D189 were replaced with aromatic amino acid residues (Chobert *et al.*, 1998b) in order to perturb the electrostatics and to amplify hydrophobic interactions of the substrate-binding site.

Caseins were and are widely used as industrial and food substrates because they are inexpensive and easily obtained in highly purified forms. β-Casein is an abundant and well-characterized milk protein with a pronounced amphiphilic character and containing a large number of prolyl residues (1 proline per about 6 amino acid residues). The peptides arising from fragmentation of β-casein have interesting physiological and biological activities (see Section III.E.). All these reasons mean that β-casein may constitute a model of choice for relatively sophisticated hydrolysis studies.

The trypsin mutants K188F, K188Y and K188W (Chobert *et al.*, 1998a) were characterized first by analyzing the kinetic parameters k_{cat}, K_m, and k_{cat}/K_m for comparable artificial tetrapeptide substrates containing arginine (Suc-Ala-Ala-Pro-Arg-*pNA*) and lysine (Suc-Ala-Ala-Pro-Lys-*pNA*) (Table VI). Compared with trypsin, mutants K188F, K188Y and K188W exhibited a 1.6- to 3-fold increase of K_m for arginine and lysine containing substrates, respectively. These mutants had k_{cat} similar to that observed with wild-type except for a 3- to 4-fold decrease for K188W mutant. While the second-order rate constant k_{cat}/K_m of K188W trypsin mutant decreased $\approx$7- to 12-fold for substrates containing arginine and lysine, respectively, 2-fold decrease was observed for K188F and K188Y trypsin mutants. Wild-type and K188Y trypsins showed 6-fold arginine/lysine preference whereas K188F and K188W trypsins preferred the arginine over the substrate containing lysine by a factor of 8 and 10, respectively.

TABLE VI
KINETIC PARAMETERS OF WILD-TYPE AND MUTANT TRYPSINS MEASURED AT pH 8

Enzyme	Substrate[a]	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} μM^{-1})
Wild-type	Arg	32.8 ± 7.1	163 ± 31	4.97 ± 1.21
K188F		55.4 ± 1.3	144 ± 15	2.63 ± 0.31
K188Y		53.4 ± 2.2	114 ± 12	2.14 ± 0.33
K188W		98.0 ± 8.1	58 ± 3	0.59 ± 0.01
WFY[b]		47.0 ± 7.0	4.9 ± 0.4	0.11 ± 0.001
Wild-type	Lys	105 ± 15	74 ± 15	0.71 ± 0.21
K188F		188 ± 20	61 ± 12	0.32 ± 0.07
K188Y		107 ± 10	43 ± 4	0.41 ± 0.03
K188W		292 ± 25	17 ± 4	0.059 ± 0.013
WFY[b]		123 ± 0.3	1.7 ± 0.1	0.013 ± 0.0005

[a]Substrates: Suc-Ala-Ala-Pro-Arg-*pNA* and Suc-Ala-Ala-Pro-Lys-*pNA*.
[b]WFY: G187W/K188F/D189Y.

The same lysyl residue 188 was replaced with histidine in order to build a metal chelation site in the substrate-binding pocket of trypsin (Briand *et al.*, 1997). K188H mutation did not affect catalytic efficiency at all. In the presence of Cu^{2+}, trypsin K188H exhibited a 30- to 100-fold increase of K_m, while k_{cat} was only slightly decreased (Table VII). Hydrolytic activity of this mutant could be fully restored by addition of EDTA. Thus, in contrast to the chelation of the active site, a different mode of metal-dependent inhibition of the activity of trypsin by building a co-ordination site in the substrate-binding pocket of the protease was achieved.

In other series of experiment, the highly conserved G187, K188 and D189 were replaced with aromatic amino acid residues in order to perturb the electrostatic interactions and to amplify hydrophobic interactions of the substrate-binding site (Chobert *et al.*, 1998b). The mutant G187W/K188F/D189Y exhibited 1.3-fold increase in K_m values for tetrapeptide synthetic substrates. This mutant shows a 30- to 40-fold decrease of its k_{cat} and its second-order rate constant k_{cat}/K_m decreased ≈40- and 55-fold for substrates containing arginine and lysine, respectively (Table VI).

Synthetic substrates allow rapid determination of the catalytic constants of an enzyme. Nevertheless, it is known that the environment of the peptide bond depends largely on physico-chemical conditions of the applied media, and imposed steric hindrance. Since these parameters are important, the hydrolysis of purified β-casein was studied at different pHs. The kinetic analysis revealed that the mutant conserved the native trypsin capacity to hydrolyze peptide bonds containing arginyl and lysyl residues. The optimal pH of activity changed considerably according to the mutation.

TABLE VII
KINETIC PARAMETERS OF WILD-TYPE AND K188H TRYPSINS MEASURED AT pH 8, WITH 0, 5 OR 200 μM Cu^{2+}

$[Cu^{2+}]$ (μM)	Substrate[a]	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} μM^{-1})
Wild-type	Arg			
0		41 ± 1	132 ± 5	3.22 ± 0.17
5		49 ± 2	109 ± 16	2.22 ± 0.43
200		42 ± 1	79 ± 5	1.88 ± 0.17
K188H				
0		30 ± 2	169 ± 12	5.63 ± 0.84
5		118 ± 3	150 ± 11	1.27 ± 0.32
200		~2200	~131 ± 10	0.06 ± 0.13
Wild-type	Lys			
0		75 ± 7	39 ± 2	0.52 ± 0.08
5		102 ± 4	34 ± 2	0.33 ± 0.03
200		104 ± 3	26 ± 5	0.25 ± 0.06
K188H				
0		74 ± 1	48 ± 5	0.65 ± 0.05
5		409 ± 42	54 ± 3	0.13 ± 0.02
200		~7500	~54 ± 5	0.007 ± 0.001

[a]Substrates: Suc-Ala-Ala-Pro-Arg-*pNA* and Suc-Ala-Ala-Pro-Lys-*pNA*.

Surprisingly, as demonstrated only by proteolysis of a natural substrate (β-casein), all aromatic mutants and the triple mutant G187W/K188F/D189Y acquired the capacity to hydrolyze β-casein at C-termini of amidated amino acids (Q and N) (Figure 9).

The use of β-casein as a test substrate presents, besides the importance of this protein in the food industry, advantages of releasing the hydrolysis from several structural limitations characteristic of many other potential native protein substrates. The use of this protein enabled a better understanding of the scope and validity of the results obtained with synthetic substrates. Additionally, the harnessing of mutated trypsins into the processing of β-casein diversified the peptide products obtained. Most of the observed new cleavage sites were located in the hydrophobic portion of the protein.

Modifications introduced by the mutations were central to the alteration of the specificities of the enzymes studied, which were capable of cleaving β-casein at many new sites, for example, hydrolyzing the fragment Arg1-Lys105, reported to be a trypsin inhibitor (Bouhallab *et al.*, 1997). Since many tryptic inhibitors contain amidated Glu and Asp, and form amyloid structures, the mutants of this type could be used for the hydrolysis of the lytically resistant protein structures.

FIG. 9 Cleavage sites obtained from 48 h of hydrolysis of β-casein with K188F (***F***), K188Y (***Y***), K188W (***W***) and G187W/K188F/D189Y (↘) at pH 8. β-Casein (1 mg/ml, initial concentration) was dissolved in 0.2 M Tris-HCl. Active enzyme was added to β-casein solution at an enzyme/substrate ratio of 0.01, 0.02, 0.02 and 1% for K188F, K188Y, K188W and G187W/K188F/D189Y (w/w), respectively. *Source*: From Haertlé and Chobert (1999), by courtesy of Food & Nutrition Press, Inc.

V. CONCLUSION

Proteins are fundamentally as important as nutrients but now they are considered to be much more. Through their unique functional properties, they can become key ingredients that determine many parameters of food quality. However, most proteins still have scope for further improvement in their physical and functional properties. The application of several simple physico-chemical and hydrolytic treatments to caseins and whey proteins can change significantly their functional properties. The simplest approach involves the modification of protein/peptide surface polarity. This may be achieved either by changing the ionization of polar amino acid side-chains or by blocking ε-amino groups by glycation or substitution of carboxylates by esterification. Esterified dairy proteins or any abundant food proteins can become almost unlimited substrates for the production of diversified and amplified peptide libraries. Relatively mild phosphorylation of dairy proteins with $POCl_3$, in the presence of natural nucleophiles such as basic amino acids, alters the net charge of substituted proteins but also grafts significant amounts of arginine and lysine through phosphoamide bonds. Simple glycations of β-lactoglobulin with reducing sugars under mild conditions (pH 6, 60°C) can substitute up to 70% of ε-amino groups and significantly improve the solubility of transformed proteins. As us known for over 90 years due to the pioneering work of L. C. Maillard, these processes also operate during food storage and aging. It is clear that they may be either beneficial to food protein functionality or harmful to their perception as food. The manipulation of disulfide bridges of abundant whey globulins such as α-lactalbumin and β-lactoglobulin through astute use of baric treatment for conformational destabilization and of appropriate redox conditions scrambles disulfide bridges creating intermolecular arrays of disulfide connections. By varying applied pressure, temperature, pH, concentrations and initiating reducer sulfide, different viscosities or gel types of the protein solutions can be obtained. Alternatively, the blocking of free cysteine in β-lactoglobulin increases its stability and eliminates the capacity to promote the aggregation process described before. The surfactant properties of proteins have been given a special attention since they determine the physical and functional properties of proteins in many food and biological systems. The sophisticated methodology available to improve the surface properties of proteins emphasizes the potential of chemical (and enzymatic) modifications of their functionality.

Enzymes have long been used for modifying food proteins and their use is more acceptable in the eye of the nonscientist than is the use of chemicals. The use of modified enzymes for processing foods is developing rapidly. In each case, the tailored proteins are used for processing purposes and not as

part of the food, *per se*, such as immobilized enzyme. The use of enzymes for preparing peptides for research purposes has been employed for the formation of opioids from food proteins such as caseins. As stated by Steijns (2001), before commencing the development of nutraceuticals from milk or whey, a number of technological and marketing issues should be addressed. The question of the cost of the process required to manufacture the desired protein or peptide should be dealt with at a very early stage, together with how to productively utilize the nonbioactive residual raw material. Food safety is also an important feature. Cost effectiveness of the ingredient and ease of incorporation into a good tasting end product are vital. Claiming a message that can be understood by consumers or is allowed by legal authorities is a further pre-requisite for successful market introduction. According to the present state of knowledge, caseinophosphopeptides and ACE-inhibitory peptides are the favorite bioactive peptides for diet supplementation and application to foodstuffs formulated to provide specific health benefits.

We have seen how oligodeoxynucleotide site-directed mutagenesis can be used to engineer the primary sequences of proteins, and examples were given of how this powerful technique can be used to engineer enzymes and their protein substrates.

The modification of the contact surfaces between substrate protein and protease triggers important variations in the hydrolytic processing of dairy proteins. This is achieved either by modifying the conformation of dairy globulins by mild physico-chemical treatments or by well-conceived site-directed mutagenesis of the S2 proteinase substrate-binding site. Finally, microbial transglutaminase, as well as other transglutaminases, can catalyze formation of the ε-(γ-glutamyl) lysine bond in many food proteins; the resulting cross-link drastically alters protein functionalities. Applications are emerging in the development of novel foods and nonfood processing methods. There may be many applications in the incorporation of various amines, amino acids, lysine-containing peptides, glutamine-containing peptides and heterologous polypeptides. There is no doubt that microbial transglutaminase technology will be an essential tool for protein modification in both food processing and nonfood processing in the future.

Additional research is necessary before successful genetic manipulation of plants and animals to tailor-make more functional proteins becomes a reality.

In summary, intact milk proteins have their specific functions such as casein micelle formation and regulation of lactose synthesis. Milk proteins exhibit various biological activities when they are partially digested as described before. They are finally a source of essential amino acids. This means milk proteins are highly functional substances.

ACKNOWLEDGEMENTS

Professor David Julian McClements (Department of Food Science, University of Massachusetts, Amherst, MA, USA) is gratefully acknowledged for helpful discussion during the preparation of the manuscript and for kindly revising the text.

REFERENCES

Adamson, N.J. and Reynolds, E.C. 1995. Characterization of tryptic casein phosphopeptides prepared under industrially relevant conditions. *Biotechnol. Bioengng.* **45**, 196–204.

Adamson, N.J. and Reynolds, E.C. 1996. Characterization of casein phosphopeptides prepared using alcalase: determination of enzyme specificity. *Enzyme Microb. Technol.* **19**, 202–207.

Adler-Nissen, J. 1986a. Enzymatic Hydrolysis of Food Proteins. Elsevier, London.

Adler-Nissen, J. 1986b. Relationship of structure to taste of peptides and peptide mixtures *In* "Protein Tailoring for Food and Medical Uses" (R.E. Feeney and J.R. Whitaker eds), pp. 97–122. Marcel Dekker, New York.

Adler-Nissen, J. and Olsen, H.S. 1979. The influence of peptide chain length on taste and functional properties of enzymatically modified soy protein *In* "Functionality and Protein Structure", Advances in Chemical Series 92 (A. Pour-El ed.), pp. 125–146. American Chemical Society, Washington, DC.

Agboola, S.O. and Dalgleish, D.G. 1996. Enzymatic hydrolysis of milk proteins used for emulsion formation. 1. Kinetics of protein breakdown and storage stability of the emulsions. *J. Agric. Food Chem.* **44**, 3631–3636.

Aguilera, J.M. 1995. Gelation of whey proteins. *Food Technol.* **49**(10), 83–89.

Al-Abed, Y., Mitsuhashi, T., and Li, H. 1999. Inhibition of advance glycation end product formation by acetaldehyde: role in the cardioprotective effect of ethanol. *Proc. Natl Acad. Sci. USA* **96**, 2385–2390.

Alcami, A. and Koszinowski, U.H. 2000. Viral mechanisms of immune evasion. *Trends Microbiol.* **8**, 410–418.

Aluko, R.E. and Mine, Y. 1999. Characterization of oil-in-water emulsions stabilized by hen's egg yolk granule. *Food Hydrocoll.* **12**, 203–210.

Ames, J.M. 1990. Control of the Maillard reaction in food systems. *Trends Food Sci. Technol.* **Dec**, 150–154.

Anese, M., Nicoli, M.C., and Lerici, C.R. 1994. Influence of pH on the oxygen scavenging properties of heat-treated glucose-glycine systems. *Ital. J. Food Sci.* **3**, 339–343.

Aoki, T., Kitahata, K., Fukumoto, T., Sugimoto, Y., Ibrahim, H.R., Kimura, T., Kato, Y., and Matsuda, T. 1997. Improvement of functional properties of β-lactoglobulin by conjugation with glucose-6-phosphate through the Maillard reaction. *Food Res. Int.* **30**, 401–406.

Asselin, J., Herbert, J., and Amiot, J. 1989. Effects of in vitro proteolysis on the allergenicity of major whey proteins. *J. Food Sci.* **54**, 1037–1039.

Baker, E.N., Anderson, B.F., Baker, H.M., Day, C.L., Haridas, M., Norris, G.E., Rumball, S.V., Smith, C.A., and Thomas, D.H. 1994. Three-dimensional structure of lactoferrin in various functional states. *Adv. Exp. Med. Biol.* **357**, 1–12.

Batt, C.A. 1997. Genetic engineering of food proteins *In* "Food Proteins and Their Applications" (S. Damodaran and A. Paraf eds), pp. 425–441. Marcel Dekker, New York.

Beckel, R.W. and Waller, G.R. 1983. Antioxidative arginine-xylose Maillard reaction products: conditions of synthesis. *J. Food Sci.* **48**, 996–997.

Benjamin, J., de Feijter, J.A., Evans, M.T.A., Graham, D.E., and Phillips, M.C. 1975. Dynamic and static properties of proteins adsorbed at the air-water interface. *Faraday Discuss. Chem. Soc.* **59**, 218–229.

Bertrand-Harb, C., Charrier, B., Dalgalarrondo, M., Chobert, J.-M., and Haertlé, T. 1990. Condensation of glycosidic and aromatic structures on amino groups of β-lactoglobulin B via reductive alkylation. Solubility and emulsifying properties of the protein derivatives. *Lait* **71**, 205–215.

Bertrand-Harb, C., Chobert, J.-M., Dufour, E., and Haertlé, T. 1991. Esterification of food proteins: characterisation of the derivatives by a colorimetric method and by electrophoresis. *Sci. Aliments* **11**, 641–652.

Bertrand-Harb, C., Baday, A., Dalgalarrondo, M., Chobert, J.-M., and Haertlé, T. 2002. Thermal modifications of structure and co-denaturation of α-lactalbumin and β-lactoglobulin induce changes of solubility and susceptibility to proteases. *Nahrung* **46**, 283–289.

Bouhallab, S., Sapin, B., Mollé, D., Henry, G., and Léonil, J. 1997. Inhibition of bovine trypsin by the phosphorylated N-terminal part β(1-105) of β-casein. *J. Peptide Res.* **49**, 23–27.

Bouhallab, S., Morgan, F., Henry, G., Mollé, D., and Léonil, J. 1999. Formation of stable covalent dimer explains the high solubility at pH 4.6 of lactose-β-lactoglobulin conjugates heated near neutral pH. *J. Agric. Food Chem.* **47**, 1489–1494.

Brantl, V., Teschemacher, H., Henschen, A., and Lottspeich, F. 1979. Novel opioid peptides derived from casein (β-casomorphins): I. Isolation from bovine casein peptone. *Hoppe-Seyler's Z. Physiol. Chem.* **360**, 1211–1216.

Brantl, V., Teschemacher, H., Blasig, J., Henschen, A., and Lottspeich, F. 1981. Opioid activities of beta-casomorphins. *Life Sci.* **28**, 1903–1909.

Brantl, V., Pfeiffer, A., Herz, A., Henschen, A., and Lottspeich, F. 1982. Antinociceptive potencies of β-casomorphin analogs as compared to their affinities towards μ and δ opiate receptor sites in brain and periphery. *Peptides* **3**, 793–797.

Briand, L., Chobert, J.-M., and Haertlé, T. 1994. Tryptic hydrolysis of esters of β-casein and β-lactoglobulin. *Milchwissenschaft* **49**, 367–371.

Briand, L., Chobert, J.-M., and Haertlé, T. 1995. Peptic hydrolysis of esterified β-casein and β-lactoglobulin. *Int. J. Pept. Protein Res.* **46**, 30–36.

Briand, L., Chobert, J.-M., Tauzin, J., Declerck, N., Léonil, J., Mollé, D., Tran, V., and Haertlé, T. 1997. Regulation of trypsin activity by Cu^{2+} chelation of the substrate binding site. *Protein Engng* **10**, 551–560.

Budtz, P. and Nielsen, P.M. 1992. Protein preparations. Int. Patent Application W092/13964.

Caessens, P.W.J.R., Visser, S., Gruppen, H., van Aken, G.A., and Voragen, A.G.J. 1999. Emulsion and foam properties of plasmin derived β-casein peptides. *Int. Dairy J.* **9**, 347–351.

Cayot, P. and Lorient, D. 1998. Structures et technofonctions des protéines du lait (Structure and functionality of milk proteins). Arilait Recherches, Tec & Doc—Lavoisier, Paris, (In French).

Chabance, B., Jollès, P., Izquierdo, C., Mazoyer, E., Francoual, C., Drouet, L., and Fiat, A.-M. 1995. Characterization of an antithrombotic peptide from kappa-casein in newborn plasma after milk ingestion. *Br. J. Nutr.* **73**, 583–590.

Cheftel, J.C. and Lorient, D. 1982. Aspects Technologiques. Les propriétés fonctionnelles des protéines laitières et leur amélioration. *Lait* **62**, 435–483.

Chen, S.X., Hardin, C.C., and Swaisgood, H.E. 1993. Purification and characterization of β-structural domains of β-lactoglobulin liberated by limited proteolysis. *J. Protein Chem.* **12**, 613–625.

Chen, S.X., Swaisgood, H.E., and Foegeding, E.A. 1994. Gelation of β-lactoglobulin treated with limited proteolysis by immobilized trypsin. *J. Agric. Food Chem.* **42**, 234–239.

Chevalier, F., Chobert, J.-M., Dalgalarrondo, M., and Haertlé, T. 2001a. Characterization of the Maillard reactions products of β-lactoglobulin glucosylated in mild conditions. *J. Food Biochem.* **25**, 33–55.

Chevalier, F., Chobert, J.-M., Genot, C., and Haertlé, T. 2001b. Scavenging of free radicals, antimicrobial, and cytotoxic activities of the Maillard reaction products of β-lactoglobulin glycated with several sugars. *J. Agric. Food Chem.* **49**, 5031–5038.

Chevalier, F., Chobert, J.-M., Mollé, D., and Haertlé, T. 2001c. Maillard glycation of β-lactoglobulin with several sugars: comparative study of the properties of the obtained polymers and of the substituted sites. *Lait* **81**, 651–666.

Chevalier, F., Chobert, J.-M., Popineau, Y., Nicolas, M.-G., and Haertlé, T. 2001d. Improvement of the functional properties of β-lactoglobulin glycated through the Maillard reaction is related to the sugar nature. *Int. Dairy J.* **11**, 145–152.

Chevalier, F., Chobert, J.-M., Dalgalarrondo, M., Choiset, Y., and Haertlé, T. 2002. Maillard glycation of β-lactoglobulin induces conformation changes. *Nahrung* **46**, 58–63.

Cho, Y., Gu, W., Watkins, S., Lee, S.P., Brady, J.W., and Batt, C.A. 1994. Thermostable variants of bovine β-lactoglobulin. *Protein Engng* **7**, 263–270.

Chobert, J.-M., Sitohy, M.Z., and Whitaker, J.R. 1987. Specific limited hydrolysis and phosphorylation of food proteins for improvement of functional and nutritional properties. *J. Am. Oil Chem. Soc.* **64**, 1704–1711.

Chobert, J.-M., Sitohy, M.Z., and Whitaker, J.R. 1988a. Solubility and emulsifying properties of caseins modified enzymatically by *Staphylococcus aureus* V8 protease. *J. Agric. Food Chem.* **36**, 220–224.

Chobert, J.-M., Bertrand-Harb, C., and Nicolas, M.-G. 1988b. Solubility and emulsifying properties of caseins and whey proteins modified enzymatically by trypsin. *J. Agric. Food Chem.* **36**, 883–892.

Chobert, J.-M., Sitohy, M., and Whitaker, J.R. 1989. Covalent attachment of phosphate and amino acids to zein. *Sci. Aliments* **9**, 749–761.

Chobert, J.-M., Touati, A., Bertrand-Harb, C., Dalgalarrondo, M., Nicolas, M.-G., and Haertlé, T. 1990. Ethyl substituted β-casein. Study of differences between esterified and reductively alkylated β-casein derivatives. *J. Agric. Food Chem.* **38**, 1321–1326.

Chobert, J.-M., Briand, L., Grinberg, V., and Haertlé, T. 1995. Impact of esterification on the folding and susceptibility to peptic proteolysis of β-lactoglobulin. *Biochim. Biophys. Acta* **1248**, 170–176.

Chobert, J.-M., Briand, L., Tran, V., and Haertlé, T. 1998a. How the substitution of K188 of trypsin binding site by aromatic amino acids can influence the processing of β-casein. *Biochem. Biophys. Res. Commun.* **246**, 847–858.

Chobert, J.-M., Briand, L., and Haertlé, T. 1998b. Influence of G187W/K188F/D189Y mutation in the substrate-binding pocket of trypsin on β-casein processing. *J. Food Biochem.* **22**, 529–545.

Chuyen, N.V. 1998. Maillard reaction and food processing. Application aspects. *Adv. Exp. Med. Biol.* **434**, 213–235.

Clare, D.A. and Swaisgood, H.E. 2000. Bioactive milk peptides: a prospectus. *J. Dairy Sci.* **83**, 1187–1195.

Corredig, M. and Dalgleish, D.G. 1995. A differential microcalorimetric study of whey proteins and their behavior in oil water emulsions. *Colloids Surface* **4**, 411–422.

Dalgalarrondo, M., Dufour, E., Chobert, J.-M., Bertrand-Harb, C., and Haertlé, T. 1995. Proteolysis of β-lactoglobulin and β-casein by pepsin in ethanolic media. *Int. Dairy J.* **5**, 1–14.

Das, K.P. and Kinsella, J.E. 1990. Stability of food emulsions: physicochemical role of protein and non-protein emulsions. *Adv. Food Nutr. Res.* **34**, 81–201.

De Man, J.M. (ed.) 1999. *In* "Principles of Food Chemistry", 3rd . Aspen Publishers, Gaithersburgh, Maryland, USA.

Dickinson, E. and Matsumura, Y. 1991. Time dependent polymerization of β-lactoglobulin through disulphide bonds at oil water interface in emulsions. *J. Biol. Macromol.* **13**, 26–31.

Dionysius, D.A. and Milne, J.M. 1997. Antibacterial peptides of bovine lactoferrin: purification and characterization. *J. Dairy Sci.* **80**, 667–674.

Einarsson, H. 1987a. The effect of time, temperature, pH and reactants on the formation of antibacterial compound in the Maillard reaction. *Lebensm.-Wiss. Technol.* **20**, 51–55.

Einarsson, H. 1987b. The effect of pH and temperature on the antibacterial effect of Maillard reaction products. *Lebensm.-Wiss. Technol.* **20**, 56–58.

Einarsson, H., Snygg, B.G., and Eriksson, C. 1983. Inhibition of bacterial growth by Maillard reaction products. *J. Agric. Food Chem.* **31**, 1043–1047.

Ellis, G.P. 1959. The Maillard Reaction. *Adv. Carbohydr. Chem.* **14**, 63–134.

Færgemand, M., Murray, B.S., and Dickinson, E. 1997. Cross-linking of milk proteins with transglutaminase at the oil-water interface. *J. Agric. Food Chem.* **45**, 2514–2519.

Feeney, R.E., Whitaker, J.R. (eds) 1977. *In* "Food Proteins: Improvement Through Chemical and Enzymatic Modification", Advances in Chemistry Series 160 . American Chemical Society, Washington, DC.

Feeney, R.E., Whitaker, J.R. (eds) 1982. *In* "Modification of Proteins: Food, Nutritional, and Pharmacological Aspects", Advances in Chemistry Series 198 . American Chemical Society, Washington, DC.

Feeney, R.E., Whitaker, J.R. (eds) 1986. *In* "Protein Tailoring for Food and Medical Uses" Marcel Dekker, New York.

Ferreira, S.H., Bartelt, C., and Greene, L.J. 1970. Isolation of bradikinin potentiating peptides from *Bothrops jararaca* venom. *Biochemistry* **9**, 2583–2593.

Fogliano, V., Monti, S.M., Visconti, A., and Ritieni, A. 1998. Identification of a β-lactoglobulin lactosylation site. *Biochim. Biophys. Acta* **1388**, 295–304.

Fraenkel-Conrat, H. and Olcott, H.S. 1945. Esterification of proteins with alcohols of low molecular weights. *J. Biol. Chem.* **161**, 259–268.

Friedman, M. 1996. Food browning and its prevention: an overview. *J. Agric. Food Chem.* **44**, 631–653.

Friedman, M. and Finot, R.A. 1990. Nutritional improvement of bread with lysine and γ-glutamyllysine. *J. Agric. Food Chem.* **38**, 2011–2020.

Fujihara, T. and Hayashi, K. 1995. Lactoferrin inhibits herpes simplex virus type-1 (HSV-1) infection to mouse cornea. *Arch. Virol.* **140**, 1469–1472.

Gezimati, J., Creamer, L.K., and Singh, H. 1997. Heat-induced interactions and gelation of mixtures of β-lactoglobulin and α-lactalbumin. *J. Agric. Food Chem.* **45**, 1130–1136.

Gist brocades (1996). Production of aminopeptidases from *Aspergillus niger*. Patent WO 96/38549.

Godovac-Zimmermann, J., Conti, A., Sheil, M., and Napolitano, L. 1990. Covalent structure of the minor monomeric β-lactoglobulin II component from donkey milk. *Biol. Chem. Hoppe-Seyler* **371**, 871–879.

Godovac-Zimmermann, J., Krause, I., Buchberger, J., Weiss, G., and Klostermeyer, H. 1990. A novel wild-type β-lactoglobulin W and its primary sequence. *Biol. Chem. Hoppe-Seyler* **371**, 255–260.

Grenby, T.H., Andrews, A.T., Mistry, M., and Williams, R.J.H. 2001. Dental caries—protective agents in milk and milk products: investigation in vitro. *J. Dentistry* **29**, 83–92.

Grobler, S.R. 1991. The effect of high consumption of citrus fruit and a mixture of other fruits on dental caries in man. *Clin. Prevent. Dent.* **13**, 13–17.

Haertlé, T. and Chobert, J.-M. 1999. Recent progress in processing of dairy proteins: a review. *J. Food Biochem.* **23**, 367–407.

Halpin, M.I. and Richardson, T. 1985. Elected functionality changes of β-lactoglobulin upon esterification of side chain carboxyl groups. *J. Dairy Sci.* **68**, 3189–3198.

Haque, Z.U. and Mozaffar, Z. 1992. Casein hydrolysate. II. Functional properties of peptides. *Food Hydrocoll.* **5**, 559–571.

Harmsen, M.C., Swart, P.J., de Béthune, M.P., Pawels, R., de Clercq, E., The, T.H., and Meijer, D.K.F. 1995. Antiviral effects of plasma and milk proteins: lactoferrin shows a potent activity

against both human immunodeficiency virus and human cytomegalovirus replication in vitro. *J. Infect. Dis.* **172**, 380–388.

Hasegawa, K., Motsuchi, W., Tanaka, S., and Dosako, S. 1994. Inhibition with lactoferrin of in vitro infection with human herpes virus. *Jpn. J. Med. Sci. Biol.* **47**, 73–85.

Hattori, M., Ogino, A., Nakai, H., and Takahashi, K. 1997. Functional improvement of β-lactoglobulin by conjugating with alginate lyase-lysate. *J. Agric. Food Chem.* **45**, 703–708.

Henschen, A., Lottspeich, F., Brantl, V., and Teschemacher, H. 1979. Novel opioid peptides derived from casein (β-casomorphins). II. Structure of active components from bovine casein peptone. *Hoppe-Seyler's Z. Physiol. Chem.* **360**, 1217–1224.

Higaki, J.N., Evnin, L.B., and Craik, C.S. 1989. Introduction of a cysteine protease active site into trypsin. *Biochemistry* **28**, 9256–9263.

Hill, A., Kleijnen, M., Schust, D., Kosinowski, U., and Ploegh, H. (1996). Murine cytomegalovirus (MCMV) encodes a 34 kDa glycoprotein, which binds to class I MHC molecules at the surface of infected cells. Abstract 410, Keystone Symposium on Cell Biology of Virus Entry, Replication and Pathogenesis. Santa Fe, NM.

Hirayama, M., Toyota, K., Hidaka, H., and Naito, H. 1992. Phosphopeptides in rat intestinal digests after ingesting casein phosphopeptides. *Biosci. Biotechnol. Biochem.* **56**, 1128–1129.

Hirotsuka, M., Taniguchi, H., Narita, H., and Kito, M. 1984. Functionality and digestibility of a highly phosphorylated soybean protein. *Agric. Biol. Chem.* **48**, 93–100.

Holt, C. 1994. The biological role of casein? *In* "Hannah Research Institute Research Reviews", pp. 60–68. Association for the Advancement of Dairy Science, Wageningen, The Netherlands.

Holt, C. and Horne, D.S. 1996. The hairy casein micelle: evolution of the concept and its implications for dairy technology. *Neth. Milk Dairy J.* **50**, 85–112.

Hooker, P.H., Munro, P.A., and O'Meara, G.M. 1982. Reduction of the viscosity of sodium caseinate solutions by enzymatic hydrolysis. *NZ J. Dairy Sci. Technol.* **17**, 35–40.

Huang, Y.T. and Kinsella, J.E. 1986. Functional properties of phosphorylated yeast proteins: solubility, water holding capacity and viscosity. *J. Agric. Food Chem.* **34**, 670–674.

Huang, Y.T. and Kinsella, J.E. 1987. Effect of phosphorylation on emulsifying and foaming properties and digestibility of yeast protein. *J. Food Sci.* **52**, 1684–1688.

Huang, X.L., Catignani, G.L., and Swaisgood, H.E. 1994. Comparison of the size and rate of formation of peptides released by limited proteolysis of β-lactoglobulins A and B with immobilized trypsin. *J. Agric. Food Chem.* **42**, 1281–1284.

Huang, X.L., Catignani, G.L., and Swaisgood, H.E. 1995. Immobilization of biotinylated transglutaminase by bioselective adsorption to immobilized avidin and characterization of the immobilized activity. *J. Agric. Food Chem.* **43**, 895–901.

Huang, X.L., Catignani, G.L., and Swaisgood, H.E. 1996. Improved emulsifying properties of the β-barrel domain peptides obtained by membrane-fractionation of a limited tryptic hydrolysate of β-lactoglobulin. *J. Agric. Food Chem.* **44**, 3437–3443.

Huang, X.L., Catignani, G.L., and Swaisgood, H.E. 1999. Modification of the rheological properties of whey protein isolates by limited proteolysis. *Nahrung* **43**, 79–85.

Huffman, L.M. 1996. Processing whey protein for use as a food ingredient. *Food Technol.* **50**, 49–52.

Hunt, J.A. and Dalgleish, D.G. 1994. Effect of pH on the stability and surface composition in emulsions made with whey protein isolate. *J. Agric. Food Chem.* **42**, 2131–2135.

Iwami, K. and Yasumoto, K. 1986. Amino-binding capacities of food proteins in transglutaminase reaction and digestibility of wheat gliadin with ε-attached lysine. *J. Sci. Food Agric.* **37**, 495–503.

Jing, H. and Kitts, D.D. 2000. Comparison of the antioxidative and cytotoxic properties of glucose-lysine and fructose-lysine Maillard reaction. *Food Res. Int.* **33**, 509–516.

Johnson, J.E. and Chiu, W. 2000. Structures of virus and virus-like particles. *Curr. Opin. Struct. Biol.* **10**, 229–235.

Jones, A.D., Tier, C.M., and Wilkins, J.P.G. 1998. Analysis of the Maillard reaction products of β-lactoglobulin and lactose in skimmed milk powder by capillary electrophoresis and electrospray mass spectrometry. *J. Chromatogr.* **822**, 147–154.

Kato, A. and Nakai, S. 1980. Hydrophobicity determined by a fluorescence probe method and its correlation with the surface properties of proteins. *Biochim. Biophys. Acta* **624**, 13–20.

Kato, H. and Suzuki, T. 1971. Bradykinin-potentiating peptides from the venom of *Agkistrodon halysblomhoffii*. Isolation of five bradykinin potentiators and the amino acid sequences of two of them, potentiators B and C. *Biochemistry* **10**, 972–980.

Kim, S.C., Olson, N.F., and Richardson, T. 1990. Polymerization and gelation of thiolated β-lactoglobulin at ambient temperature induced by oxidation by potassium iodate. *Milchwissenschaft* **45**, 627–630.

Kim, S.Y., Park, P.S.W., and Rhee, K.C. 1990. Functional properties of proteolytic enzyme modified soy protein isolate. *J. Agric. Food Chem.* **38**, 651–656.

Kinsella, J.E. and Whitehead, D.M. 1989. Protein in whey: chemical, physical and functional properties. *Adv. Food Nutr. Res.* **33**, 343–438.

Kirimura, J., Shimizu, A., Kimizuka, A., Ninomiya, T., and Katsuya, N. 1969. The contribution of peptides and amino acids to the taste of foodstuffs. *J. Agric. Food Chem.* **17**, 689–695.

Kitabatake, N., Cuq, J.-L., and Cheftel, J.-C. 1985. Covalent binding of glycosyl residues to β-lactoglobulin: effects on solubility and heat stability. *J. Agric. Food Chem.* **33**, 125–130.

Kitts, D.D., Wu, C.H., Stich, H.F., and Powrie, W.D. 1993. Effect of glucose-lysine Maillard reaction product on bacterial and mammalian cell mutagenesis. *J. Agric. Food Chem.* **41**, 2353–2358.

Klemaszewski, J.L., Das, K.P., and Kinsella, J.E. 1992. Formation and covalence stability of emulsions stabilized by different milk proteins. *J. Food Sci.* **57**, 366–371.

Le Denmat, M., Anton, M., Beaumal, V., and Gandemer, G. 1999. Characterization of the interface of oil-in-water emulsions prepared with hen egg yolk at different pH and NaCl concentrations. Proceedings VIII European Symposium on the Quality of Eggs and Eggs Products, Bologna, Italy. pp. 325–332.

Ledl, F. and Schleicher, E. 1990. New aspects of the Maillard reaction in foods and in the human body. *Angew. Chem. Int. Ed. Engl.* **29**, 565–594.

Lee, P. and Swaisgood, H.E. 1998. Cloning and expression of a streptavidin-lipase fusion gene in *Escherichia coli* and characterization of the immobilized fusion protein. *Enzyme Microb. Technol.* **22**, 246–254.

Lee, Y.S., Noguchi, T., and Naito, H. 1980. Phosphopeptides and soluble calcium in the small intestine of rats given a casein diet. *Br. J. Nutr.* **43**, 457–467.

Lee, Y.S., Noguchi, T., and Naito, H. 1983. Intestinal absorption of calcium in rats given diets containing casein or an amino acid mixture: the role of casein phosphopeptides. *Br. J. Nutr.* **49**, 67–76.

Lee, S.P., Cho, Y., and Batt, C.A. 1993. Enhancing the gelation of β-lactoglobulin. *J. Agric. Food Chem.* **41**, 1343–1348.

Lee, S.P., Kim, D.S., Watkins, S., and Batt, C.A. 1994. Reducing whey syneresis in yogurt by the addition of a thermolabile variant of β-lactoglobulin. *Biosci. Biotechnol. Biochem.* **58**, 309–315.

Léonil, J., Mollé, D., Fauquant, J., Maubois, J.-L., Pearce, R.J., and Bouhallab, S. 1997. Characterization by ionization mass spectrometry of lactosyl β-lactoglobulin conjugates formed during heat treatment of milk and whey and identification of one lactose-binding site. *J. Dairy Sci.* **80**, 2270–2281.

Loimaranta, V., Laine, M., Soderling, E., Vasara, E., Rokka, S., Marnila, P., Korhonen, H., Tossavainen, O., and Tenovuo, J. 1999. Effect of bovine immune and non-immune whey preparations on the composition and pH response of human dental plaque. *Eur. J. Oral Sci.* **107**, 244–250.

Lorenzen, P.C., Mautner, A., and Schlimme, E. 2000. Enzymatic crosslinking of proteins in the production of milk products *In* "Proceedings of the 1st International Symposium on Enzymatic Protein Processing" (H. Gruppen and W. van Hartingsveldt eds), pp. 163–169. TNO Nutrition and Food Research Institute, Zeist, The Netherlands.

Lottspeich, F., Henschen, A., Brantl, V., and Teschemacher, H. 1980. Novel opioid peptides derived from casein (β-casomorphins). III. Synthetic peptides corresponding to components from bovine casein peptone. *Hoppe-Seyler's Z. Physiol. Chem.* **361**, 1835–1839.

Mahmoud, M.I., Malone, W.T., and Cordle, C.T. 1992. Enzymatic hydrolysis of casein: effect of degree of hydrolysis on antigenicity and physical properties. *J. Food Sci.* **57**, 1223–1229.

Maillard, L.C. 1912. Action des amines sur les sucres: formation des mélanoïdines par voie méthodique. *C. R. Acad. Sci.* **154**, 66–68.

Makowski, L. 1994. Phage display: structure, assembly and engineering of filamentous bacteriophage. *Curr. Opin. Struct. Biol.* **4**, 225–230.

Marchetti, M., Longhi, C., Conte, M.P., Pisani, S., Valenti, P., and Seganti, L. 1996. Lactoferrin inhibits herpes simplex virus type 1 adsorption to Verop cells. *Antiviral Res.* **29**, 221–231.

Marvin, D.A. 1998. Filamentous phage structure, infection and assembly. *Curr. Opin. Struct. Biol.* **8**, 150–158.

Matheis, G. 1991. Phosphorylation of food proteins with phosphorus oxychloride: improvement of functional and nutritional properties. *Food Chem.* **39**, 13–26.

Matheis, G., Penner, H.M., Feeney, R.E., and Whitaker, J.R. 1983. Phosphorylation of casein and lysozyme by phosphorus oxychloride. *J. Agric. Food Chem.* **31**, 379–387.

Mattarella, N.L. and Richardson, T. 1983. Physicochemical and functional properties of positively charged derivatives of bovine β-lactoglobulin. *J. Agric. Food Chem.* **31**, 972–978.

Mattarella, N.L., Creamer, K.L., and Richardson, T. 1983. Amidation or esterification of bovine β-lactoglobulin to form positively charged proteins. *J. Agric. Food Chem.* **31**, 968–972.

McCrae, C.H., Law, A.J.R., and Leaver, J. 1999. Emulsification properties of whey proteins in their natural environment: effect of whey protein concentration at 4 and 18% milk fat. *Food Hydrocoll.* **13**, 389–399.

McGookin, B.J. and Augustin, M.A. 1991. Antioxidant activity of casein and Maillard reaction product from casein-sugar mixtures. *J. Dairy Res.* **58**, 313–320.

McKenzie, H.A. 1971. Milk Proteins. Chemistry and Molecular Biology, Vol. II. Academic Press, New York.

McKenzie, H.A. and Sawyer, W.H. 1967. Effect of pH on β-lactoglobulins. *Nature* **214**, 1101–1104.

McSwiney, M., Singh, H., and Campanella, O.H. 1994. Thermal aggregation and gelation of bovine β-lactoglobulin. *Food Hydrocoll.* **8**, 441–453.

Means, G.E. and Feeney, R.E. 1971. Chemical Modification of Proteins. Holden Day, San Francisco.

Medina, A.L., Colas, B., Le Meste, M., Renaudet, I., and Lorient, D. 1992. Physicochemical and dynamic properties of caseins modified by chemical phosphorylation. *J. Food Sci.* **57**, 617–620.

Meisel, H. 1986. Chemical characterization and opioid activity of an exorphin isolated from in vivo digests of casein. *FEBS Lett.* **196**, 223–227.

Meisel, H. 1997. Biochemical properties of bioactive peptides derived from milk proteins; potential nutraceuticals for food and pharmaceutical applications. *Livestock Prod. Sci.* **50**, 125–138.

Meisel, H. 1998. Overview on milk proteins-derived peptides. *Int. Dairy J.* **8**, 363–373.

Meisel, H. and Frister, H. 1989. Chemical characterization of bio-active peptides from in vivo digests of casein. *J. Dairy Res.* **56**, 343–349.

Meisel, H. and Schlimme, E. 1990. Milk proteins: precursors of bioactive peptides. *Trends Food Sci. Technol.* **1**, 41–43.

Meisel, H. and Schlimme, E. 1996. Bioactive peptides derived from milk proteins: ingredient for functional foods? *Kieler Milchwirtsch. Forschungsber.* **48**, 343–357.

Miclo, L., Perrin, E., Driou, A., Papadopoulos, V., Boujrad, N., Vanderesse, R., Boudier, J. F., Desor, D., Linden, G., and Gaillard, J.-L. 2001. Characterization of α-casozepine, a tryptic peptide from bovine α_{S1}-casein with benzodiazepine-like activity. *FASEB J.* ((Express Article 10.1096/fj.00-0685fje published online June 8, 2001)).

Mietsch, F., Feher, J., and Halasz, A. 1989. Investigation of functional properties of partially hydrolyzed proteins. *Nahrung* **33**, 9–15.

Mine, Y. 1998. Emulsifying characterization of hen's egg yolk proteins in oil-in-water emulsions. *Food Hydrocoll.* **12**, 409–415.

Mitchell, J., Irons, L., and Palmer, G.J. 1970. A study of the spread and adsorbed films of milk proteins. *Biochim. Biophys. Acta* **200**, 138–150.

Mohri, M. and Matsushita, S. 1984. Improvement of water absorption of soybean protein by treatment with bromelain. *J. Agric. Food Chem.* **32**, 486–490.

Monahan, F.R., McClements, D.J., and Kinsella, J.E. 1993. Polymerisation of whey protein-stabilised emulsions. *J. Agric. Food Chem.* **41**, 1826–1829.

Morgan, F., Léonil, J., Mollé, D., and Bouhallab, S. 1997. Nonenzymatic lactosylation of bovine β-lactoglobulin under mild heat treatment leads to structural heterogeneity of the glycoforms. *Biochem. Biophys. Res. Commun.* **236**, 413–417.

Morgan, F., Léonil, J., Mollé, D., and Bouhallab, S. 1999a. Modification of bovine β-lactoglobulin by glycation in a powdered state or in an aqueous solution: effect on association behavior and protein conformation. *J. Agric. Food Chem.* **47**, 83–91.

Morgan, F., Mollé, D., Henry, G., Vénien, A., Léonil, J., Peltre, G., Levieux, D., Maubois, J.-L., and Bouhallab, S. 1999b. Glycation of bovine β-lactoglobulin: effect on the protein structure. *Int. J. Food Sci. Technol.* **34**, 429–435.

Morgan, F., Vénien, A., Bouhallab, S., Mollé, D., Léonil, J., Peltre, G., and Levieux, D. 1999c. Modification of bovine β-lactoglobulin by glycation in a powdered state or in aqueous solution: immunological characterization. *J. Agric. Food Chem.* **47**, 4543–4548.

Morimoto, M., Okubo, S., Tomita, F., and Marumo, H. 1981. Gilvocarcins, new antitumor antibiotics. 3. Antitumor activity. *J. Antibiot. (Tokyo)* **34**, 701–707.

Motoki, M. and Seguro, K. 1998. Transglutaminase and its use for food processing. *Trends Food Sci. Technol.* **9**, 204–210.

Mullally, M.M., Meisel, H., and FitzGerald, R.J. 1997. Identification of a novel angiotensin-I-converting enzyme inhibitory peptide corresponding to a tryptic fragment of bovine beta-lactoglobulin. *FEBS Lett.* **402**, 99–101.

Nacka, F., Chobert, J.-M., Burova, T., Léonil, J., and Haertlé, T. 1988. Induction of new physicochemical and functional properties by the glycosylation of whey proteins. *J. Protein Chem.* **17**, 495–503.

Nagasawa, K., Takhashi, K., and Hattori, M. 1996. Improved emulsifying properties of β-lactoglobulin by conjugating with carboxymethyl dextran. *Food Hydrocoll.* **10**, 63–67.

Naito, H. and Suzuki, H. 1974. Further evidence for the formation in vivo of phosphopeptide in the intestinal lumen from dietary β-casein. *Agric. Biol. Chem.* **38**, 1543–1545.

Nakamura, S., Kato, A., and Kobayashi, K. 1991. New antimicrobial characteristics of lysozyme-dextran conjugate. *J. Agric. Food Chem.* **39**, 647–650.

Nakamura, T., Sado, H., Syukunobe, Y., and Hirota, T. 1993. Antigenicity of whey protein hydrolysates prepared by combination of two proteases. *Milchwissenschaft* **48**, 667–670.

Nakano, H., Matsuda, Y., Ito, K., Ohkubo, S., Morimoto, M., and Tomita, F. 1981. Gilvocarcins, new antitumor antibiotics. 1. Taxonomy, fermentation, isolation and biological activities. *J. Antibiot. (Tokyo)* **34**, 266–270.

Ney, K.H. 1971. Bitterness of peptides: amino acids composition and chain length *In* "Food Taste Chemistry", Advances in Chemistry Series 115 (J.C. Boudreau ed.), pp. 149–173, American Chemical Society, Washington, DC.

Ney, K.H. and Rezzlaff, G. 1986. A computer program predicting the bitterness of peptides, especially in protein hydrolysates, based on amino acid composition and chain length (computer Q) *In* "Shelf Life of Foods and Beverages", Proceedings of the 4th International Flavour Conference (G. Charalambous ed.), pp. 543–550. Elsevier, The Netherlands.

Nonaka, M., Tanaka, H., Okiyama, A., Motoki, M., Ando, H., Umeda, K., and Matsuura, A. 1989. Polymerization of several proteins by Ca^{2+}-independent transglutaminase derived from microorganisms. *Agric. Biol. Chem.* **53**, 2619–2623.

Nonaka, M., Sakamoto, H., Toiguchi, S., Kawajiri, H., Soeda, T., and Motoki, M. 1992. Sodium caseinate and skim milk gels formed by incubation with microbial transglutaminase. *J. Food Sci.* **57**, 1214–1218.

Nonaka, M., Matsuura, Y., and Motoki, M. 1996. Incorporation of lysine dipeptides into α_{s1}-casein by Ca^{2+}-independent microbial transglutaminase. *Biosci. Biotechnol. Biochem.* **60**, 131–133.

Novo Nordisk. 1998. Carboxypeptidase from *Aspergillus orizae* and nucleic acids encoding the same. Patent WO 98/14599.

O'Brien, J. and Morrissey, P.A. 1989. Nutritional and toxicological aspects of the Maillard browning reaction in foods. *Crit. Rev. Food Sci. Nutr.* **28**, 211–248.

Ochirkhuyag, B., Chobert, J.-M., Dalgalarrondo, M., Choiset, Y., and Haertlé, T. 1998. Characterization of whey protein from Mongolian yak, khainak, and bactrian camel. *J. Food Biochem.* **22**, 105–124.

Oh, S., Catignani, G.L., and Swaisgood, H.E. 1993. Characteristics of an immobilized form of transglutaminase: a possible increase in substrate specificity by selective interaction with a protein spacer. *J. Agric. Food Chem.* **41**, 1337–1342.

O'Sullivan, M.M., Kelly, A.L., and Fox, P.F. 2002. Effect of transglutaminase on the heat stability of milk: a possible mechanism. *J. Dairy Sci.* **85**, 1–7.

Pawlett, D. and Bruce, G.N. 1996. Debittering of protein hydrolysates *In* "Industrial Enzymology", 2nd (T. Godfret and S. West eds), pp. 177–186. Macmillan, U.K..

Pearce, K.N. and Kinsella, J.E. 1978. Emulsifying properties of proteins: evaluation of a turbidimetric technique. *J. Agric. Food Chem.* **26**, 716–723.

Perona, J.J. and Craik, C.S. 1995. Structural basis of substrate specificity in the serine proteases. *Protein Sci.* **4**, 337–360.

Pihlanto-Leppälä, A., Rokka, T., and Korhonen, H. 1998. Angiotensin I converting enzyme inhibitory peptides derived from bovine milk proteins. *Int. Dairy J.* **8**, 325–331.

Popineau, Y. and Pineau, F. 1993. Emulsifying properties of wheat gliadins and gliadin peptides *In* "Food Proteins. Structure and Functionality" (K.D. Schwenke and R. Mothes eds), pp. 290–296. VCH, Weinheim.

Puddu, P., Borghi, P., Gessani, S., Valenti, P., Belardelli, F., and Seganti, L. 1998. Antiviral activity of bovine lactoferrin saturated with metal ions on early steps of human immunodeficiency virus type 1 infection. *Int. J. Biochem. Cell Biol.* **30**, 1055–1063.

Puski, G. 1975. Modification of functional properties of soy proteins by proteolytic enzyme treatment. *Cereal Chem.* **52**, 655–664.

Reynolds, E.C. and Del Rio, A. 1984. Effect of casein and whey protein solutions on the caries experience and feeding patterns of the rat. *Arch. Oral Biol.* **29**, 927–933.

Reynolds, E.C., Cain, C.J., Webber, F.L., Black, C.L., Riley, P.F., Johnson, I.H., and Preich, J.W. 1995. Anticariogenicity of calcium phosphate complexes of tryptic casein phosphopeptides in the rat. *J. Dent. Res.* **74**, 1271–1279.

Roefs, S.P.F.M. and de Kruif, K.G. 1994. A model for the denaturation and aggregation of β-lactoglobulin. *Eur. J. Biochem.* **226**, 883–889.

Röhm GmbH. 1997. Rekombinant hergestellte Leucinaminopeptidase aus *Aspergillus soja*. Patent C 12 N 15/80.

Roy, G. (ed.) 1997. *In* "Recent overview of the mechanisms of bitter taste", Modifying Bitterness , pp. 139–160. Technomic Publishing Co. Inc., USA.

Sakai, K., Ogawa, M., Takashima, H., Mizunuma, T., and Manabe, S. 1997. Peptic digestibility of proteins in different heat-treated commercial cow's milks. *J. Jpn. Soc. Food Sci. Technol.* **45**, 330–333.

Sambrook, J., Fritsch, E.F., and Maniatis, T. 1989. 2nd, "Molecular cloning". A Laboratory Manual, Vol. I. Cold Spring Harbor Laboratory, New York, 4.1–4.23 and A.3..

Sano, T. and Cantor, C.R. 1991. Expression vectors for streptavidin-containing chimeric protein. *Biochem. Biophys. Res. Commun.* **176**, 571–577.

Sato, R., Shindo, M., Noguchi, T., and Naito, H. 1991. Characterization of phosphopeptide derived from bovine β-casein: an inhibitor to intra intestinal precipitation of calcium phosphate. *Biochim. Biophys. Acta* **1077**, 413–415.

Sato, K., Nakamura, M., Nishiya, T., Kawanari, M., and Nakajima, I. 1995. Preparation of a gel of partially heat-denatured whey protein by proteolytic digestion. *Milchwissenschaft* **50**, 389–392.

Sawyer, L. and Holt, C. 1993. The secondary structure of milk proteins and their biological functions. *J. Dairy Sci.* **76**, 3062–3078.

Sawyer, L., Brownlow, S., Polikarpov, I., and Wu, S.-Y. 1998. β-Lactoglobulin: structural studies, biological clues. *Int. Dairy J.* **8**, 65–72.

Schanbacher, F.L., Talhouk, R.S., Murray, F.A., Gherman, L.I., and Willett, L.B. 1998. Milk-born bioactive peptides. *Int. Dairy J.* **8**, 393–403.

Scholz-Ahrens, K.E. and Schrezenmeir, J. 2000. Effects of bioactive substances in milk on mineral and trace element metabolism with special reference to casein phosphopeptides. *Brit. J. Nutr.* **84**(suppl. 1), 147–153.

Schuepbach, P., Nesser, J.R., Golliard, M., Rouvet, M., and Guggenheim, B. 1996. Incorporation of casein glycomacropeptides and casein phosphopeptides into the salivary pellicle inhibits adherence of mutans Streptococci. *J. Dent. Res.* **75**, 1779–1788.

Seguro, K., Kumazawa, Y., Kuraishi, C., Sakamoto, H., and Motoki, M. 1996. The ε-(γ-glutamyl)lysine moiety in cross-linked casein is an available source of lysine for rats. *J. Nutr.* **126**, 2557–2562.

Shih, F.F. 1992. Modification of food proteins by non-enzymatic methods *In* "Biochemistry of Food Proteins" (B.J.F. Hudson ed.), pp. 235–270. Elsevier Applied Science Publishers, London.

Shimizu, M., Kamiya, T., and Yamauchi, K. 1981. The adsorption of whey proteins on the surface of emulsified fat. *Agric. Biol. Chem.* **45**, 2491–2496.

Shimizu, M., Saito, M., and Yamauchi, K. 1985. Emulsifying and structural properties of β-lactoglobulins at different pHs. *Agric. Biol. Chem.* **49**, 189–194.

Shimizu, K., Matsuzawa, H., Okada, K., Tazume, S., Dosako, S., Kawasky, Y., Hashimoto, K., and Koga, Y. 1996. Lactoferrin-mediated protection of the host from murine cytomegalovirus infection by a T-cell-dependent augmentation of natural killer cell activity. *Arch. Virol.* **141**, 1875–1889.

Singh, A.M. and Dalgleish, D.G. 1998. The emulsifying properties of hydrolysates of whey proteins. *J. Dairy Sci.* **81**, 918–924.

Sitohy, M., Chobert, J.-M., and Haertlé, T. 1994. Influence of the reaction conditions on the chemical phosphorylation of milk proteins. *Milchwissenschaft* **49**, 610–615.

Sitohy, M., Chobert, J.-M., and Haertlé, T. 1995a. Physico-chemical characterization of monomeric chemically phosphorylated caseins. *J. Food Biochem.* **19**, 97–108.

Sitohy, M., Chobert, J.-M., and Haertlé, T. 1995b. Amino acid grafting on β-lactoglobulin mediated with phosphorus oxychloride. *Int. J. Biol. Macromol.* **5**, 269–272.

Sitohy, M., Chobert, J.-M., and Haertlé, T. 1995c. Phosphorylation of β-lactoglobulin under mild condition. *J. Agric. Food Chem.* **43**, 59–62.

Sitohy, M., Chobert, J.-M., and Haertlé, T. 2000. Study of factors influencing protein esterification using β-lactoglobulin as a model. *J. Food Biochem.* **24**, 381–398.

Sitohy, M., Chobert, J.-M., and Haertlé, T. 2001a. Simplified short-time method for the esterification of milk proteins. *Milchwissenschaft* **56**, 127–131.

Sitohy, M., Chobert, J.-M., and Haertlé, T. 2001b. Peptic hydrolysis of methyl-, ethyl- and propyl-ester derivatives of β-casein and α-lactalbumin. *Milchwissenschaft* **56**, 303–307.

Sitohy, M., Chobert, J.-M., and Haertlé, T. 2001c. Improvement of solubility and of emulsifying properties of milk proteins at acid pHs by esterification. *Nahrung* **45**, 87–93.

Sitohy, M., Chobert, J.-M., and Haertlé, T. 2001d. Study of the formation of complexes between DNA and esterified dairy proteins. *Int. Dairy J.* **11**, 875–885.

Sitohy, M., Chobert, J.-M., Dalgalarrondo, M., and Haertlé, T. 2001e. Factors influencing pepsinolysis of methyl-, ethyl- and propyl-ester derivatives of β-lactoglobulin. *J. Food Biochem.* **25**, 181–198.

Slattery, H. and FitzGerald, R.J. 1998. Functional properties and bitterness of sodium caseinate hydrolysates prepared with a *Bacillus* proteinase. *J. Food Sci.* **63**, 418–422.

Smacchi, E. and Gobetti, M. 2000. Bioactive peptides in dairy products: synthesis and interaction with proteolytic enzymes. *Food Microb.* **17**, 129–141.

Smithers, G.W., Ballard, F.J., Copeland, A.D., and de Silva, K.J. 1996. Symposium: advances in dairy foods proceeding and engineering. New opportunities from the isolation and utilization of whey proteins. *J. Dairy Sci.* **79**, 1454–1459.

Smyth, M. and FitzGerald, R.J. 1998. Relationship between some characteristics of WPC hydrolysates and the enzyme complement in commercially available proteinase preparations. *Int. Dairy J.* **8**, 819–827.

Stecchini, M.L., Giavedoni, P., Sarais, I., and Lerici, C.R. 1993. Antimicrobial activity of Maillard reaction products against *Aeromonas hydrophilia*. *Ital. J. Food Sci.* **2**, 147–150.

Steijns, J.M. 2001. Milk ingredients as neutraceuticals. *Int. J. Dairy Technol.* **54**, 81–88.

Sung, H.-Y., Chen, H.-J., Liu, T., and Su, J.-C. 1983. Improvement of functionality of soy protein isolate through chemical phosphorylation. *J. Food Sci.* **48**, 716–721.

Suzuki, H., Kajimoto, Y., and Kumagai, H. 2002. Improvement of the bitter taste of amino acids through the transpeptidation reaction of bacterial γ-glutamyltranspeptidase. *J. Agric. Food Chem.* **50**, 313–318.

Swaisgood, H.E. 1991. Immobilized enzymes: applications to bioprocessing of food *In* "Food Enzymology" (P.F. Fox ed.), Vol. 2, pp. 309–341. Elsevier Applied Science, New York.

Tanford, C. 1980. Formation of micelles and biological membranes *In* "The Hydrophobic Effect" (Wiley and Sons ed.), pp. 139–146. Wiley and Sons, New York.

Tanford, C., Bunvile, L.G., and Nozaki, Y. 1959. The reversible transformation of β-lactoglobulin at pH 7.5. *J. Am. Chem. Soc.* **81**, 4032–4036.

Tani, F., Iio, K., Chiba, H., and Yoshikawa, M. 1990. Isolation and characterization of opioid antagonist peptides derived from human lactoferrin. *Agric. Biol. Chem.* **54**, 1803–1810.

Teschemacher, H. and Koch, G. 1990. β-Casomorphins: possible physiological significance *In* "Proceedings First International Symposium on β-casomorphins and related peptides" (F. Nyberg and V. Brantl eds), pp. 143–149. Fyris-Tryck AB, Uppsala, Sweden.

Turgeon, S.L., Gautier, S.F., and Paquin, P. 1992. Emulsifying properties of whey peptide fractions as a function of pH and ionic strength. *J. Food Sci.* **57**, 601–604 (, 634).

Vagnarelli, P., de Sario, A., Cuzzoni, M.T., Mazza, P., and de Carli, L. 1991. Cytotoxic and clastogenic activity of ribose-lysine browning model system. *J. Agric. Food Chem.* **39**, 2237–2239.

Van Wezenbeek, P.M.G.F., Hulsebos, T.J.M., and Schoenmakers, J.G.G. 1980. Nucleotide sequence of the filamentous bacteriophage M13 DNA genome: comparison with phage fd. *Gene* **11**, 129–135.

Vieths, S., Reindl, J., Mueller, U., Hoffmann, A., and Haustein, D. 1999. Digestibility of peanut and hazelnut allergens investigated by a simple in vitro procedure. *Eur. Food Res. Technol.* **209**, 379–388.

Waissbluth, M.D. and Grieger, R.A. 1974. Alkaline denaturation of β-lactoglobulins. Activation parameters and effect on dye binding site. *Biochemistry* **13**, 1285–1288.

Walsh, M.K. and Swaisgood, H.E. 1994. An *Escherichia coli* plasmid vector system for production of streptavidin fusion proteins: expression and bioselective adsorption of streptavidin-β-galactosidase. *Biotechnol. Bioengng* **44**, 1348–1354.

Waniska, R.D. and Kinsella, J.E. 1988. Foaming and emulsifying properties of glycosylated β-lactoglobulin. *Food Hydrocoll.* **2**, 439–449.

Warner, E.A., Kanekanian, A.D., and Andrews, A.T. 2001. Bioactivity of milk proteins: 1. Anti-cariogenicity of whey proteins. *Int. J. Dairy Technol.* **54**, 151–153.

Wijngaards, G., Maagd, J.M., Boumans, H., de Jong, G.A.H., and Bol, J. 2000. Transglutaminase based cross-linking phenomena in protein and food systems. International Symposium on Enzymatic Protein Processing (H. Gruppen and W. van Hartingsveldt eds), pp. 154–162. TNO Nutrition and Food Research Institute, Zeist, The Netherlands.

Wilcox, P.E. 1967. Esterification. *Methods Enzymol.* **11**, 605–617.

Xavier, K.A., Eder, P.S., and Giordano, T. 2000. RNA as a drug target: methods for biophysical characterization and screening. *TIBTECH* **18**, 349–365.

Yamamoto, N. 1997. Antihypertensive peptides derived from food proteins. *Biopolymers* **43**, 129–134.

Yamauchi, K., Schimizu, M., and Kamiya, T. 1980. Emulsifying properties of whey proteins. *J. Food Sci.* **45**, 1237–1242.

Yen, G.-C. and Hsieh, P.-P. 1995. Antioxidative activity and scavenging effects on native oxygen of xylose-lysine Maillard reaction products. *J. Sci. Food Agric.* **67**, 415–420.

Yoshikawa, M., Sasakai, R., and Chiba, H. 1981. Effect of chemical phosphorylation of bovine casein components on the properties related to casein micelle formation. *Agric. Biol. Chem.* **45**, 909–914.

Zeidler, R., Eissener, G., Meissner, P., Uebel, S., Tampe, R., Lazis, S., and Hammerschmidt, W. 1997. Down regulation of TAP1 in B lymphocytes by cellular and Epstein-Barr virus-encoded interleukin-10. *Blood* **90**, 2390–2397.

Zhu, V., Rinzema, A., and Tramper, J. 1995. Microbial transglutaminase. A review of its production and application in food processing. *Appl. Microbiol. Biotechnol.* **44**, 277–282.

Zioudrou, C., Streaty, R.A., and Klee, N.A. 1979. Opioid peptides derived from food proteins, the exorphin. *J. Biol. Chem.* **254**, 2446–2451.

Zucht, H.-D., Raida, M., Adermann, K., Mägert, H.-J., and Forssmann, W.-G. 1995. Casocidin-I: a casein-α_{S2} derived peptide exhibits antibacterial activity. *FEBS Lett.* **372**, 185–188.

THE NUTRITIONAL SIGNIFICANCE, METABOLISM AND TOXICOLOGY OF SELENOMETHIONINE

GERHARD NORBERT SCHRAUZER

Biological Trace Element Research Institute
2400 Boswell Rd., Suite 200
Chula Vista, CA 91914
USA

ADVANCES IN FOOD AND NUTRITION RESEARCH VOL 47
ISSN: 1043-4526
DOI: 10.1016/S1043-4526(03)47002-2

I. INTRODUCTION

Selenomethionine (SeMet), the selenium (Se-) analog of methionine (Met), was first suspected in the 1930s to be one of the toxic principles of seleniferous plants (Franke and Painter, 1938), although definite proof for its existence became possible only in 1947, after synthetic SeMet had become available (Klosterman and Painter, 1947). Chromatographic evidence also suggested SeMet to be present in seleniferous wheat protein extracts (Smith, 1949), which was confirmed by Peterson and Butler (1962), Butler and Peterson (1967) and Olson *et al.* (1970). Concurrent studies established that SeMet was also synthesized by certain marine algae (Shrift, 1954 a,b), *Escherichia coli* (Tuve and Williams, 1961), *Saccharomyces cerevisiae* (Blau, 1961), *Candida albicans* (Hedegaard *et al.*, 1963), and by rumen bacteria (Hidiroglou *et al.*, 1968). In 1962, ^{75}Se-labeled SeMet was introduced as a radioactive tracer and pancreatic radioimaging agent (Blau and Bender, 1962). The discovery of the nutritional essentiality of Se by Schwarz and Foltz (1957) created the need for an appropriate supplemental form of Se to prevent Se deficiency diseases in farm animals. Since synthetic SeMet was prohibitively expensive, sodium selenite and -selenate were approved for use as feed additives. These inorganic Se salts are readily absorbed and utilized for selenoenzyme synthesis but are less effective in raising blood Se levels than SeMet (Thomson and Stewart, 1974; Griffiths *et al.*, 1976; Robinson *et al.*, 1978; Thomson *et al.*, 1978; Van Rij *et al.*, 1979). To provide an economical food source of SeMet for use in human and animal nutrition, methods for the industrial production of Se-enriched yeast were developed by leading yeast manufacturers on initiative of Nutrition 21, a California nutritional supplement company. Presently, Se yeast is manufactured in many countries. In 1984, synthetic SeMet became available at a cost comparable to Se yeast on a *per se* basis. Methods of production of alternative food sources of SeMet such as high-Se wheat grains and of other Se-enriched crops were also devised (Gupta and MacLoed, 1998; Djusic *et al.*, 1998; Kivisaari, 1998; Whanger *et al.*, 1998).

II. PROPERTIES OF SELENOMETHIONINE

Selenomethionine [*2-amino-4-(methylseleno)-butanoic acid*], $CH_3-Se-CH_2CH_2CH(NH)_2-COOH$, ($C_5H_{11}NO_2Se$; C, 30.62%, H, 5.65%, N,

7.14%, O, 16.32%, Se, 40.26%, FW 196.11), Chemical Abstracts Number 2578-28-1, is a colorless compound with a musty odor, and crystallizes from aqueous acetone in the form of hexagonal crystals. SeMet exists in three forms. The L-form occurs naturally; the D-enantiomer and DL-mixture are obtained synthetically. Pure L-SeMet melts with decomposition at 274°C, DL-SeMet at 265°C, making it thermally somewhat less stable than its sulfur analog. SeMet is less soluble in water than Met due to the greater hydrophobicity of the CH_3-Se- as compared to the CH_3-S- group (Shepherd and Huber, 1969); at 30°C and pH 7.0. For example, saturated solutions of SeMet and Met are 0.108 and 0.386 M, respectively. In 2N HCl ($c = 0.02$), L-SeMet has an $[\alpha]_D$ of $+21.2°$ (Koch and Burchardt, 1993), its half-time of racemization of 19–20 days in aqueous solution at 100°C at pH 7.4 is similar to that of Met (Boehm and Bada, 1985; Mendez *et al.*, 1999). On acid hydrolysis, SeMet is significantly less stable than Met. In 6N HCl at 110°C, hydrolysis of SeMet was complete after 7 h, a treatment that leaves the sulfur analog Met still essentially unchanged (Chiao and Peterson, 1953). SeMet is also more oxygen-sensitive than Met, although the rates of oxidation and the role of promoters and inhibitors in oxidation remain to be determined. Methionine selenoxide, $CH_3-Se(O)-CH_2CH_2CH(NH)_2-COOH$, is formed on reaction of SeMet with hydrogen peroxide (Walter *et al.*, 1973). The oxidation also occurs efficiently with peroxynitrite (Assmann *et al.*, 1998). Whereas methionine sulfoxide is difficult to reduce to Met under physiological conditions, methionine selenoxide is easily converted back to SeMet on reaction with reducing agents such as glutathione (GSH), leading to the suggestion that SeMet acts catalytically as a cellular antioxidant (Walter *et al.*, 1973; Arteel *et al.*, 1999).

III. METHODS OF ANALYSIS

Pure SeMet may be distinguished from Met by its infrared spectrum (Shepherd and Huber, 1969). Identification of SeMet in protein hydrolysates is possible by means of standard amino acid analyzers (Sliwkowski, 1984). SeMet elutes near to, or with, leucine. Major factors affecting the resolution of these two amino acids are temperature and pH.

Identification of SeMet after being separated from other amino acids by means of paper chromatography and electrophoresis is facilitated by spraying spots with H_2O_2 or preferably by exposure to cyanogen bromide (Shepherd and Huber, 1969). Pre-derivatization of SeMet through reaction with *o*-benzoquinone facilitates separation and identification in the presence of Met. Both SeMet and Met react with *o*-benzoquinone at pH 1 to form phenolic sulfonium- or selenonium derivatives whose UV absorption spectra differ.

At pH 2, only Met reacts, allowing a distinction between the two compounds (Raju *et al.*, 1981). Reversed-phase high-performance liquid chromatography (RP-HPLC) involving post-column-derivatization with *o*-phthalaldehyde (OPA) was employed for SeMet determinations in high-Se yeast (Schrauzer, 1998a,b). For a description of state-of-the-art ion-exchange HPLC, ion-pair HPLC and RP-HPLC methods of analysis, see Bird *et al.* (1997a,b). For determinations by gas chromatography with inductively-coupled mass spectrometry (GC-ICP-MS), the carboxylic group of SeMet is esterified using propan-2-ol, followed by the acylation of the amino group with trifluoroacetic acid anhydride. Alternatively, the esterification and acylation of SeMet can be achieved in one step by a reaction with ethyl chloroformate–ethanol catalyzed by pyridine (Vasquez-Pelaez *et al.*, 2000). A method was described (Montes-Bayon *et al.*, 2001), allowing the detection of L- and D-SeMet by reversed-phase liquid chromatography following chiral derivatization with 1-fluoro-2,4,-dinitrophenyl-5-L-alanine amide (Marfey's Reagent), and inductively-coupled plasma spectrometry (ICP-MS) for detection.

Reliable enzymatic assays for SeMet are not available as specific SeMet metabolizing enzymes have not been identified and enzymes such as glutamine transaminase react with Met equally as well as with SeMet (Blazon *et al.*, 1994). However, with some enzymes reaction rates for SeMet and Met differ sufficiently to be of some use in SeMet analysis. For example, SeMet is a better substrate than Met for the α,γ-elimination by L-methionine γ-lyase of *Pseudomonas putida* (Esaki *et al.*, 1979). The adenosyl methionine transferase from rat liver reacts with L-SeMet at 51% of the rate with L-Met, and with the corresponding D-isomers at only 13 and 10% of the rate of L-Met (Pan and Tarver, 1967). Other adenosyl methionine transferases, such as that from yeast, react with SeMet more rapidly and with higher stereoselectivity than with Met, providing an indirect means for SeMet determination (Mudd and Cantoni, 1957; Sliwkowski, 1984; Uzar and Michaelis, 1994).

Indirect methods for SeMet determination utilize the facile reaction of SeMet with cyanogen bromide. The reaction of SeMet with BrCN yields CH_3SeCN, which can be measured either via gas chromatography (Zheng and Wu, 1988) or determined chemically (Zheng *et al.*, 1989). Applying this method to blood and grain, SeMet recoveries of 92.3–96.7% were achieved (Yang *et al.*, 1997a,b).

Use of appropriate methods of protein hydrolysis is of key importance irrespective of the method of SeMet determination used. Conditions must be chosen to achieve complete protein hydrolysis with minimal concomitant destruction of SeMet. In one study (Sliwkowski, 1984), SeMet recovery was 60–70% after heating the protein (thiolase from *C. kluyveri*) for 40 min at 155°C in 3 M mercaptoethanesulfonic acid. Recoveries of SeMet ranging

from 55 to 85% were obtained using 6N HCl under anaerobic conditions or pronase and prolidase for yeast or wheat protein hydrolysis (Beilstein and Whanger, 1986). SeMet recoveries of >90% are routinely accomplished with performic acid followed by the reduction of oxidized SeMet (Schrauzer, 1998a,b). The sample is first oxidized with performic acid followed by addition of HBr to remove excess performic acid. Performic acid oxidation converts SeMet to the selenium analog of a sulfone, which is more stable than SeMet during the subsequent 4 h hydrolysis/reduction step in 6N HCl and ascorbic acid addition at 145°C in a N_2-purged, sealed ampoule. SeMet is determined by RP-HPLC using post-column-derivatization with OPA. Primary nonaryl amines yield a strongly fluorescent derivative with OPA and 2-mercaptoethanol at pH 9 (borate buffer). Monitoring of the eluted peaks using UV prior to OPA treatment is used to augment the OPA-fluorescence detection.

IV. SYNTHESIS

Early syntheses of SeMet were tedious, non-stereospecific or limited to small-scale preparations. Klosterman and Painter (1947), for example, first reacted 5-(β-bromoethyl)-hydantoin with benzyl selenol to yield γ-benzylselenohomocysteine. The latter was converted to the sodium salt of DL-selenohomocysteine with sodium in liquid ammonia, and reacted with methyl iodide to yield DL-SeMet. Plieninger (1950) obtained DL-SeMet by the reaction of sodium selenomethyl mercaptide with α-amino-γ-butyrolactone in an inert solvent at 170°C. A synthesis of DL-SeMet from acrolein was also described (Zdansky, 1968). The first stereospecific synthesis of L-SeMet via esters of tosylated homoserine was reported by Pande *et al.* (1970).

In the newest method of synthesis, which allows the large-scale production of SeMet, L-Met is first converted to the methyl-methionium derivative, which is hydrolyzed to homoserine and converted to α-amino-γ-butyrolactone. The latter is reacted with HBr to 2-amino-4-bromobutanoic acid, from which L-SeMet is obtained on reaction with $LiSeCH_3$ (Koch and Burchardt, 1993; Krief *et al.*, 1994).

V. NATURAL OCCURRENCE AND BIOSYNTHESIS

A. PLANTS

Although Se is not known to be required for the growth of plants, most assimilate it when grown on seleniferous soils, or in Se-containing

hydroponic growth media. The degree of Se uptake is species-dependent. Primary Se-accumulators such as some species of *Astragalus*, *Stanleya*, and *Morinda* may, in high-Se regions, reach Se contents from hundreds to thousands of μg of Se/kg dry weight (Terry *et al.*, 2000). The tolerance of these plants to Se is attributed to their ability to convert Se into compounds that are not incorporated into the plant proteins and which, therefore, do not interfere with plant growth and metabolism. Detected Se compounds include SeMethylselenocysteine, selenocystathionine and γ-glutaminyl-SeMethylselenocysteine. Secondary Se-accumulators, such as cereals and forage crops, convert Se predominantly into SeMet. On normal soils, their Se contents range from 0.01 to 1 mg Se/kg dry weight, but levels toxic to foraging animals of up to 63 and 180 mg Se/kg dry weight were observed in wheat grown in high-Se areas of Canada and Columbia, respectively (Benavides and Silva, 1973). Selenate was sublethal to wheat seedlings when grown under experimental conditions in Keyport clay loam at levels of 15 to about 30 ppm Se, giving rise to a snow-white chlorosis, which, in sand cultures, is accompanied by a pink coloration (Hurd-Karrer, 1934). However, the toxicity of selenium to plants cannot be stated accurately as it is determined by the amount of sulfur available: whereas wheat seedlings in water cultures in the absence of added sulfate suffer distinct injury at selenium concentrations as low as 0.1 ppm, 18 ppm of Se as selenate was required to produce visible injury at sulfate-sulfur levels of 192 ppm (Hurd-Karrer, 1934). In mature secondary Se-accumulator plants, the SeMet is stored mainly in the grain and the roots, while smaller amounts are found in the stems and leaves (Beath, 1937). In plant tissue of the grassland legume species *Melilotus indica* L., for example, SeMet content increased with increasing soil Se concentrations until it accounted for more than 50% of the total selenoamino acids of the plant. In seleniferous corn, wheat and soybeans, SeMet contents ranged from 81 to 82% of the total Se (Yang *et al.*, 1997a,b). The biosynthesis of SeMet proceeds in analogy to that of Met via selenocysteine and selenocystathione according to Figure 1. The accumulation of SeMet in the protein of seleniferous plants is possible because $tRNA^{Met}$ does not discriminate between SeMet and its sulfur analog, Met; thus allowing its non-specific incorporation into proteins (Burnell, 1981; Eustice *et al.*, 1981). In contrast, replacement of cysteine (Cys) by selenocysteine (SeCys) would alter protein structure and does not occur to any significant extent. Moreover, since SeCys does not charge $tRNA^{Cys}$, the SeCys content of plants increases only moderately with increasing soil Se (from 5.07 to 22.02 mg/kg) and soon reaches a plateau. Other selenoamino acids, namely methylselenocysteine and γ-glutamyl-SeMethylselenocysteine, also remain at relatively low levels in secondary Se-accumulator plants, irrespective of the soil Se content (Guo and Wu, 1998). Plant-derived SeMet was detected in μg/kg levels in soil

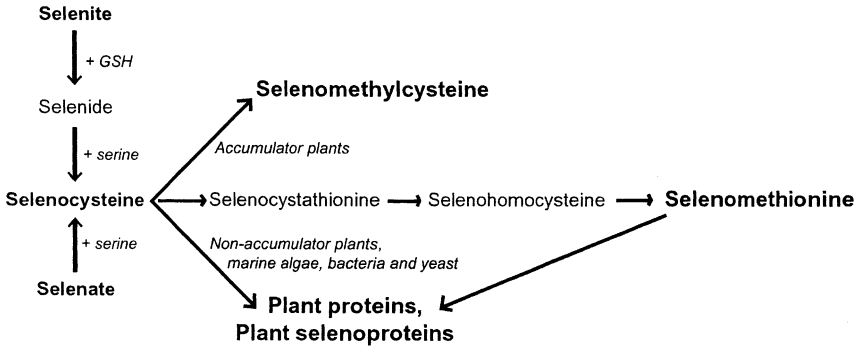


FIG. 1 Biosynthesis of selenomethionine; adapted from Marschner (1995); Schrauzer (2000).

extracts, indicating considerable resistance to further degradation, and suggesting that it could be an important source of plant-available Se (Abrams and Burau, 1989). Residual SeMet in soils could play a role as a dormancy-breaking agent for certain plants (Barros and De Paula-Freitas, 2001). The direct assimilation of SeMet by higher plants such as Indian mustard (*Brassica junctea*) has been demonstrated (Zayed *et al.*, 1998). SeMet in certain plants is methylated further to SeMethylselenonio-methionine (SeMethylSeMet) and cleaved to the volatile dimethylselenide (DMSe) (Figure 2). The latter may also be produced from dimethylselenoniopropionate (DMSeP), formed from SeMethylSeMet by decarboxylation, transamination and betaine aldehyde dehydrogenase.

The ability of plants to degrade SeMet to the DMSe is strongly species-dependent and represents a detoxification mechanism. However, Se volatilization may also result in losses of Se from food crops, aggravating Se deficiency in low-Se regions. Rice, broccoli and cabbage volatilized at the highest rates (200–350 μg Se/m^2 leaf area per day); and sugar beet, bean, lettuce and onion volatilized at lowest rates (Terry *et al.*, 1992).

B. MARINE PHYTOPLANKTON

Marine algae also convert Se predominantly into SeMet (Bottino *et al.*, 1984), Se uptake is dependent on the Se/S ratio in the medium and on the chemical form supplied. *Chlorella vulgaris* and *Chaetoceros calcitrans*, for example, take up Se(IV) preferentially over Se(VI). Some of the organoselenium compounds produced by these algae are not incorporated into cellular proteins but are extruded into the culture medium (Hu *et al.*, 1997). *Chlorella* also takes up SeMet; and its incorporation is antagonized by Met (Shrift, 1954a,b).

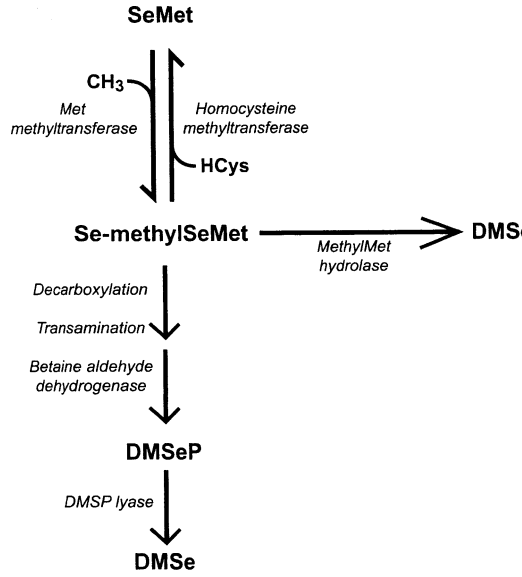


FIG. 2 Production of dimethylselenide (DMSe) from SeMet in plants via dimethylselenoniopropionate (DMSeP), adapted after Terry *et al.* (2000).

Spirulina incorporates Se when grown in Se-containing media. The resulting Se-rich spirulina contained the majority of the Se in the form of SeMet in two selenoproteins of 20–30 and 80 kDa. However, compared to the free SeMet and selenite, spirulina Se was less effective in restoring tissue Se levels and glutathione peroxidase (GSH-Px) activities (Cases *et al.*, 2001). The growth of spirulina in media containing 70–80 mg Se/l as SeO_2 was affected by the S:Se ratio and optimal at S : Se = 3 : 1. The Se content of biomass was 390 mg/kg at the Se concentration of 80 mg/l (as SeO_2) and the lowest sulfur concentration of 28 mg/l (Gradova *et al.*, 2001).

C. YEAST

S. cerevisiae converts inorganic Se to SeMet and incorporates it into the cellular protein in place of Met. The biosynthesis of SeMet proceeds via SeCys in analogy to that of Met, as was demonstrated in a study of a mutant strain of yeast requiring Met for growth due to a lack of homocysteine methyl transferase activity. When grown in Se-containing media, this strain produces SeCys, but no SeMet (Mason, 1994). While most of the SeCys is synthesized without involving Se-specific enzymes, recent studies indicate that some of the SeCys is also produced by a specific tRNA and incorporated into a 25 kDa

cytosolic protein. This protein migrates with the same velocity as the GSH-Px subunit isolated from rat liver and exhibits GSH-Px activity (Kyriakopoulos *et al.*, 1998). With currently available strains of *S. cerevisiae*, Se contents of up to 3000 ppm can be reached, at which level approximately half of the normally present cellular Met is replaced by SeMet. Commercial Se yeasts typically contain from 500 to 2000 ppm Se (Korhola *et al.*, 1986; Schrauzer, 1998a,b; Demirci *et al.*, 1999; Suhajda *et al.*, 2000). The Se is present predominantly (94 ± 5%) in the form of protein-bound SeMet (Schrauzer, 1998a,b). Other Se compounds include, in percentage of total Se: SeCys (0.5%), selenocystathionine (0.5%), methylselenocysteine (0.5%), γ-glutamyl-SeMethylselenocysteine (0.5%), Se-adenosyl-selenohomocysteine (2–5%) and inorganic Se (1%) (Schrauzer, 2000), bringing the Se balance in terms of identified Se compounds to 100 ± 5%. Previously reported SeMet contents of Se yeast were usually lower, largely, however, because of analytical problems and not because of differences in the actual SeMet contents. Lower than actual SeMet contents may result because of incomplete yeast protein hydrolysis and losses of SeMet due to hydrolysis and oxidation during sample digestion. The publication of these data led some authors to suspect that Se yeast was an unpredictable source of SeMet and was poorly characterized. Because Se yeast was used as a source of supplemental Se in the cancer prevention trial by Clark *et al.* (1996), the results of this trial were also called into question (Ip, 1998; Whanger, 2002). Analyses of water extracts of Se yeast (Bird *et al.*, 1997a,b) were initially interpreted similarly to suggest that the Se yeast contained only about 23% of the total Se in the form of SeMet, along with more than 20 unidentified Se-compounds (Ip, 1998; Whanger, 2002). However, water extracts of Se yeast analyzed contain only a fraction of the total Se (less than 10%), as most of the SeMet in Se yeast is protein-bound and insoluble in water. Accordingly, these results do not reflect the composition of Se yeast as a whole. Korhola *et al.* (1986) previously reported Se yeast to contain 50% of the total Se in the form of SeMet. However, even though the yeast in this study was hydrolyzed with acid under relatively vigorous conditions, the presence of unidentified Se-containing peaks in the chromatograms indicates that protein hydrolysis was incomplete. Beilstein and Whanger (1986) hydrolyzed Se yeast in 6N HCl in an inert atmosphere and achieved SeMet recoveries of 55–85%. Assuming an average recovery of 70%, they calculated the SeMet content of Se yeast to 93% of the total Se present, in agreement with the result of Schrauzer (1998a, b), who used performic acid for protein hydrolysis followed by the reduction of oxidized SeMet back to SeMet (see Section III). Oxidation of SeMet may occur during the enzymatic hydrolysis of Se yeast and may lower the SeMet recoveries. Thus, HPLC analysis of Se yeast after digestion with β-glucosidase revealed SeMet and what appeared to be SeMet oxide as the two

major Se compounds present (Larsen *et al.*, 2001). After Se yeast protein digestion with Protease XIV (Sigma, St. Louis), SeMet contents (85% of total Se) were obtained (Ip *et al.*, 2000). While Se yeast is generally a reliable source of SeMet, a cautionary note is necessary inasmuch as 'inorganic' Se yeast preparations containing only selenite or selenate are also marketed, often under the same name. The need for distinguishing between the two types has been emphasized (Schrauzer and McGinness, 1979; Gössler *et al.*, 1999; Schrauzer, 2001).

VI. SELENOMETHIONINE-CONTAINING PROTEINS AND ENZYMES

A. EFFECTS OF REPLACEMENT OF METHIONINE BY SELENOMETHIONINE

The replacement of Met by SeMet as a rule does not alter the protein structure, as evidenced from X-ray crystallographic studies of SeMet-substituted proteins. However, the presence of SeMet in place of Met may cause changes of contacts nearest to SeMet, altering the space group, as was recently demonstrated with oritidine 5′-monophosphate decarboxylase (Poulsen *et al.*, 2001). Since the CH_3-Se group of SeMet is more hydrophobic than the CH_3-S-moiety of Met, the kinetic reactivity of some enzymes changes if the replacement occurs in the vicinity of the active site. The SeMet-substituted thymidylate synthase of *E. coli*, for example, exhibited a 40% higher specific activity than the normal enzyme (Boles *et al.*, 1991). The SeMet-substituted phosphomannose isomerase from *C. albicans*, which normally contains four Met residues in the vicinity of the active site, had a four-fold higher K_M and a similarly increased inhibition constant for zinc ion, suggesting interference of substrate access due to the SeMet (Bernard *et al.*, 1995). In β-galactosidase of *E. coli*, replacement of more than half of the 150 Met residues by SeMet resulted in inactivity of this enzyme (Huber and Criddle, 1967). Compared to the normal enzyme, the thermal stability of the SeMet-substituted thymidylate synthase of *E. coli* was lowered eight-fold and its sensitivity to dissolved oxygen was significantly enhanced (Boles *et al.*, 1991).

The excessive replacement of Met by SeMet lowers the protein stability *in vitro*, but not necessarily *in vivo*. At the low levels of SeMet normally present in culture media, the substitution of Met by the more oxygen-sensitive SeMet is unlikely to affect the properties of enzymes in an adverse manner. Moreover, since methionine selenoxide is readily converted back to SeMet on reaction with biogenic thiols such as GSH, a mechanism of repair of this type of oxidative damage is available. The reversibility of SeMet oxidation in the

presence of GSH led to the suggestion that SeMet has the potential of being catalytically active as an antioxidant (Walter *et al.*, 1973), specifically against peroxynitrite (Arteel *et al.*, 1999). Since SeMet has radioprotective properties *in vitro* (Shimazu and Tappel, 1964), its presence in proteins could also directly or indirectly increase the resistance of cells to high-energy radiation UV light *in vivo*. Topical SeMet protects against UV light induced skin damage and skin cancer in mice (Burke *et al.*, 1992a,b).

B. SELENOMETHIONINE IN ORGANS AND TISSUES

In rats supplemented with Se in the form of sodium selenite, only SeCys, but no SeMet, was detected (Olson and Palmer, 1976). Since higher animals and man are unable to synthesize SeMet, any detectable amount in their organs and tissues must arise only from dietary sources. SeMet is incorporated into tissue proteins in place of Met (Martin and Hurlbut, 1976; Deagan *et al.*, 1987), especially in the skeletal muscles, the liver and the testes. The skeletal muscles are major Se-storage organs, accounting for about 46.9% of the total Se in the human body (Oster *et al.*, 1988). Se contents of skeletal muscle from Japanese adults were the highest (1700 ng/g), followed by those of Canadians (370 ng/g) and Americans (240 ng/g). Low-Se values (61 ng/g) were observed in skeletal muscle of New Zealand adults, reflecting their generally low-Se (and SeMet) intakes. Since erythrocytes incorporate SeMet mainly into hemoglobin (Waschulewski and Sunde, 1988), and in plasma SeMet is found primarily in the albumin fraction, Se levels in human blood also reflect the dietary SeMet intakes. In two samples of blood from Chinese men residing in a low-Se region, SeMet contents of 28.3 and 53.4 ng/g were observed, corresponding to 20–30% of the total Se (Yang *et al.*, 1997a,b). While the albumin of Chinese men with low-Se status contained 20% of the total Se, this percentage increased to 47 ± 5% in the albumin of men residing in a high-Se region. Their main dietary sources of SeMet were locally grown corn and rice and their average plasma Se level was 494 ng/ml (Beilstein *et al.*, 1992; Whanger and Butler, 1994). The same plasma Se concentration within experimental error (517 ± 84 ng/ml), and the identical percentage of plasma Se distribution, were observed in a study with Rhesus monkeys (*Macaca mulatta*) after they had been receiving 0.5 mg Se/l as SeMet in the drinking water during the first month, and 0.25 mg Se/l during the following 11 months (Butler *et al.*, 1990). In the livers, erythrocytes, hair and muscle of these animals, the Se concentrations were 3-, 5-, 7- and 11 times higher than in the corresponding organs of animals exposed to the equivalent amount of selenite Se. However, GSH-Px-activities in the plasma and in the erythrocytes were not different between the two treatment groups, re-affirming that SeMet is incorporated into protein to a higher extent than selenite. Similarly, in rats

fed a basal Se-deficient diet containing 2 ppm Se as SeMet, the level of Se in the muscle was 10 times that of the amount in rats receiving the equivalent amount of selenite or selenocysteine. In the liver, kidney and testes, the increase was 1.3–3.6-fold (Deagen *et al.*, 1987). In another study (Shearer, 1975), the muscle (thigh) of pregnant rats and of their pups contained approximately twice the amount of Se than the muscle of other animals when exposed to Se from the ninth day of pregnancy to the day of parturition to 0.2 ppm of SeMet in the drinking water (with some additional ^{75}Se–SeMet), than with the equivalent amount of selenite. The pup/mother ratio of Se contents of organs ranged mostly from 0.8 to 0.9, but was substantially higher for the teeth (molars: 7.71; incisors: 1.64) and bone (1.91). The high level of Se in the molars of the pups exposed to SeMet *in uteri* indicates that the incorporation of Se occurred endogenously during tooth development. In growing pigs, SeMet supplied in the form of Se yeast at the level of 0.3 ppm Se was about twice as effective as selenite in increasing the Se content of the loin muscle. SeMet in yeast also raised Se levels in porcine serum and liver significantly more than inorganic Se (Suomi and Alaviuhkola, 1992). Hair and fingernails of human subjects supplemented with SeMet contained a higher percentage of alkali-extractable Se than those receiving the same amount of Se in the form of selenate, indicating that SeMet is transported, deposited and metabolized differently than selenate (Whanger and Butler, 1994).

C. ORGAN DISTRIBUTION AFTER INJECTION

Injected ^{75}Se–SeMet produced high levels of radioactivity in the pancreas, the liver, the kidneys, in the stomach and the gastrointestinal mucosa. Lower but significant activities were observed in the salivary gland, the seminal vesicles, the mammary glands and bone marrow; the distribution pattern was similar to that observed with ^{35}S-methionine and ^{14}C-phenylalanine (Hansson and Jacobsson, 1966), indicating that SeMet is incorporated into organs with high rates of protein synthesis. Injection of the same amount of ^{75}Se in the forms of selenate or selenite resulted in much lower radioactivities in the respective organs as inorganic Se is converted only to SeCys in specific selenoproteins and is not incorporated into protein in place of Cys. The disappearance of intraperitoneally injected SeMet Se in tissues was slower than that of Se injected as selenite or selenate, especially from the brain. For example, in rats, ^{75}Se from ^{75}Se-sodium selenite disappeared from the cerebellum, the cerebral hemisphere, and the spinal cord very rapidly, 70% within 48 h, followed by a slower phase. The $t_{1/2}$ of Se disappearance was about 12 days for the cerebellum and the cerebral hemisphere, and 13 days from the spinal cord. Of an equivalent dose of ^{75}Se–SeMet, 20–30% disappeared during the first

48 h, the remainder with a $t_{1/2}$ of 25 ± 4 days from cerebellum and the cerebral hemisphere, and 29 ± 4 days from the spinal cord (Grønbaek and Thorlacius-Ussing, 1992). The high affinity of SeMet for the brain was also observed in rats after oral administration, suggesting that SeMet behaves like an active form of Se for incorporation in brain tissue. Selenomethionine was thus judged to serve as a better chemical form for Se-supplementation than either selenite or selenate (Wang *et al.*, 1992).

D. UPTAKE, RETENTION AND EXCRETION

Ingested SeMet is absorbed in the small intestine. The absorption occurs via the Na^+-dependent neutral amino acid transport system; and Met and other amino acids mutually inhibit SeMet absorption (Wolffram *et al.*, 1989; Vendeland *et al.*, 1994). The whole-body turnover rate of SeMet is slower than that of selenite-Se, indicating that the SeMet is incorporated into a long-term body pool. The reported average whole-body half-lives of SeMet and selenite in humans are 252 and 102 days, respectively, indicating that SeMet is extensively utilized and re-utilized (Patterson *et al.*, 1989; Swanson *et al.*, 1991). In a study with New Zealand women with low-Se status (Griffiths *et al.*, 1976), the intestinal absorption of a small oral dose of ^{75}Se–SeMet of approximately 20 mCi containing <2 μg Se was 95.5–97.3%. Approximately, 3% of this dose was excreted with the urine during the first day and 6–9% during the first 2 weeks. Urinary excretion of ^{75}Se continued to decrease gradually and at 44 weeks of the trial was about 0.08% dose/day. SeMet–Se was steadily incorporated into erythrocytes during a period of 8–12 weeks, with some radioactivity still persisting at 36 weeks. Plasma Se reached a maximum 3–4 h after administration and about 4–8 h sooner than after the administration of an equivalent dose of selenite. The urinary excretion rate of selenite was approximately twice that of SeMet and the fecal excretion of selenite Se was also faster initially, reaching 1.3–1.7 % of the dose in the first 14 days and dropping to less than 0.1% by weeks 10–19. After a single large oral dose of SeMet corresponding to 1 mg Se, 5–22% of dose was eliminated in 24 h in the urine and 3.5% in the feces. During the same period, 41–85% of the equivalent dose of selenite was excreted in the urine, and 11–13% with the feces. The total recovery of selenite reached 82–95% of the ingested dose, and 26% for SeMet (Thomson *et al.*, 1978). On continuing the supplementation for 11 weeks at 0.1 mg Se/day, SeMet caused a steady increase in blood Se from 0.08 to 0.18 μg Se/ml, the rate of increase being 0.009 μg Se/ml per week. The equivalent dose of selenite increased the blood Se more slowly until a plateau was reached after 7–8 weeks at 0.11 μg/ml. SeMet increased plasma Se more rapidly than erythrocyte Se, from 0.073 to 0.13 μg/ml, causing plasma Se to reach a higher concentration

than erythrocyte Se during the first two weeks of supplementation (Robinson *et al.*, 1978). SeMet is incorporated nonspecifically into albumin and becomes a part of the methionine pool (Burk *et al.*, 2001). Although the rates at which steady-state conditions are reached vary for different organs, protein turnover prevents accumulation of SeMet to toxic levels in the organism, causing blood Se levels to be linearly dependent on dietary Se intake over a wide range of intakes (Schrauzer and White, 1978; Yang *et al.*, 1989a). Similarly, hair, toenail and fingernail Se levels are proportional to the (long-term average) dietary Se intakes (Yang *et al.*, 1989a,b).

VII. METABOLISM OF SELENOMETHIONINE

A. ENZYMATIC DEGRADATION

SeMet is metabolized along with Met by the same enzymes and at similar rates until SeCys is formed. SeMet metabolism is rapid, as evidenced from the similar rates of appearance and disappearance of reactive Se metabolites in rat serum after oral administration of SeMet or selenite (Wang *et al.*, 1992). In contrast to Cys, which can be re-utilized for protein synthesis, SeCys is not appreciably incorporated into protein but further degraded in the liver to selenide. The latter is converted either to selenophosphate and used for the synthesis of SeCys-containing selenoproteins by specific tRNAs, or methylated to DMSe or the trimethylselenonium ion (TMSe) and excreted (Figure 3). Studies with rats and mice also demonstrated that SeMet is directly degraded by the action of α,γ-lyases in the liver to methylselenol (CH_3SeH), which is then converted to TMSe (Nakamuro *et al.*, 1997; Okuno *et al.*, 2001). The presence of the SeMet α,γ-lyases in the liver suggests that these serve primarily for SeMet detoxification. The adenoviral introduction of a bacterial α,γ-lyase gene to achieve this mode of SeMet degradation in tumor cells has been suggested as an approach to cancer gene therapy into tumor (Miki *et al.*, 2001).

Glutamine transaminase from bovine liver, one of the enzymes involved in methionine catabolism, utilizes SeMet as well as methionine (Blazon *et al.*, 1994). However, with some enzymes, differences in the reaction rates for SeMet and Met have been observed. For example, SeMet is a better substrate than Met for the α,γ-elimination by L-methionine γ-lyase of *Pseudomonas putida* (Esaki *et al.*, 1979). The adenosyltransferase from rat liver reacts with L(+)-SeMet at 51% of the rate with L(+)-Met, and with the corresponding D(−) isomers at only 13 and 10% of the rate of L-Met (Pan and Tarver, 1967). The adenosyl transferase from yeast, on the other hand, is more active with SeMet than with Met (Mudd and Cantoni, 1957). This enzyme produces the

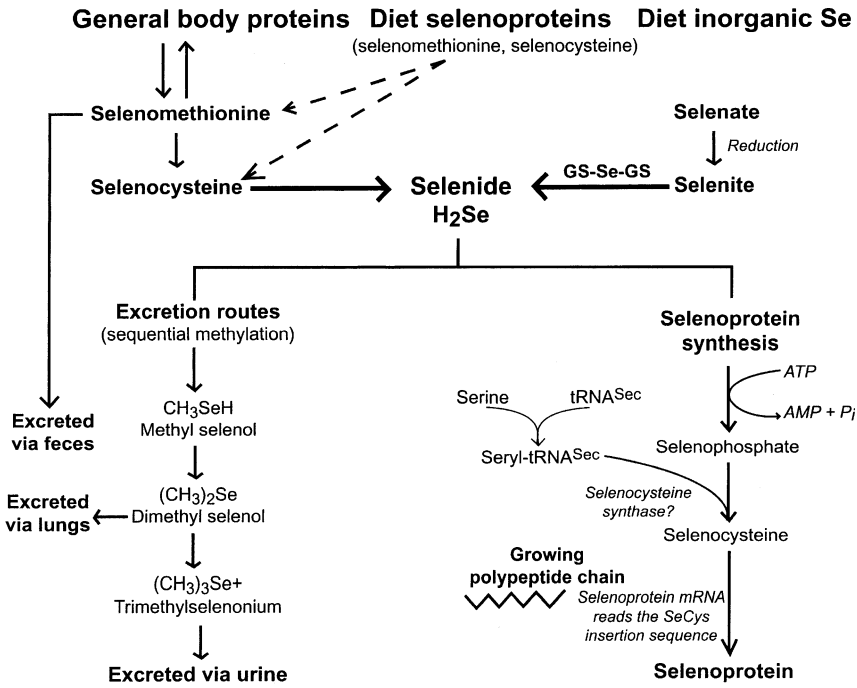


FIG. 3 Routes of selenium metabolism in animals; adapted from Jacques (2001), after Schrauzer (2000); Low and Berry (1996), and Daniels (1996).

adenosyl derivative of Met with low stereoselectivity due to the isomerization of Met during the incubation, while the corresponding formation of the Se-derivative occurs with >99% stereoselectivity (Uzar and Michaelis, 1994). SeMet neither blocks the metabolism of Met, nor the synthesis of DNA, RNA, or of proteins when added to the culture medium. Low SeMet levels (10 μM) replace Met and support cell growth in the absence of Met (Kajander *et al.*, 1991). In studies with human lymphoblast cells from subjects with trans-sulfuration defects, SeMet was poorly metabolized, as evidenced by the significantly higher levels required to induce GSH-Px activity, although these cells utilized selenite and selenocystine as well as normal cells (Beilstein and Whanger, 1992).

B. UTILIZATION FOR GLUTATHIONE PEROXIDASE SYNTHESIS

Glutathione peroxidases (GSH-Px) are enzymes catalyzing the reduction of hydrogen peroxide to water, and of lipid hydroperoxides to alcohols, with

GSH as the reductant. As GSH-Px was the first selenoenzyme to become known (Rotruck *et al.*, 1973), quantitative assessments of the bioavailabilities of different chemical forms of Se were initially based almost exclusively on the determination of the minimum amount of the Se compound required for the induction of GSH-Px in serum, erythrocytes, or liver. In interpreting the results of such determinations, it must be considered that they depend on the experimental conditions, the test animal and the diets chosen. In rats, for example, SeMet was as active as selenite in protecting against the development of liver necrosis. In chicks, SeMet was four times more active than selenite in protecting against pancreatic fibrosis, but was less effective than selenite in the prevention of exudative diathesis (Cantor *et al.*, 1975a; Mutanen, 1986). While liver GSH-Px activities induced by SeMet were slightly lower relative to selenite in the above-mentioned experiments, they were the same in the livers of nursing rat dams maintained on a low-Se diet to which 0.15 μg Se/g was added as either SeMet or as sodium selenite, and higher in the livers, kidneys and eyes of 14-day-old nursing pups from SeMet-supplemented dams than in pups of the same age from dams receiving Se as selenite. GSH-Px activities in the hearts were the same in both groups (Lane *et al.*, 1991). In Se-deficient heifers, supplementation with SeMet or Se yeast almost doubled the GSH-Px activity as compared to the equivalent amount of inorganic Se (Pehrson *et al.*, 1989). When ^{75}Se–SeMet was fed to ewes, the milk produced contained more Se available to the pre-ruminant lamb than milk from ewes receiving the equivalent amount of ^{75}Se in the form of selenite (Jenkins and Hidiroglou, 1971). The release of SeMet from body proteins during Se depletion causes SeMet-supplemented animals to maintain higher activities of selenoenzymes for longer periods than in animals supplemented with selenite. Thus, in SeMet- or Se yeast-supplemented mice, liver GSH-Px activities declined more slowly during the 7th to the 11th week of Se depletion than in mice that had previously received the equivalent amount of Se as sodium selenite (Spallholz and Rafferty, 1987). In nursing mothers, SeMet supplied as such or in Se yeast prevented the decline of plasma Se and GSH-Px activity, as well as the decline of Se in milk during lactation. In addition, significantly more Se appeared in the milk of mothers receiving SeMet than selenite (McGuire *et al.*, 1993; Alaejos and Romero, 1995). SeMet was detected in human milk (Michalke and Schramel, 1997).

C. FACTORS INFLUENCING TISSUE DEPOSITION AND BIOAVAILABILITY

In the living organism' structural and functional proteins are continuously synthesized and degraded within hours to days (Mitch and Goldberg, 1996).

In general, functional proteins and enzymes have higher turnover rates than structural proteins. The protein turnover rates are also dependent on age and are the highest during early development. Protein synthesis and degradation occur in separate compartments. Proteins are synthesized in the RNA-rich ribosomes, while the degradation of structural proteins occurs notably in pathological states such as starvation or following injury involving lysosome-localized proteases such as the cathepsins. Since some cells lack lysosomes and some structural proteins are too large to be internalized, additional means of degradation are available, which include cytosolic proteases such as the calpains and those in organelles, called proteasomes (Klasing, 1998). During stress, the activity of proteasomes increases to provide amino acids needed for the formation of immune defense cells and enzymes, including the Se-dependent GSH-Px and the thioredoxin reductases. During such times food intake is reduced, requiring reserves of amino acids and minerals to be utilized to a higher degree than in health or under stress-free conditions. It is at this point that the presence of SeMet in proteins becomes especially important since the SeMet released during protein catabolism provides a source of Se needed for the synthesis of GSH-Px and other selenoenzymes (Figure 4). The SeMet in the free amino acid pool is either incorporated into body proteins or degraded (Figure 4). Whereas the incorporation of SeMet into proteins is reversible, allowing SeMet to be re-used, its degradation is irreversible. The incorporation of SeMet into proteins and its degradation are dependent on the Met content of the diet. In one study, the Se-contents of muscle and erythrocytes of Met-deficient, SeMet-supplemented rats were shown to be significantly higher than those in SeMet-supplemented, Met-adequate animals (Sunde *et al.*, 1981). In another study (Butler *et al.*, 1989), a direct correlation was observed between the dietary Met and the percentage of Se associated with GSH-Px. In adult Chinese men with low-Se status with adequate but presumably limiting dietary Met intakes, supplemental Met increased the RBC GSH-Px activity (Luo *et al.*, 1987), indicating that less SeMet was deposited in protein, increasing the amount of Se available for GSH-Px synthesis.

On supplementing SeMet, GSH-Px activities reach a maximum value and then plateau, while tissue Se levels continue to rise in proportion to the dosage (Deagen *et al.*, 1987). The release of Se from SeMet also depends on vitamin B_6 status. In rats maintained on a feed supplemented with 0.25 mg Se/kg of diet in the form of SeMet, GSH-Px activities in erythrocytes, muscle and heart were lower in vitamin B_6-deficient than in vitamin B_6-supplemented animals (Yin *et al.*, 1996). Vitamin B_6 also significantly reduced lipid peroxide contents in tissues. These results indicate that dietary vitamin B_6 is involved in the metabolic breakdown of SeMet.

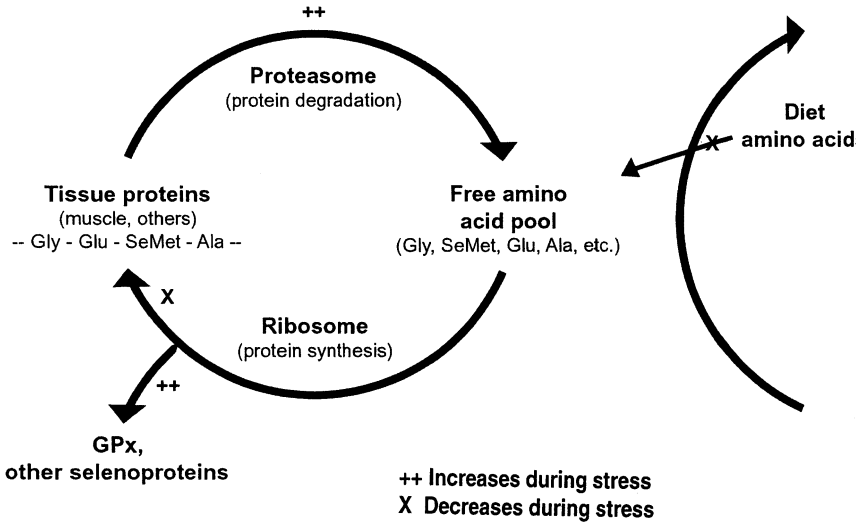


FIG. 4 Protein turnover releases stored selenomethionine (SeMet) to the free amino acid pool (Gly = glycine, Glu = glutamic acid, Ala = alanine). Adapted from Jacques, 2001.

D. SELENOMETHIONINE AND IMMUNE CELL FUNCTION

Selenium is well-known to be required for the maintenance of the functions of the immune system (McKenzie *et al.*, 2001). The immunomodulatory effects of Se depend on dosage and chemical form of Se administered. However, the required dosages of Se tend to be higher than the estimated minimum dietary requirements. In previous studies, supplementation of Se-replete subjects with 200 μg Se/day as sodium selenite resulted in increased T-lymphocyte proliferation and activity (Roy *et al.*, 1994). In gut failure patients on home parenteral nutrition, a daily parenteral dose of 200 μg Se in the form of L-SeMet elicited a significant increase of the antibody production against common mitogens (PWM and PHA) (Peretz *et al.*, 1994). There is also evidence that cells of the immune system discriminate between the different chemical forms of Se. Addition of SeMet to the medium of lymphocyte cultures, for example, produced no signs of cytotoxicity and increased the immunoglobulin G (IgG) production at levels of 0.5–2 μM, while selenite at equivalent concentrations decreased IgG production and exerted cytotoxic effects (Borella *et al.*, 1995). L-SeMet and sodium selenite also differ in their relative effectiveness in modulating GSH-Px activities of blood cells, as evidenced in a recent study with 45 healthy men and women from the UK

(Brown *et al.*, 2000). The enzyme activities measured include GSH-Px1, the cytosolic glutathione peroxidase; GSH-Px3, the extracellular glutathione peroxidase, and GSH-Px4, the phospholipid hydroperoxide GSH-Px. At a daily dose of 50 μg Se as SeMet, lymphocyte GSH-Px1 activities increased during the first two weeks of supplementation to an approximately 20% higher level than with the equivalent dose of selenite. The platelet GSH-Px1 activity also increased during the first two weeks of supplementation and more so with SeMet than with selenite, while no significant changes occurred in the placebo group. During the remaining two weeks of supplementation no further changes occurred in the SeMet and selenite groups, while a decline of the GSH-Px1 activities occurred in the placebo group. The granulocyte GSH-Px1 activity declined during the first 7 days of supplementation in the SeMet group, but subsequently increased during the following three weeks. In the selenite group, the granulocyte GSH-Px1 activities increased during the first week, declined to nearly the baseline value in the second week, only to increase again to the value measured after the first week in the following two weeks. The lymphocyte GSH-Px4 activities increased in the selenite-supplemented group during the first week of supplementation, and then declined during the following three weeks. In the SeMet-supplemented group, the lymphocyte GSH-Px4 activities increased slightly during the first week, declined during the second week, but after four weeks of supplementation, reached its maximum value. The granulocyte GSH-Px4 activities in the selenite group were also increased after the first week, lowered after the second, and increased again after the fourth week. With SeMet, the granulocyte GSH-Px4 activities increased during the first two weeks of supplementation and subsequently remained constant. Platelet GSH-Px4 activities increased in the selenite group during the first two weeks and subsequently declined. However, in the SeMet group, they remained essentially unchanged throughout the four weeks of supplementation. This was interpreted to suggest that immune cells take up and metabolize selenite-Se more rapidly than SeMet, and that the conversion of SeMet into reactive inorganic forms of Se may be an important regulator of Se bioavailability. The authors, furthermore, noted that the SeMet supplementation failed to increase plasma and erythrocyte GSH-Px activities in half of their subjects, which they attributed to possibly deficient dietary intakes of Met (Brown *et al.*, 2000).

VIII. SELENOMETHIONINE IN HUMAN SE SUPPLEMENTATION

The Se in SeMet was shown in numerous studies to be as bioavailable as naturally occuring nutritional sources of Se, and superior to inorganic Se in

improving the Se status of humans during lactation, in pregnancy and in infant nutrition (Schrauzer and White, 1978; Levander *et al.*, 1983; Kumpulainen *et al.*, 1985; Beilstein and Whanger, 1986; Korhola *et al.*, 1986; Spallholz and Rafferty, 1987; Vinson and Bose, 1987; Yang *et al.*, 1989a,b; McGuire *et al.*, 1993). Se yeast was used as the source of food-form Se in several human cancer prevention trials (Yu *et al.*, 1988; Clark *et al.*, 1996). The Clark study (Clark *et al.,* 1996) is especially relevant as it involved a daily administration of 200 μg of Se as Se yeast over a period of up to 7 years to conservatively treated nonmelanoma cancer patients. Whereas the Se-supplementation had no effect on skin cancer recurrence, it significantly reduced the incidences of cancers of prostate, lung, colon and rectum. At the dosage chosen, Se yeast was well tolerated. Mean serum Se levels at the steady state reached 190 ± 50 μg/l and did not result in accumulation to toxic levels. In a Chinese study (Yu *et al.*, 1988), a daily supplement of 200 μg Se as Se yeast provided to subjects at risk of developing primary liver cancer caused a substantial decline of liver cancer incidence within a year. Recently, synthetic SeMet was chosen as the source of Se in SELECT, the largest (prostate) cancer prevention study ever to be conducted (Klein *et al.*, 2001). Se yeast and SeMet were also tested in a number of small observational studies. In HIV-infected patients, supplemental SeMet (in yeast) produced symptomatic improvements (Olmsted *et al.*, 1988). Several studies are presently underway to further explore the effect of supplemental Se in HIV-infected patients (Baum *et al.*, 2001).

IX. SE YEAST AS AN ANIMAL FEED SUPPLEMENT

A. DAIRY COWS

Se yeast was shown to be superior to inorganic Se in improving the Se status of farm animals (Power, 1994; Lyons and Oldfield, 1996; Mahan and Kim, 1996; Hemken and Jacques, 1998; Ortman and Pehrson, 1999; Pavlata *et al.*, 2001). Since SeMet is synthesized by rumen bacteria from inorganic sources of Se (Hidiroglou *et al.*, 1968), selenite or selenate should improve the Se status of cattle equally as well as supplemental SeMet. However, the bacterial SeMet synthesis in the rumen is apparently either inefficient or the SeMet incorporated in the bacterial protein is poorly absorbed since, in dairy cows, SeMet supplied in yeast was more effective in increasing the concentration of Se in milk than added sodium selenite or -selenate. At feed Se contents of 0.21–0.31 mg/kg DM, milk Se reached 31.2 μg/l in the Se yeast group, but only 16.4 μg/l in the selenite and selenate groups (Ortman and Pehrson, 1999). Supplementation of dairy cows with Se yeast and vitamin E also

affected milk production, increasing it by 13.8% (Zemanova *et al.*, 2000). Previously, Conrad and Moxon (1979) showed that lactating cows transferred 19.8% of the Se in brewer's grains into the milk but only less than 4.8% of the Se supplied as selenite. Supplementation of nursing cows with Se yeast also improved the Se status of the calves more so than the equivalent amount of selenite (Mahan and Kim, 1996; Pehrson *et al.*, 1999), and improved the performance and meat quality in feedlot steers (Clyburn *et al.*, 2001).

Note added with proof: On Sept. 3, 2003, the selenium yeast product "Sel-Plex" (Alltech) was approved by the U.S. Food and Drug Administration as a source of selenium for beef and dairy cattle. See: Federal Register 68, No. 170.

B. PIGS

Addition of Se yeast to sow feed at 0.9 mg Se/sow/day starting three days prepartum increased the milk Se concentration 10-fold compared to controls (0.421 versus 0.046 mg Se/l). In 7-day-old piglets, the mean blood Se concentration was almost five times higher than in controls (0.221 versus 0.046 mg Se/l). In the experimental group, the weight gain of the piglets was higher and their mortality lower than in the controls. The Se yeast supplement also influenced the conception rate size and weight of the next litter (Kolacz *et al.*, 2001). Increasing the organic Se content of sow feed also has a vitamin E-sparing effect. Supplemental Se (0.2 ppm) in the form of Se yeast and vitamin E (20 IU) as DL-α-tocopherol acetate improved the litter size of sows; it also increased the number of piglets born alive compared to sows in the control group maintained on a diet with a Se content of only 0.1 ppm and a vitamin E content of 37 IU (Chen *et al.*, 2001). Se yeast (Sel-Plex™) fed to pigs at the FDA-approved dietary concentration of 0.3 ppm Se, alone or in combination with vitamin E (200 ppm) for 60 days prior to slaughter, decreased the metmyoglobin formation, especially in the psoas major muscle. In addition, the level of thiobarbituric acid-reactive substances in psoas major muscle was reduced after 7 days of carcass cool storage. A positive effect of Se plus vitamin E was also seen with respect to malondialdehyde formation in homogenates of longissimus dorsi muscle sampled after slaughter (Krska *et al.*, 2000).

C. POULTRY

SeMet as the source of Se restored appetite and liver GSH-Px activity in Se-deficient chicks more rapidly than selenite (Bunk and Combs, 1980) and was especially effective against pancreatic fibrosis (Cantor *et al.*, 1975b,c). In broiler chickens, Se yeast as the source of Se improved feathering rate in slow-feathering animals as compared to animals fed selenite or no Se. The Se

yeast also increased body weights and yields of leg and thigh meat and improved the efficiency of feed conversion. In addition, SeMet in yeast increased the stability of erythrocyte membranes, suggesting that this is the mechanism by which moisture retention is increased in the breast muscle from male broilers. Organic Se supplementation also improved the meat quality of broiler males and reduced drip loss from breast meat, while selenite Se appeared to increase drip loss. Furthermore broilers receiving yeast Se showed diminished levels of heat shock protein 70 (HSP 70) following challenge by enteropathogenic *E. coli*, or by acute heat stress (Edens, 2001). In June 2000, Se yeast (Sel-Plex™) was approved for broiler production in the United States (Anon, 2000) and approvals for other species are pending (Edens, 2001). Organic Se from yeast (Sel-Plex™) is readily transferred into the egg and is incorporated predominantly in egg albumin, while the Se from selenite and selenocysteine is deposited preferentially in the yolk (Latshaw and Osman, 1975; Latshaw and Biggert, 1981). Selenium supplementation has a sparing effect on vitamin E, causing the yolks of Se-supplemented hens to have significantly higher vitamin E contents (Surai, 2000), up to 27 mg per large egg compared to 0.70 mg in a regular egg (Slaugh, 2002). 'Designer eggs' with a high content of omega-3 fatty acids, obtained by feeding hens a 2% flax ration, require a higher vitamin E content than regular eggs to prevent the oxidation of the acids. Supplementing the diet of the layer hens with Se yeast (Sel-Plex™) prevented the oxidation of the omega-3 fatty acids and the loss of vitamin E of the eggs during a two-week storage period significantly more than the equivalent amount of Se as selenite. Supplementation of the feed with Se yeast also increased the duration of freshness of the eggs on storage (Wakebe, 1998). Addition of Se to the feed at the level of 0.3 ppm increased the albumin content of the eggs. Since SeMet is preferentially incorporated into albumin this may explain why the eggs from Se yeast-supplemented hens maintained better albumin quality on storage (Slaugh, 2002).

X. TOXICITY OF SELENOMETHIONINE

A. STORAGE AND HANDLING INSTRUCTIONS

Note: *Instructions were compiled from current Material Safety Data Sheets; for information only, not purported to be all-inclusive and to be used only as a guide.*

SeMet is classified as a very toxic substance. It should be stored in closed containers below 4°C in the freezer. SeMet should only be handled in a well-ventilated hood with rubber gloves, protective goggles and facial mask. To reduce the risk of accidental poisoning in the food and feed industry, pre-

mixes of SeMet rather than the pure substance should be used. First aid measures include immediately flushing the eyes with plenty of water for at least 15 min, occasionally lifting the upper and lower lids. Skin should be flushed with plenty of soap and water for at least 15 min while removing contaminated clothing and shoes. Upon accidental ingestion, do not induce vomiting. Allow the victim to rinse his mouth and then to drink 2–4 cupfuls of water and seek medical advice. On inhalation, remove victim from exposure to fresh air immediately. Exposure limits (NIOSH and OSHA), as Se: 0.2 mg/m^3.

B. ACUTE TOXICITY

As no human case of SeMet poisoning has been reported, all acute toxicity data for SeMet are derived from studies with animals. In general, the acute response to SeMet was delayed and not as severe as observed with selenite. Whereas sodium selenite, at 3.0 mg Se/kg, caused fatal selenium toxicosis in swine 2.5 h after intravenous injection, the equivalent amount of Se as SeMet was fatal after 14 h, with both compounds producing pulmonary edema as the main lesion (Herigstad *et al.*, 1973). The liver and kidney Se concentrations as determined by these authors reached 10.2 and 7.28 ppm in the animals injected with selenite, 11.3 and 10.8 ppm, in the animals given SeMet. The same authors showed that young pigs fed diets containing 60, 120 and 600 ppm of organic Se (SeMet) developed symptoms of acute toxicity after 125, 140 and 38 h on these feeds with a total of 49, 44 and 33 mg of Se being consumed. Liver Se levels reached 17.95, 34.5 and 27.4 ppm while kidney Se levels, 13,7, 11.1 and 12.8 ppm, respectively.

In rats, the LD_{50} of SeMet on intraperitoneal injection is 4.25 mg Se/kg body wt (Klug *et al.*, 1949). In mice, the LD_{50} of SeMet on intravenous and intracervical injection was determined to be 8.86 ± 1.38 and 5.24 ± 0.23 mg Se/kg, whereas the corresponding LD_{50} of sodium selenite was much lower, 2.28 ± 0.28 and 0.13 ± 0.04 mg Se/kg, respectively (Ammar and Couri, 1981).

C. CHRONIC TOXICITY

The chronic toxicity of SeMet is lower than that of sodium selenite, presumably because its incorporation into tissue proteins removes a portion from the circulatory system. The diminished toxicity of SeMet at dietary Se levels exceeding 5 ppm as compared to selenite was apparent in pigs in the grower–finisher period (Kim and Mahan, 2001a). In mature pigs, 5 ppm of Se as selenite as well as organic Se (SeMet in yeast) elicited similar toxic effects. However, in animals under conditions of high protein turnover such as during pregnancy, SeMet stored in tissues may re-enter circulation, resulting in increased toxicity as compared to selenite. High levels of organic Se also

affected gestation and parturition performance of sows more than the equivalent amount of Se as selenite. Especially at levels of >7 ppm Se, fewer piglets were born and pig weights at parturition and weaning were lower from sows fed organic Se than sodium selenite. This was attributed to the increased tissue turnover from the organic Se source (Kim and Mahan, 2001b). In addition, diminished placental transfer of inorganic as compared to organic Se could have been responsible, as evidenced by the lower tissue Se concentrations in the piglets from sows fed selenite compared to those sows fed SeMet. Nursing piglets from sows fed toxic levels of SeMet are at a further disadvantage compared to those from sows fed selenite due to the higher Se content of the milk from sows fed organic Se. In pigs fed SeMet as in Sel-Plex Se yeast, the higher Se content was evident already at the 0.3 ppm level. At the chronically toxic level of 7 ppm, the milk on day 14 reached 4.14 ppm compared to 0.76 ppm in the milk of animals receiving 7 ppm Se as sodium selenite. The greater deposition of SeMet into the organs of animals fed organic Se was evident at all levels between 0.3 and 10 ppm, and was particularly pronounced in the loin: at 10 ppm of SeMet Se in the feed, its Se content reached 5.33 ppm, compared to 0.23 ppm in the animals on selenite, corresponding to a factor of 23. Hair and hoofs of the animals fed SeMet contained 3.7 and 6.13 times more Se than of the animals on selenite, livers and kidneys 2–3 times more (Kim and Mahan, 2001b). In female mice, the injection of SeMet at 2.0 mg Se/kg body wt induced a transient decrease of the number of circulating leukocytes, which was more extensive and of longer duration than with selenate. The agranulocyte/granulocyte ratio also increased to a lesser degree with SeMet than with selenate (Hogan, 1998). The chronic toxicity of SeMet is lower than that of selenite. Rats fed high levels of Se (16 ppm) as SeMet (selenium yeast) for 8 weeks showed no signs of Se toxicity, while the same amount of Se as sodium selenite produced severe hepatotoxity, cardiotoxicity and splenomegaly (Spallholz and Rafferty, 1987). SeMet on oral administration did not produce toxic effects in rats at 0.5 and 1.0 mg SeMet (0.2 and 0.4 mg Se)/kg body wt/day in a 13-week study (NCI, 1993). However, in the same study, weight loss, decreased food consumption, liver abnormalities and toxic hepatitis occurred at 2, 3 and 4.5 mg SeMet (0.8, 1.2 and 1.8 mg Se)/kg body wt/day. Pancreatitis and atrophy or degeneration of pancreatic acini occurred at 0.8, 1.2 and 1.8 mg Se/kg body wt/day. At 1.2 and 1.8 mg Se/kg body wt/day, bile duct hyperplasia, telangiectasis, hemorrhage, necrosis, inflammation, vacuolar changes and brown pigment in hepatocytes, increased extramedullary hematopoiesis in livers and spleen were seen at 3 and 4.5 mg/kg body weight/day. Female rats were more sensitive than males with all of 10 females dead during 13 weeks, but only 1 male dead at 3 mg SeMet/kg body wt/day (1.2 mg Se/kg/day). In beagle dogs, no toxic effects of oral SeMet were

observed at 0.1 and 0.3 mg SeMet (0.04 and 0.12 mg Se)/kg body wt/day. At 1.0 mg SeMet (0.4 mg Se/kg body wt)/kg body wt/day, toxicity resulted in elevations of aspartate amino transferase (AST), glutamate pyruvate transaminase (GPT) and alanine amino transferase (ALT) activities; inflammation, telangiectasis, hemorrhage, vacuolar changes and brown pigment in hepatocytes, thymic atrophy, lymphocyte depletion in tonsils and intestine, gastrointestinal hemorrhage (NCI, 1993).

D. TOXICITY AND BIOPOTENCY OF THE D- AND L-ISOMERS

Additions of SeMet (D or L) at 6.25 mg/kg (corresponding to 2.5 mg Se/kg) over six weeks to the diet of rats produced no evidence of depressed growth or diminished survival. The same result was obtained with diets containing equivalent amounts of Se in the form of selenite or selenate (McAdam and Levander, 1987). However, severe growth depression and death of rats within 29 days occurred at 26 mg of D- or L-SeMet (10 mg Se) per kg of diet, as well as with the corresponding amounts of Se as selenite or selenate. In rats, D- and L-SeMet exhibited the same chronic toxicities at 13 mg of SeMet (5 mg Se) per kg of diet. At this level, the two isomers produced the same concentrations of Se in skeletal muscle, heart, liver and erythrocytes. Only the plasma Se levels were lower in the animals receiving L-SeMet (McAdam and Levander, 1987). Similarly, little difference in the retention of Se by liver and muscle tissue was seen when nutritional levels of D- or L-SeMet were fed to Se-depleted rats (McAdam and Levander, 1986). However, at the subtoxic level of 6.25 mg SeMet/kg diet, the Se concentrations were significantly higher in skeletal muscle, heart and liver in the animals receiving the D-isomer, indicating that the L-form is catabolized more rapidly under these conditions. Since adenosylation is the first step in the trans-sulfurization pathway of Met as well as of SeMet, this could cause the accumulation of D-SeMet in tissues and organs of species lacking an efficient mechanism of the conversion of D-Met into the L-form. In rats, which are known to utilize D-Met as well as L-Met, biopotency, acute toxicity and tissue retention of injected or dietary doses of both isomers of SeMet were about the same (McAdam *et al.*, 1985; McAdam and Levander, 1986,1987). In other species, the L-isomer was better utilized and more toxic than the D-isomer. In mallard ducklings, for example, Se as L-SeMet at 30 ppm in the diet was significantly more toxic than DL-SeMet, but concentrations of Se in the livers were about the same (Heinz *et al.*, 1996; Hoffman *et al.*, 1996). While normal mice metabolize both the isomers of SeMet equally well, more of the D-isomer was observed in the tumors of tumor-bearing mice. Within 48 h of administration, the *in vivo* uptake of D-SeMet was several times higher than that of the L-isomer in Ehrlich solid tumor, and in sarcoma 180 solid tumor. Since the uptake of both isomers was

about the same in the pancreas, this suggested the presence of a transport system specific for D-SeMet in tumor cells, in addition to a transport system common to both the D- and L- forms (Goto *et al.*, 1987). Furthermore experiments with cultured murine and human lymphoid cells demonstrated that the cells of the immune system also discriminate between the two enantiomeric forms of SeMet. Thus, DL-SeMet was only about half as cytotoxic than L-SeMet, and only L-SeMet was found to be a good substrate for adenosylmethionine synthetase and was effectively metabolized by trans-methylation reactions and in polyamine synthesis. The differential responses of the normal cells to L- and D-SeMet are thus attributable primarily to the initial steps of SeMet metabolism, which involve stereospecific trans-sulfurization enzymes preferentially utilizing L-SeMet. Differences in the activity of trans-methylation enzymes may also affect the cytotoxicity and the invasiveness behavior of tumor cells. For example, while pre-incubation with selenite resulted in a dose-dependent decrease in the ability of HeLa or NIH OVCAR-3 cells to invade a layer of a re-constituted basement membrane preparation, indicating diminished invasiveness, SeMet had no such effect (Gong and Frenkel, 1994).

E. MAXIMUM TOLERATED DOSE

In a 30-day trial with long-tailed female Macaques (*Macaca fascicularis*) receiving 0–600 μg of L-SeMet/kg day by nasogastric intubation, SeMet was well tolerated until the second to third week of the study at which time two animals given 600 μg/kg day died. One animal from the 300 μg/kg day group developed Se-induced hypothermia. Six animals in the 188 μg/kg day level or greater required supplements of fruit and *Sustagen* (Mead Johnson & Co, Evansville, IN), a powdered nutritional supplement for human consumption that is used to supply anorexic primates with necessary nutritional and caloric intake during periods when animals refuse their normal diet. With increasing SeMet dosage and duration of exposure, the incidence of anorexia, gastrointestinal distress, mucocutaneous toxicity, and frequency of reduced body temperature increased. A dose-dependent weight-loss was also observed. At the higher dosage, disturbances in the menstrual function were evident, and were accompanied by the absence of serum progesterone concentrations above 1.0 ng/ml, reduced luteal phase lengths, increased intermenstrual intervals, and lowered estrogen excretion. A "maximum tolerated dose" of 150 μg/kg day of L-SeMet for 30 days was identified based on mean body weight reduction, hypothermia, dermatitis, xerosis, cheilitis, disturbances in menstruation, and the need of dietary intervention to prevent death at doses of 188 μg/kg day or greater (Cukierski *et al.*, 1989). The same highest tolerated dose of L-SeMet (150 μg/kg day) on nasogastric intubation

was established with pregnant long-tailed Macaques (Hawkes *et al.*, 1992). In this study, the weight loss at a dosage of 300 μg Se/(kg day) of L-SeMet was three times greater than in the 150 μg Se/(kg day) group, and clear signs of Se toxicity (anorexia and vomiting) were apparent. The No-Effect Level was determined to the 375 μg SeMet/kg body wt day. At twice this dose, all animals developed anorexia, vomiting, and weight loss. Plasma Se levels increased in proportion to SeMet dosage only until day 21 and subsequently plateaued. Plasma GSH-Px activities increased in proportion to SeMet dosage and began to plateau after 20 days of administration. A steady state was reached at the two dosage levels when plasma Se concentrations had reached 1.91 and 3.86 μg/ml, respectively. However, erythrocyte and hair Se levels continued to increase until day 30 and for about two weeks after cessation of supplementation. Erythrocyte GSH-Px levels increased in proportion up to the SeMet dosage of 150 μg/kg body wt/day. At the toxic level of 300 μg/kg/day, erythrocyte GSH-Px activity was lower, and during the 30 days of dosing dropped to values only as high as in the 25 μg Se/kg body wt/day control group. However, in the 150- and 300 μg SeMet/kg body wt day animals, erythrocyte GSH-Px activity continued to increase for two weeks after cessation of supplementation, suggesting the release of SeMet from body storage organs. Erythrocyte Se, plasma Se and hair Se levels of >2.3 μg/ml, >2.8 μg/ml and >27 μg/g, respectively, were associated with increased weight loss due to Se toxicity. These values for Se toxicity are consistent with the cutoff values for humans subsisting on a predominantly vegetarian diet, as determined in a Chinese study (Liu and Li, 1987).

F. REFERENCE VALUES

From observations of populations residing in high-Se regions and evidence from supplementation studies, a *Reference Dose* (RfD) for selenium has recently been set to 350 μg Se/day for humans of 70 kg body weight periods (Patterson and Levander, 1997). This value corresponds to 5 μg Se/kg BW or five times the "Recommended Dietary Allowance" (RDA) for Se and represents the total intake of selenium from nutritional sources which are safe for indefinite periods, while still below the "maximal safe intake" of 450 μg/day, as defined by the British Committee on Medical Aspects of Food Policy (Department of Health, 1991). Another reference value is the "Tolerable Upper Intake Level," UL, representing "the highest level of daily selenium intake that is likely to pose no risk or adverse health effects to almost all individuals in the general population." The UL for selenium was set at 400 μg/day for adult, corresponding to one half of the No-Observed-Adverse-Effect-Level (NOAEL) of 800 μg/day. Although neither the RfD nor the UL specify

the chemical form of Se, both may be assumed to apply primarily to SeMet, the major nutritional form of Se. The RfD and UL thus set a safe limit for nutritional Se supplementation. In the cancer prevention trial of Clark *et al.* (1996), for example, the supplementary dose of 200μg Se/day, which consisted mainly of SeMet, increased the Se intakes of the study participants from 100–150 to 300–350 μg/day, causing the average plasma Se concentrations to increase, in 6–9 months, from 114 ± 22 to 190 ± 50μg/l. Selenium intakes of 450 μg/day or more were reached by the study participants of a Chinese liver cancer chemoprevention trial and produced no adverse effects (Yu *et al.*, 1988). In this study, supplementary Se at 400 μg Se/day was given, causing whole blood Se levels to reach 0.386 μg/ml after one year of supplementation. The Observed-NOAEL was provisionally estimated to 750–850 μg/day, based largely on observations with Chinese subjects residing in the high-Se areas. At 850 μg Se per day, blood Se levels reach 1 μg/ml, blood GSH concentrations were diminished and prothrombin time was reduced, with more significant signs of Se toxicosis appearing in one subject at 880 μg Se/day (Yang *et al.*, 1989a,b; Yang and Xia, 1995). However, in subjects from another area in China with Se intakes of 1457 ± 554 μg/day, selenosis was seen only occasionally (Yang and Zhou, 1994). From these observations an "Adverse Effect Level" (AEL), or "Individual Toxic Level", for dietary Se, of 1595 or 1600 μg/day was derived. This level of intake "seems capable of causing the development of chronic overt toxic selenosis after long-term intake" (Yang and Zhou, 1994; Yang and Xia, 1995). Earlier studies on subjects residing in high-Se regions of the United States suggested that mild, reversible signs of Se toxicity occur after 2400–3000 μg of Se/day for many months (NRC, 1976), suggesting that Se toxicity is influenced by diet' and particularly, the total protein intake; that high protein diets reduce Se toxicity is well known. Rats on a feed containing 35% casein and 35 ppm Se showed no signs of selenosis (Moxon and Rhian, 1943). The NOAELs and AELs for Se thus could be rendered more precise by relating them to the level of protein in the diet consumed. This could explain why adults receiving 1600 μg of nutritional Se per day for several months need not show overt signs of selenosis, while exhibiting blood Se levels of 1.61 μg/ml. Although the concentrations of Se in whole blood, plasma, hair, nails and urine are linearly dependent on the dietary Se-intakes (Schrauzer and White, 1978; Yang *et al.*, 1989a), these linear relationships are obeyed only if Se is ingested predominantly in the form of SeMet. If the ingested Se is in an inorganic form, blood and hair Se levels become unreliable indices of exposure and hence for diagnosis of selenosis, nail changes are the most significant signs (Yang *et al.*, 1989a,b).

XI. SELENIUM REQUIREMENTS AND RECOMMENDED DIETARY INTAKES

In 1980 the "Estimated Safe and Adequate Daily dietary Intake" (ESADDI) for adults and children above the age of 7 years was set at 50–200 μg Se/day (Food and Nutrition Board, 1980). Subsequently, based on Se balance studies, proposed 80 μg/day for men and 55 μg/day for women as safe and adequate Se intakes, corresponding to 1 μg Se/kg BW/day. In 1989, the RDA for selenium was lowered to 70 μg/day for adult men. This new intake recommendation was derived from the minimal amount of Se required for 100% saturation of the plasma GSH-Px activity. For adult Chinese males of 60 kg body weight, this amount had previously been determined to be 40 μg/day (Yang *et al.*, 1988). From this value, the RDA for North American males was calculated by taking into account their higher body weight (79 kg) and by increasing it further through multiplication with a safety factor. For adult women the RDA for selenium was maintained at 55 μg/day. For pregnant women, the RDA was set at 65 μg Se/day, for lactating women at 75 μg Se/day, assuming an average body weight of 63 kg.

In 1996, expert committees of the World Health Organization (WHO) derived basal and normative requirements for vitamins and minerals. The basal requirement was defined as "the intake needed to prevent pathologically relevant and clinically detectable signs of impaired functions attributable to inadequacy of the nutrient," and the normative intake as "the level of intake that serves to maintain a level of tissue storage or other reserve that is judged by the Expert Consultation to be desirable" (WHO, 1996). For selenium, the basal Se requirement was derived from the minimal amount needed to protect against Keshan disease, and was set at 21 μg/day for men and at 16 μg/day for women. The normative Se intakes were derived primarily under the assumption that two thirds of the maximum plasma GSH-Px activity affords sufficient antioxidant protection, corresponding to 40 and 30 μg/day for men and women, respectively. In the year 2000, the panel on dietary antioxidants and related compounds of the U.S. Food and Nutrition Board replaced the RDAs by "Dietary Reference Intakes" (DRIs), which for the most part numerically resembled the 1989 RDAs. All currently recommended Se intakes would be insufficient if selenium adequacy were assessed on immunological criteria such as antibody production or the cancer preventive effects of Se. In home-TPN patients receiving 50 μg Se per day, antibody production against common antigens was low compared to patients supplemented with 200 μg Se as SeMet (Peretz *et al.*, 1991). Similarly, 200 μg Se as SeMet in yeast was shown to be an effective dose of Se for cancer prevention, see Section F before. Obviously, all intake recommendations depend on the definitions used to derive them; they are not to be

considered final and must be re-evaluated and revised periodically. Specifically for Se, revisions of current intake recommendations are likely to be forthcoming in the light of rapidly advancing research and the growing recognition of its protective effects at supranutritional intakes.

XII. SUMMARY AND CONCLUSIONS

SeMet is a naturally occurring toxic amino acid but at the same time represents the major nutritional source of selenium for higher animals and humans. The ability of SeMet to be incorporated into the body proteins in place of Met furthermore provides a means of reversible Se storage in organs and tissues. This property is not shared by any other naturally occurring selenoamino acid and thus could be associated with a specific physiological function of SeMet. Since higher animals cannot synthesize SeMet, yet from it all needed forms of Se are produced, SeMet meets the criteria of an essential amino acid. Accordingly, SeMet, or enriched food sources thereof, are appropriate forms of Se for human nutritional Se supplementation. However, while SeMet or Se yeast are already widely used in over-the-counter nutritional supplements, infant formulas and parenteral feeding mixtures still contain Se in the form of sodium selenate or sodium selenite, even though these are not the normal nutritional forms of Se. In animal nutrition, these inorganic selenium salts are increasingly replaced by food sources of SeMet such as Se yeast. Synthetic SeMet could also be employed as a feed additive, but its regulatory status is as yet undetermined. The optimal nutritional levels of SeMet for different animal species still need to be determined. The expectation is that lower additions to feedstock of equivalent levels of SeMet will suffice to achieve adequacy than currently approved maximum levels of Se in the form of inorganic Se salts.

REFERENCES

Abrams, M.M. and Burau, R.G. 1989. Fractionation of selenium and detection of selenomethionine in a soil extract. *Commun. Soil Sci. Plant Anal.* **20**, 221–237.

Alaejos, M.S. and Romero, C.D. 1995. Selenium in human lactation. *Nutr. Rev.* **53**, 159–166.

Ammar, E.M. and Couri, D. 1981. Acute toxicity of sodium selenite and selenomethionine in mice after ICV or IV administration. *Neurotoxicology* **2**, 383–386.

Anonymous, 2000. Food additives permitted in feed and drinking water of animals; selenium yeast. *Fed. Register* **56**(109), 35823–35824.

Arteel, G.E., Gavin, E., Bribiva, K., and Sies, H. 1999. Protection against peroxynitrite. *FEBS Lett.* **445**(2,3), 226–230.

Assmann, A., Briviba, K., and Sies, H. 1998. Reduction of methionine selenoxide to selenomethionine by glutathione. *Arch. Biochem. Biophys.* **349**, 201–203.

Barros, R.S. and De Paula-Freitas, A.W. 2001. Selenomethionine as a dormancy-breaking agent in seeds of *Stylosanthes humilis*. *Acta Physiol. Plant.* **23**(3), 279–284.

Baum, M.K., Campa, A., Miguez-Burbano, M.J., Burbano, X., and Shor-Posner, G. 2001. Role of selenium in HIV/AIDS. *In* "Selenium—Its Molecular Biology and Role in Human Health" (D.L. Hatfield, ed.), pp. 247–255. Kluwer Academic Publishers, Boston/Dordrecht/London.

Beath, O.A. 1937. The occurrence of selenium and seleniferous vegetation in Wyoming. II. Seleniferous vegetation in Wyoming. *Wyoming Agric. Exp. Stn Bull.* **221**, 29–64.

Beilstein, M.A. and Whanger, P.D. 1986. Deposition of dietary organic and inorganic selenium in rat erythrocyte proteins. *J. Nutr.* **116**, 1701–1710.

Beilstein, M.A. and Whanger, P.D. 1992. Selenium metabolism and glutathionine peroxidase activity in cultured human lymphoblasts. *Biol. Trace Elem. Res.* **35**, 105–118.

Beilstein, M.A., Whanger, P.D., and Yang, G.O. 1992. Chemical forms of corn and rice grown in a high selenium area of China. *Biomed. Environ. Sci.* **4**, 392–398.

Benavides, S.T., Silva, F. 1973. Mojica Selenosis, Instituto Geografico 'Augustin Codazzi', Bogota, Columbia (1965), see Jaffé W.G., Qualitas Planarum, Plant Food for Human Nutrition. XXIII: 191.

Bernard, A.R., Wells, T.N., Cleasby, A., Borlat, F., Payton, M.A., and Proudfoot, A.E. 1995. Selenomethionine labelling of phosphomannose isomerase changes its kinetic properties. *Eur. J. Biochem.* **230**, 111–118.

Bird, S.M., Ge, H., Uden, P.C., Tyson, J.F., Block, E., and Donoyer, E. 1997a. High performance liquid chromatography of selenoamino acids and organo selenium compounds. Speciation by inductively coupled plasma mass spectrometry. *J. Chromatogr.* **789**, 349–359.

Bird, S.M., Uden, P.C., Tyson, J.F., Block, E., and Denier, A. 1997b. Speculation of and compounds in selenium-enriched yeast using high-performance liquid chromatography-inductively coupled plasma mass spectrometry. *J. Anal. At. Spectrom.* **12**, 785–788.

Blau, M. 1961. Biosynthesis of (^{75}Se)selenomethionine and (^{75}Se)selenocystine. *Biochem. Biophys. Acta* **49**, 389–390.

Blau, M. and Bender, M.A. 1962. (^{75}Se)selenomethionine for visualization of the pancreas by isotope scanning. *Radiology* **78**, 974–979.

Blazon, C., Coccia, R., Pensa, B., Cini, C., and De Marco, C. 1994. Selenomethionine as substrate for glutamine transaminase. *Biochem. Mol. Biol. Int.* **32**, 79–86.

Boehm, M.F. and Bada, J.L. 1985. The racemization rate of selenomethionine and methionine in yeast at 100°C and neutral pH. *Anal. Biochem.* **145**, 273–276.

Boles, J.O., Censors, R.J., Weir, M.S., Doom, J.D., Villa franca, J.E., and Dunlap, R.B. 1991. Purification and characterization of selenomethionyl thymidylate synthase from *Escherichia coli*; comparison with the wild-type enzyme. *Biochemistry* **30**(46), 11073–11080.

Borella, P., Bargellini, A., and Medici, C.I. 1995. Chemical form of selenium greatly affects metal uptake and responses by cultured human lymphocytes. *Biol. Trace Elem. Res.* **51**, 43–54.

Bottino, N.R., Banks, C.H., Irgolic, K.J., Micks, P., Wheeler, A.E., and Zingaro, R.A. 1984. Selenium containing amino acids and proteins in marine algae. *Phytochemistry* **23**, 2445–2452.

Brown, K.M., Pickard, K., Nicol, F., Beckett, G.J., Duthie, G.G., and Arthur, J.R. 2000. Effects of organic and inorganic selenium supplementation on selenoenzyme activity in blood lymphocytes, granulocytes, platelets and erythrocytes. *Clin. Sci.* **98**, 593–599.

Bunk, M.J. and Combs, J.F. 1980. Effect of selenium on appetite in the selenium-deficient chick. *J. Nutr.* **110**, 743–749.

Burk, R.F., Hill, K.E., and Motley, A.K. 2001. Plasma selenium in specific and non-specific forms. *BioFactors* **14**(1–4), 107–114.

Burke, K.E., Bedford, R.G., Combs, G.F. Jr., French, I.W., and Skeffington, D.R. 1992a. The effect of topical L-selenomethionine on minimal erythema dose of ultraviolet irradiation in humans. *Photodermatol. Photoimmunol. Photomed.* **9**, 52–57.

Burke, K.E., Combs, G.F., Gross, E.G., Bhuyan, K.C., Gross, E.G., and Abu Lideh, H. 1992b. The effects of topical and oral L-selenomethionine on pigmentation and skin cancer induced by ultraviolet irradiation. *Nutr. Cancer* **17**, 123–137.

Burnell, J.N. 1981. Methionyl-tRNA synthetase from *Phaseolus aureus*: purification and properties. *Plant Physiol.* **67**, 316–324.

Butler, G.W. and Peterson, P.J. 1967. Uptake and metabolism of inorganic selenium-75 by *Spirondela oligorhizza*. *Aust. J. Biol. Sci.* **20**, 77–86.

Butler, J.A., Beilstein, M.A., and Whanger, P.D. 1989. Influence of dietary methionine on the metabolism of selenomethionine in rats. *J. Nutr.* **119**, 1001–1009.

Butler, J.A., Whanger, P.D., Kaneps, A.J., and Patton, N.M. 1990. Metabolism of selenite and selenomethionine in the rhesus monkey. *J. Nutr.* **120**, 751–759.

Cantor, A.H., Langevin, M.L., Moguchi, T., and Scott, M.L. 1975a. Efficacy of selenium in selenium compounds and feedstuffs for prevention of pancreatic fibrosis in chicks. *J. Nutr.* **105**, 106–111.

Cantor, A.H., Scott, M.L., and Noguchi, T. 1975b. Biological availability of selenium in feedstuffs and selenium compounds for prevention of pancreatic fibrosis in chicks. *J. Nutr.* **105**, 106–111.

Cantor, A.H., Langevin, M.L., Noguchi, T., and Scott, M.L. 1975c. Efficacy of selenium in selenium compounds in feedstuffs and selenium compounds for prevention of exudative diathesis in chicks. *J. Nutr.* **105**, 106–111.

Cases, J., Vacchina, V., Napolitano, A., Caporiccio, B., Besancon, P., Lobinski, R., and Rouanet, J.-M. 2001. Selenium from selenium-rich spirulina is less bioavailable than from sodium selenite and selenomethionine in selenium-deficient rats. *J. Nutr.* **131**(9), 2343–2350.

Chen, S.C., Chen, S.D., Wang, G.S., Liou, J.J., and Chang, F.C. 2001. On-farm trials of organic selenium to improve sow herd fertility. *Taiwan Tangye Yanjiuso Yanjiu Huibao* **170**, 45–54.

Chiao, J.S. and Peterson, W.H. 1953. Yeasts: methionine and cystine contents. *Agric. Food Chem.* **1**(15), 1005–1008.

Clark, L.C., Combs, G.F., Turnbull, B.W., Slate, E.H., Chalker, D.K., Chow, J., Davis, L.S., Glover, R.A., Graham, G.F., Gross, E.G., Krongrad, A., Lesher, J.L. Jr., Park, H.K., Sanders, B.B. Jr., Smith, C.L., and Taylor, J.R. 1996. Effects of selenium supplementation for cancer prevention in patients with carcinoma of the skin. *J. Am. Med. Assoc.* **276**, 1957–1963.

Clyburn, B.S., Richardson, C.R., Montgomery, J.L., Pollard, G.V., Herring, A.D., and Miller, M.F. 2001. Effect of selenium source and vitamin E level on performance and meat quality of feedlot steers. *In* "Science and Technology in the Feed Industry" (T.P. Lyons and K.A. Jacques, eds), pp. 377–392. Nottingham University Press, Nottingham, UK.

Conrad, H.R. and Moxon, A.L. 1979. Transfer of dietary selenium to milk. *J. Dairy Sci.* **62**, 404–411.

Cukierski, M.J., Willhite, C.C., Lasley, B.L., Hendrie, T.A., Book, S.A., Cox, D.N., and Hendricks, A.G. 1989. 30-day oral toxicity study of L-selenomethionine in female long-tailed macaques (*Macaca fascicularis*). *Fundam. Appl. Toxicol.* **13**, 26–39.

Daniels, L.A. 1996. Selenium metabolism and bioavailability. *Biol. Trace Elem. Res.* **54**, 185–199.

Deagen, J.T., Butler, J.A., Beilstein, M.A., and Whanger, P.D. 1987. Effects of dietary selenite, selenocysteine and selenomethionine on selenocysteine lyase and glutathione peroxidase activities in rat tissues. *J. Nutr.* **117**, 91–98.

Demirci, A., Pometto, A.L., and Cox, D.J. 1999. Enhanced organically bound selenium yeast production by fed-batch fermentation. *J. Agric. Food Chem.* **47**, 2496–2500.

Department of Health, UK. 1991. Dietary reference values for food energy and nutrients for the United Kingdom (Report on Health and Social Subjects, No. 41). London, Her Majesty's Stationery Office.

Djusic, I.S., Djermanovic, V., and Milovac, M. 1998. Foliar supplementation with selenium the efficient way to increase Se content in wheat grain, its products and human consumers. *In* "Proc. 6th Internat. Symp. Uses of Selenium and Tellurium" (Y. Palmieri, ed.), pp. 129–134, Scottsdale, AZ. Grimbergen.

Edens, F.W. 2001. Involvement of Sel-Plex in physiological stability and performance of broiler chickens. *In* "Science and Technology in the Feed Industry" (T.P. Lyons and K.A. Jacques, eds), pp. 349–376. Nottingham University Press, Nottingham, UK.

Esaki, N., Tanaka, H., Uemura, S., Suzuki, T., and Soda, K. 1979. Catalytic action of L-methionine gamma-lyase on selenomethionine and selenols. *Biochemistry* **18**, 407–410.

Eustice, D.C., Krull, F.J., and Shrift, A. 1981. Selenium toxicity: aminoacylation and peptide bond formation with selenomethionine. *Plant Physiol.* **67**, 1054–1058.

Food and Nutrition Board, 1980. Committee on Dietary Allowances. *In* Recommended Dietary Allowances, 9th Revised Edition, National Academy Press, Washington D.C., p. 185.

Franke, K.W. and Painter, E.P. 1938. A study of the toxicity and selenium content of the seleniferous diets: with statistical consideration. *Cereal Chem.* **15**, 1–24.

Gössler, W., Zheng, J., and Kosmus, W. 1999. Speziation von Selen in Supplementierungspräparaten. *In* "Spurenelemente—Speziationsanalyse, Supplementierung und Therapie mit Spurenelementen" (D. Meißner, ed.), Wissenschaftliche Verlagsgesellschaft mbH Stuttgart, pp. 61–64.

Gong, Y. and Frenkel, G.D. 1994. Effect of selenite on tumor cell invasiveness. *Cancer Lett.* **78**, 195–199.

Goto, R., Unno, K., Okada, S., and Tamemasa, O. 1987. Tumor accumulation of D-selenomethionine-75Se in tumor-bearing mice. *J. Pharmacobio-Dyn.* **10**(9), 456–461.

Gradova, N.B., Sukhinina, E.A., Babusenko, E.S., Gusarova, N.A., and Baum, I.F. 2001. Growth of Spirulina on selenium containing media. *Biotekhnologia* **2001**(5), 40–44.

Griffiths, N.M., Stewart, R.D.H., and Robinson, M.F. 1976. The metabolism of (^{75}Se)selenomethionine in four women. *Br. J. Nutr.* **35**, 119–127.

Grønbaek, H. and Thorlacius-Ussing, O. 1992. Selenium in the central nervous system of rats exposed to 75-Se selenomethionine and sodium selenite. *Biol. Trace Elem. Res.* **35**, 119–127.

Guo, X. and Wu, L. 1998. Distribution of free seleno-amino acids in plant tissue of *Melilotus indica* L. grown in selenium-laden soils. *Ecotoxicol. Environ. Saf.* **39**(3), 207–214.

Gupta, U.C. and MacLoed, J.A. 1998. Selenium enrichment of crops and selenium in ground water following selenium fertilization. *In* "Proc. 6th Internat. Symp. Uses of Selenium and Tellurium" (Y. Palmieri, ed.), pp. 119–126, Scottsdale, AZ. Grimbergen.

Hansson, E. and Jacobsson, S.-O. 1966. Uptake of [^{75}Se]selenomethionine in the tissues of the mouse studied by whole-body autoradiography. *Biochim. Biophys. Acta* **115**, 285–293.

Hawkes, W.C., Willhite, C.C., Craig, K.A., Omaye, S.T., Cox, D.N., Choy, W.N., and Hendrickx, A.G. 1992. Effects of excess selenomethionine on selenium status indicators in pregnant long-tailed Macaques (*Macaca fascicularis*). *Biol. Trace Elem. Res.* **35**, 281–297.

Hedegaard, J., Falcone, G., and Calabro, S. 1963. Incorporation de selenium dans des analogues des acides amines soufr dans *Candida albicans*. *C. R. Soc. Biol.* **157**, 280–285.

Heinz, G.H., Hoffman, D.J., and LeCaptain, L.J. 1996. Toxicity of seleno-L-methionine, seleno-D,L-selenomethionine, high selenium wheat, and selenized wheat to mallard ducklings. *Arch. Environ. Contam. Toxicol.* **31**(1), 93–99.

Hemken, R.W. and Jacques, K.A. 1998. Bioavailability of selenium yeast (Sel-Plex) vs sodium selenite for dairy cattle. Proceedings of the 6th International Symposium on the Uses of Selenium and Tellurium, Selenium-Tellurium Development Association, May 10–12, 1998, Scottsdale, AZ, USA. pp 161–162.

Herigstad, R.R., Whitehair, C.K., and Olson, O.E. 1973. Inorganic and organic selenium toxicosis in young swine: comparison of pathologic changes with those in swine with vitamin E-selenium deficiency. *Am. J. Vet. Res.* **34**, 1227–1238.

Hidiroglou, M., Heaney, D.P., and Jenkins, K.J. 1968. Metabolism of inorganic selenium in rumen bacteria. *Can. J. Physiol. Pharmacol.* **46**(2), 229–232.

Hoffman, D.J., Heinz, G.H., LeCaptain, L.J., Eiseman, J.D., and Pendleton, G.W. 1996. Toxicity and oxidative stress of different forms of organic selenium and dietary protein in mallard ducklings. *Arch. Environ. Contam. Toxicol.* **31**(1), 120–127.

Hogan, G.R. 1998. Selenate- and selenomethionine-induced leukopenia in ICR female mice. *J. Toxicol. Environ. Health, Part A* **53**, 113–119.

Hu, M., Yang, Y., Martin, J.M., Yin, K., and Harrison, P.J. 1997. Preferential uptake of Se(IV) over Se(VI) and the production of dissolved organic Se by marine. *Mar. Environ. Res.* **44**, 225–231.

Huber, R.E. and Criddle, R.S. 1967. The isolation and properties of beta-galactosidase from *Escherichia coli* grown in sodium selenate. *Biochim. Biophys. Acta* **141**, 587–599.

Hurd-Karrer, A.M. 1934. Selenium injury to wheat plants and its inhibition by sulphur. *J. Agric. Res.* **49**, 343–357.

Ip, C. 1998. Lessons from basic research in selenium and cancer prevention. *J. Nutr.* **128**, 1845–1854.

Ip, C., Birringer, M., Block, E., Kotrebai, M., Tyson, J.F., Uden, P.C., and Lisk, D.J. 2000. Chemical speciation influences comparative activity of selenium-enriched garlic and yeast in mammary cancer prevention. *J. Agric. Food Chem.* **48**, 2062–2070.

Jacques, K.A. 2001. Selenium metabolism in animals: the relationship between dietary selenium form and physiological response. *In* "Science and Technology in the Feed Industry" (T.P. Lyons and K.A. Jacques, eds), pp. 319–348. Nottingham University Press, Nottingham, UK.

Jenkins, K.J. and Hidiroglou, M. 1971. Transmission of selenium as selenite and as selenomethionine from ewe to lamb via milk using selenium-75. *Can. J. Anim. Sci.* **51**, 389–403.

Kajander, E.O., Harvima, R.J., Eloranta, T.O., Martikainen, H., Kantola, M., Kaerenlampi, S.O., and Akerman, K. 1991. Metabolism, cellular actions, and cytotoxicity of selenomethionine in cultured cells. *Biol. Trace Elem. Res.* **28**(1), 57–68.

Kim, Y.Y. and Mahan, D.C. 2001. Comparative effects of high dietary levels of organic and inorganic selenium on selenium toxicity of growing-finishing pigs. *J. Anim. Sci.* **79**, 942–948.

Kim, Y.Y. and Mahan, D.C. 2001. Prolonged feeding of high dietary levels of organic and inorganic selenium to gilts from 25 kg body weight through one parity. *J. Anim. Sci.* **79**, 956–966.

Kivisaari, S. 1998. Selenium in NPK fertilizers: twelve years experience in its effects on crop production. *In* "Proc. 6th Internat. Symp. Uses of Selenium and Tellurium" (Y. Palmieri, ed.), pp. 113–118, Scottsdale, AZ. Grimbergen.

Klasing, K.C. 1998. Comparative Avian Nutrition. CAB International, New York, NY.

Klein, E.A., Thompson, I.M., Lippman, S.M., Goodman, P.J., Albanes, D., Taylor, P.R., and Coltman, C. 2001. SELECT: the next prostate cancer prevention trial. Selenum and Vitamin E Cancer Prevention Trial. *J. Urol.* **166**(4), 1311–1315.

Klosterman, H.J. and Painter, E.P. 1947. An improved synthesis of the selenium analog of methionine and homocystine. *J. Am. Chem. Soc.* **69**, 2009–2010.

Klug, H.L., Petersen, D.F., and Moxon, A.L. 1949. The toxicity of selenium analogues of cystine and methionine. *Proc. S. D. Acad. Sci.* **28**, 117.

Koch, T. and Burchardt, O. 1993. Synthesis of L(+)-selenomethionine. *Synthesis*, 1065–1067.

Kolacz, R., Dobrzanski, Z., and Grudnik, T. 2001. Effect of selenium yeast feed supplement on selenium level in sow milk and piglet blood serum and on production performance. *Folia Univ. Agric. Stetinensis* **224**, 77–81.

Korhola, M., Vainio, A., and Edelmann, K. 1986. Selenium yeast. *Ann. Clin. Res.* **18**, 65–68.

Krief, A., Trabelsi, M., and Shastri, M. 1994. Efficient synthesis of (D,L)- and D-selenomethionine. *In* "Proc. of STDA's Fifth Internatl. Symposium" (S.C. Carapella, J.E. Oldfield, and Y. Palmieri, eds), pp. 107–109, Brussels, May 8–10.

Krska, P., Lahucky, R., Palanska, O., Kuchenmeister, U., Kuhn, G., Demo, P., and Bahelka, I. 2000. Effect of organic selenium on fattening ability and meat quality in pigs. *J. Farm Anim. Sci.* **33**, 153–159.

Kumpulainen, J., Salmenperä, L., Simes, M.A., Koivistonen, P., and Perheentupa, J. 1985. Selenium status of exclusively breast-fed infants as influenced by maternal organic and inorganic selenium supplementation. *Am. J. Clin. Nutr.* **42**, 829–835.

Kyriakopoulos, A., Rothlein, D., Pfeifer, H., Gessner, H., and Behne, D. 1998. Selenium-containing compounds in backerUs yeast (*Saccharomyces cerevisiae*). *In* "Proc. Mengen-Spurenelemente Arbeitstagung, 18th" (M. Anke, ed.), pp. 740–745. Harald Schubert Verlag, Leipzig, Germany.

Lane, H.W., Strength, R., Johnson, J., and White, M. 1991. Effect of chemical form of selenium on tissue glutathione peroxidase activity in developing rats. *J. Nutr.* **121**, 80–86.

Larsen, E.H., Hansen, M., Fan, T., and Vahl, M. 2001. Speciation of selenoamino acids, selenonium ions and inorganic selenium ion by exchange HPLC with mass spectrometric detection and its application to yeast and algae. *J. Anal. At. Spectrom.* **16**(12), 1403–1408.

Latshaw, J.D. and Biggert, M.D. 1981. Incorporation of selenium into egg proteins after feeding selenomethionine or sodium selenite. *Poult. Sci.* **60**, 1309–1313.

Latshaw, J.D. and Osman, M. 1975. Distribution of selenium in egg white and yolk after feeding natural and synthetic selenium compounds. *Poult. Sci.* **54**(4), 1244–1252.

Levander, O.A., Alfthan, G., Arvilommi, H., Gref, C.G., Huttunen, J.K., Kataja, M., Koivistoinen, P., and Pikkarainen, J. 1983. Bioavailability of selenium in Finnish men as assessed by glutathione peroxidase activity and other blood parameters. *Am. J. Clin. Nutr.* **37**, 887–897.

Liu, B.-S. and Li, S.-S. 1987. Endemic selenosis, and fluorosis. *In* "Selenium in Biology and Medicine, Part B" (G.F. Combs, J.E. Spallholz, O.A. Levander, and J.E. Oldfield, eds), pp. 708–711. AVI Van Nostrand Reinhold Company, New York.

Low, S.C. and Berry, M.J. 1996. Knowing when not to stop. Selenocysteine incorporation in eukaryotes. *TIBS* **21**, 203–208.

Luo, X.M., Wei, H.J., Yang, C.L., Xing, J., Liu, X., Qiao, C.H., Feng, Y.M., Liu, Y.X., and Wu, O. 1987. Bioavailability of selenium to residents in a low-selenium area of China. *In* "Selenium in Biology and Medicine, Part A" (G.F. Combs, J.E. Spallholz, O.A. Levander, and J.E. Oldfield, eds), pp. 436–444. AVI Van Nostrand Reinhold Company, New York.

Lyons, T.P. and Oldfield, J.E. 1996. The case for organic selenium. *Bull. Selenium-Tellurium Dev. Assoc.* **June Issue**, 1–3 (Grimbergen Belgium).

Mahan, D.C. and Kim, Y.Y. 1996. Effect of inorganic and organic selenium at two dietary levels on reproductive performance and tissue selenium concentrations in first parity gilts and their progeny. *J. Anim. Sci.* **74**, 2711–2718.

Marschner, H. 1995. "Mineral Nutrition of Higher Plants". Academic Press/Harcourt Brace & Company, London/New York.

Martin, J.L. and Hurlbut, J.A. 1976. Tissue selenium levels and growth responses of mice fed selenomethionine, Se-methylselenocysteine or sodium selenite. *Phosphorus and Sulfur* **1**, 295–300.

Mason, A.C. 1994. Selenium incorporation into selenocysteine in yeast proteins. *FASEB J.* **8**(4), A541.

McAdam, P.A. and Levander, O.A. 1986. Comparative biopotency of dietary D- or L-selenomethionine (SeMet) with or without added methionine (Met) in rats. *Am. J. Clin. Nutr.* **43**, 672.

McAdam, P.A. and Levander, O.A. 1987. Chronic toxicity of dietary selenium fed to rats as D- or L-selenomethionine, selenite or selenate. *Nutr. Res.* **7**, 601–610.

McAdam, P.A., Wood, L.E., and Levander, O.A. 1985. Comparative acute toxicity and nutritional biopotency of injected doses of D- versus L-selenomethionine (SeMet) in rats. *Am. J. Clin. Nutr.* **41**, 864.

McGuire, M.K., Burgert, S.L., Milner, J.A., Glass, L., Kummer, R., Deering, R., Boucek, R., and Picciano, M.F. 1993. Selenium status of lactating women is affected by the form of selenium consumed. *Am. J. Clin. Nutr.* **58**, 649–652.

McKenzie, R.C., Rafferty, T., Beckett, G.J., and Arthur, J.R. 2001. Effect of selenium on immunity and aging. *In* "Selenium in Molecular Biology and Role in Human Health" (D.L. Hatfield, ed.), pp. 257–272. Kluwer Academic Publishers, Boston/Dordrecht/London.

Mendez, S.P., Bayon, M.M., Gonzalez, E.B., and Sanz-Medel, A. 1999. Selenomethionine chiral speciation in yeast and parenteral solutions by chiral phase capillary gas chromatography-ICP-MS. *J. Anal. At. Spectrom.* **14**(9), 1333–1337.

Michalke, B. and Schramel, P. 1997. Selenium speciation in human milk with special respect to quality control. *Biol. Trace Elem. Res.* **59**, 45–56.

Miki, K., Xu, M., Gupta, A., Ba, Y., Tan, Y., Al-Refaie, W., Bouvet, M., Makuuchi, M., Moossa, A.R., and Hoffman, R.M. 2001. Methioninase cancer gene therapy with selenomethionineas suicide prodrug substrate. *Cancer Res.* **61**(18), 6805–6810.

Mitch, M.E. and Goldberg, A.L. 1996. Mechanisms of muscle of wasting. *N. Engl. J. Med.* **335**(25), 1897–1905.

Montes-Bayon, M., B'Hymer, C., Ponce de Leon, C., and Caruso, J.A. 2001. Resolution of seleno-amino acid optical isomers using chiral derivatization and inductively coupled plasma mass spectrometric (ICP-MS) detection. *J. Anal. At. Spectrom.* **16**(9), 945–950.

Moxon, A.L. and Rhian, M. 1943. Selenium poisoning. *Physiol. Rev.* **23**, 305–327.

Mudd, S.H. and Cantoni, G.L. 1957. Selenomethionine in enzymatic transmethylations. *Nature* **180**, 1052.

Mutanen, M. 1986. Bioavailability of selenium. *Ann. Clin. Res.* **18**, 48–54.

Nakamuro, K., Nakanishi, K., Okuno, T., Hasegawa, T., and Sayato, Y. 1997. Comparison of methylated selenium metabolites in rats after oral administration of various selenium compounds. *Jpn. J. Toxicol. Environ. Health* **43**, 182–189.

NCI. 1993. "Proc. Workshop on Selenium Compounds in Cancer Chemo Prevention Trials." Division of Cancer Prevention and Control, National Cancer Institute, National Institutes of Health, Bethesda, MD, September 20–21.

National Research Council. 1976. Selenium. *In* "Medical and Biologic Effects of Environmental Pollutants", pp. 28–35. National Academy of Sciences, Washington, DC.

Okuno, T., Kubota, T., Kuroda, T., Ueno, H., and Nakamuro, K. 2001. Contribution of an enzymatic α, γ-elimination reaction in detoxification pathway of selenomethionine in mouse liver. *Toxicol. Appl. Pharmacol.* **176**(1), 18–23.

Olmsted, L., Schrauzer, G.N., Flores-Arce, M., and Dowd, J. 1988. Selenium supplementation of symptomatic immunodeficiency virus infected patients. *Biol. Trace Elem. Res.* **15**, 167–177.

Olson, O.E. and Palmer, I.S. 1976. Selenoamino acids in tissues of rats administered inorganic selenium. *Metabolism* **25**, 299.

Olson, O.E., Novacek, E.J., Whitehead, E.I., and Palmer, I.S. 1970. Investigations on selenium in wheat. *Phytochemistry* **9**, 1181–1188.

Ortman, K. and Pehrson, B. 1999. Effect of selenate as a feed supplement to dairy cows in comparison to selenite and selenium yeast. *J. Anim. Sci.* **77**(12), 3365–3370.

Oster, O., Schmiedel, G., and Prellwitz, W. 1988. The organ distribution of selenium in German adults. *Biol. Trace Elem. Res.* **15**, 23–45.

Pan, F. and Tarver, H. 1967. Comparative studies on methionine, selenomethionine and the ethyl analogues as substrates for methionine adenosyltransferase from rat liver. *Arch. Biochem. Biophys.* **119**, 429–434.

Pande, C.S., Rudick, J., and Walter, R. 1970. Synthesis and application in peptide chemistry of amino acids possessing an optically active selenohomocysteine skeleton. *J. Org. Chem.* **35**, 1440–1445.

Patterson, B. and Levander, O.A. 1997. Naturally occurring selenium compounds in cancer chemoprevention trials: a workshop summary. *Cancer Epidemiol. Biomark. Prevent.* **6**, 63–69.

Patterson, B., Levander, O., Helzsouer, K., McAdam, P., Lewis, S., Taylor, P., Veillon, C., and Zech, L.A. 1989. Human selenite metabolism. A kinetic model. *Am. J. Physiol.* **257**, R556–R567.

Pavlata, L., Illek, J., and Pechova, A. 2001. Blood and tissue selenium concentrations in calves treated with inorganic or organic selenium compounds. *Acta Vet. Brno* **70**(1), 19–26.

Pehrson, B., Knutsson, M., and Gyllensward, M. 1989. Glutathione peroxidase activity in heifers fed diets supplemented with organic and inorganic selenium compounds. *Swed. J. Agric. Res.* **19**, 53–59.

Pehrson, B., Ortman, K., Madjid, N., and Trafikowska, U. 1999. The influence of dietary selenium as selenium yeast or sodium selenite on the concentration of selenium in the milk of suckler cows and the selenium status of their calves. *J. Anim. Sci.* **77**(12), 3371–3376.

Peretz, A., Nève, J., Duchateau, J., Siderova, V., Huygen, K., Framaey, J.-P., and Carpentier, Y.A. 1994. Effects of selenium supplementation on immune parameters in gut failure patients on home parenteral nutrition. *Nutrition* **7**, 215–221.

Peterson, P.J. and Butler, G.W. 1962. The uptake and assimilation of selenite by higher plants. *Aust. J. Biol. Sci.* **15**, 126–146.

Plieninger, H. 1950. The cleavage of γ-butyrolactone and α-amino-γ-butyrolactone with sodium methylmercaptide or selenide. A synthesis of methionine. *Chem. Ber.* **83**, 265–268.

Poulsen, J.-C.N., Harris, P., Jensen, K.F., and Larsen, S. 2001. Selenomethionine substitution of oritidine-5′-monophosphate decarboxylase causes a change in crystal contacts and space group. *Acta Crystallogr., Sect. D: Biol. Crystallogr.* **D57**(9), 1251–1259.

Power, R.F. 1994. Selenium enriched yeast: form and applications. "Proc. STDA 5th Internatl. Symp.", pp. 89–94156. May 8–10. Brussels, STDA, Brussels.

Raju, K., Gopinath, R., and Gupta, M.N. 1981. Reaction of selenomethionine with *o*-benzoquinone. *Anal. Biochem.* **118**, 227–230.

Robinson, M.F., Rea, H.M., Friend, G.M., Stewart, R.D.H., Snow, P.C., and Thomson, C.D. 1978. On supplementing the selenium intake of New Zealanders. 2. Prolonged metabolic experiments with daily supplements of selenomethionine, selenite and fish. *Br. J. Nutr.* **39**, 589–600.

Rotruck, J.T., Pope, A.E., Ganther, H.E., Swanson, A.B., Hafeman, D.G., and Hoekstra, W.G. 1973. Selenium: biochemical role as a component of glutathionine peroxidase. *Science* Washington **179**, 588.

Roy, M., Kiremidjian-Schumacher, L., Wishe, H., Cohen, M.W., and Stotzky, G. 1994. Supplementation with selenium and human immune cell functions. I. Effect on lymphocyte proliferation and interleukin 2 receptor expression. *Biol. Trace Elem. Res.* **41**, 103–114.

Schrauzer, G.N. 1998a. Selenomethionine and selenium yeast: appropriate forms of selenium for use in infant formulas and nutritional supplements. *J. Med. Foods* **1**, 201–206.

Schrauzer, G.N. 1998b. Characterization of selenium yeasts for nutritional selenium supplementation. *In* "Proc. Sixth Internatl. Symp. on the Uses of Selenium and Tellurium" (Y. Palmieri, ed.), pp. 77–79, May 10–12. Scottsdale, AZ.

Schrauzer, G.N. 2000. Selenomethionine: a review of its nutritional significance, metabolism and toxicity. *J. Nutr.* **130**, 1653–1656.

Schrauzer, G.N. 2001. Nutritional selenium supplements: product types, quality, and safety. *J. Am. Coll. Nutr.* **20**, 1–4.

Schrauzer, G.N. and White, D.A. 1978. Selenium in human nutrition: dietary intakes and effects of supplementation. *Bioinorg. Chem.* **8**, 303–318.

Schrauzer, G.N. and McGinness, J.E. 1979. Observations on human selenium supplementation. *Trace Subst. Environ. Health XIII*, 64–67.

Schwarz, K. and Foltz, C.M. 1957. Selenium as an integral part of factor 3 against dietary necrotic liver degeneration. *J. Am. Chem. Soc.* **79**, 3292–3293.

Shearer, T.E. 1975. Developmental and post developmental uptake of dietary organic and inorganic selenium into the molar teeth of rats. *J. Nutr.* **105**, 338–347.

Shepherd, L. and Huber, R.E. 1969. Some chemical and biochemical properties of selenomethionine. *Can. J. Biochem.* **47**, 877–881.

Shimazu, F. and Tappel, A.L. 1964. Selenoamino acids decrease radiation damage to amino acids and proteins. *Science* **143**, 369–371.

Shrift, A. 1954a. Sulfur-selenium antagonism. I. Antimetabolite action of selenate on the growth of *Chlorella vulgaris*. *Am. J. Bot.* **41**, 351–358.

Shrift, A. 1954b. Sulfur-selenium antagonism II. Antimetabolite action of selenomethionine on the growth of *Chlorella vulgaris*. *Am. J. Bot.* **41**, 345–352.

Slaugh, B. 2002. Nutrition-specific eggs: an even more perfect food. *In* "Nutritional Biotechnology in the Feed and Food Industries" (T.P. Lyons and K.A. Jacques, eds), pp. 289–293. University of Nottingham Press, Nottingham, UK.

Sliwkowski, M.X. 1984. Characterization of selenomethionine in proteins. *Methods Enzymol.* **107**, 620–623.

Smith, A.L. 1949. Separation of the selenium compounds in seleniferous plant protein hydrolysates by paper partition chromatography. Thesis in partial fulfillment of the requirements for the degree of Master of Science, South Dakota State College of Agriculture and Mechanic Arts, August 1949.

Spallholz, J.E. and Rafferty, A. 1987. Nutritional, chemical and toxicological evaluation of high-selenium yeast. *In* "Selenium in Biology and Medicine, Part A" (G.F. Combs, J.E. Spallholz, O.A. Levander, and J.E. Oldfield, eds), pp. 516–529. AVI Van Nostrand Reinhold Company, New York.

Suhajda, A., Hegoczki, J., Janzso, B., Pais, I., and Vereczkey, G. 2000. Preparation of selenium yeast I. Preparation of selenium-enriched *Saccharomyces cerevisiae*. *J. Trace Elem. Med. Biol.* **14**(1), 43–47.

Sunde, R.A., Gutzke, G.E., and Hoekstra, W.G. 1981. Effect of dietary methionine on the biopotency of selenite and selenomethionine in the rat. *J. Nutr.* **111**, 76–86.

Suomi, K. and Alaviuhkola, T. 1992. Response to organic and inorganic selenium on the performance and blood Se content of growing pigs. *Agric. Sci. Finl.* **1**, 211–215.

Surai, P.F. 2000. Organic selenium: benefits to animals and humans, a biochemist's view. *In* "Biotechnology in the Feed Industry, Proceedings of Alltech's 16th Annual Symposium" (T.P. Lyons and K.A. Jacques, eds), pp. 205–260. Nottingham University Press,, Nottingham, UK.

Swanson, C.A., Patterson, B.H., Levander, O.A., Vellon, C., Taylor, P., Helzsouer, K., McAdam, P.A., and Zech, L.A. 1991. Human [^{74}Se]selenomethionine metabolism: a kinetic model. *Am. J. Clin. Nutr.* **54**, 917–926.

Terry, N., Carlson, C., Raab, T.K., and Zayed, A.M. 1992. Rates of selenium volatilization among crop species. *J. Environ. Qual.* **21**, 342–344.

Terry, N., Zayed, A.M., de Souza, M.P., and Tarun, A.S. 2000. Selenium in higher plants. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **51**, 401–432.

Thomson, C.D. and Stewart, R.D.H. 1974. The metabolism of (^{75}Se)selenite in young women. *Brit. J. Nutr.* **32**, 47–57.

Thomson, C.D., Burton, C.E., and Robinson, M.F. 1978. On supplementing the selenium intake of New Zealanders. I. Short experiments with large doses of selenite or selenomethionine. *Br. J. Nutr.* **39**, 579–587.

Tuve, T. and Williams, H.H. 1961. Metabolism of selenium by *Escherichia coli*: biosynthesis of selenomethionine. *J. Biol. Chem.* **236**, 597–601.

Uzar, H.G. and Michaelis, J. 1994. The metabolism of L-methionine/L-selenomethionine: NMR-studies. *GIT Fachz. Lab.* **38**(12), 1214 (see also pp. 1216–1218).

Van Rij, A.M., Thomson, C.D., McKenzie, J.M., and Robinson, M.F. 1979. Selenium deficiency in total parenteral nutrition. *Am. J. Clin. Nutr.* **32**, 2076–2085.

Vasquez-Pelaez, M., Montes Bayon, M., Garcia Alonso, J.I., and Sanz-Medel, A. 2000. A comparison of different derivatization approaches for the determination of selenomethionine by GS-ICP-MS. *J. Anal. At. Spectrom.* **15**(9), 1217–1222.

Vendeland, S.C., Deagen, J.T., Butler, J.A., and Whanger, P.D. 1994. Uptake of selenite, selenomethionine and selenate by brush border membrane vesicles isolated from rat small intestine. *BioMetals* **7**, 305–312.

Vinson, J.A. and Bose, P. 1987. *In* "Selenium in Biology and Medicine, Part B" (G.F. Combs, J.E. Spallholz, O.A. Levander, and J.E. Oldfield, eds), pp. 708–711. AVI Van Nostrand Reinhold Company, New York.

Wakebe, M. 1998. Feed for meat chickens and feed for laying hens. Japanese Patent Office, Application Heisei 8-179629. Jan. 27.

Walter, R., Schwartz, I.L., and Roy, L. 1973. Can selenoamino acids act as reversible biological antioxidants? *Ann. NY Acad. Sci.* **107**, 175–180.

Wang, Z.-J., Zhou, J., and An, P. 1992. Metabolic differences and similarities of selenium in blood and brain of the rat following the administration of different selenium compounds. *Biol. Trace Elem. Res.* **33**, 135–143.

Waschulewski, I.H. and Sunde, R.A. 1988. Effect of dietary methionine on tissue selenium and glutathione peroxidase on tissue selenium and glutathione peroxidase (EC 1.11.1.9) activity given selenomethionine. *Br. J. Nutr.* **60**, 57–68.

Whanger, P.D. 2002. Selenocompounds in plants and animals and their biological significance. *J. Am. Coll. Nutr.* **21**, 223–232.

Whanger, P.D. and Butler, J.A. 1994. Can physiological responses be used to assess the chemical forms of selenium consumed by humans, *In* "Proc. STDA 5th Internatl. Symp.", pp. 153–156, May 8–10. Brussels, STDA, Brussels.

Whanger, P.D., Green, J.L., and Butler, J.A. 1998. Should selenium enriched vegetables be consumed for the prevention of cancer. *In* "Proc. 6th Internat. Symp. Uses of Selenium and Tellurium" (Y. Palmieri, ed.), pp. 57–61, Scottsdale, AZ. Grimbergen.

WHO, 1996. *Report of a Joint FAO/IAEA/WHO Expert Consultation, Trace Elements in Human Nutrition and Health*, World Health Organization, Geneva, p. 343.

Wolffram, S.B., Berger, B., and Scharrer, E. 1989. Transport of selenoaminoacids and their sulfur analogues across the intestinal brush border membrane. *J. Nutr.* **119**, 706–712.

Yang, G. and Xia, Y. 1995. Studies on human dietary requirements and safe range of dietary intakes of selenium in China and their application in the prevention of related endemic diseases. *Biomed. Environ. Sci.* **8**, 187–201.

Yang, G. and Zhou, R. 1994. Further observations on the human maximum safe dietary selenium intake in a seleniferous region of China. *J. Trace Elem. Electrol. Health Dis.* **8**, 159–165.

Yang, G., Zhou, R., Yin, S., Gu, L., Liu, Y., and Liu, X. 1989a. Studies of safe maximal dietary Se intake in a seleniferous area in China. I. Selenium intake and tissue selenium levels of the inhabitants. *J. Trace Elem. Electrol. Health Dis.* **3**, 77–89.

Yang, G., Yin, S., Zhou, R., Gu, L., Liu, Y., and Liu, X. 1989b. Studies of safe maximal dietary Se intake in a seleniferous area in China. II. Relation between Se intake and the manifestation of clinical signs and certain biochemical alterations in blood and urine. *J. Trace Elem. Electrol. Health Dis.* **3**, 123–130.

Yang, X., Tian, Y., Ha, P., and Gu, L. 1997a. Determination of the selenomethionine content in grain and human blood. *Wei Sheng Yen Chiku and J. Hyg. Res.* **26**(2), 113–116.

Yang, X., Zhou, R., Ha, P., Tian, Y., Gu, L., and Xia, Y. 1997b. Method of cyanogen bromide-fluorimetric determination of trace amount of selenomethionine in grain and blood. *Weishung Yanjiu* **26**, 56–59.

Yin, S., Jin, Y., and Kenji, Y. 1996. Effect of dietary vitamin B6 on selenium bioavailability. IV. Effect of dietary vitamin B6 on selenium bioavailability from different chemical forms and lipid peroxidation in rats. *Weishung Yanjiu* **25**(5), 296–300.

Yu, S.Y., Chu, Y.J., and Li, W.G. 1988. Selenium chemoprevention of liver cancer in animals and possible human applications. *Biol. Trace Elem. Res.* **15**, 231–241.

Zayed, A.M., Lytle, C.M., and Terry, N. 1998. Accumulation and volatilzation of different chemical species of selenium by plants. *Planta* **206**, 284–294.

Zdansky, G. 1968. Studies in the synthesis of selenoamino acids. *Acta Univ. Upsalensis* **123**.

Zemanova, D., Simek, M., Illik, J., and Pavlata, L. 2000. Organic trace elements (Se) in rations for dairy cows. *In* "Mengen- und Spurenelemente, Arbeitstagung, 20th, Jena, Germany, Dec. 1–2, 2000" (M. Anke, ed.), pp. 636–644.

Zheng, O. and Wu, J. 1988. The determination of selenomethionine in selenium yeast by cyanogen bromide gas chromatography. *Biomed. Chromatogr.* **2**, 258–259.

Zheng, O., Wu, J., and Xie, L. 1989. A method for the indirect determination of trace bound selenomethionine in plants and some biological materials. *Anal. Biochem.* **178**, 77–81.

ECHINACEA AS A FUNCTIONAL FOOD INGREDIENT

CLIFFORD HALL III

324 IACC, Department of Cereal and Food Sciences
North Dakota State University
Fargo, ND 58105
USA

I. INTRODUCTION

Echinacea (pronounced ek-a-NAY-sha) or purple coneflower is a perennial plant in the Compositae or daisy family (Foster, 1985). Traditional literature has reported that nine species of *Echinacea* exists in nature. However, under a new reclassification system, eight varieties are categorized under only four species (Binns *et al.*, 2002a). *Echinacea* (*E.*) *pallida* var. *angustifolia* (hereafter referred to as *E. angustifolia*) and *E. purpurea* are the most common species of *Echinacea*. *E. angustifolia* is a wild flower in North

ADVANCES IN FOOD AND NUTRITION RESEARCH VOL 47
ISSN: 1043-4526
DOI: 10.1016/S1043-4526(03)47003-4

Dakota and other parts of the North American great plains that range from Texas to Saskatchewan whereas *E. purpurea* can be found from Georgia to Michigan and into Kansas in the west (Foster, 1991). The flower size and color vary among species of *Echinacea* but in general they can be characterized by hues of purple ray florets that surround an orange-brown colored head containing numerous achene, i.e., fruiting bodies that contain seeds (Foster, 1985; Schulthess *et al.*, 1991). *E. purpurea* (L.) Moench., *E. angustifolia* DC, and *E. pallida* var. *pallida* Nutt. (hereafter referred to as *E. pallida*) are the *Echinacea* species most widely used for medicinal purposes and are commercially cultivated. In the western United States and Canada, *E. purpurea* (L) Moench. and *E. angustifolia* DC. account for 80 and 20%, respectively, of the cultivated *Echinacea* (Li, 1998). Other species (*E. laevigata*, *E. atrorubens*) and varieties (*E. tennesseensis*, *E. sanguinea*, *E. simulata*, and *E. paradox*) are adapted to specific growing regions and are thus not exploited commercially.

In 1909, the American Medical Association dropped *Echinacea* from the list of approved medicinal agents (Wills *et al.*, 2000). In contrast, the European Community embraced *Echinacea* as a pharmaceutical agent. By 1930, German researchers began exploring the chemical constituents of *Echinacea* in the hope of identifying the component(s) responsible for the biological activity (Wills *et al.*, 2000). Over the past 70 years, a number of components have been identified as having biological activity. It is widely accepted today that the active constituents are grouped into the unsaturated lipophilic compounds (Figure 1), caffeic acid phenols (Figure 2), and polysaccharides (Figure 3) categories. However, synergistic activities may exist with other components of *Echinacea* to provide bioactivity.

Around the turn of the 20th century, *Echinacea* was being prescribed as a treatment for a number of aliments, which included snake bites, typhus, dysentery and cancer (Wills *et al.*, 2000). Today, *Echinacea* is promoted as an immunostimulatory agent (Bauer, 1999a, 2000) and is one of the most popular dietary supplements in the United States. In addition, *Echinacea* has been used successfully in Germany as evidenced by the two million prescriptions filled by German physicians annually (Barrett *et al.*, 1999). Supplements or preparations of *Echinacea* are derived from the herbal (including seeds or flowers) and root or rhizome parts of the plant. Numerous preparations exist in the market, which include fresh and dried plant materials, expressed juices, ethanol tinctures and glycerin extracts.

Most of the available *Echinacea* preparations could be incorporated into food systems to create a functional food. Teas, beverages, and confections, for example, have been targeted as delivery agents of *Echinacea* phytochemicals in food systems (Wills *et al.*, 2000). The use of *Echinacea* as a component in functional foods has come under fire because *Echinacea* is not considered

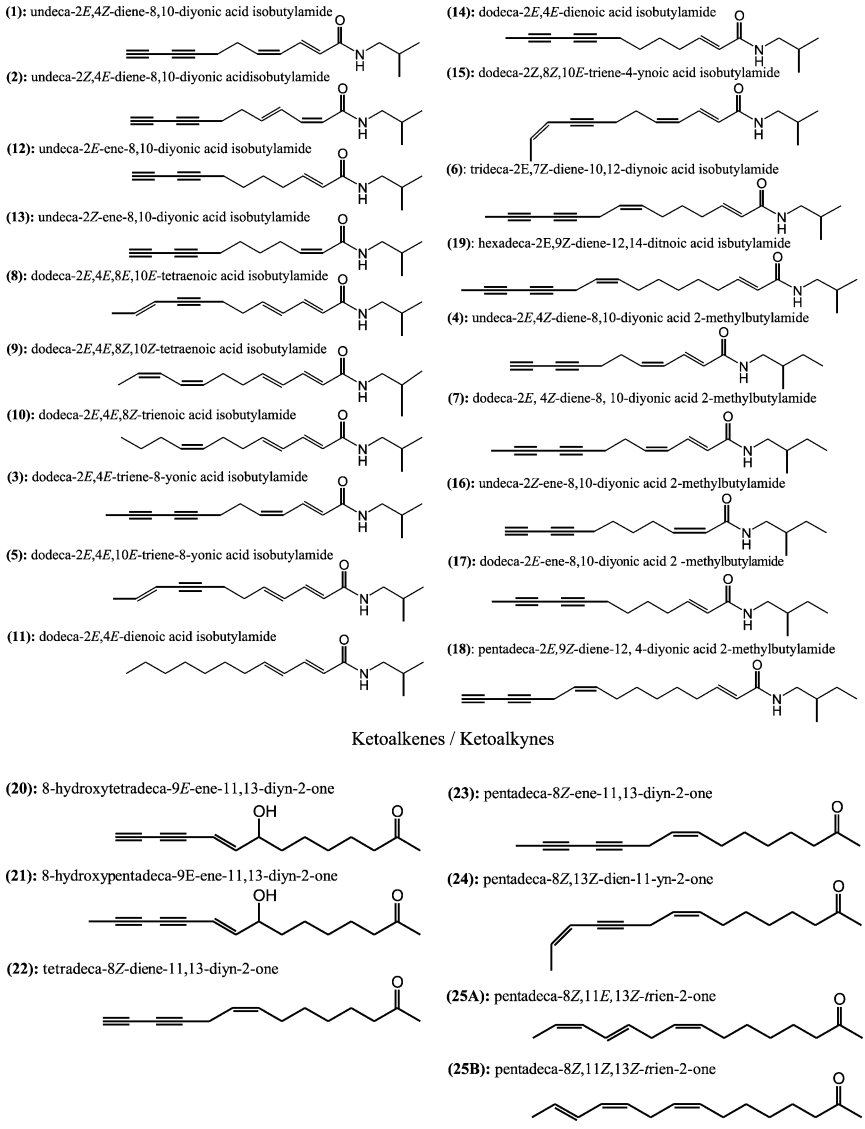


FIG. 1 Alkamides and ketoalkenes/alkynes identified in *Echinacea*. Numbers in parentheses refer to the numbers assigned by Bauer and Remiger (1989).

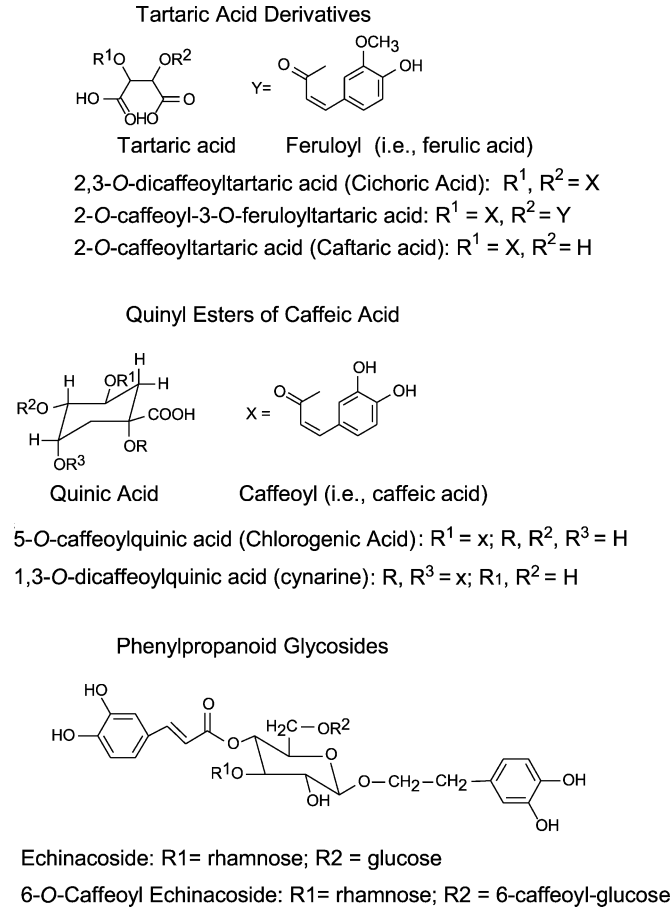


FIG. 2 Major caffeic acid phenols (CAP) found in *Echinacea*.

a "generally recognized as safe" (GRAS) ingredient in the United States. However, Health Canada does support the use of *Echinacea* in food products and functional foods could be developed for the Canadian market (Health Canada, 1999). The economic importance of *Echinacea* in the dietary and medicinal markets is significant, and thorough studies need to be completed to assess the risk/benefits of consuming *Echinacea*. In addition, phytochemical stability assessments are critical if *Echinacea* is to be incorporated into food systems. This chapter will highlight the phytochemical constituents of *Echinacea* and will include information relevant to the biological activity,

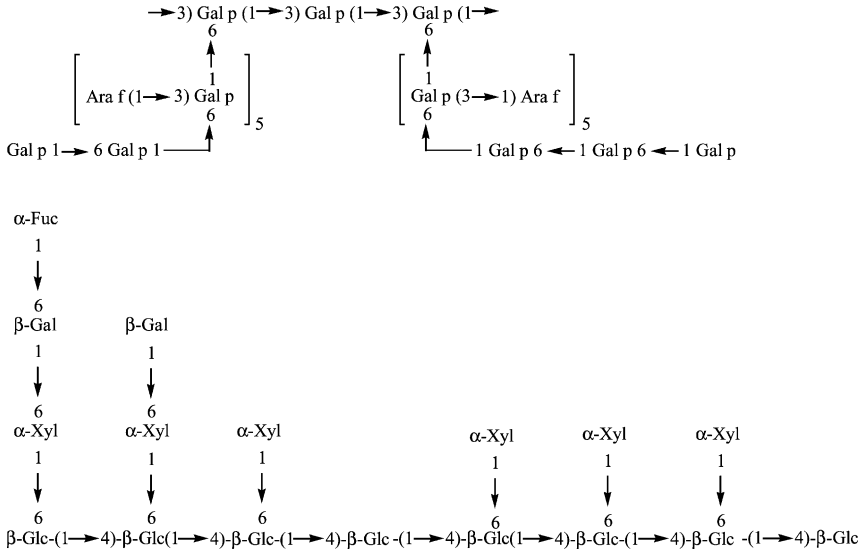


FIG. 3 Example of polysaccharide obtained from *Echinacea* and cell cultures of *Echinacea*. Partially characterized structures (adapted from Wagner *et al.*, 1988; Melchart and Linde, 1999).

potential safety concerns, detection methods and the effects of processing on phytochemicals, including potential functional activities such as antioxidant and antimicrobial activities. Limited information will be provided for growing and harvesting of *Echinacea*. It is the author's goal to present the most current information; thus a great number of studies may not be presented in which the author suggests that the reader seek for additional background. In addition, a variety of studies showing both positive and negative results have been presented in the hope that a more standard method be adopted for assessing the biological activity of *Echinacea*.

II. PHYTOCHEMICAL CONSTITUENTS

A. GENERAL

Unsaturated lipophilic compounds, caffeic acid phenols (CAP) and polysaccharides are believed to be responsible for the observed immunostimulatory activity. The alkamides and ketoalkenes/alkynes (Figure 1) are the two major groups in the unsaturated lipophilic compound category.

Approximately, 20 alkamides and seven ketoalkenes/alkynes have been characterized (Bohlmann and Hoffman, 1983; Bauer *et al.*, 1987; Bauer and Remiger, 1989; He *et al.*, 1998; Dietz and Bauer, 2001). Twelve (12) CAP (Figure 2) have been characterized and classified as quinyl esters of caffeic acid, tartaric acid derivatives, or phenylpropanoid glycosides (Becker *et al.*, 1982; Becker and Hsieh, 1985; Bauer *et al.*, 1988a,b; Cheminat *et al.*, 1988). In addition, at least eight phenolic acids have been identified (Glowniak *et al.*, 1996). The polysaccharides are the least characterized of the three main categories; however, approximately five polysaccharides and three glycoproteins have been characterized (Proksch and Wagner, 1987; Wagner *et al.*, 1988; Roesler *et al.*, 1991a; Bauer, 1999a; Classen *et al.*, 2000). Other components such as flavonoids and essential oils are present in various parts of the plant and could act to enhance the biological activity of *Echinacea*.

B. LIPOPHILIC COMPOUNDS

1. Concentrations and composition

The immune-enhancing activity of the lipophilic compounds is attributed to the alkamides. Isobutylamides and 2-methylbutylamides are the two general classes of alkamides (Bauer and Remiger, 1989) based on the isobutyl or methylbutyl moiety linked via an amide bond to an unsaturated hydrocarbon (Figure 1). The ketoalkenes/alkynes lack the butylamide moiety and could be artifacts formed during storage (Bauer *et al.*, 1987). The most common methods for the extraction of lipophilic constituents include the use of hexane (Bauer *et al.*, 1989; He *et al.*, 1998) or acetonitrile (Perry *et al.*, 1997). Most researchers use HPLC to separate the alkamides. Typical conditions include a reverse-phase column and a mobile phase of water and acetonitrile (40–80%, gradient) and detection at 254 nm (Bauer *et al.*, 1988c, 1989).

Total alkamide levels in *Echinacea* are variable and limited data are available for direct comparisons between laboratories (Table I). However, most researchers agree that the roots contain higher alkamide levels than the aerial parts (Bauer and Remiger, 1989; Perry *et al.*, 1997; Wills and Stuart, 1999; Stuart and Wills, 2000a,b; Binns *et al.*, 2002b). Stuart and Wills (2000a) reported that approximately 70% of the alkamides were found in the roots followed by the flower (20%), stem (10%) and leaves (1%). Further dissection of the *Echinacea* plant gave similar alkamide distributions with 24% of the alkamides being obtained from the reproductive stem, followed by the roots (22%), flowers (19%), rhizomes (17%), vegetative stems (14%) and leaves (4%) (Perry *et al.*, 1997).

Wills and Stuart (1999) and Stuart and Wills (2000a) completed a series of experiments to evaluate alkamide levels in *E. purpurea* grown in Australia.

TABLE I
AVERAGE ALKAMIDE CONTENT (mg/g PLANT PART DRY WEIGHT) OF VARIOUS PLANT PARTS OF *E. PURPUREA*

	Plant part					
Location	Roots	Rhizome	Flower	Leaf	Veg. stem[a]	Rep. stem[b]
Australia[c]	8.9	n/r	2.6	0.10	n/r	0.75
Australia[d]	3.9[e]	n/r	0.66[f]	–[f]	–[e]	–[f]
New Zealand[g]	6.2	8.0	2.9	0.24	19.0	1.52
United States[h]	12.9	n/r	5.63[i]	n/r	n/r	–[i]

[a]Vegetative stem.
[b]Reproductive stem; n/r = not reported.
[c]Wills and Stuart (1999).
[d]Rogers *et al.* (1998).
[e]Includes vegetative stems.
[f]Reported as aerial parts but may include flower, leaf and reproductive stem parts.
[g]Perry *et al.* (1997).
[h]Binns *et al.* (2002a).
[i]Reported as inflorescences (flower and reproductive stem parts).

Of the 62 commercial samples tested, 50% of the samples had alkamide levels in the range 6–9 mg/g roots (d.w.b.) and 35% fell in the range 3–6 mg alkamide/g root material. In addition, 90% of the samples derived from the aerial parts (leaves, stems, flowers) had alkamide levels in the range 0.2–1.4 mg/g dried aerial parts (Wills and Stuart, 1999). This same study showed that alkamide levels were higher in samples obtained in the areas north of the 32°S latitude in Australia. Further studies showed that coastal (32°S latitude, sea level, 13–23°C) growing sites had slightly higher, but not significant, levels of alkamides than the sub-tropic tableland (32°S latitude, 1030 m altitude, 13–23°C) during the first growing season (Stuart and Wills, 2000a). However, the level of alkamides in the tableland flower tissue collected during the second season, and at the mature growth stage, was significantly higher than the alkamide levels from flower tissue of coastal *E. purpurea*. Stuart and Wills (2000a) also found that alkamide levels decreased as the tissue aged during the growing season. For example, the concentration of alkamides dropped from 11.7 mg/g root tissue, collected at the pre-flowering stage, to 10.2, 9.5, and 9.0 mg/g during the flowering, mature and senescent growth stages, respectively. However, an overall accumulation of alkamides was found per plant due to an increase in biomass during the growing season.

Rogers *et al.* (1998) reported alkamide levels in *E. purpurea* between 0.24 and 1.1 mg/g aerial parts for samples collected at various locations within Australia. Again, the growing location did not have a significant effect

on alkamide formation. These authors also noted that the vegetative stem and root portions gave higher alkamide levels (3.9 mg/g), which is in agreement with the other researchers. The average alkamide levels in the root of *E. angustifolia* grown in the United States and Australia were 1.2 and 0.59 mg/g root, respectively (Rogers *et al.*, 1998). However, only a few samples were evaluated in this study and in one Australian sample the alkamide level of 1.1 mg/g root was found, again indicating that the growing location may not significantly influence alkamide concentrations.

Binns *et al.* (2002b) evaluated the alkamide levels in cultivated and wild populations of various *Echinacea* species and varieties. In most samples, the concentration of alkamides was highest in the root material. However, no clear trend was observed across all species and varieties under all growing conditions. Only the flowering parts of *E. pallida* obtained from plants grown from wild-harvested seeds in the greenhouse (referred to as germlings) had alkamide levels higher than the root material of the same plant. With the exceptions of *E. purpurea* and *pallida*, the roots of the germlings had total alkamide levels higher than wild-harvested roots (Table II). Transplanting of the wild species/varieties and growing under greenhouse conditions generally favored the production of alkamides (Binns *et al.*, 2002b). The most interesting samples were that of *E. atrorubens* var. *atrorubens*. The cultivated samples had significantly higher alkamide levels than the wild-harvested samples. For example, the cultivated samples had alkamide levels of 59.5 and 22.6 mg/g root tissue for the germlings and transplants, respectively; whereas, the wild-harvested had only 4.9 mg alkamides/g root tissue (Table II). For a general summary of the total alkamides in the roots of *Echinacea*, please see Table II and for additional details please refer to the report by Binns *et al.* (2002b). In addition, wild-harvested

TABLE II

AVERAGE TOTAL ALKAMIDE[a] CONTENT (mg/g DRY ROOT) OF VARIOUS *ECHINACEA* SPECIES AND VARIETIES GROWN UNDER CULTIVATED AND WILD ENVIRONMENTS

	Germlings[b]	Wild-harvested	Wild-transplant
E. purpurea	12.9	14.2	17.4
E. pallida var *angustifolia*	11.1	10.7	24.1
E. pallida var *pallida*	1.3	8.6	2.3
E. pallida var *sanguinea*	53.1	36.1	14.3
E. pallida var *tennesseensis*	28.6	14.2	16.2
E. atrorubens var *atrorubens*	59.5	4.9	22.6

Adapted from Binns *et al.* (2002a).

[a]Estimated average total alkamide levels.

[b]Plants grown from wild-harvested seeds in a greenhouse.

E. atrorubens var. *paradox* and *E. atrorubens* var. *neglecta* had significantly higher levels of ketoalkenes/alkynes than other wild-harvested *Echinacea* (Binns *et al.*, 2002b). Cultivating the *E. atrorubens* var. *neglecta* in the greenhouse had little effect on the production of the ketoalkenes/alkynes, as noted by the slight reduction in root alkamide levels from 6.7 to 4.3 mg/g in wild-harvested and cultivated samples, respectively. In contrast, cultivating of *E. pallida* slightly promoted the formation of ketoalkenes/alkynes from 1.0 in wild-harvested to 1.9 and 3.3 mg/g root tissue for transplants and germlings, respectively. For most other *Echinacea* species/varieties, the cultivated samples had lower ketoalkenes/alkynes levels than the wild-harvested plants. Similar to the alkamide, the root tissue had higher concentrations of the ketoalkenes/alkynes than did the flower tissue (Binns *et al.*, 2002b).

The concentration of individual alkamides appeared to depend more on the tissue type than on the growing location and conditions. Twenty (20) alkamides have been identified in *Echinacea* and not one species/variety contains all 20 alkamides (Bohlmann and Hoffman, 1983; Bauer *et al.*, 1987; Bauer and Remiger, 1989; He *et al.*, 1998; Dietz and Bauer, 2001). In general, alkamides in *E. purpurea* possess a 2,4-dienoic moiety, whereas the 2-monene moiety is more common in *E. angustifolia*. The 2-ketoalkenes and 2-alkynes lack an isobutylamide moiety and are common for *E. pallida* (Bauer *et al.*, 1988c; Bauer and Remiger, 1989; Schulthess *et al.*, 1991). Regardless of species/variety, the roots appear to have a more diverse alkamide profile than the herbal/aerial parts. However, dodeca-2*E*,4*E*,8*Z*, 10*Z*-tetraenoic acid isobutylamide and dodeca-2*E*,4*E*,8*Z*,10*E*-tetraenoic acid isobutylamide (hereafter referred to as the tetraenoic alkamides) are the major alkamides in *Echinacea*.

Bauer and Remiger (1989) reported that the tetraenoic alkamides content was 0.04–0.39 mg/g root of *E. purpurea* and 0.09–1.51 mg/g root of *E. angustifolia*. The aerial parts had less than 0.01–0.3 mg/g of these alkamides in samples obtained from the United States and Europe. Rogers *et al.* (1998) found similar levels of tetraenoic alkamides in Australian-grown *E. purpurea* and *E. angustifolia*. Perry *et al.* (1997) observed that the tetraenoic alkamides accounted for 27% of the alkamides in the roots, 71% in the rhizome and 74% in the vegetative stem. These three parts are often harvested as a root and thus collectively the root system accounted for 84% of the total tetraenoic alkamides in the *E. purpurea* plant tissue. In the aerial parts of the plant, 64% of tetraenoic alkamides were found in the flower, and 31 and 5% were found in the reproductive stem and leaves, respectively. In *E. purpurea*, the tetraenoic alkamides accounted for 45 and 76% of the alkamides from the roots and aerial parts, respectively (Wills and Stuart, 1999). Additional research showed that the tetraenoic alkamides dodeca-2*E*, 4*E*, 8*Z*, 10*Z*-tetraenoic acid isobutylamide and

dodeca-2*E*, 4*E*, 8*Z*, 10*E*-tetraenoic acid isobutylamide accounted for 13 and 32% of the total alkamides in the root tissue (or 29 and 71% of tetraenoic alkamides), respectively (Stuart and Wills, 2000a). Binns *et al.* (2002b) also reported that the tetraenoic alkamides were the predominant alkamides in all *Echinacea* species/varieties except *E. pallida* var. *tennesseensis*. In this particular variety, undeca-2*Z*-ene-8, 10-diynoic acid isobutylamide was the major (45%) alkamide in the root tissue. However, the tetraenoic alkamides were the predominant alkamides in the aerial parts in all the species/varieties. With a few exceptions, the alkamide distribution was similar between the cultivated and wild-harvested plants (Binns *et al.*, 2002b).

Other predominant alkamides include undeca-2*Z*, 4*E*-diene-8, 10-diynoic acid isobutylamide and dodeca-2*E*,4*Z*-diene-8,10-diynoic acid isobutylamide (Wills and Stuart, 1999; Stuart and Wills, 2000a; Binns *et al.*, 2002b). In *E. purpurea*, these alkamides accounted for 15–25 and 13–27% of the root alkamides, respectively. However, neither compound was found in the aerial parts (Wills and Stuart, 1999; Stuart and Wills, 2000a; Binns *et al.*, 2002b). Undeca-2*E*,4*Z*-diene-8,10-diynoic acid isobutylamide accounted for 20% of the alkamides in the aerial parts and only 6% in the root of *E. purpurea* (Wills and Stuart, 1999). This observation was further supported by Binns *et al.* (2002b) who found that undeca-2*E*,4*Z*-diene-8,10-diynoic acid isobutylamide was predominantly present in the aerial parts for most species/varieties tested. These authors found that this alkamide accounted for 29% of the total alkamides from the aerial parts of *E. atrorubens* var. *atrorubens*, which was the highest average percentage among the plants tested. Additionally, they found that this alkamide was more prevalent in the cultivated plants and that most wild *Echinacea* plants tested lacked this alkamide (Binns *et al.*, 2002b). The cultivation of *Echinacea* is widely practiced and the higher alkamide levels found in cultivated plants provide sufficient evidence to eliminate the wild-harvesting practices.

2. Alkamide isolation and key structural features

Although a great number of advances have been made in the understanding of *Echinacea*, only limited information is available on the health benefits, chemical interaction, and safety of individual components or mixtures of components. From a practical perspective, using single components has little significance considering the make-up of *Echinacea* preparations currently used for pharmaceutical purposes. However, from a basic scientific perspective many additional questions are still not fully answered, such as the bioavailability and characterization of potential degradation products. Information on the isolation and characterization of components for basic studies is provided below. Additional information on how the components

are affected by processing/extraction of *Echinacea* will be discussed in later sections.

Two of the most common methods for the extraction of alkamides involve the use of hexane (Bauer *et al.*, 1989; He *et al.*, 1998) or acetonitrile (Perry *et al.*, 1997); however, ethanol-based extractions have also proven successful (Livesey *et al.*, 1999; Stuart and Wills, 2000b). In our laboratory at North Dakota State University, we use a series of solvents starting with hexane, then ethanol and finally 70% ethanol to extract the various families of compounds (e.g., lipophilic). Regardless of the extraction method, separation of individual alkamides can be completed using the thin layer chromatography (TLC) method of Bauer and Remiger (1989). Typically, hexane extracts are yellow and can be fractionated over silica gel using ethyl acetate–hexane (1:5) or by TLC using ethyl acetate–hexane (1:2). Bauer and Remiger (1989) used silica gel plates containing fluorescence indicator (F_{254}) and anisaldehyde/sulfuric acid spray, for detection, as a screening method on fractions collected during column chromatography. They found that the 2-monoenamide structures gave a yellow color while a violet color was formed when 2,4-dienamides were present. The 2,4-dienamides fluoresce at 254 nm, thus the plate can be viewed under UV light and used as a method for preliminary identification. Further purification of the alkamides can be completed on a reverse-phase semi-preparative column using water and acetonitrile (40–80%) gradient and detected at 254 nm (Bauer *et al.*, 1988c, 1989). When separating natural products, confirmation of the structural features is needed if an authentic source is not readily available. Nuclear magnetic resonance (NMR) data for the alkamides are similar because most contain an isobutylamide moiety and an unsaturated carbon chain. The variation in NMR data reflects the position and type of chemical bonds present in the alkamide.

The isobutylamide region of the alkamides (Figure 1) is unique and can be used to characterize the alkamides from the 2-methyl alkamides and other olefins. The broad singlet at *ca.* 5.5 ppm represents the proton on the nitrogen (N–H) whereas the doublet at 0.93 ppm represents the methyl protons ($2CH_3$). The methylene proton next to the nitrogen ($-NH-CH_2-$) is represented by a double doublet at 3.18 ppm and the multiplet signal at 1.80 ppm represents the methine protons ($-NH-CH_2-CH-$). If the isobutylamide feature were replaced by a 2-methyl-butylamide substitution (Figure 1), the most obvious change would be the additional triplet signal at 0.91 ppm, which represents the protons of the methyl group adjacent to the methylene group. The presence of a multiplet signal at 1.41 and 1.16 ppm represents the methylene protons adjacent to the methyl and methine groups. The proton signal at 1.58 ppm represents the methine group next to nitrogen.

The unsaturated carbon moiety will produce signals in two primary regions. The signals between 5.87 and 7.51 ppm represent the olefin protons

while the allylic methylene protons resonate at 2.18 and 2.55 ppm. A proton on terminal acetylene (i.e., triple bond) would have a signal near 1.98 ppm. Coupling constants of 11 and 15 Hz correspond to the *Z* and *E* configurations, hand are thus important for establishing the stereochemistry of the alkamide. For additional discussion on proton NMR for the alkamides, see Bauer *et al.* (1988c, 1989). Perry *et al.* (1997) provided ^{13}C NMR data that showed a characteristic carbonyl carbon at 166 ppm, which corresponds to the carbon attached to the nitrogen via an amide bond. The aliphatic carbon signals can be found between 11 and 47 ppm whereas carbon signals associated with the double and triple bonds of the unsaturated chain can be found between 119–136 and 64–77 ppm, respectively.

Mass spectral data is a useful analytical tool for characterizing structural features of the alkamides. Typically, the mass to charge (*m*/*z*) ratios ranged from 229 to 278 for the alkamides (He *et al.*, 1998). The isobutylamides and 2-methyl-butylamides can be differentiated by evaluating the fragmentation patterns. Isobutylamides include $[M - 57]^+$, $[M - 72]^+$, and $[M - 100]^+$, while 2-methyl-butylamides have $[M - 29]^+$, $[M - 86]^+$, and $[M - 114]^+$. The loss of 57, 72 and 100 mass units indicates a loss of the isobutyl group $[C_4H_9]^+$, $[C_4H_{10}N]^+$ and $[C_4H_{10}NCO]^+$, respectively. The loss of 29 mass units from 2-methyl-butylamides represents a $[C_2H_5]^+$ or ethyl ($-CH_2-CH_3$) unit. The loss of $[C_5H_{12}N]^+$ and $[C_5H_{12}NCO]^+$ are indicated by a loss of 86 and 144 *m*/*z* units from the parent ion.

3. Role of processing on alkamide recovery and stability

Because of the unsaturated nature of the alkamides, the oxidative stability of the alkamides may be a potential concern during the storage and processing of *Echinacea*. Bauer *et al.* (1988b) found a reduced alkamide level in stored *E. pallida*. They also noted that the ketoalkenes/alkynes undergo oxidative reactions, particularly in chopped samples. The storage of herbal medicines containing *Echinacea*, in dry form, would be expected to have similar alkamide reductions. Thus, reducing the potential for oxidation during storage and processing is critical for maintaining optimal alkamide levels in *Echinacea*-containing products.

Alkamide concentrations increased when *Echinacea* was subjected to various physical treatments that included cutting, compression or crushing (Wills and Stuart, 2000). The alkamide concentration increased from 6.0 mg/g in undamaged plant tissue to 9.6 mg/g in cut plant tissue. Bruising of the tissue prior to drying did not enhance alkamide retention; however, bruising followed by cutting significantly enhanced alkamide retention to about 9.2 mg/g (Wills and Stuart, 2000). These authors hypothesized that the higher alkamide concentrations observed in the physically abused samples

were due to the faster drying time as opposed to a biosynthetic production of alkamides. The physically abused samples were dried to less than 12% moisture in 12 h at 40°C whereas the whole plant required 48 h to reach the same moisture level. Kabganian *et al.* (2002) observed that the drying rate had two phases in which the moisture reduction was initially slow, but increased rapidly when the moisture content of the plant material was approximately 35%. These authors attributed the increase in drying rate to a cell-disruption phenomenon such that during the initial drying process a molecular diffusion across membranes was the mechanism for dehydration. The second phase of drying was attributed to a convective mass transfer where the cells disrupt allowing for the release and evaporation of water more quickly, thus increasing the drying rate. For example, drying *Echinacea* from 57 to 10% moisture at 30°C required 103.4 h whereas 5.3 h was required for drying at 70°C.

In addition, Kabganian *et al.* (2002) reported that the alkamide levels were not significantly affected by the drying process, which supports the findings of Wills and Stuart (2000) who found that the more rapid the drying time, the greater the alkamide retention. Total alkamide levels of *E. purpurea* roots were not significantly affected by drying (32°C for 48 h) from 69.2 to 6.8% moisture (Perry *et al.*, 2000). However, a significant reduction in the concentrations of two alkamides was found in the chopped *E. purpurea* roots. Dodeca-2*E*,4*Z*-diene-8,10-diynoic acid isobutylamides and dodeca-2*E*,4*Z*-diene-8,10-diynoic acid isobutylamide levels each dropped by 13% simply by chopping the root and rhizome tissue. A greater reduction (16 and 23%, respectively) was found after the samples had been chopped and then dried for 48 h.

The storage length and temperature are critical factors in the oxidative stability of polyunsaturated compounds. This also held true for the alkamides. Bauer *et al.* (1988b) noted that 8-hydroxy-9-ene derivatives were formed during the storage of *E. pallida*. They noted that the ketoalkenes/alkynes undergo oxidative reactions and suggested that the root remains whole and that the extract remains in solution to prevent oxidative degradation. Rogers *et al.* (1998) found that the storage of powdered *E. angustifolia* roots in a sealed bag at room temperature, under desiccation, resulted in a reduction in alkamide levels by 13%. A loss of 40, 55 and 80% in alkamide levels were found in the chopped *E. purpurea* samples stored at −18, 3 or 24°C respectively for 64 weeks, (Figure 4; Perry *et al.*, 2000). Livesey *et al.* (1999) reported a significant loss in the alkamide levels in powdered *E. purpurea* roots as temperatures increased from −20 to 40°C. These authors reported alkamide levels of 7.5 mg/g root for powders stored at −20°C; whereas, alkamide levels of 1.1 and 0.2 mg/g root were found in samples stored at 25 and 40°C, respectively. In contrast, no significant reductions of alkamides

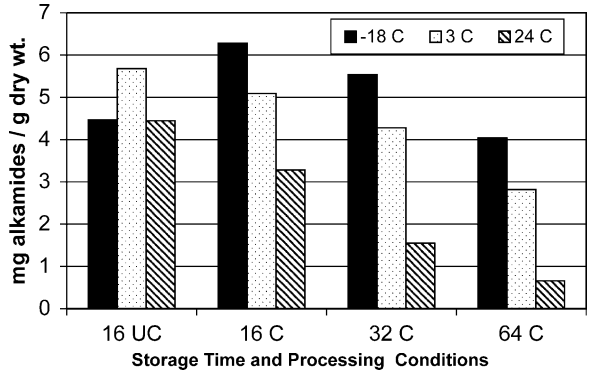


FIG. 4 The effect of storage time (days) and processing (chopped (C) or unchopped (UC)) conditions on alkamide retention (adapted from Perry *et al.*, 2000).

were found when alkamides were stored under acetonitrile (Perry *et al.*, 2000) or methanol (He *et al.*, 1998), which is further supported by the observation that alkamide stored under dry conditions (i.e., free of solvent) were unstable (Jacobson, 1954). Although acetonitrile would not be used in tinctures, the alkamides may behave in a similar manner under ethanol-preserved tinctures as demonstrated by Stuart and Wills (2000b). No significant change in alkamide levels were found in ethanol (55%) extracts stored at temperatures between −20 and 40°C (Livesey *et al.*, 1999).

Alkamide concentration increased during storage of *E. purpurea* roots at 20°C and 60% relative humidity (RH) over a 20-day storage period (Wills and Stuart, 2000). However, a loss, although not significant, of alkamides was reported after an additional 10-day storage. Storage of the aerial parts under the same conditions did not affect the alkamide retention over the 30-day storage. In contrast, 70 and 55% reductions in alkamide concentrations were found in dried, crushed *E. purpurea* stored at 20°C in the presence of light or at 30°C in the dark over 60 days, respectively (Wills and Stuart, 2000).

In addition to storage length, the method of drying plant tissue can have an impact on alkamide retention. Freeze-dried *E. purpurea* roots had significantly higher total alkamide levels compared to vacuum (50 mmHg) microwave-dried, air-dried at 70°C, and vacuum microwave-dried at partial vacuum (200 mmHg) products Kim *et al.* (2000a). In the partial vacuum-dried samples, the total alkamides and tetraenoic acid isobutylamides were significantly lower than other dried samples. In contrast, the total alkamide concentrations were higher in the air-dried (50°C) *E. purpurea* leaves than for the freeze-dried and vacuum microwave-dried samples.

Bauer (1999b) found that the alkamide, dodeca-2*E*,4*E*,8*Z*,10*E*/*Z*-tetraenoic acid isobutylamide, level was influenced by the preparation method. Nonthermal preparations appeared to have slightly higher levels of the tested alkamide than thermally treated products. Thus, the drying process may not be the best method for preparing *Echinacea* products. Pressing of the plant material to obtain an expressed juice is a common preparation method; however, preservation of the juice with ethanol is required. Direct ethanol extraction of the plant material can be used in place of the pressing operation.

Stuart and Wills (2000b) evaluated a series of ethanol:water mixtures as well as solvent:solute ratio and extraction temperatures as a means to identify the optimal solvent extraction conditions. These authors found that high ethanol levels favored alkamide extractions and that the 90:10 (ethanol:water v/v) removed 70 and 50% of the alkamides in the roots and aerial parts, respectively (Figure 5). In general, higher alkamide recovery was reported for the root tissue as compared to the aerial tissue. Although the alkamide recovery was quite high, the phenolic content of the extract was poor suggesting that only optimal extraction conditions were met for the alkamides. In general, the authors suggested a solvent ratio of 60:40 (ethanol:water v/v) for optimal extraction of alkamides and phenolic compounds. Dropping the ethanol content from 90 to 60% resulted in a 9% reduction in alkamide extraction (Stuart and Wills, 2000b). These observations were similar to the findings of Bergeron *et al.* (2000) and Binns *et al.* (2002b) who reported that a 70% ethanol solution was optimal for extracting various *Echinacea* phytochemicals; however, 95% ethanol was ideal for extracting alkamides. Sun *et al.* (2002) reported that the presence of

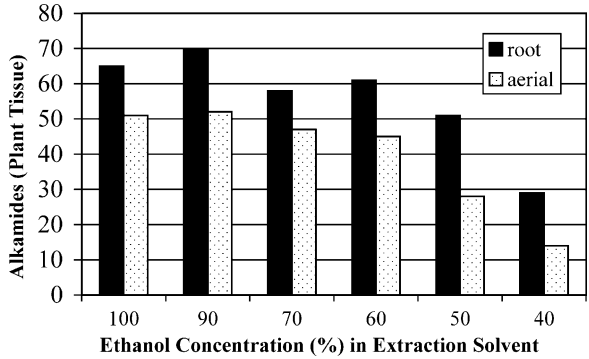


FIG. 5 The effect of ethanol concentration on alkamide recovery from *Echinacea* tissue (adapted from Stuart and Wills (2000b)).

water hindered the extraction of alkamides by supercritical fluid (SF) carbon dioxide. However, no difference in alkamide extraction was noted for roots having moisture contents of 8.4 and 4.9%. They hypothesized that the presence of water (75%) in the fresh *Echinacea* sample altered the polarity of the supercritical carbon dioxide, thus reducing the extraction of the lipophilic alkamides.

The ratio of solvent:solute is also important as noted by Stuart and Wills (2000b). They noted that as the solvent:solute ratio increased from 2:1 to 8:1, alkamide in the extract increased from 37 to 68% for the roots and from 27 to 53% for the aerial parts. As the ratio of solvent:solute increases, so does the ethanol level thus enhancing alkamide recovery. In SF extractions, the addition of ethanol (10–12%) as a modifier to the supercritical carbon dioxide enhances the total extract or oleoresin yield but does not significantly influence the alkamide concentrations (Catchpole *et al.*, 2002). Sun *et al.* (2002) also noted that a 5% ethanol addition did not significantly affect the alkamide recovery.

Stuart and Wills (2000b) also reported that an extraction temperature of 20°C was optimal for extracting alkamides when a 60:40 ratio of ethanol:water was used as the solvent. Under these conditions, 61 and 45% of the alkamides were extracted from the roots and aerial tissues, respectively (Figure 6). In contrast, Sun *et al.* (2002) reported the higher temperatures favored alkamide extraction during SF extraction of *E. angustifolia* under constant pressures. These authors estimated that 2–3 times more alkamides could be extracted as the temperature of the SF extractor increased from 45 to 60°C, regardless of the vessel pressure. Catchpole *et al.* (2002) also reported similar findings in that increasing the temperature favored alkamide extraction when constant pressures were maintained in the SF extractor.

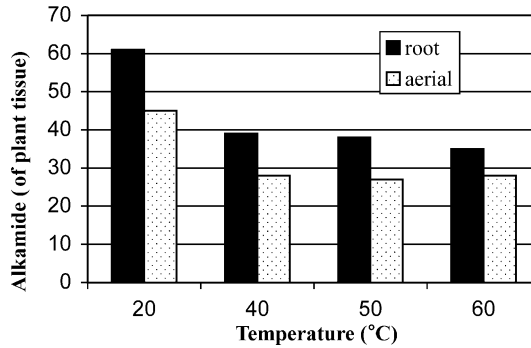


FIG. 6 The effect of temperature on alkamide recovery from *Echinacea* tissue (adapted from Stuart and Wills (2000b)).

Stuart and Wills (2000b) reported a decrease, from 60 to 15%, in alkamide content of extracts prepared from *Echinacea* root with increasing particle size, from 300 to 4000 μm. Particle size is also important in SF extractions as noted by Sun *et al.* (2002). These authors noted a 10-fold increase in alkamide extraction simply due to grinding the sample.

C. CAFFEIC ACID PHENOLS

1. Concentrations and composition

Caffeic acid phenols (CAP; Figure 2) make up the largest percentage of the *Echinacea* phenolic compounds with flavonoids, phenolic acids, and anthocyanins contributing a smaller percentage. The tartaric acid derivatives of the CAP are believed to be responsible for some of the immune-enhancing activity of *Echinacea*, with cichoric (also referred to as chicoric) acid being the most important (Bauer *et al.*, 1989). Chlorogenic acid, verbascoside and echinacoside are present in significant quantities but have no immune-enhancing activity. However, these compounds do possess antioxidant activity and are hence potentially important phytochemicals that could act as synergists to enhance the biological activity of *Echinacea*.

A qualitative determination of the CAP in *E. pallida* showed that the roots contain high echinacoside levels; whereas, cichoric acid was the predominant CAP in the flowers and leaves (Cheminat *et al.*, 1988). This qualitative evaluation is a reasonable approximation of the CAP distribution in plant tissue; however, the level of the individual CAP is dictated by the *Echinacea* species evaluated. Pietta *et al.* (1998) presented a qualitative evaluation on the CAP in *E. purpurea*, *E. pallida* and *E. angustifolia* using micellar electrokinetic chromatography (MEKC). These authors noted that echinacoside was present in the roots and aerial parts of the *E. pallida* and *E. angustifolia,* but not *E. purpurea*. Cichoric acid is the predominant CAP in *E. purpurea* roots and aerial parts but is virtually absent in all parts of *E. angustifolia* and roots of *E. pallida*. The presence of other CAP in plant tissues is variable and species-variety-dependent (Tables III–V).

Becker and Hsieh (1985) reported that the roots and aerial parts contained 7.6 and 13 mg cichoric acid/g dried plant material, respectively. Bauer and colleagues reported that the range of cichoric acid levels in aerial parts was 1.2–3.1% and, in roots, 0.6–2.1% for German-grown *Echinacea* (Bauer *et al.*, 1988a; Bauer and Wagner, 1990). Wills and Stuart (1999) reported that the average cichoric acid levels for Australian *E. purpurea* roots and aerial parts were 13.2 (1.32%) and 12.9 mg/g (1.29%), respectively. Furthermore, chromatographic separation indicated that cichoric acid accounted for 63 and 67% of the phenols in the root and aerial parts, respectively. Similar to the

TABLE III
AVERAGE CICHORIC ACID CONTENT (mg/g PLANT PART DRY WEIGHT) OF VARIOUS PLANT PARTS OF *E. PURPUREA*

	Plant part			
Location	Roots	Flower	Leaf	Stem[a]
Stuart and Wills (2000a)	21	34	19	7.5
Becker and Hseih (1985)	7.5	13[b]	–[b]	n/r
Bauer and colleagues[c]	14	22	10	4
Binns *et al.* (2002a)[d]	8.3	6.6[e]	n/r	n/r

[a]Vegetative and reproductive stem; n/r = not reported.
[b]Reported as aerial parts but may include flower, leaf and stem parts.
[c]Bauer *et al.* (1988a) and Bauer and Wagner (1990).
[d]Binns *et al.* (2002a)—average cichoric acid content for *E. purpurea* grown under cultivated and wild environments.
[e]Reported as inflorescences (flower and reproductive stem parts).

alkamides, the *Echinacea* samples obtained from areas north of the 32°S latitude in Australia had higher levels of cichoric acid (Wills and Stuart, 1999). Fifty percent (50%) of the 62 samples tested had cichoric acid levels in the range 10–15 mg/g roots and 90% fell in the range 5–25 mg cichoric acid/g root material. In addition, 90% of the herbal samples had cichoric acid levels in the range 5–20 mg/g dried aerial parts (Wills and Stuart, 1999).

TABLE IV
CONTENT[a] AND DISTRIBUTION[b] OF CICHORIC ACID IN VARIOUS PLANT TISSUE OF MATURE *E. PURPUREA* GROWN OVER TWO SEASONS AT DIFFERENT LOCATIONS IN AUSTRALIA

	Location			
	Coastal		Tableland	
Plant Part	Year 1	Year 2	Year 1	Year 2
Roots	37[b]	31	35	44
Flower	24	32	17	7
Leaf	24	15	34	43
Stem	16	22	14	6
Total cichoric acid/plant[a]	1380	2120	847	2058

Adapted from Stuart and Wills (2000a).
[a]mg/plant dry weight.
[b]Contribution (%) to total cichoric acid/plant by each plant part.

TABLE V
AVERAGE TOTAL CAFFEIC ACID PHENOL CONTENT (mg/g DRY ROOT) OF VARIOUS *ECHINACEA* SPECIES AND VARIETIES GROWN UNDER CULTIVATED AND WILD ENVIRONMENTS[a]

	Germlings[b]	Wild-harvested	Wild-transplant
E. purpurea	10.5	8.0	6.5
E. pallida var *angustifolia*	12.9	3.2	2.0
E. pallida var *pallida*	2.5	2.5	0.5
E. pallida var *sanguinea*	0.2	1.0	0.5
E. pallida var *tennesseensis*	6.7	0.8	0.2
E. atrorubens var *atrorubens*	0.2	0.3	0.6

[a]Adapted from Binns *et al.* (2002a). Estimated total alkamide levels.
[b]Plants grown from wild-harvested seeds in a greenhouse.

Bauer *et al.* (1988a) noted that the *E. purpurea* had higher levels of cichoric acid than *E. angustifolia* DC. In addition, average cichoric acid contents in mg/g plant tissue of *E. purpurea* were 22, 14, 10 and 4 for the flower, root, leaf, and stem segments, respectively (Table III). The average cichoric acid content in Australian-grown *E. purpurea* was 34, 21, 19, and 8 mg/g dried flower, root, leaf, and stem segments, respectively (Stuart and Wills, 2000a). Binns *et al.* (2002b) also reported that the flowers had higher concentrations of cichoric acid than the roots of wild-harvested *Echinacea* obtained in the United States. Although the data suggests that geographic location may have a slight effect on cichoric acid concentration, the accumulation of cichoric acid in specific plant parts was not affected by location. In these studies, the flowers had the highest level of cichoric acid at all locations, followed by root, leaf, and stem segments (Table III).

E. purpurea grown in the coastal (32°S latitude, sea level, 13–23°C) and sub-tropic tableland (32°S latitude, 1030 m altitude, 13–23°C) growing sites in Australia had similar cichoric acid levels (Stuart and Wills, 2000a). The average cichoric acid levels were 34, 23, 20 and 9 mg/g plant part for the flowers, roots, leaves and stems, respectively, in mature *E. purpurea* plants grown in coastal areas. The tableland-grown, mature *E. purpurea* had average cichoric acid levels of 31, 18, 17 and 8 mg/g plant part for the flowers, roots, leaves and stems, respectively. In contrast, significantly higher levels of cichoric acid per plant were found in *E. purpurea* plants grown in coastal areas, due in part to the higher plant mass. Stuart and Wills (2000a) reported a total cichoric acid content of 1750 and 1452 mg/plant for *E. purpurea* grown in coastal and tableland areas, respectively (Table IV). The contribution of each plant part to the total cichoric acid level was similar between growing locations. The root tissue accounted for 37 and 35% of the total cichoric acid

in coastal and tableland grown plants, respectively, harvested at the mature stage of growth, followed by the stem (24 and 34%), flowers (23 and 17%), and leaves (16 and 14%). However, the contribution of each plant part harvested at the mature stage varied between two growing seasons (Stuart and Wills, 2000a). For example, the flower tissue accounted for 8% of the total cichoric acid levels in year one and 32% after the second growing season (Table IV). In general, cichoric acid levels decreased as the plant entered the senescent stage of the growing cycle (Stuart and Wills, 2000a). Kim *et al.* (2000b) noted a slight reduction in CAP in the samples harvested between August and October. Cichoric acid decreased significantly in freeze-dried samples between harvest periods, again indicating the importance of harvesting at the correct stage of the growing cycle. Perry *et al.* (2001) reported that cichoric acid contents were highest in summer and lowest in autumn-harvested plants, thus supporting the trends observed by other researchers. The mean cichoric acid levels of 2.0 and 2.3 mg/g root or aerial parts, respectively, were found in *E. purpurea* during the summer harvest, but decreased to 1.7 and 0.34 mg/g root or aerial parts, respectively, in the autumn-harvested *E. purpurea* (Perry *et al.*, 2001). These studies indicate the importance of harvest time on CAP retention.

Binns *et al.* (2002b) determined the CAP levels in cultivated and wild populations of various *Echinacea* species and varieties. Similar to the alkamides, no clear trend was observed across all species and varieties under all growing conditions (Table V). The CAP concentration was highest in the flower tissue in most samples. The flowers of the wild-harvested samples had the highest CAP levels, followed by the transplants and lastly the germlings. In contrast, the roots of the germlings had the highest CAP levels followed by wild-harvested samples and lastly the transplants (Binns *et al.*, 2002b). With a few exceptions, the CAP concentration was highest in *E. purpurea* samples and ranged from 6.5 to 10.5 mg/g of root material. Cichoric acid was the predominant CAP in *E. purpurea* roots; whereas, echinacoside predominated in all the other species and varieties tested (Table V). Cynarin also accounted for a significant proportion of the CAP in root tissue of cultivated *E. angustifolia* and *E. pallida* var. *tennesseensis* but contributed to a lesser proportion of the CAP in the flowers of these varieties (Binns *et al.*, 2002b). Cichoric acid was the predominant flower CAP in all *Echinacea* samples evaluated except *E. atrorubens* var. *atrorubens*, where chlorogenic acid was the predominant CAP. Dietz and Bauer (2001) reported similar findings for this species but did not observe cichoric acid or echinacoside, as did Binns *et al.* (2002b). Other *E. atrorubens* varieties contained significant levels of chlorogenic acid, which supports the work of Bauer and Foster (1991) who noted the presence of chlorogenic acid and echinacoside, but no cichoric acid, in the roots of *E. atrorubens* var. *paradox*. Chlorogenic acid was also in

significant levels in the flowers of *E. angustifolia* and *E. pallida* (Binns *et al.*, 2002b). *E. purpurea* had a chlorogenic acid content at 125 μg/g; whereas, 26 μg/g was found in *E. angustifolia* (Glowniak *et al.*, 1996).

Phenolic acids are structurally similar to the CAP. A phenolic acid content of 190 and 870 μg/g was reported for *E. angustifolia* and *E. purpurea*, respectively (Glowniak *et al.*, 1996). Caffeic acid is part of the structural features of the CAP but can also be found as a free acid. Binns *et al.* (2002b) reported caffeic acid levels of 10 and 70 μg/g root in *E. purpurea* and *E. pallida* var. *tennesseensis*, respectively. These authors also noted that *E. pallida* var. *tennesseensis* contained 70 μg caffeic acid/g flower. However, substantially higher (320 μg/g) caffeic acid levels were reported in the aerial parts of *E. purpurea* (Glowniak *et al.*, 1996). Vanillic acid was present in the aerial parts of *E. angustifolia* but not *E. purpurea*. In contrast, 86 μg of vanillic acid/g roots of *E. purpurea* was found using an analytical method which utilized MEKC (Pomponio *et al.*, 2002). Protocatechuic, *p*-hydroxybenzoic, *p*-coumaric and ferulic acids were common to both *E. angustifolia* and *E. purpurea*.

2. *CAP isolation and key structural features*

The extraction of CAP can be completed using alcohol or alcohol/water mixtures (Bauer *et al.*, 1988b; Cheminat *et al.*, 1988; Wills and Stuart, 1999; Bergeron *et al.*, 2000). A common extraction approach uses a Soxhlet apparatus, methanol, and an extraction time of 12–24 h. Cheminat *et al.* (1988) completed an in-depth structural evaluation of 12 CAP isolated from *Echinacea* using NMR and grouped the CAP as quinyl esters of caffeic acid, tartaric acid derivatives, and phenylpropanoid glycosides. This grouping can be advantageous to individuals interested in obtaining standards because preliminary fractionation can be based on groups of similar compounds instead of single components. Recently, a chemical synthesis of cichoric acid has been reported (Lamidey *et al.*, 2002), thus providing an alternative source of cichoric acid for biological and stability studies.

The extraction and separation of the CAP can best be accomplished using the protocol of Cheminat *et al.* (1988), unless a preparative HPLC is available for separations. In this protocol, *Echinacea* was extracted with methanol: water (4:1) for 12 h followed by removal of methanol and extraction of the remaining aqueous phase with petroleum ether and chloroform. The aqueous phase was acidified, extracted with ethyl acetate and then *n*-butanol. After concentration of the ethyl acetate and *n*-butanol fractions, CAP were fractionated over a Sephadex LH20 column using methanol:water (4:1) followed by separation over silica gel, TLC or HPLC. Unlike other CAP,

cichoric acid can be isolated in a purified form after separation on Sephadex LH20 column. Echinacoside isolation can be achieved using a polyamide column and eluting with water followed by ascending methanol concentrations.

In our laboratory, we use a series of solvents to extract compounds with similar physical properties. Typically, an extraction protocol includes extracting the dried root or aerial parts with hexane to remove alkamides followed by 100% ethanol and 70% ethanol for the removal of phenolic and phenolic glycosides, respectively (Hall *et al.*, 2001). Bergeron *et al.* (2000) reported that 70% alcohol solutions were optimal for extracting the various *Echinacea* phytochemicals. A unique feature of their method was the use of ultrasound promoted extractions, which provided similar alkamide but higher CAP than traditional Soxhlet. Unlike Cheminat *et al.* (1988), we (Hall *et al.*, 2001) used reverse-phase or C_{18} packing material as the stationary phase for the separation of the CAP and a mobile phase of ethanol and 0.5% acetic acid under gradient elution. Bergeron *et al.* (2000) reported the use of reverse-phase stationary phase and gradient elution with methanol and water to purify echinacoside. Johnson *et al.* (2002) reported a three-phase method for concentrating an ethanol:water extract of *Echinacea*. In phase one, ethanol was removed via pervaporation followed by precipitation of the alkamides by microfiltration and osmotic distillation.

The variation in NMR data between CAP is due to the non-caffeic acid moiety and should then be used as a part of the identification process. In general, the caffeic acid moiety will have chemical shifts between 6.22 and 7.65 ppm with the aromatic proton ranging from 6.81 to 7.08 ppm. The hydrogens on the carbons neighboring the aromatic ring have chemical shifts near 7.59 ppm. The olefin hydrogens on the carbons next to the carbonyl have chemical shifts around 6.25 ppm, and are readily observed by the large associated coupling constants ($\sim$16 Hz) (Becker *et al.*, 1982; Becker and Hsieh, 1985; Cheminat *et al.*, 1988). The tartaric acid moiety is characterized by a proton resonance at 5.67 ppm. The elimination of one caffeic acid molecule from the tartaric acid moiety (i.e., caftaric acid) results in two chemical shifts at 4.57 and 5.34 ppm.

Echinacoside contains a caffeic acid, a β-(3,4-dihydroxyphenyl)-ethoxy substitution, two glucose groups, and a rhamnose group. The caffeic acid moiety has chemical shifts similar to those listed above while the aromatic proton chemical shifts for the β-(3,4-dihydroxyphenyl)-ethoxy substitution are found at 6.54, 6.66, and 6.67 ppm. The ethyl hydrogens on the carbon attached to the aromatic ring produce a signal at 2.73 ppm, whereas chemical shifts of 3.67 and 3.92 ppm are characteristic of protons on the carbon attached to the glucose moiety. For the glucose and rhamnose moieties, the majority of chemical shifts are between 3.14 and 3.75 ppm.

Fast atom bombardment mass spectrometry (FAB-MS), in the negative-ion mode, was used to elucidate the structure of CAP (Facino *et al.*, 1993). The negative ions ($[M - H]^-$) of 473, 353, and 311 *m/z* correspond to cichoric acid (MW 474), chlorogenic acid (MW 354) and caftaric acid (MW 312), respectively. FAB-MS mass spectrometry (FAB-MS/MS) of cichoric acid produced $[M - H]^-$ daughter ions of 311, 293, 179, 149 and 113 *m/z*. Cleavage of the ester bond results in the release of caftaric acid and a caffeoyl residue (160 *m/z*). The subsequent loss of a second caffeoyl or caffeic acid results in 149 and 113 *m/z* ions, which correspond to the tartaric acid moiety. Chlorogenic acid is a quinic acid ester of caffeic acid, thus, one would expect the loss of a caffeoyl unit. A daughter ion at 191 *m/z* represents the loss of caffeic acid from chlorogenic acid to give quinic acid. Minor ions at 309 and 147 *m/z* are indicative of a loss of carbon dioxide (44 *m/z* units) from chlorogenic acid and quinic acid, respectively. Other CAP can be evaluated using a similar approach.

3. Role of processing on CAP recovery and stability

A 1,2-dihydroxy moiety is a structural feature of molecules susceptible to the enzymatic degradation by polyphenol oxidase (PPO). Cichoric acid contains this structural feature and is thus susceptible to enzymatic degradation (Bauer, 1997). The immunostimulatory activity of *Echinacea* is partly due to the cichoric acid, and hence protection against enzymatic degradation is critical for retaining the potency of *Echinacea* preparations.

Cichoric acid content of six commercially available *E. purpurea* expressed juice preparations, preserved with ethanol (20%) or by thermal processing, varied significantly between preservation methods (Bauer, 1999b). The thermally treated preparations had an average cichoric acid content of 0.24 g/100 ml of extract. In contrast, the cichoric acid content of the five ethanol preserved preparations was 0.075 g/100 ml extract. Several preparations had no cichoric acid whereas one ethanol-preserved preparation contained 0.14 g cichoric acid/100 ml extract. Within this preparation, the cichoric acid content of several lots varied from 0 to 0.34 g/100 ml extract. Bauer (1999b) hypothesized that the inactivation of PPO by heat may account for the difference in cichoric acid content found between heated and non-heated preparations. Any expressed juice would be expected to contain enzyme inherent to the plant tissue; thus, additional processing of the expressed juice preparation is critical for CAP retention.

Kreis *et al.* (2000) characterized a PPO from the aerial part of *E. purpurea* as a diphenolase with a high affinity for caffeic acid. In addition, the PPO lacked monophenolase activity. The PPO was reported to be a 47–54 kDa protein having an optimal activity near pH 6.0 and reversibly inhibited by

metal-chelating agents. Copper was found to be essential for activity as the addition of excess copper could reverse the inactivation by metal chelators. Bergeron *et al.* (2002) noted that the addition of the metal chelators, citric and malic acids, greatly enhanced the stability of CAP in glycerin extracts of *E. purpurea*. In the control (i.e., without chelators) glycerin extracts, approximately 50, 72 and 80% reductions in caffeic, caftaric and cichoric acids were found, respectively after 4 months of storage; whereas a 21% increase in 2-*O*-feruloyl-tartaric acid was found during the same storage period. Citric and malic acids were found to be the most effective in preventing CAP degradation at the 0.5% level. A hibiscus extract at 15% was also found to be an effective antioxidant (Bergeron *et al.*, 2002). Cichoric acid levels in hibiscus, citric acid and malic acid protected glycerin extracts decreased by 44, 32 and 31%, respectively after 4 months of storage, which is significantly better than the 80% loss observed in the control sample. Other CAP were protected by the addition of the metal chelators/antioxidants. Additional studies showed that the addition of ascorbic acid (AA) (50 mM) or soaking of the plant material in a nitrogen-rich environment prevented cichoric acid degradation (Nüsslein *et al.*, 2000). These authors also found that the degradation of cichoric acid was promoted by protein extracts of *E. purpurea* during incubation at 40°C whereas heat processing inactivated the protein extract.

Perry *et al.* (2001) reported 50% reduction of cichoric acid and other CAP within minutes after water had been added to ground roots and aerial parts. They noted that the extraction solvent of 70% ethanol did not promote degradation and theorized that the PPO had been denatured. Nüsslein *et al.* (2000) reported that the ethanol concentration (22%) typically found in alcohol-preserved preparations, plus ethanol levels as high as 40%, were not able to prevent cichoric acid degradation. However, the addition of AA to the ethanol extracts did reduce the rate of loss but did not inhibit the degradation completely. Within 1 week, cichoric acid levels dropped by 20%, and by 4 weeks of storage 70% of the cichoric acid was gone from extracts containing 22% ethanol and 100 mM AA. In contrast, 80 and 90% of the cichoric acid in 30 and 40% ethanol-preserved extracts containing AA remained after a 4-week study, respectively. Nüsslein *et al.* (2000) recommended that 40% ethanol and 50 mM AA be used for the preservation of expressed juice products. They observed that the cichoric acid level in the chemically preserved preparations equalled those of thermal process preparations. In contrast, the addition of 30% ethanol and 50 mM AA to pressed juice was insufficient to inhibit the degradation of cichoric acid. Nüsslein *et al.* (2000) proposed that the degradation was caused by an esterase and not PPO. They observed an increase in caftaric and caffeic acids in stored extracts, indicating the hydrolysis of the ester bond between the caffeoyl

moiety and tartaric acid (Figure 7). If PPO were responsible for the degradation, one would not expect to find caffeic acid in the extracts because caffeic acid has been shown to be a very good substrate for PPO (Kreis *et al.*, 2000) and would undergo polymerization.

The loss of CAP during the growing season indicates that the enzymatic activity exists in the plant and that handling of the plant could further the degradation of CAP. Thus, handling of the plant is critical if high levels of CAP are to be retained. Post-harvest handling of *Echinacea* is as important for the production of dried products as is the thermal or extraction process is to pressed juice products. *E. purpurea* processed using various drying methods showed that freeze drying and vacuum microwave drying of flowers had significantly higher total cichoric and caftaric acid levels compared to air drying (Kim *et al.*, 2000b). Significantly higher CAP were retained in the samples air-dried at 40°C than at 25 and 70°C. Li and Wardle (2001) reported that the cichoric acid levels were significantly higher in *E. purpurea* dried at 45°C than at 40 or 35°C. Similar results were reported for *E. pallida* except that no significant differences in cichoric acid levels were found between 40 or 35°C. No correlation was found between the drying temperature and retention of echinacoside in dried *E. angustifolia* and *E. pallida* roots. Compared to a freeze-dried control, no significant reduction in echinacoside level was found in the roots of *E. angustifolia* dried at 23°C (Kabganian *et al.*, 2002). However, a 28 and 55% reduction in echinacoside was found at dry temperatures of 30 and 60°C, respectively.

Wills and Stuart (2000) found that cichoric concentration was not significantly altered when *Echinacea* was subjected to various physical treatments that included cutting, compression or crushing. These results appear to contradict other reports (Nüsslein *et al.*, 2000; Perry *et al.*, 2001) regarding the instability of the cichoric acid after harvesting. However, the method of damage may not have been sufficient to cause cell disruption normally caused by the pressing operation. Subsequently, there may not have been sufficient substrate–enzyme interactions to promote a high degree of

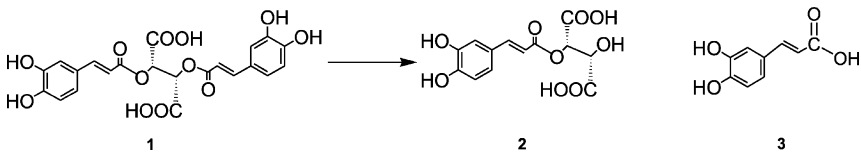


FIG. 7 The hydrolysis of the ester bond between the caffeoyl moiety and tartaric acid in cichoric acid (**1**) to give caftaric (**2**) and caffeic (**3**) acids as proposed by Nüsslein *et al.* (2000).

browning, although Wills and Stuart (2000) did observe some enzymatic browning. Kim *et al.* (2000b) reported that *Echinacea* product with a moisture content of 9.3% had a significantly lower cichoric acid content compared to the ones dried to 6.1% prior to storage. In contrast, Li and Wardle (2001) noted that cichoric acid levels were the greatest for *E. purpurea* samples having moisture contents of 10–15%. Although insignificant, higher echinacoside levels were reported for *E. angustifolia* roots dried to 10% moisture content. *E. pallida* roots dried to 5% moisture had significantly lower echinacoside levels than those dried to 10–15%.

Storage of freshly harvested *Echinacea* at 20°C and 60% RH did not significantly affect the concentration of cichoric acid over a 30-day storage period (Wills and Stuart, 2000). However, an 80% reduction in cichoric acid was reported for samples stored at 5°C and 80% RH. Although the storage temperature was significantly lower, the higher RH was thought to be the main reason for the enhanced cichoric acid degradation. For example, the moisture content of dried *Echinacea* stored at 20°C and 60% remained constant (10%) over the course of 60 days. In contrast, the moisture content of the dried sample stored at 5°C and 80% RH increased from 10 to 25% over the 60-day storage (Wills and Stuart, 2000). These authors proposed that drying *Echinacea* to a moisture content of 10–12% was critical for inhibiting enzyme activity. Alternatively, blanching prior to storage at 5°C and 80% RH can be an effective method for preserving cichoric acid (Wills and Stuart, 2000). Storage after 7 months showed that powdered extracts of *E. purpurea* roots were more stable than a 55% ethanol preparation (Livesey *et al.*, 1999). In powdered samples, cichoric acid was not significantly affected during 7 months of storage. However, samples stored at higher temperatures did contain lower levels of cichoric acid. For example, the cichoric acid level was 27% lower in samples stored at 25°C as opposed to storage at −20°C. In contrast, storage of the extracts was significantly affected by storage temperature. The greatest cichoric acid reduction (78%) occurred in the samples stored at 40°C (Livesey *et al.*, 1999).

Stuart and Wills (2000b) and Bergeron *et al.* (2000) evaluated several extraction techniques and conditions that involved a series of alcohol:water mixtures. Both groups of researchers found that alcohol levels of 60–70% favored CAP removal. Cichoric acid was extracted slightly more efficiently by 70% methanol than 70% ethanol but both solvents were significantly more efficient extraction solvents than their corresponding 100% counterparts (Bergeron *et al.*, 2000). Ultrasonic-promoted extractions significantly enhance cichoric acid extraction when 70% alcohol was used as the solvent (Table VI). In contrast, a reduction in cichoric acid extraction was found in the ultrasonic-promoted extraction using 100% alcohol solvents. Echinacoside recovery was not significantly affected by solvent type or extraction condition (Table VI).

TABLE VI
COMPARISON BETWEEN SOXHLET AND ULTRASOUND-PROMOTED EXTRACTION OF CICHORIC ACID AND ECHINACOSIDE FROM *E. PURPUREA* AND *E. ANGUSTIFOLIA*, RESPECTIVELY

Extraction condition	*E. angustifolia* roots Echinacoside	*E. purpurea* root Cichoric acid	*E. purpurea* top Cichoric acid
Soxhlet[a]—100% MeOH	14.0[b]	19.5	19.0
Ultrasound[c]—100% MeOH	13.5	12.0	14.0
Ultrasound—70% MeOH	14.0	43.0	30.0
Ultrasound—70% EtOH	13.5	38.0	27.0

Adapted from Bergeron *et al.* (2000).
[a]Soxhlet—1 h extraction in methanol (MeOH).
[b]mg/g tissue (approximations).
[c]Ultrasound—sample extracted three times using methanol or ethanol (EtOH) under 5 min exposure to ultrasound.

Stuart and Wills (2000b) reported that 60% ethanol was optimal for recovering cichoric acid from the roots and aerial parts of *E. purpurea*. These authors reported that cichoric acid accounted for 37% of the material in the 60% ethanol extract for both the root and aerial parts, but only 23–25% of the extract obtained from 70% ethanol extraction. Significant degradation of cichoric acid was noted as the extraction residues contained very little cichoric acid. The only exception to this trend was in the residues of the samples extracted with 100% ethanol (Stuart and Wills, 2000b). The denaturation of PPO probably contributed to the stability of cichoric acid in the residue. Enzyme inactivation further supported the observed increase in cichoric acid recovery as temperatures during the extraction increased from 20 to 60°C (Figure 8). Furthermore, cichoric acid recoveries were enhanced by increasing the solvent:solute ratio from 2:1 to 8:1 and by reducing the particle size from 4000 to 300 μm (Stuart and Wills, 2000b).

In contrast to alkamides, alternative extraction solvents such as SF carbon dioxide appear to be ineffective as an extraction solvent for CAP removal (Catchpole *et al.*, 2002; Sun *et al.*, 2002). Conditions evaluated by these researchers include pressures of 31–55 MPa and temperatures between 41 and 60°C. In both studies, ethanol was used as a solvent modifier, but the supercritical carbon dioxide was not modified sufficiently to promote the extraction of CAP. The addition of 10% methanol to the supercritical carbon dioxide at 25 MPa and 60°C was sufficient to promote the extraction of rosmarinic acid, a compound with similar structure features as cichoric acid (Bicchi *et al.*, 2000). Thus, additional work is needed to determine if SFE can be used as a method to remove CAP.

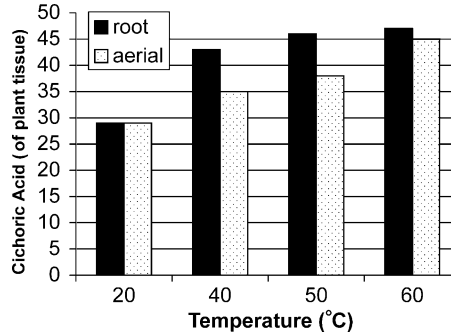


FIG. 8 The effect of temperature on cichoric acid recovery from *Echinacea* tissue (adapted from Stuart and Wills (2000b)).

D. GLYCOPROTEINS AND POLYSACCHARIDES

1. Concentrations and composition

Unlike the alkamides and CAP, the knowledge regarding concentrations and composition of glycoproteins and polysaccharides is limited. The roots of *E. purpurea* and *E. angustifolia* contain several glycoproteins in the 10–70 kDa MW range and recently a 1200 kDa glycoprotein has been isolated (Bauer, 1999a, 2000; Classen *et al.*, 2000). The glycoproteins of molecular weights, 17, 21 and 30 kDa comprised approximately 3% protein in which aspartate, glycine, glutamine and alanine are the predominant amino acids (Beuscher *et al.*, 1987). The sugar moieties include arabinose (64–84%), galactose (1.9–5.3%) and glucosamine (6%) (Bauer, 1999a, 2000). Classen *et al.* (2000) determined that the 1200 kDa glycoprotein was an arabinogalactan-protein (AGP) with a protein content of approximately 7%. Serine (16%) was the most abundant amino acid followed by alanine, hydroxyproline, and aspartate (Table VII). These authors also estimated that the AGP made up about 0.4% of the dry weight of the pressed juice. The AGP was approximately 83% polysaccharide with galactose and arabinose accounting for 90% of the monosaccharides (Table VIII). Reconstruction of the purified AGP indicated that the carbohydrate moiety had an arabino-3,6-galactan structure. The backbone of the polysaccharide consisted of β-D-galactopyranosyl residues with a high degree of branching at the C-6 oxygen via (1 → 6) to β-D-galactopyranosyl containing terminal α-L-arabinofuranosyl residues (Classen *et al.*, 2000). These authors estimated that 700 amino acid and 7000 glycosyl residues make up the structural features of the AGP

TABLE VII
AMINO ACID DISTRIBUTION[a] IN AN ARABINOGALACTAN-PROTEIN FROM *E. PURPUREA*

Amino acid	%
Serine	16.0
Alanine	12.5
Hydroxyproline	11.5
Asparagine/aspartic acid	10.6
Threonine	10.5
Glutamine/glutamic acid	9.4
Arginine	7.5
Glycine	4.6
Valine	4.1
Histidine	3.7
Lysine	3.3
Leucine	2.5
Isoleucine	2.2
Phenylalanine	1.6

Adapted from Classen *et al.* (2000).
[a]% of isolated protein.

isolated and that the galactose and hydroxyproline residues link the polysaccharide and protein moiety together.

The polysaccharides can be grouped into one of the three general categories that include fructans, pectic polysaccharides or arabinogalactans. A 35 kDa polysaccharide, 4-*O*-methyl-glucuronoarabinoxylan, isolated from the hemicellulosic fraction of *E. purpurea* was characterized by Wagner *et al.* (1984, 1985) and Proksch and Wagner (1987). These authors noted that xylose, galactose and arabinose made up the largest proportion of the

TABLE VIII
MONOSACCHARIDE DISTRIBUTION[a] IN AN ARABINOGALACTAN-PROTEIN FROM *E. PURPUREA*

Monosaccharide	%
Galactose	59.1
Arabinose	33.2
Glucosamine	4.0
Mannose	2.6
Rhamnose	1.1

Adapted from Classen *et al.* (2000).
[a]% of isolated polysaccharide.

monosaccharides whereas glucose, mannose and rhamnose were minor constituents. An arabinose:xylose ratio of 1:5 and a 4-*O*-methyl-glucuronic acid:xylose of 1:5.7 were observed. β-(1 → 4)Xylopyranosyl residues make up the backbone of the acidic arabinoxylan, with branch points at C-2 and C-3 oxygens. Arabinofuranosyl residues are linked at the C-3 oxygen via α-(1 → 5) bonds while a β-linked 4-*O*-methyl-glucuronic occupied the C-2 location (Proksch and Wagner, 1987). Emmendörffer *et al.* (1999) stated that a second immunostimulating polysaccharide, a 50 kDa acidic arabinorhamnogalactan, was characterized from the herbal parts of *E. purpurea*. In this polysaccharide, the molar ratios of monosaccharides were 1:0.8:0.6:0.6 for arabinose, rhamnose, galactose and glucose, respectively.

An acidic arabinogalactan (75 kDa) and two fucogalactoxyloglucans (10 and 25 kDa) have been isolated from the cell cultures of *E. purpurea* and were found to be characteristically different from the arabinogalactan from the *Echinacea* plants (Wagner *et al.*, 1988). An arabinose:xylose ratio of 1:1 and approximately 16% galacturonic acid were observed in the cell culture-derived arabinogalactan. Galactose accounted for 75% of the hexose sugars and was predominantly linked 1 → 3 to other galactose units to make up the polygalactan backbone. The β-D-galactan backbone was linked 1 → 6 to galactose at branch points on every second galactose. Approximately, 7 → 8 galactose residues in the side-chains are terminally linked 1 → 3 to arabinose. Other branch points may include a 1 → 2 or 1 → 4-linked rhamnosyl or galacturonic acid residue, respectively (Wagner *et al.*, 1988). These authors also noted that larger arabinan side-chains existed and that 1 → 5 linkages predominated. The fucogalactoxyloglucans both had the same molar ratios of glucose, xylose, galactose and fucose (1.5:1.0:0.4:0.1) and a 1 → 4-linked glucan backbone. Branch points were predominant (65%) at the C-6 oxygen. Recently, a hexasaccharide identified by Wagner *et al.* (1988) has been chemically synthesized (Csává *et al.*, 2001). For additional discussion on the polysaccharides, see Bauer (1999a, 2000) and Emmendörffer *et al.* (1999).

Inulin was identified as a major (5.9%) component of the roots of *E. angustifolia* (Heyl and Stanley, 1914). Giger *et al.* (1989) noted that polymerization of fructans occurred over the course of winter as observed by the reduction in fructose from October and May. The rate of polymerization was faster in *E. purpurea* than *E. angustifolia*, suggesting that other polysaccharides may develop in a similar manner. Additional research is needed to identify the effects of harvest time on polysaccharide composition.

2. *Glycoprotein and polysaccharide isolation and key structural features*

Of the phytochemical components in *Echinacea*, the glycoproteins and polysaccharide have been the least characterized. A general protocol

(Proksch and Wagner, 1987; Wagner *et al.*, 1988; Classen *et al.*, 2000) for separating the polysaccharides included pressing of the plant tissue, heating or acid hydrolysis, methanolysis, reduction and oxidation reactions. For complete NMR and sugar distribution data, see Proksch and Wagner (1987), Wagner *et al.* (1988), Classen *et al.* (2000) and Csává *et al.* (2001).

3. *Role of processing on polysaccharide recovery and stability*

In general, polysaccharides tend to be more stable than alkamides and CAP, but their presence in *Echinacea* preparations is somewhat dictated by processing conditions. For example, one would expect dried plant material to contain the highest polysaccharide levels, followed by expressed juice preparation and alcohol tincture. Limited data are available regarding the effects of post-harvest handling and processing on polysaccharide levels.

In the roots of *E. angustifolia* or *E. pallida*, the level of polysaccharides decreased as the drying temperature increased (Li and Wardle, 2001). Although the decrease in polysaccharide levels was not significant, a 7 and 15% reduction in polysaccharides was observed in *E. angustifolia* roots as the drying temperature increased from 35 to 40 or 45°C, respectively. In *E. pallida*, only 2 and 3% of the polysaccharides were lost as the temperatures increased from 30 to 40 or 45°C, respectively. In contrast, higher drying temperatures did not affect the polysaccharide retention in *E. purpurea*. There was a significant increase in polysaccharide levels as drying temperature increased from 35 to 40 or 45°C. For example, the concentration of polysaccharides increased from 24 to 28.6 mg/g root as the drying temperature increased from 35 to 45°C, (Li and Wardle, 2001). A reduction in polysaccharide content (dry weight basis) was noted in roots that had been dried to 15% moisture compared to 5%, regardless of the *Echinacea* species. For example, 1.3 times more polysaccharides were present in the roots of *E. pallida* dried to 5% moisture as opposed to roots dried to 15% moisture (Li and Wardle, 2001).

The low concentration of polysaccharides in alcoholic tinctures would be expected due to the insolubility of the polysaccharides in ethanol. Solvents containing approximately 50% v/v ethanol can be used to precipitate polysaccharides (Bauer, 1999a). Although no data were given, Bergeron *et al.* (2000) reported that 70% ethanol extracted lower polysaccharide levels than 70% methanol. A 65% glycerin extract had higher polysaccharides than a 50% ethanol extract (Bergeron *et al.*, 2002). In contrast to high alcohol levels, low to intermediate alcohol levels can be a viable extraction solvent for polysaccharides. Gahler *et al.* (2001) patented a method for extracting polysaccharides using 20% ethanol and a solvent temperature of 55°C. Ethanol concentrations greater than 20% and temperatures lower than 55°C reduced

polysaccharide recovery. An extract containing 120 mg polysaccharides/ml was produced using vacuum evaporation at 50 mbar and 60°C for 15 h (Gahler *et al.*, 2001).

III. STANDARDIZATION, QUALITY ASSURANCE AND REGULATIONS

A. IS THERE A NEED FOR STANDARDIZATION?

As already indicated throughout the chapter, the type and quantity of *Echinacea* phytochemicals vary significantly. A number of factors such as growing season, part of the plant utilized in the preparation of the commercial product, the species/variety (i.e., *purpurea*, *angustifolia* DC, *pallida*, etc.) of *Echinacea* used, method of harvest and processing can affect phytochemical concentrations in *Echinacea* products (Bauer, 1997). Bauer (1996) stated that the products should be classified according to plant species and processing methods. Bauer (1999b) noted differences in cichoric acid and alkamide concentrations between thermally treated and ethanol-preserved preparation. A survey of 46 samples from 25 commercial brands of *Echinacea* showed that alkamides varied significantly among products (Osowski *et al.*, 2000). These authors found that the alkamides and cichoric acid ranged from 0 to 60.5 μg/ml and from 0 to 4600 μg/ml, respectively. Tablets and pressed juice products had the greatest variations in cichoric acid whereas homeopathic tinctures had the greatest alkamide variation. Similar observations, with regard to product variability, were found in an Australian study that evaluated 32 *Echinacea* products (Wills and Stuart, 1998). For dry products (i.e., tablets, capsules), the cichoric acid content ranged from 0.2 to 6 mg/ml (200–6000 μg/ml) whereas liquid products obtained from retail outlets and naturopaths contained 0–3.9 and 0.1–4.7 mg/ml, respectively. The range in alkamide concentrations was similar among products; however, the average alkamide content was 1.7 times higher in samples obtained from naturopaths than from retail outlets (Wills and Stuart, 1998). These authors also noted that 28% of the samples had alkamide levels near zero; whereas, 16% of the samples had cichoric acid levels near zero, suggesting that alkamides may be more prone to degradation.

The variation in phytochemical levels between *Echinacea* preparations is apparent. Many researchers have suggested that standardization of phytochemicals in *Echinacea* products is needed. However, standardization of the products is difficult due to the complexity of the phytochemicals, the lack of understanding regarding interaction between individual phytochemicals

and the lack of agreement as to which phytochemical(s) should be used for standardization.

B. QUALITY ASSURANCE OF *ECHINACEA* PRODUCTS

1. Standardization

The CAP are commonly used as marker compounds and labeled as "phenolics" on many dietary supplements. However, general spectroscopic methods used for determining total phenols can sometimes give erroneous readings. Targeting specific compounds would be more advantageous and provide additional information such as plant species/variety and parts of the plants used in the preparation.

Echinacoside has been used to standardize many preparations, but the lack of immune-enhancing activity of this component suggests that it should not be used as a component for standardization. However, this compound could be used as a marker compound for authenticating the species of *Echinacea* used in the preparation. For example, the lack of echinacoside in an extract would indicate that *E. purpurea* would be the species most likely used in that preparation. The standardization of *Echinacea* products based on cichoric acid and alkamides has been proposed, due to the biological activity of these compounds (Bauer, 1999a,b; Perry *et al.*, 2000). Binns *et al.* (2002c) used the statistical method, canonical discriminant analysis, to differentiate growing locations among nine populations of *E. angustifolia*. One population was characterized by having traces of cynarin, and three alkamides and high amounts of echinacoside and tetradeca-8*Z*-ene-11,13-diyn-2-one (a marker ketone for *E. pallida*). Binns *et al.* (2002c) speculated that this population may have been a hybrid between *E. pallida* (Nutt.) and *E. angustifolia* DC. This statistical tool was able to distinguish between growing locations and was able to differentiate species/varieties. Additional work should be done using this statistical method to determine differences among other population of *Echinacea*, as a method to differentiate commercial products, and eventually to determine the relationships between potency and active constituents. Until additional research can pinpoint the specific phytochemical(s) and biologically active concentrations, no uniform standardization method will be used by all manufacturers. However, there is a growing consensus among researchers that CAP (specifically cichoric acid) and alkamides should be targeted as the phytochemicals for standardization. Polysaccharides do have biological activity; however, the analytical methods have not been sufficiently optimized for routine quality assurance evaluations. In addition, polysaccharides are often absent in alcohol-based products.

2. *Analytical methods for quality assurance*

A reverse-phase HPLC method can be routinely used for evaluating CAP and alkamides in *Echinacea* preparations. If dried products are evaluated, the CAP and alkamides must first be extracted prior to HPLC analysis. In contrast, some liquid preparations may need to be concentrated for example, via solid-phase extraction, prior to analysis. We have found in our laboratory that a sequential extraction of dried products with hexane (100%), ethanol (100%), and lastly ethanol:water (70:30 v/v) was an effective method to fractionate the various phytochemicals (Hall *et al.*, 2001). An alternative extraction protocol using only 70% ethanol as the extraction solvent has been reported (Bergeron *et al.*, 2000). In some cases, concentration of the alkamides may be needed prior to HPLC analysis. A separation of alkamides from CAP can be completed using C-18 solid-phase extraction columns (Schieffer, 2000; Schieffer and Kohn, 2002). Briefly, the CAP elute from the column in the methanol:water (30:70 v/v) solvent while the alkamides are removed using acetonitrile:water (90:10 v/v). Liquid preparations with low alkamide concentrations are ideally suited for this method because this technique concentrates the alkamides, thus increasing the sensitivity. However, the simultaneous analysis of CAP and alkamides using HPLC-photodiode array-electrospray mass spectrometry (Luo *et al.*, 2003) may hold promise as a rapid analytical method for analyzing the phytochemical constituents.

The method of Bauer (1999b) illustrated the most common method to evaluate alkamides using reverse-phase HPLC. The separation was completed using a gradient elution of water (eluent A) and acetonitrile (eluent B) linearly from 40 to 80% eluent B at 1 ml/min over a C-18 reverse-phase column and detection at 254 nm. TLC using silica 60 plates with indicator ($F_{254\ nm}$) can be used as an alternative to HPLC. However, this method is less adaptable for large number of samples requiring a rapid turn-around typically associated with quality assurance programs. In contrast, a high-performance TLC (HPTLC) method is being evaluated as a rapid screening method for the identification of *Echinacea* species present in commercial products and potential adulterants (Reich *et al.*, 2002).

A reversed-phase (LiChroCART 125-4 column) system with gradient elution is an effective method for separating the CAP within 12 min (Bauer, 1999b). The gradient system included water plus 0.1% orthophosphoric acid (85%) as eluent A and acetonitrile plus 0.1% orthophosphoric acid (85%) as eluent B. A linear gradient from 10 to 30% eluent B was completed within 20 min at 1 ml/min and detection at 330 nm. Bergeron *et al.* (2000) modified the method to include a pH adjustment to 2.80 for a solvent system containing phosphoric acid (solvent A) and 1% phosphoric acid (1 M) in acetonitrile (solvent B).

Alternative methods for assessing *Echinacea* phytochemicals include the use of MEKC or near infrared reflectance (NIR) spectroscopy. Pietta *et al.* (1998) used a 25 mM tetraborate buffer containing 30 mM SDS at pH 8.6 for the separation of CAP. The MEKC separation was completed within 20 min with good resolution. Goti *et al.* (2002) reported a simultaneous separation of both the CAP and alkamides using MEKC technique and variable wavelength detection. The separation was completed in 10 min using a mobile phase of SDS (110 mM) and hydroxypropyl-β-cyclodextrin (100 mM) in Britton–Robinson buffer (10 mM, pH 8.0). The advantage of this method was that the cost of operation is less than HPLC, since organic solvents were not needed. Recently, several NIR spectroscopy techniques have been reported for the analysis of adulteration and cichoric acid (Laasonen *et al.*, 2002; Gray *et al.*, 2001). Laasonen *et al.* (2002) reported that NIR could discriminate between *E. purpurea* and 10% adulterated *E. purpurea* samples. These authors also noted that about 10% of the *E. purpurea* samples and 0% of the adulterated samples were misidentified. Gray *et al.* (2001) evaluated 169 root samples using NIR and found between 1.8 and 19.1 mg cichoric acid/g root. These authors cautioned that the partial least squares regression used in the calculations requires additional samples from various locations before the method can be adopted for routine cichoric acid determinations. Schulz *et al.* (2002) also reported the use of NIR to measure the echinacoside content in *E. angustifolia* and *E. pallida* roots.

Gas chromatography (GC)–MS coupled with multivariate statistical analysis proved valuable in verifying the authenticity of *Echinacea* species (Lienert *et al.*, 1998). Similar root extracts could be grouped, based on the identified compounds from the GC-run, by principal component and cluster analysis. The correct grouping of the *Echinacea* species (i.e., *purpurea*, *angustifolia*, and *pallida*) was not influenced by the extraction method or by the aging process of the roots.

C. PRODUCT REGULATIONS

Echinacea is sold as a dietary supplement in the United States and as a natural health product in Canada. However, Health Canada does support the use of *Echinacea* in food products; thus functional foods could be developed for the Canadian market. In the United States and Canada, there are no restrictions on the species/varieties used in products. In Germany and many European countries, *Echinacea* products are sold as drugs in pharmacies (Bauer, 2000). In addition, not all products are approved for use in all countries. For example, *E. purpurea* aerial parts and *E. pallida* roots are approved in Germany; whereas *E. angustifolia* and *E. purpurea* roots are not (Blumenthal, 1998).

The regulation of *Echinacea* products in the United States would fall under the "Dietary Supplement Health and Education Act of 1994" (DSHEA). In the United States, statutory 403 (a)(1) of the Federal Food, Drug, and Cosmetic Act "prohibits labeling that is false or misleading." Under DSHEA, a structure/-function claim can be made, provided the claim meets the criteria set forth in statutory 403(r)(6). Structure/function claims are permissible if a specific disease is not targeted, unless reviewed by FDA. A structure/function claim such as "supports the immune system" would be acceptable whereas "alleviates the common cold or flu" would not be acceptable for *Echinacea* because the claim targets a specific disease state (i.e., cold or flu).

Echinacea must first be approved as a GRAS ingredient before it can legally be added to food; thus functional foods containing *Echinacea* cannot be legally sold. Creating a functional food and marketing it, as a dietary supplement is also not legal if the food resembles traditional food such as soup. In many European countries, health claims are not permitted on food products while in other countries, such as, Japan, claims are allowed under the "Foods for Specified Health Use" (FOSHU) system. The benefits of *Echinacea* in laboratory studies show promise and potential functional foods be derived from this research provided the studies support the safety of *Echinacea*. Health Canada has explicit definitions for *Echinacea* products and labeling requirements (Health Canada, 1999).

One issue not resolved by the DSHEA, specifically for botanical or herbal dietary products, is the lack of standardization of the active component. As mentioned previously, the standardization of *Echinacea* products is based on the level of plant material rather than one specific compound. In many cases, the specific compound responsible for the health benefit has not been fully characterized. In addition, many phytochemical constituents may participate in an observed health benefit. The lack of standardized preparations may, in fact, be responsible for the conflicting reports surrounding the biological activity of *Echinacea*.

IV. BIOLOGICAL AND TOXICOLOGICAL ACTIVITIES

A. BIOLOGICAL ACTIVITIES

1. Immunological investigations

Echinacea is promoted as an immune-enhancing herbal product and could be easily incorporated into cereal-based products (Wilson, 1998) to create a functional food. There are over 300 research articles dealing with the biological activity of *Echinacea*. In addition, over two million prescriptions

are filled by German physicians annually (Barrett *et al.*, 1999), thus providing some evidence of the safety of *Echinacea* products. Before *Echinacea* can be considered as a component in functional foods, additional research is needed to support the biological activity in a clinical setting and to determine the adverse effects of consuming *Echinacea*. The following discussion highlights only a fraction of the studies completed to date. The author suggests the reviews of Bauer (1999a, 2000), Emmendörffer *et al.* (1999) and Melchart and Linde (1999) for more detailed information.

Improving immune response has been the most widely documented benefit of *Echinacea* (Vömel, 1985; Bauer *et al.*, 1988d; Erhard *et al.*, 1994; Burger *et al.*, 1997; See *et al.*, 1997; Rehman *et al.*, 1999). Most studies support the *in vitro* nonspecific immune-enhancing activity while *in vivo* studies are less convincing. The expressed juice of *E. purpurea* has been the primary plant preparation used in many of the biological investigations. Alcohol-diluted expressed juice and ethanol extracts of the plant are other preparations used in immune-enhancing studies. Specific pure compounds have also been used in some studies.

Natural killer (NK) cells are responsible for nonspecific immunity by targeting virus-containing cells or tumor cells via phagocytosis. Cytokines such as TNF-α and IL-1 enhance NK cell activity whereas prostaglandins are inhibitory (Currier and Miller, 2001). Thus, compounds that promote macrophages to secrete cytokines or compounds that inhibit prostaglandin production would be expected to enhance immunity. Phagocytosis (i.e., the first immune reaction against invading foreign substances) of erythrocytes was significantly enhanced upon treatment with extracts of *Echinacea* (Vömel, 1985). Coeugniet and Elek (1987) found that the pressed juice of *E. purpurea* enhanced nonspecific cell mediated immunity at concentrations between 1 and 100 μg/g, whereas 1000 μg/g treatments were ineffective. These authors noted that the 1000 μg/g concentration promoted leukocytes mortality but suggested that this concentration was clinically irrelevant. A dose-dependent increase in the percentage of phagocytic granulocytes was found after treatment of white blood cell suspensions with lyophilized expressed juice of *E. purpurea* (Stotzem *et al.*, 1992). Lyophilized juice at concentrations of 1 and 5 mg/ml was most effective whereas the phagocytic activity at the 12.5 mg/ml concentration was not significantly different from the control. Only the 5 mg/ml had a significantly higher phagocytosis (i.e., number of phagocytosed starch grains per granulocytes) index (Stotzem *et al.*, 1992). These authors did note that the lack of activity at the highest lyophilized expressed juice concentration (12.5 mg/ml) was due to a cytotoxic effect on the granulocytes. Gaisbauer *et al.* (1990) also supported the importance of concentration on phagocytosis. In contrast, no immune-enhancing activity was found in mice fed various *Echinacea* products (South and Exon, 2001).

Ethanol extracts of *E. purpurea* and *E. angustifolia* roots were also found to enhance immune indices (Erhard *et al.*, 1994; Currier and Miller, 2000). Erhard *et al.* (1994) determined the mean channel of fluorescence intensity, which is proportional to the number of latex particles ingested or adhering to single granulocytes as one measure of immune response. The second measure, phagocytosis index, was also determined as the difference between the average numbers of latex particles ingested by the test compound and those adhering to the granulocytes after treatment with cytochalsin D (a compound that inhibits ingestion of latex particles but not adhesion). Collectively, phagocytosis incorporated both measurements. Vitamin C and a root extract of *E. angustifolia* were the only two treatments that significantly enhanced phagocytosis (Erhard *et al.*, 1994). The addition of *Lachesis muta*, *Aconitum napellus*, or *Apis mellifica* extracts to *E. angustifolia* root extract further enhanced phagocytosis. Wagner and Jurcic (1991) also found enhanced phagocytosis when combinations of *Eupartorium perfoliatum*, *Baptisia tinctoria* and *Arnica montana* were added to *E. angustifolia*. In contrast, no immune-enhancing activity was noted in patients given an extract containing *E. angustifolia*, *Eupartorium perfoliatum* and *Thuja occidentalis* after curative surgery for different malignant tumors (Elsässer-Beile *et al.*, 1996).

Burger *et al.* (1997) reported that cytokine production was enhanced in peripheral blood macrophages after treatment with an ethanol extract from the aerial parts of *Echinacea*. A dose-dependent interleukin 1 (IL-1) production was not found within or among all the fresh pressed juice lots of *Echinacea* tested. However, the 1.20 μg/ml concentration in two lots did stimulate the macrophage production of IL-1 to a greater extent. A third lot of fresh pressed juice had a maximum IL-1 production at 0.20 μg/ml. In contrast, the highest concentration (10 μg/ml) of the dried juice produced the greatest levels of IL-1 (Burger *et al.*, 1997). In the fresh pressed juice products, the 0.05 μg/ml dose promoted the greatest TNF-α production whereas 10 μg/ml dose produced the greatest TNF-α level of a third lot. The 0.03 μg/ml concentration of dried juice gave the greatest TNF-α production. The composition of the extracts may account for the variability in cytokine production (Burger *et al.*, 1997). Additional work by these authors showed that, at low doses (0.012–0.1 μg/ml), a dose-dependent cytokine production by macrophages was apparent irrespective of the product tested (i.e., fresh pressed or dried juice).

Administration, via mouse chow, of *E. purpurea* root extracts at 0.45 mg/day per mouse was found to enhance the number of NK cells in healthy young and old mice (Sun *et al.*, 1999; Currier and Miller, 2000) and leukemic mice (Currier and Miller, 2001, 2002). The number of NK cells increased in both the spleen and bone marrow, and paralleled cell cytolytic

function (Sun *et al.*, 1999; Currier and Miller, 2000). In aged mice, the number of NK cells returned to levels typically found in younger mice (Currier and Miller, 2000). In the spleen, a 30% increase in NK cells was found in mice fed *Echinacea* root extracts for 14 days compared with the *Echinacea*-free diet (i.e., control). Currier and Miller (2001) found a 2.5-fold increase in the number of NK cells in the spleen after 9 days of *E. purpurea* administration. After 3 months, the number of NK cells was 2–3 times that of normal, nonleukemic mice. The addition of melatonin to the *Echinacea*-containing diet resulted in a decrease in the NK cell numbers in the spleen (Currier and Miller, 2001). In contrast, bone marrow NK cells returned to levels similar to nonleukemic mice when melatonin and *Echinacea* were co-administered in the diet. No leukemic mice versus treated mice remained alive by 3 months; thus, the administration of *Echinacea* significantly enhanced the survival of the leukemic mice. However, the addition of melatonin to the *Echinacea*-containing diet significantly increased the survival rate, with 50% of the leukemic mice surviving to and beyond 3 months. Further immune-enhancing activity was noted by a significant increase in NK cells when mice were immunized with dead leukemia cells 5 weeks prior to administration of *Echinacea* in the diet (Currier and Miller, 2002). See *et al.* (1997) reported that extracts of dried *E. purpurea* significantly enhanced the NK cells in healthy subjects and those suffering from chronic fatigue syndrome and acquired immunodeficiency syndrome, at concentrations greater than 0.1 μg/ml.

Combination of low doses of cyclophosphamide, *E. purpurea* extract (Echinacin®) and thymostimulin enhanced the immune functions of terminally ill cancer patients (Lersch *et al.*, 1990, 1992). NK cells increased between 17 and 29% whereas lymphokine activated killer cell activity increased from 180 to 195%. In contrast, polysaccharides isolated from cell cultures of *E. purpurea* did not enhance the phagocytic activity of granulocytes in chemotherapy patients (Melchart *et al.*, 2002).

Bauer *et al.* (1988d) found that ethanol extracts (1:10) of *E. purpurea*, *E. pallida* and *E. angustifolia* roots significantly enhanced *in vitro* phagocytosis of granulocytes. Partitioning of the ethanol extract of *E. purpurea* and *E. pallida* into chloroform and water gave lipophilic and polar fractions, respectively. The lipophilic fraction was significantly more active than the whole ethanol extract and the polar fraction at enhancing phagocytosis. Bauer *et al.* (1988d) hypothesized that the isobutylamides and polyacetylenes in the lipophilic fraction were responsible for the granulocyte phagocytosis. In addition, concentration of the extracts had varying degrees of activity. The ethanol extract of *E. purpurea* root was the most effective at 1 μg/g (10^{-4}% as reported by Bauer *et al.* (1988d)) whereas the 100 and 10 μg/g concentrations of the extract were most effective for *E. pallida*

and *E. angustifolia*, respectively. Irrespective of the *Echinacea* species/varieties, the 1 μg/g concentration of the lipophilic fraction obtained from the ethanol extract was found to have the highest activity. Furthermore, the *in vivo* granulocyte phagocytosis results correlated with the *in vitro* results (Bauer *et al.*, 1988d). Goel *et al.* (2002) reported that alkamides significantly enhanced (60%) the phagocytic activity of mice alveolar macrophages at a dose of 12 μg/kg/day. The phagocytic activity of the cells treated with 0.5 and 4 μg/kg/day levels were not significantly higher than the activity of the control. Significantly, more TNF-α and nitric oxide were generated by the alveolar macrophages treated with *Echinacea* components and stimulated, *in vitro*, with a lipopolysaccharide from *E. coli*. Again, the optimal concentration was 12 μg/kg/day for the alkamides (Goel *et al.*, 2002). However, the levels of TNF-α, interferon-γ (IFN-γ) and interleukin 2 (IL-2) released by rat splenocytes were not significant.

Polar constituents of *Echinacea* include polysaccharides and CAP. Wagner *et al.* (1985, 1988) reported that *in vivo* and *in vitro* experiments supported the immune-enhancing activity of a polysaccharide fraction. These authors reported that a polysaccharide fraction from *E. purpurea* herb and root enhanced phagocytosis of yeast particles by polymorphonuclear neutrophil cells (PMNC) by 27% at concentrations of 1 and 10 μg/ml, respectively. A 32% increase in phagocytosis by PMNC was reported after treatment with a 10 μg/ml polysaccharide solution obtained from *E. angustifolia*. Wagner *et al.* (1985) originally reported on *E. angustifolia*; however, Bauer (1999a) stated that *E. pallida* could possibly be the species/variety evaluated based on misidentification that commonly occurred prior to 1985. Carbon-clearance assay showed, in mice, that phagocytosis was enhanced at a dose of 10 mg/kg body weight, thus supporting *in vitro* observations (Wagner *et al.*, 1985). A 4-*O*-methyl-glucuronoarbinoxylan (35 kDa) was found to enhance phagocytic activity *in vitro* by 23% at concentrations as low as 10^{-4} mg/ml. However, no *in vivo* activity was observed (Proksch and Wagner, 1987). Polysaccharides isolated from cell cultures of *E. purpurea* were found to stimulate phagocytosis by macrophages, with the fucogalactoxyloglucan (25 kDa) having the greatest phagocytic activity (Wagner *et al.*, 1988). In addition, an arabinogalactan (75 kDa) stimulated TNF-α excretion from the macrophage (Wagner *et al.*, 1988). An arabinogalactan also stimulated the release of IL-1, TNF-α and IFN in macrophages, *in vitro* (Luettig *et al.*, 1989; Roesler *et al.*, 1991a). However, the same immune responses were not significantly enhanced after human subjects were injected intravenously with a polysaccharide solution, although nonspecific immune functions could be shown (Roesler *et al.*, 1991a). Goel *et al.* (2002) reported that a polysaccharide dosage

of 3000 μg/kg/day was required to induce a significant increase in TNF-α production by rat alveolar macrophage.

Another polar constituent, cichoric acid, did not enhance TNF-α production (Goel *et al.*, 2002) at levels up to 120 μg/kg/day. Schumacher and Friedberg (1991) noted that, over the concentrations (2.5–250 mg/kg) and delivery (injection, oral) methods tested, no immune-enhancing activity was found in mice given the lyophilized water extracts of *E. angustifolia*. In addition, the water-soluble CAP, echinacoside, was not found to enhance immune function over the concentrations of 1–250 mg/kg. Bauer *et al.* (1989) also reported that echinacoside, along with verbascoside and 2-caffeoyl tartaric acid, did not stimulate phagocytosis. However, these authors did find that cichoric acid was effective in stimulating phagocytosis at concentrations of 10^{-5} mg/ml.

To date, all the studies evaluating the immunostimulating activity of *Echinacea* have focused on extracts or purified compounds as they exist in nature. The conflicting results between *in vivo* and *in vitro* studies may be caused by the test system used in the studies. Rininger *et al.* (2000) developed an innovative approach to evaluate the activity of *Echinacea* preparation using an *in vitro* protocol designed to stimulate *in vivo* conditions. The unique feature of this protocol was that the *Echinacea* raw material was subjected to a simulated digestive process. The samples were first subjected to digestion by gastric juice for 2 h at 37°C, neutralized with sodium hydroxide and finally incubated in simulated intestinal fluid for 2 h at 37°C. Viability and proliferation of human peripheral mononuclear cells (PBMC) and release of cytokines from murine macrophages were used to characterize immunostimulatory activity. Rininger *et al.* (2000) reported a 10–15% variation in inter-assay TNF-α demonstrating a good reproducibility for the digestion assay. The production of TNF-α and nitric oxide by macrophages stimulated with digested *Echinacea* preparation were dose-dependent over the range 0–1280 μl/ml. Treatment of murine macrophages with various levels (5–320 μl/ml) of digested *Echinacea* preparation also promoted a dose-dependent secretion of other cytokines (IL-1, IL-6, IL-10). Various nondigested *Echinacea* products, including commercial products standardized for phenolics, at concentrations greater than 250 μl/ml were unable to stimulate cytokine production in murine macrophages (Rininger *et al.*, 2000). The viability and proliferation of PBMC was enhanced by digested *Echinacea* products only at concentrations found to stimulate cytokine secretion. This study suggests that the immune-enhancing components can survive the digestive process and possibly act as *in vivo* immunostimulating agents (Rininger *et al.*, 2000).

2. *Antimicrobial and antiviral activity*

Related to the immunostimulatory activity is the ability of macrophages and granulocytes to eliminate bacterial and viral infections. *Echinacea* has been found to promote resistance to viruses. Wacker and Hilbig (1978) found that mouse L 929 cells treated with methanol or aqueous extracts of *Echinacea* were 50–80 times more resistant to influenza, herpes and vesicular stomatitis viruses than nontreated cells. The treatment was effective for 24 h; however, the cells became sensitive to the viruses after 48 h suggesting that repeated treatment would be required. A dose-dependent antiviral activity of cichoric acid, echinacoside and caffeic acid was observed in mouse L 929 cells (Cheminat *et al.*, 1988). The authors also reported a substantial reduction in cell growth as the concentration of the components increased, with caffeic acid causing the most inhibition. The optimal cichoric acid concentration for reducing the infectiousness of vesicular stomatitis viruses by 50% was 125 μg/ml. Cichoric and caftaric acids inhibited the hyaluronidase enzyme; an enzyme produced by pathogenic organisms to penetrate tissue and cause infection (Facino *et al.*, 1995). Thus, this may partly explain the reduction in viral infections in the presence of *Echinacea*.

The CAP have been reported to inhibit HIV type 1 (HIV-1) integrase and HIV-1 replications at concentrations as low as 10 μM (McDougall *et al.*, 1998; King *et al.*, 1999; Reinke *et al.*, 2002). Cichoric acid inhibited 50% of the integrase activity and blocked HIV-1 infections by 50% at concentrations of 0.3 and 4 μM, respectively (Robinson *et al.*, 1996a,b). Hexane extracts of *Echinacea* roots were found to have antiviral activity against the Herpes simplex virus type 1 at a 0.12 mg/ml concentration (Binns *et al.*, 2002d).

Wildfeuer and Mayerhoer (1994) noted a 45% increase in phagocytosis of *Candida albicans* by granulocytes treated with *E. purpurea*. Macrophages eliminate *Listeria monocytogens* and *Candida albicans* from internal organs of animals after polysaccharides, isolated from cell cultures, of *Echinacea* were added to model test systems (Roesler *et al.*, 1991b; Steinmüller *et al.*, 1993). Roesler *et al.* (1991b) found that 1 mg/ml concentration of polysaccharides enhanced the production of reactive oxygen intermediates (ROI) by 270% in J774-macrophage cells. However, concentrations greater than 1 mg/ml suppressed ROI. Liver cell macrophages treated with 0.2 mg/ml of polysaccharide suppressed the growth of *Candida albicans* by approximately 95%. White blood cell counts were also found to significantly increase 2.5 h after injecting the mice with 0.2 mg of polysaccharides. The same polysaccharide dose was sufficient to protect 90 and 100% of the mice infected with a LD_{80} dose of *C. albicans* and *L. monocytogens*, respectively (Roesler *et al.*, 1991b). However, delaying the polysaccharide treatment for more than 18 h after administration of the microorganism to the mice was

ineffective in preventing animal death. A dose-dependent reduction in colony-forming units was reported in the spleen, liver and kidney after treatment with polysaccharides (Roesler *et al.*, 1991b; Steinmüller *et al.*, 1993).

The *in vitro* antifungal activity of the lipophilic extracts and commercial tinctures of *Echinacea* was enhanced after exposure to UV irradiation (Binns *et al.*, 2000). These authors noted that *E. purpurea* flowers and a commercial tea product were the only products that lacked antifungal activity after UV exposure. The photooxidation of the alkamide, ketoalkenes and ketoalkynes contributed to the enhanced antifungal activity (Binns *et al.*, 2000).

3. *Anti-oxidant and anti-inflammatory activities*

Rininger *et al.* (2000) noted that standardized *Echinacea* extracts to 3–4% phenolic acids did not necessarily equate to a greater radical scavenging activity. Product type appeared to have a greater impact on radical scavenging activity than the actual standardization process. Cichoric acid and other CAP were found to act as radical scavengers (Facino *et al.*, 1995). Although not tested, verbascoside and 2-caffeoyl-cichoric acid would be expected to have similar radical scavenging activity. Facino *et al.* (1995) reported that *Echinacea* protected collagen from free radical damage by scavenging the reactive oxygen species. Echinacoside and cichoric acid were found to prevent collagen degradation best followed by cynarin ≈ caffeicacid and chlorogenic acid. Alcohol extracts of roots had greater radical scavenging activity than the leaf extracts irrespective of *Echinacea* species/variety (Sloley *et al.*, 2001). However, *E. purpurea* had the highest radical scavenging activity among the three species. The radical scavenging activity of the alcohol extract was equivalent to 1–4% of that of pure AA. In contrast, pure cichoric acid has three times more scavenging activity than AA (Sloley *et al.*, 2001). Alcohol root extracts of *E. pallida* prevented malondialdehyde (MDA) formation in catecholaminergic neuroblastoma SH-SY5Y cells better than *E. purpurea* and *E. angustifolia*. However, the MDA levels were not significantly lower than that observed in the control. The addition of iron to the test system resulted in a significant increase in MDA formation for all extracts and the control. The increase in MDA in the *Echinacea*-treated cells was significantly lower than that of the control. Again, cells treated with *E. pallida* had the lowest MDA formation. Irrespective of the *Echinacea* species/variety, the concentration of 0.5 mg/ml prevented MDA formation more than the 1 mg/ml concentration in the iron-free test system whereas the opposite was true for the iron-containing test system (Sloley *et al.*, 2001). In contrast to the radical scavenging assay, the leaf extracts inhibited the formation of MDA greater than the root extracts. No clear trends were apparent with regard to *Echinacea* species/variety or concentration of the

additive having the greatest activity (Sloley *et al.*, 2001). However, Hu and Kitts (2000) reported that *E. angustifolia* and *E. pallida* were more effective than *E. purpurea* in various *in vitro* assays and suggested that the antioxidant capacity was due to the high levels of echinacoside.

The anti-inflammatory property of *Echinacea* has been associated with the alkamides and polysaccharides. However, a standardized extract containing 4% phenolic acids was found to be more effective at inhibiting prostaglandin synthesis than the *E. purpurea* herb (Rininger *et al.*, 2000). Recently, echinacoside has been shown to inhibit inflammation and wound healing in rats (Speroni *et al.*, 2002). *Echinacea angustifolia* root polysaccharide fraction (EPF) effectively inhibited a croton oil-induced edema when applied topically while an intravenous injection (0.5 mg/kg) inhibited a carrageenan-induced edema (Tubaro *et al.*, 1987). The intravenous injection was slightly more effective than topical applications. Water extracts of *E. angustifolia* root (EAE) were less effective than the polysaccharide fraction at inhibiting carrageenan-induced edema. To achieve similar inhibition rates, 100 times more EAE was required compared to EPF. The application of *Echinacea* alkamides was shown to inhibit 5-lipoxygenase, a key enzyme in arachidonic acid metabolism to prostaglandins, via a competitive inhibition of the enzyme mechanism (Müller-Jakic *et al.*, 1994). Alternatively, radical scavenging activity of the highly unsaturated alkamides may reduce the oxidative reactions that may occur during arachidonic acid metabolism. Dodeca-2*E*,4*E*,8*Z*,10*E*/*Z*-tetraenoic acid isobutylamides had the highest inhibitory activity (62.6%) against 5-lipoxygenase. However, a hexane extract of *E. angustifolia* root inhibited 81.8% of the 5-lipoxygenase activity, suggesting that additional components are involved in the enzyme inhibition (Müller-Jakic *et al.*, 1994). Alkamides (50 μg/ml) from *E. angustifolia* root also were potent cyclooxygenase (COX) inhibitors (Müller-Jakic *et al.*, 1994). Alkamides from *E. purpurea* roots were found to inhibit two COX isozymes (COX-I and COX-II) at a concentration of 100 μg/ml (Clifford *et al.*, 2002; Raso *et al.*, 2002). Alkamides inhibited the COX-I and COX-II by 36–60% and 15–46%, respectively.

B. CLINICAL EVALUATIONS

In general, the majority of the *in vitro* investigations support the biological activities of *Echinacea*. However, results from *in vivo* investigations are somewhat contradictory. More specifically, clinical investigations are less convincing due in part to the poor experimental design of the trials and the variability of *Echinacea* products tested. Virtually, all the clinical investigations prior to 1993 are flawed in some way or another. Only a few studies prior to 1993 used proper methodology such as randomized,

double-blind and placebo-controlled (RDBPC) studies (Dorn, 1989; Reitz, 1990; Schmidt *et al.*, 1990; Bräunig *et al.*, 1992; Bräunig and Knick, 1993). For further details regarding trials prior to 1993, see the reviews of Wills *et al.* (2000) and Melchart *et al.* (1994). Only clinical trials relevant to upper respiratory infections (URI) or the common cold will be highlighted below.

Hydroalcohol tincture of *E. purpurea* and *E. pallida* roots effectively reduced the duration and symptoms of URI (Bräunig *et al.*, 1992; Bräunig and Knick, 1993; Dorn *et al.*, 1997). These clinical investigations were RDBPC studies that showed that 90 drops of *E. purpurea* or *E. pallida* root tincture/day (i.e., equivalent to 900 mg root/day) shortened the duration of the URI by 3.2 and 3.9 days for bacterial and viral infections, respectively. However, daily doses of 450 mg *Echinacea* did not significantly affect URI (Bräunig *et al.*, 1992). Brinkeborn *et al.* (1999) used a four-armed RDBPC to evaluate three *Echinacea* products and a placebo as treatment for common cold. Subjects (246) were randomly placed into 1 of 4 groups and were instructed to take the preparation (2 tablets, 3 times daily) at the first onset of cold and visit a physician within 2 days of the cold onset. Group one received *E. purpurea* extract (6.78 mg, Echinaforce®—95% herb and 5% root), group two, a concentrated fresh *E. purpurea* (95% herb and 5% roots; 48.3 mg), and the third group, a root extract of *E. purpurea* (29.6 mg). The fourth group was given a placebo. Reduction in mean physician assessed symptom index from day 1 or 2 to day 7 was 29% for the placebo, 45% for group three, and 63 and 64% for groups one and two, respectively. Groups one and two's physician assessments were significantly higher than group three and the placebo. Based on the dosage, groups one, two and three had daily *Echinacea* intakes of 41,290 and 178 mg, respectively, suggesting that *Echinacea* intake was not the driving force behind the improved physician assessments observed in group one compared to group three (Brinkeborn *et al.*, 1999). The efficacy of the treatments in groups one and two, as judged by physician was approximately 68 and 78%, respectively, and by patients was 78 and 84%, respectively. This study supports the earlier report that significant reduction in clinical cold symptoms was observed when patients were given 1500 mg *E. purpurea*/day (Brinkeborn *et al.*, 1998). Again, the validity of the data can be supported by the use of RDBPC experimental design. Hoheisel *et al.* (1997) and Schulten *et al.* (2001) evaluated the pressed juice product Echinacin® under a RDBPC study to determine clinical relevance of this *Echinacea* preparation. Treatment with Echinacin® shortened the duration of the cold by 4 days, with fewer symptoms and with 20% fewer subjects developing fully expressed colds (Hoheisel *et al.*, 1997). Schulten *et al.* (2001) found that the duration of the cold was reduced by 3 days for subjects treated with Echinacin®; however, full expression of the common cold was not significantly different between the treatment group and the placebo group.

The treatment group did have significantly lower scores for rhinorrhea, nasal congestion and sore throats, suggesting that treatment with *Echinacea* could alleviate some symptoms of the common cold. Wüstenberg *et al.* (1999) reported similar results using the preparation Esberitox®, a product made with *Echinacea* root, wild indigo root and white cedar leaves.

In contrast to the positive outcomes provided before, several reports have shown that *Echinacea* does not significantly reduce URI. Schöneberger (1992) found no significant differences in the frequency (although lower) and intensity of URI between the treatment group and placebo group. This study used the pressed juice of *E. purpurea* at a dose of 8 ml/day over 8 weeks and involved 108 patients. In five randomized placebo-controlled studies involving the same 134 patients, relative phagocytic activity of PNG and leukocytes number in peripheral blood cells were enhanced in only two of the five studies (Melchart *et al.*, 1995). Of the two studies testing positive, only one used *Echinacea* as the sole source of immune-enhancing ingredient. In this study, ethanol extracts of *E. purpurea* (1000 mg) were taken orally for five days by the 134 patients. A 54% increase in phagocytosis was measured. Treatments containing various levels of ethanol extracts of *E. purpurea* root, *E. pallida* root and *E. purpurea* herb did not enhance phagocytosis or leukocytes in peripheral blood (Melchart *et al.*, 1995). Schwarz *et al.* (2002) also reported that *E. purpurea* herb did not enhance phagocytosis activity of polymorphonuclear leukocytes. A three-armed RDBPC involving 302 patients was conducted to evaluate the effects of *E. purpurea* and *E. angustifolia* root extracts on URI (Melchart *et al.*, 1998). Approximately 50 drops (91 mg) of the extracts were administered twice daily. Time to the first infections were 65, 66 and 69 days for the groups given a placebo, *E. angustifolia* root extract or *E. purpurea* root extract, respectively. The percentage of the patients infected were 37, 32 and 29% for groups given a placebo, *E. angustifolia* root extract or *E. purpurea* root extract, respectively. All data were not significantly different from the placebo groups, thus no benefit was observed (Melchart *et al.*, 1998). In an experimental rhinovirus common cold model, patients treated with 900 mg/day dose of *E. purpurea* during a period of 14 days prior to and five days after viral challenge were not significantly protected from infection (Turner *et al.*, 2000). Rhinovirus infection occurred in 44% of the subjects treated, compared to the 57% in the placebo group ($p = 0.3$). A common cold developed in 50 and 59% of subjects in the *Echinacea* and placebo groups, respectively. Grimm and Müller (1999) reported that 65 and 74% of *Echinacea* and placebo groups, respectively, had at least one cold or respiratory infection during the 8-week study. The average number of colds was 0.78 and 0.93 for the *Echinacea* and placebo groups, respectively. The duration of the colds was reduced by two days in the group

treated with the expressed juice of *Echinacea* diluted to 22% w/alcohol (8 ml/day). However, this reduction in cold duration was insignificant.

From the clinical evaluations reported to date, no clear recommendation can be drawn with regard to the effectiveness of *Echinacea* in clinical environments. However, the use of *Echinacea* as a treatment for colds or symptoms of colds are more strongly supported by clinical studies than using *Echinacea* as a prophylactic. The use of standardized extracts and dosages may be a better approach for evaluating *Echinacea* in clinical studies.

C. SAFETY/TOXICOLOGY

1. Safety of Echinacea

Echinacea enhances the immune system via nonspecific, antigen-independent mode of action (Wüstenberg *et al.*, 1999). With few exceptions, *Echinacea* has been found to be safe for consumption, with the number of adverse effects being minimal in many clinical investigations. However, the toxicological properties of *Echinacea* have not been fully explored. Only one report (Mengs *et al.*, 1991) systematically evaluated the toxicological properties of *E. purpurea*, whereas other reports are afterthoughts with regard to safety.

Mengs *et al.* (1991) used rat and mice models to assess the acute and subacute toxicities, mutagenicity and carcinogenicity. The LD_{50} in rats was $>15{,}000$ and >5000 mg *Echinacea*/kg when administered orally and intravenously, respectively. The *Echinacea* dosage needed to reach the LD_{50} in mice was even higher at concentrations $>30{,}000$ and $>10{,}000$ mg/kg for oral and intravenous administration, respectively. Subacute toxicity was determined using oral doses of *Echinacea* at 800, 2400 or 8000 mg/kg daily (Mengs *et al.*, 1991). A significant reduction in alkaline phosphatase was found in the male rats treated with 2400 and 8000 mg/kg; whereas, a significant rise in prothrombin time was found in female rats fed 2400 and 8000 mg *Echinacea*/kg. Necropsy results and histology failed to show the differences between the treated and control animals; thus no subacute toxicity was noted. No toxicity was found in the bacterial mutagenicity assays used between 8 and 5000 μg *E. purpurea*/plate. No statistical increase in mutation frequency was found in mouse lymphoma assays at concentrations up to 5000 μg/ml. In the human lymphocyte assay, mitotic inhibition was not detected at concentrations up to 5000 μg/ml; however, a significant increase in cells with aberrations was noted only at the 20 h sampling and in the 5000 μg/ml treatment. Nonsignificance was retained at the 44 h measurement. No significant increase in polychromatic erythrocytes was observed after the mice were administered a single oral dose of 25,000 mg *E. purpurea*/kg (Mengs *et al.*, 1991). The *in vitro* carcinogenicity confirmed the nontoxic effect

of other assays. See *et al.* (1997) reported that a 1000 μg/ml concentration of *Echinacea* was nontoxic based on viability of PBMC assays.

No significant differences were observed in the number of malformations, live births, or spontaneous abortions between the control group and study group (Gallo *et al.*, 2000). The study group consisted of 206 women who used *Echinacea* during pregnancy. Of the 206 women, 54% consumed *Echinacea* during the first trimester whereas only 8% consumed *Echinacea* in all three trimesters. *Echinacea* consumption varied between 250 and 1000 mg/day in tablets and from 5 to 30 drops of tincture per day. Gallo *et al.* (2000) recommended further testing on larger populations and suggested that standardized dosage may provide improved statistical power.

2. *Adverse reaction to Echinacea*

The use of *Echinacea* is not recommended for patients with autoimmune conditions, such as multiple sclerosis and AIDS, or those taking drugs to suppress immune response (e.g., corticosteroids) (Blumenthal, 1998; Gruenwald *et al.*, 2000). However, these recommendations appear to be based on speculation more than rigorous, peer-reviewed research. Research is needed to further support or refute the claim that individuals with autoimmune conditions should not use *Echinacea*.

The most notable adverse reaction to *Echinacea* was reported in Australia (Mullins, 1998, 2000). In this case, the subject orally consumed twice the recommended dose (patient consumed one teaspoon) of a 40% ethanol in water extract of *Echinacea*, which was the equivalent to 3825 mg of *E. angustifolia* (whole plant) and 150 mg dried *E. purpurea* root. Initial symptoms include burning of the mouth and throat, which is not an uncommon sensation when consuming *Echinacea*, followed by chest tightness, general urticaria and diarrhea. The subject recovered 2 h after self-administration of promethazine. A 3 mm flare was observed in a skin prick test using the same extract ingested by the subject whereas a 3 mm wheal and 5 mm flare formed in the test from a glycerin extract from the same manufacturer (Mullins, 1998). Other dietary components consumed by the subject gave negative reactions. Additional testing using radioallergosorbent (RAST) revealed *Echinacea*-binding immunoglobulin E (IgE) in the subject's serum. The patient did decline a rechallenge of the other dietary supplements taken around the time of the *Echinacea*-associated anaphylaxis. Subsequent testing of 84 subjects, with asthma or allergic rhinitis, using the above mentioned *Echinacea* products resulting in 16 (or 19%) positive responses in the skin prick test. Only two of the 16 subjects had previously consumed *Echinacea*, raising the question of potential cross-reactivity of *Echinacea* proteins and other allergens.

Further evaluation of adverse reactions to *Echinacea* has been recently reported (Mullins and Heddle, 2002). In addition to the case (i.e., case 1) given above, four additional case studies showed that *Echinacea* caused adverse reaction in subjects. An acute asthma attack occurred along with itchy and watery eyes and runny nose after the subject ingested an *Echinacea*-containing tea. The skin prick test using the same extract as in case 1 produced a 3 mm wheal. A third case involved a health professional who noted general urticaria, facial and upper airway angioedema, difficulty in swallowing and dizziness after consuming a tablet containing *Echinacea*. The skin prick test was negative to the same extract as in case 1, except that the extract was 1-year-old at the time the test was administered. However, a positive RAST score was reported. A third case involved the onset of asthma after ingestion of *Echinacea*-containing tablets. A 2 mm wheal formed after exposure to the 6-month-old extract used in case 1. The RAST test was negative for this subject. The final case involved the development of a pruritic rash after consumption of tablets containing *Echinacea*. Exposure to a 5-month-old extract, used in case 1, in the skin prick test gave negative results. In addition to these five case studies, Mullins and Heddle (2002) summarized 51 reports of adverse reaction to *Echinacea*. Twenty-six (26) of the 51 reports were possibly IgE-mediated hypersensitivity.

Other components in the *Echinacea* products may contribute to many of the adverse reactions reported, not only Australian, but also the United States, United Kingdom, Canada and New Zealand. Additional studies are needed to further support the IgE-mediated hypersensitivity reported in *Echinacea*-containing products. Studies could include growing and processing of *Echinacea* under environmentally controlled conditions, with the intent to minimize foreign contaminants, and administration of extracts to patients under supervision. Although this would not represent real-world conditions, it may further support or refute the IgE-mediated hypersensitivity observed in subjects taking commercial *Echinacea* products. Additional work is also needed to identify the component responsible for the IgE-mediated hypersensitivity.

V. *ECHINACEA* AS A FUNCTIONAL FOOD ADDITIVE

Several reports have recently been published on the *in vivo* (Sloley *et al.*, 2001—see section IV, subsection 3) and *in vitro* (Hu and Kitts, 2000; Rininger *et al.*, 2000) antioxidant activity. Hu and Kitts (2000) found that a methanol extract of *E. pallida* had a higher antioxidant activity than *E. purpurea* and *E. angustifolia*. In addition, the relationship between CAP and antioxidant activity was established. The CAP concentrations of 2.61, 1.11, and 0.49% were found in *E. pallida*, *E. angustifolia*, and *E. purpurea*,

respectively, which correlated to the antioxidant activity. Hu and Kitts (2000) hypothesized that echinacoside may account for the antioxidant activity based on the observation that the *E. purpurea* lacked echinacoside and had the lowest antioxidant potential. Many of their antioxidant assays were applicable to biological systems other than foods.

In an effort to find more efficient food antioxidants, the Food Chemistry Research Laboratory at North Dakota State University (Hall *et al.*, 2001) has preliminarily evaluated the antioxidant capacity of *Echinacea* in oil systems. The oxidative stability of sunflower oil, using Rancimat (110°C), was determined as a means to evaluate the antioxidant efficiencies of the ground herbal and root portions of *E. purpurea* and *E. angustifolia*. With the exception of *E. angustifolia* root, all antioxidant activity increased as the concentration of *Echinacea* increased from 0.05 to 1% (Figure 9). The antioxidant activity of the 0.5 and 1% addition level of *E. angustifolia* root was lower than the 0.05 and 0.1% concentrations suggesting that sufficient concentrations of other components such as metals may have contributed to the limited antioxidant activity. Up to 480 ppm of iron can be found in the taproot of *E. angustifolia* (Hobbs, 1996). The roots of *E. purpurea* is more rhizome-like than a true taproot, which may result in a lower iron content and the lack of the observed decrease in antioxidant activity as concentration increased.

A second study involved the addition of hexane and ethanol extracts of *E. purpurea* and *E. angustifolia* to stripped corn oil (50°C) and oxidation

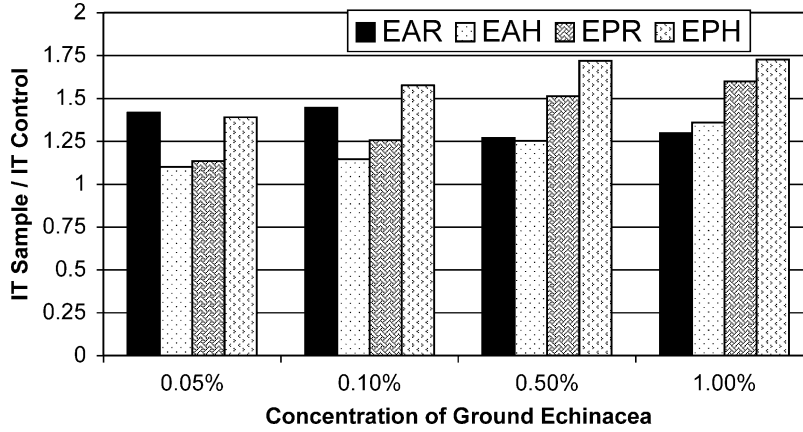


FIG. 9 Sunflower oil stability (induction time (IT) sample/IT control) under Rancimat (110°C) conditions and in the presence of ground *Echinacea* (0.05–1%): *E. purpurea* herb (EPH) and root (EPR), and *E. angustifolia* herb (EAH) and root (EAR).

assessed using peroxide value. The ethanol extracts of the *Echinacea* roots significantly inhibited the oxidation of stripped corn oil to a greater extent than the other treatments (Figure 10). The 0.5% level of the ethanol root extracts was significantly better than the 0.05% concentration at preventing oxidation. The 0.5% concentration of the ethanol extracts of the herb was less efficient than the 0.05%; however, the peroxide values for both the treatments were higher than the control and thus were pro-oxidants. All other extracts of the herbal parts of *Echinacea* promoted the oxidation (Figure 10). A positive relationship existed between phenolic content of the extracts and antioxidant activity. A qualitative assessment using HPLC showed that the CAP levels for *E. angustifolia* root were higher than other *Echinacea* products (Hall *et al.*, 2001).

In addition to oil-stability evaluations, AA retention was evaluated in orange juice treated with *E. purpurea* and *E. angustifolia* extracts and stored at room temperature. Our initial studies at North Dakota State University showed that the addition of ground *Echinacea* plant tissue was ineffective at preventing AA loss. For example, only 8% of the AA remained after 48 h in the orange juice treated with dried aerial parts. A 30% loss of AA after 48 h was found in the control juice. In contrast, 91 and 94% of AA was retained in the orange juice treated with ethanol extracts of the aerial parts and roots, respectively. In a subsequent study,

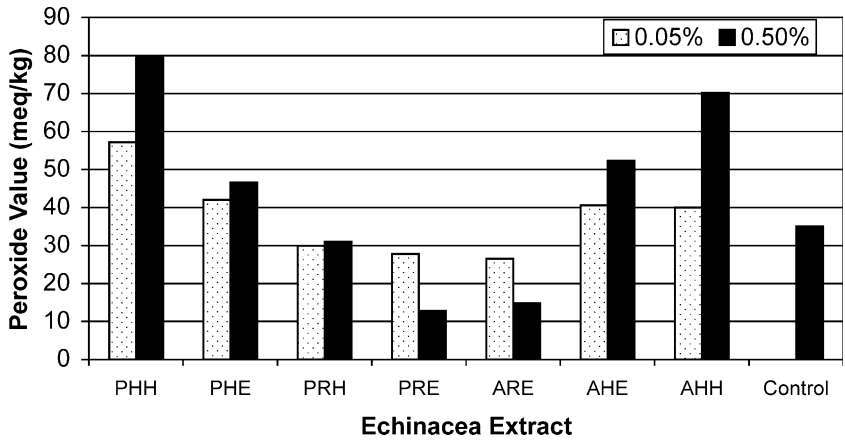


FIG. 10 Peroxide values of corn oil treated with various *Echinacea* extracts and concentration (0.05 and 0.5%). Hexane extracts of *E. purpurea* herb (PHH) and root (PRH). Ethanol extracts of *E. purpurea* herb (PHE) and root (PRE). Hexane extracts of *E. angustifolia* herb (AHH). Ethanol extracts of *E. angustifolia* herb (AHE) and root (ARE).

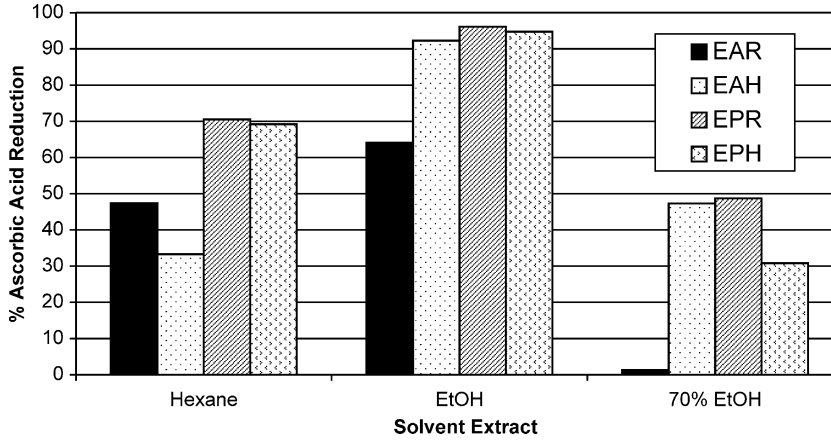


FIG. 11 Ascorbic acid reduction in orange juice treated with various *Echinacea* extracts (1%): *E. purpurea* herb (EPH) and root (EPR), and *E. angustifolia* herb (EAH) and root (EAR).

the plant materials were extracted sequentially using hexane, ethanol (95%) and ethanol:water (70:30), and the resulting extracts added separately to orange juice at 1%. At the 5-day measurement, the 70% ethanol extracts protected AA best with an average reduction in AA of 32%, compared to 55 and 87% for hexane and ethanol (95%) extracts (Figure 11). In general, the *E. angustifolia* root provided the most protection against AA loss. We are currently characterizing the phytochemical constituents in the extracts to determine if a relationship exists between phenolic content or individual phenols and AA protection. In general, the addition of *Echinacea* had a positive effect on AA retention.

VI. CONCLUSION

Echinacea has been used for centuries as a medicinal plant and has been promoted recently as an immunostimulant. Research from the last two decades has shown that *Echinacea* can enhance the immune system using *in vitro* and *in vivo* indicators. Jager *et al.* (2002) reported that dodeca-2*E*,4*E*,8*Z*,10*E*/*Z*-tetraenoic acid isobutylamides could cross biological barriers via passive diffusion, suggesting that the alkamides may contribute to the *in vivo* effects noted by researchers. In contrast, conflicting results have

been reported during clinical evaluations of *Echinacea*, thus there is a need to standardize formulas to truly evaluate the effectiveness of *Echinacea* in biological systems. In addition, further research is needed to establish the safety of *Echinacea* in light of the reports published on adverse reactions to *Echinacea*. Recently, the National Institutes of Health awarded the University of Iowa and Iowa State University a 5-year, $6 million grant to establish a center to investigate *Echinacea* and St. Johns Wort as dietary supplements. This level of funding indicates the seriousness to which answers are needed regarding the true benefits and risks of consuming *Echinacea*.

ACKNOWLEDGEMENTS

The author thanks Dr. Jurgen Schwarz from the University of Maryland for his assistance in translating, to English, the manuscripts written in German. The author also thanks Penny Neisen for her assistance in producing the figures and references.

REFERENCES

Barrett, B., Kiefer, D., and Rabago, D. 1999. Assessing the risks and benefits of herbal medicine: an overview of scientific evidence. *Altern. Ther. Health Med.* **5**(4), 40–49.

Bauer, R. 1996. *Echinacea* Drogen Wirkungen und Wirksubstanzen. *Z. Aerztl. Fortbild.* **90**, 111–115.

Bauer, R. 1997. Standardisierung von *Echinacea purpurea*-Preßsaft auf cichoriensäure und alkamide. *Z. Phytother.* **18**, 270–276.

Bauer, R. 1999a. Clinical investigations of *Echinacea* phytopharmaceuticals. *In* "Immunomodulatory Agents from Plants" (H. Wagner, ed.), pp. 41–88. Birkhäuser Verlag, Basel, Switzerland.

Bauer, R. 1999b. Standardization of *Echinacea purpurea* expressed juice with reference to cichoric acid and alkamides. *J. Herbs Spices Med. Plants* **6**, 51–61.

Bauer, R. 2000. Chemistry, pharmacology, clinical applications of *Echinacea* products. *In* "Herbs, Botanicals and Teas as Functional Foods and Nutraceuticals" (G. Mazza and B.D. Oomah, eds), pp. 45–73. Technomic Publishing, Inc., Lancaster, PA.

Bauer, R. and Foster, S. 1991. Analysis of alkamides and caffeic acid derivatives from *Echinacea simulata* and *E. Paradoxa* roots. *Planta Med.* **57**, 447–449.

Bauer, R. and Remiger, P. 1989. TLC and HPLC analysis of alkamides in *Echinacea* drugs. *Planta Med.* **55**, 367–371.

Bauer, R. and Wagner, H. 1990. *Echinacea*: Ein Handbuch für Ärzte, Apotheker und andere Naturwissenschaftler. Wissenschaftliche Veragsgesellschaft, Stuttgart.

Bauer, R., Khan, I., Wray, V., and Wagner, H. 1987. Two acetylenic compounds from *Echinacea pallida* roots. *Phytochemistry* **26**, 1198–1200.

Bauer, R., Remiger, P., and Wagner, H. 1988a. Vergleichende DC-und HPLC Analyse der Herba-Drogen von *Echinacea purpurea*, *E. pallida*, und *E. angustifolia*. *Dtsch. Apoth. Ztg* **128**, 174–180.

Bauer, R., Khan, A., and Wagner, H. 1988b. TLC and HPLC analysis of *Echinacea pallida* and *E. angustifolia* roots. *Planta Med.* **54**, 426–430.

Bauer, R., Remiger, P., and Wagner, H. 1988c. Alkamides from the roots of *Echinacea purpurea*. *Phytochemistry* **27**, 2339–2342.

Bauer, R., Jurcic, K., Puhlmann, J., and Wagner, H. 1988d. Immunologische *in-vivo* und *in-vitro* untersuchungen mit *Echinacea*—Extrakten. *Arzneim.-Forsch.* **38**, 276–281.

Bauer, R., Remiger, P., and Wagner, H. 1989. Alkamides from the roots of *Echinacea angustifolia*. *Phytochemistry* **28**, 505–508.

Becker, H. and Hsieh, W. 1985. Chocoree-Saüre und deren Derivate aus *Echinacea*-Arten. *Z. Fuer Naturfuschung* **40**, 585–587 (Section C).

Becker, H., Hsieh, W., Wylde, R., Laffite, C., and Andary, C. 1982. Structure of Echinacoside. *Z. Fur Naturfuschung* **37**, 351–353 (Section C).

Bergeron, C., Livesey, J., Awang, D., Arnason, J., Rana, J., Baum, B., and Letchamo, W. 2000. A quantitative HPLC method for the quality assurance of *Echinacea* products on the North American market. *Phytochem. Anal.* **11**, 207–215.

Bergeron, C., Gafner, S., Batcha, L., and Angerhofer, C. 2002. Stabilization of caffeic acid derivatives in *Echinacea purpurea* L. glycerin extract. *J. Agric. Food Chem.* **50**, 3967–3970.

Bicchi, C., Binello, A., and Rubiolo, P. 2000. Determination of phenolic diterpene antioxidants in Rosemary (*Rosmarinus officinalis* L.) with different methods of extraction and analysis. *Phytochem. Anal.* **11**, 236–242.

Binns, S., Purgina, B., Bergeron, C., Smith, M., Ball, L., Baum, B., and Arnason, J. 2000. Light-mediated antifungal activity of *Echinacea* extracts. *Planta Med.* **66**, 241–244.

Binns, S., Baum, B., and Arnason, J. 2002a. A taxonomic revision of *Echinacea* (Asteraceae: Heliantheae). *Syst. Bot.* **27**, 610–632.

Binns, S., Livesey, J., Arnason, J., and Baum, B. 2002b. Phytochemical variation in *Echinacea* from roots and flowerheads of wild and cultivated populations. *J. Agric. Food Chem.* **50**, 3673–3687.

Binns, S., Arnason, J., and Baum, B. 2002c. Phytochemical variation within populations of *Echinacea angustifolia* (Asteraceae). *Biochem. Syst. Ecol.* **30**, 837–854.

Binns, S., Hudson, J., Merali, S., and Arnason, J. 2002d. Antiviral activity of characterized extracts from *Echinacea* spp. (Heliantheae: Asteraceae) against Herpes simplex virus (HSV-I). *Planta Med.* **68**, 780–783.

Blumenthal, M. 1998. The Complete German Commission E. Monographs: Therapeutic Guide to Herbal Medicines. America Botanical Council, Austin, TX.

Bohlmann, F. and Hoffman, H. 1983. Further amides from *Echinacea purpurea*. *Phytochemistry* **22**, 1173–1175.

Bräunig, B. and Knick, E. 1993. Therapeutische Erfahrungen mit Echinacea pallidae bei Grippalen Infekten. *Naturheilpraxis* **1**, 72–75.

Bräunig, B., Dorn, M., and Knick, E. 1992. *Echinaceae purpureae* Radix: zur Stärkung der Körpereigene Abwehr bei Grippalen Infekten. *Z. Für Phytother.* **13**, 7–13.

Brinkeborn, R., Shah, D., and Degenring, F. 1999. Echinaforce® and other *Echinacea* fresh plant preparations in the treatment of the common cold. *Phytomedicine* **6**, 1–5.

Burger, R.A., Torres, A.R., Warren, R.P., Caldwell, V.D., and Hughes, B.G. 1997. *Echinacea*-induced cytokine production by human macrophages. *Int. J. Immunopharmacol.* **19**, 371–379.

Catchpole, O., Perry, N., da Silva, B., Grey, J., and Smallfield, B. 2002. Supercritical extraction of herbs I: Saw Palmetto, St. John's Wort, Kava Root and *Echinacea*. *J. Supercrit. Fluids* **22**, 129–138.

Cheminat, A., Zawatzky, R., Becher, H., and Brouillard, R. 1988. Caffeoyl conjugates from *Echinacea* species: structures and biological activity. *Phytochemistry* **27**, 2787–2794.

Classen, B., Witthohn, K., and Blaschek, W. 2000. Characterization of an arabinogalactan-protein isolated from pressed juice of *Echinacea purpurea* by precipitaion of the β-Glucosy Yariv Reagent. *Carbohydr. Res.* **327**, 497–504.

Clifford, L., Nair, M., Rana, J., and Dewitt, D. 2002. Bioactivity of alkamides isolated from *Echinacea purpurea* (L.) Moench. *Int. J. Phytother. Pharmacol.* **3**, 249–253.

Coeugniet, E. and Elek, E. 1987. Immunomodulation with *Viscum album* and *Echinacea purpurea* extracts. *Onkologie* **10**(Suppl. 3), 27–33.

Csává, M., Borbás, A., Jánossy, L., Batta, G., and Lipták, A. 2001. Synthesis of the α-L-Araf-(1 → 2)-β-D-Galp-(1 → 6)-β-Galp-(1 → 6)-[α-L-Araf-(1 → 2)]-β-D-Galp-(1 → 6)-β-Gal hexasaccharide as a possible repeating unit of the cell-culture exudates of *Echinacea purpurea* arabinogalactan. *Carbohydr. Res.* **336**, 107–115.

Currier, N. and Miller, S. 2000. Natural killer cells from aging mice treated with extracts from *Echinacea purpurea* are quantitatively and functionally rejuvenated. *Exp. Gerontol.* **35**, 627–639.

Currier, N. and Miller, S. 2001. *Echinacea purpurea* and malatonin augment natural-killer cells in leukemic mice and prolong life span. *J. Altern. Complemen. Med.* **7**, 241–251.

Currier, N. and Miller, S. 2002. The effect of immunization with killed tumor cells, with/without feeding of *Echinacea purpurea* in an erythroleukemic mouse model. *J. Altern. Complement. Med.* **1**, 49–58.

Dietz, B. and Bauer, R. 2001. The constituents of *Echinacea atrorubens* roots and aeria. *Pharm. Biol.* **39**, 11–15.

Dorn, M. 1989. Milderung grippaler effekte durch ein pflazliches immunstimulans. *Nat. und Ganzheitsmedizin* **2**, 314–319.

Dorn, M., Knick, E., and Lewith, G. 1997. Placebo-controlled, double-blind study of *Echinacea pallidae* radix in upper respiratory infections. *Complement. Ther. Med.* **3**, 40–42.

Elsässer-Beile, U., Willenbacher, W., Bartsch, H., Gallati, H., Monting, J., and Von Kleist, S. 1996. Cytokine production in leukocyte cultures during therapy with *Echinacea* extract. *J. Clin. Lab. Anal.* **10**, 441–445.

Emmendörffer, A., Wagner, H., and Lohmann-Matthes, M.L. 1999. Clinical Investigations of *Echinacea* phytopharmaceuticals. *In* "Immunomodulatory Agents from Plants" (H. Wagner, ed.), pp. 89–104. Birkhäuser Verlag, Basel, Switzerland.

Erhard, M., Kellner, J., Wild, J., Losch, U., and Hatiboglu, F.S. 1994. Effect of *Echinacea* aconitum, lachesis and apis extracts, their combinations on phagocytosis of human granulocytes. *PTR, Phytother. Res.* **8**, 14–17.

Facino, R.M., Carini, M., Aldini, G., Marinello, C., Arlandini, E., Franzoi, L., Colombo, M., Pietta, P., and Mauri, P. 1993. Direct characterization of caffeoyl esters with antihyaluronidase activity in crude extracts from *Echinacea angustifolia* roots by fast atom bombardment tandem mass spectrometry. *Farmaco* **48**, 1447–1461.

Facino, R.M., Carini, M., Aldini, G., Saibene, L., Pietta, P., and Mauri, P. 1995. Echinacoside and caffeoyl conjugates protect collagen from free radical-induced degradation: a potential use of *Echinacea* extracts in the prevention of skin photodamage. *Planta Med.* **61**, 510–514.

Foster, S. 1985. *Echinacea*: the purple coneflower. *Am. Hortic.* Aug., 14–17.

Foster, S. 1991. The species: identification and distribution. *In* "*Echinacea*, Nature's Immune Enhancer", pp. 107–115. Healing Arts Press, Rochester, VT.

Gahler, R., Barton, R., Slama, J., Chang, C. 2001. *Echinacea* supplement and method of manufacture. World Intellectual Property Organization, *International Publication Number* WO 01/22977.

Gaisbauer, M., Schleich, Th., Stickl, H., and Wilczek, I. 1990. Untersuchungen zum eijfluß von *Echinacea purpurea* moench auf die phagozytose von granulozyten mitels messung der chemilunineszenz. *Arzneim.-Forsch. (Drug Res.)* **40**, 594–597.

Gallo, M., Sarkar, M., Au, W., Pietrzak, K., Comas, B., Smith, M., Jaeger, T., Einarson, A., and Koren, G. 2000. Pregnancy outcome following gestational exposure to *Echinacea*. *Arch. Intern. Med.* **160**, 3141–3143.

Giger, E., Keller, F., and Baumann, T. 1989. Fructans in *Echinacea* and in its phytotherapeutic preparations. *Planta Med.* **55**, 638–639.

Glowniak, K., Zgorka, G., and Kozyra, M. 1996. Solid-phase extraction and reversed-phase high-performance liquid chromatography of free phenolic acids in some *Echinacea* species. *J. Chromatogr. A* **730**, 25–29.

Goel, V., Chang, C., Slama, J., Barton, R., Bauer, R., Gahler, R., and Basu, T. 2002. Alkylamides of *Echinacea purpurea* stimulate alveolar macrophage function in normal rats. *Int. Immunopharmacol.* **2**, 381–387.

Goti, R., Pomponio, R., Bertucci, C., and Cavrini, V. 2002. Simultaneous analysis of the lipophilic and hydrophilic markers of *Echinacea* plant extracts by capillary electrophoresis. *J. Sep. Sci.* **25**, 1079–1086.

Gray, D., Roberts, C., Rottinghaus, G., Garrett, H., and Pallardy, S. 2001. Quantification of root chicoric acid in purple coneflower by near infrared reflectance spectroscopy. *Crop Sci.* **41**, 1159–1161.

Grimm, W. and Müller, H. 1999. A randomized controlled trial of the effect of fluid extract of *Echinacea purpurea* on the incidence and severity of colds and respiratory infections. *Am. J. Med.* **106**, 138–143.

Gruenwald, J., Brendler, T., and Jaenicke, C. 2000. *In* "PDR for Herbal Medicines", 2nd Ed., pp. 261–266. Medical Economics Company, NJ.

Hall III, C., Schwarz, J., Shultz, K., 2001. The antioxidant activity of the purple coneflower (*Echinacea*). Abstracts of the 92nd AOCS Annual Meeting & Expo, May 13–16, Minneapolis, MN. (http://www.aocs.org/archives/am2001wp.htm).

He, X., Lin, L., Bernart, M.W., and Lian, L. 1998. Analysis of alkamides in roots and achenes of *Echinacea purpurea* by liquid chromatography–electrospray mass spectrometry. *J. Chromatogr. A* **815**, 205–211.

Health Canada, 1999. *Echinacea* Root Labelling Standards. Health Canada Therapeutic Products Programme, Ottawa, Ontario.

Heyl, F. and Stanley, J. 1914. Analyses of two *Echinacea* roots. *Am. J. Pharm.* **86**, 450–455.

Hobbs, C. 1996. "*Echinacea*: The Immune Herb", pp. 31–35. Botanic Press, Santa Cruz, CA.

Hoheisel, O., Sandberg, M., Bertram, S., Bulitta, M., and Schäfer, M. 1997. *Echinacea* treatment shortens the course of the common cold: a double-blind placebo-controlled clinical trial. *Eur. J. Clin. Res.* **9**, 261–269.

Hu, C. and Kitts, D. 2000. Studies on the antioxidant activity of *Echinacea* root extract. *J. Agric. Food Chem.* **48**, 1466–1472.

Jacobson, M. 1954. Occurrence of a pungent insecticidal principle in American Coneflower roots. *Science* **120**, 1028–1029.

Jager, H., Meinel, L., Dietz, B., Lapke, C., Bauer, R., Merkle, H., and Heilmann, L. 2002. Transport of alkamides from *Echinacea* species through Caco-2 monolayers. *Planta Med.* **68**, 469–471.

Johnson, R., Sun, J.C., and Sun, J. 2002. A pervaporation-microfiltration-osmotic distillation hybrid process for concentration of ethanol–water extracts of the *Echinacea* plant. *J. Membr. Sci.* **209**, 221–232.

Kabganian, R., Carrier, D., and Sokhansanj, S. 2002. Physical characteristic and drying rate of *Echinacea* root. *Drying Technol.* **20**, 637–649.

Kim, H., Durance, T., Scaman, C., and Kitts, D. 2000a. Retention of alkamides in dried *Echinacea purpurea*. *J. Agric. Food Chem.* **48**, 4187–4192.

Kim, H., Durance, T., Scaman, C., and Kitts, D. 2000b. Retention of caffeic acid derivatives in dried *Echinacea purpurea*. *J. Agric. Food Chem.* **48**, 4182–4186.

King, P., Ma, G., Miao, W., Jia, Q., McDougall, B., Reinecke, M., Cornell, C., Kuan, J., Kim, T., and Robinson, W. 1999. Structure–activity relationships: analogues of the dicaffeoylquinic and dicaffeoyltartaric acids as potent inhibitors of human immunodeficiency virus type 1 integrase and replication. *J. Med. Chem.* **42**, 497–509.

Kreis, W., Sußner, U., and Nüsslein, B. 2000. Reinigung und Charakterisierung Einer Polyphenoloxidase aus der Arzneidroge *Echinacea purpurea* herba (Sonnenhutkraut). *J. Appl. Bot. (Angew. Bot.)* **74**, 106–112.

Laasonen, M., Harmia-Pulkkinen, T., Simard, C., Michiels, E., Rasanen, K., and Vuorela, H. 2002. Fast identification of *Echinacea purpurea* dried roots using near-infrared spectroscopy. *Anal. Chem.* **74**, 2493–2499.

Lamidey, A., Fernon, L., Pouysegu, L., Delattre, C., Quideau, S., and Pardon, P. 2002. A convenient synthesis of the *Echinacea*-derived immunostimulator and HIV-1 integrase inhibitor (−)-(2*R*, 3*R*)-chicoric acid. *Helv. Chim. Acta* **85**, 2328–2334.

Lersch, C., Zeuner, M., Bauer, A., Siebenrock, K., Hart, R., Wagner, F., Fink, U., Dancygier, H., and Classen, M. 1990. Stimulation of the immune response in outpatients with hepatocellular carcinomas by low doses of cyclophosphamide (LDCY), *Echinacea purpurea* extracts (Echinacin®) and thymostimunlin. *Arch. Geschwulstforsch.* **60**, 379–383.

Lersch, C., Zeuner, M., Bauer, A., Siemens, M., Hart, R., Drescher, M., Fink, U., Dancygier, H., and Classen, M. 1992. Nonspecific immunostimulation with low doses of cyclophosphamide (LDCY), Thymnostimulin, *Echinacea purpurea* extracts (Echinacin®) in patients with far advanced colorectal cancers: preliminary results. *Cancer Investig.* **10**, 343–348.

Li, T.S.C. 1998. *Echinacea*: cultivation and medicinal value. *HortTechnology* **8**, 122–129.

Li, T.S.C. and Wardle, D. 2001. Effects of root drying temperature and moisture content on the levels of active ingredients in *Echinacea roots*. *J. Herbs Spices Med. Plants* **8**, 15–22.

Lienert, D., Anklam, E., and Panne, U. 1998. Gas chromatography–mass spectral analysis of roots of *Echinacea* species and classification by multivariate data analysis. *Phytochem. Anal.* **9**, 88–98.

Livesey, J., Awang, D., Arnason, J., Letchamo, W., Barrett, M., and Pennyroyal, G. 1999. Effect of temperature on stability of marker constituents in *Echinacea purpurea* root formulations. *Phytomedicine* **6**, 347–349.

Luettig, B., Steinmüller, C., Gifford, G., Wagner, H., and Lohmann-Matthes, M. 1989. Macrophage activation by the polysaccharide arabinogalactan isolated from plant cell cultures of *Echinacea purpurea*. *J. Natl Cancer Inst.* **81**, 669–675.

Luo, X., Chen, B., Yao, S., and Zeng, J. 2003. Simultaneous analysis of caffeic acid derivatives and alkamides in roots and extracts of *Echinacea purpurea* by high performance liquid chromatography-photodiode array detection-electrospray mass spectrometry. *J. Chromatogr. A* **986**, 73–81.

McDougall, B., King, P., Wu, B., Hostomsky, Z., Reinecke, M., and Robinson, W. 1998. Dicaffeoylquinic and dicaffeoyltartaric acids are selective inhibitors of human immunodeficiency virus type 1 integrase. *Antimicrob. Agents Chemother.* **42**, 140–146.

Melchart, D. and Linde, K. 1999. Clinical investigations of *Echinacea* phytopharmaceuticals. *In* "Immunomodulatory Agents from Plants" (H. Wagner, ed.), pp. 105–118. Birkhäuser Verlag, Basel, Switzerland.

Melchart, D., Linde, K., Worku, F., Bauer, R., and Wagner, H. 1994. Immunomodulation with *Echinacea*—a systematic review of controlled clinical trials. *Phytomedicine* **1**, 245–254.

Melchart, D., Linde, K., Worku, F., Sarkady, L., Holzmann, M., Jurcic, K., and Wagner, H. 1995. Results of five randomized studies on the immunomodulatory activty of preparations of *Echinacea*. *J. Altern. Complement. Med.* **1**, 145–160.

Melchart, D., Walther, E., Linde, K., Brandmaier, R., and Lersch, C. 1998. *Echinacea* roots extracts for the prevention of upper respiratory tract infections: a double-blind, placebo-controlled randomized trial. *Arch. Fam. Med.* **7**, 541–545.

Melchart, D., Clemm, C., Weber, B., Draczynski, T., Worku, F., Linde, K., Weidenhammer, W., Wagner, H., and Saller, R. 2002. Polysaccharide isolated from *Echinacea purpurea* herba cell cultures to counteract undesired effects of chemotherapy—a pilot study. *Phytother. Res.* **16**, 138–142.

Mengs, U., Clare, C., and Poiley, J. 1991. Toxicity of *Echinacea purpurea*. Acute, subacute and genotoxicity studies. *Arzneim.-Forsch. (Drug Res.)* **41**, 1076–1081.

Müller-Jakic, B., Breu, W., Probstle, A., Redl, K., Greger, H., and Bauer, R. 1994. *In vitro* inhibition of cyclooxygenase and 5-lipoxygenase by alkamides from *Echinacea* and *Achillea* species. *Planta Med.* **60**, 37–40.

Mullins, R. 1998. *Echinacea*-associated anaphylaxis. *Med. J. Aust.* **168**, 170–171.

Mullins, R. 2000. Allergic reactions to *Echinacea*. *J. Allergy Clin. Immunol.* **105**, S340–S341.

Mullins, R. and Heddle, R. 2002. Adverse reactions associated with *Echinacea*: the Australian experience. *Ann. Allergy Asthma Immun.* **88**, 42–51.

Nüsslein, B., Kurzmann, M., Bauer, R., and Kreis, W. 2000. Enzymatic degradation of cichoric acid in *Echinacea purpurea* preparations. *J. Nat. Prod.* **63**, 1615–1618.

Osowski, S., Rostock, M., Bartsch, H., and Massing, U. 2000. Zur Pharmazeutischen Vergleichbarkeit von Therapeutisch Verwendeten Echinacea-Praparaten. *Forschende. Komplementearmedizin Klassische Naturheilkunde (Res. Complement. Nat. Classical Med.)* **7**, 294–300.

Perry, N.B., Van Klink, J.W., Burgess, E.J., and Parmenter, G.A. 1997. Alkamide levels in *Echinacea purpurea*: a rapid analytical method revealing differences among roots, rhizomes, stems, leaves and flowers. *Planta Med.* **63**, 58–62.

Perry, N., Van Klink, J.W., Burgess, E.J., and Parmenter, G.A. 2000. Alkamide levels in *Echinacea purpurea*: effects of processing, drying, storage. *Planta Med.* **66**, 54–56.

Perry, N., Burgess, E., and Glennie, V. 2001. *Echinacea* standardization: analytical methods for phenolic compounds and typical levels in medicinal species. *J. Agric. Food Chem.* **49**, 1702–1706.

Pietta, P., Mauri, P., and Bauer, R. 1998a. MEKC analysis of different *Echinacea* species. *Planta Med.* **64**, 649–652.

Pomponio, R., Gotti, R., Hudaib, M., and Cavrini, V. 2002. Analysis of phenolic acids by micellar electrokinetic chromatography: application of *Echinacea purpurea* plant extracts. *J. Chromatogr. A* **945**, 239–247.

Proksch, A. and Wagner, H. 1987. Structural analysis of a 4-*O*-methyl-glucuronoarabinoxylan with immuno-stimulating activity from *Echinacea purpurea*. *Phytochemistry* **26**, 1989–1993.

Raso, G., Pacilio, M., Di-Carlo, G., Esposito, E., Pinto, L., and Meli, R. 2002. *In-vivo* and *in-vitro* anti-inflammatory effect of *Echinacea purpurea* and *Hypericum perforatum*. *J. Pharm. Pharamacol.* **54**, 1379–1383.

Rehman, J., Dillow, J.M., Carter, S.M., Chou, J., Le, B., and Maisel, A.S. 1999. Increased production of antigen-specific immunoglobulins G and M following *in vivo* treatment with the medicinal plants *Echinacea angustifolia* and *Hydrastis canadensis*. *Immunol. Lett.* **68**, 391–395.

Reich, E., Blatter, A., Jorns, R., Kreuter, M., and Thiekotter, K. 2002. An AOAC peer-verified method for identification of *Echinacea* species by HPTLC. *J. Planar Chromatogr.* **15**, 244–251.

Reinke, R., King, P., Victoria, J., McDougall, B., Ma, G., Mao, Y., Reinecke, M., and Robinson, W. 2002. Dicaffeoyltartaric acid analogues inhibit human immunodeficiency virus type 1 (HIV-1) integrase and HIV-1 replication at nontoxic concentrations. *J. Med. Chem.* **45**, 3669–3683.

Reitz, H. 1990. Immunomodulation mit Pflanzlichen Wirkstoffen: Eine Wissenschaftliche Studie am Beispiel Esberitox N. *Notabene Med.* **20**, 362–366.

Rininger, J., Kickner, S., Chigurupati, P., McLean, A., and Franck, Z. 2000. Immunopharmacological activity of *Echinacea* preparations following simulated digestion of murine macrophages and human peripheral blood mononuclear cells. *J. Leukoc. Biol.* **68**, 503–510.

Robinson, W., Reinecke, M., Abdel-Malek, S., Jia, Q., and Chow, S. 1996a. Inhibitors of HIV-1 replication that inhibit HIV integrase. *Proc. Natl Acad. Sci. USA* **93**, 6326–6331.

Robinson, W., Cordeiro, M., Abdel-Malek, S., Jia, Q., Chow, S., Reinecke, M., and Mitchell, W. 1996b. Dicaffeoylquinic acid inhibitors of human immunodeficiency virus integrase: inhibition of the core catalytic domain of human immunodeficiency virus integrase. *Mol. Pharmacol.* **50**, 846–855.

Roesler, J., Emmendörffer, A., Steinmüller, C., Luettig, B., Wagner, H., and Lohmann-Matthes, M. 1991a. Application of purified polysaccharides from cell cultures of the plant *Echinacea purpurea* to test subjects mediates activation of phagocyte system. *Int. J. Immunopharmacol.* **13**, 931–941.

Roesler, J., Steinmüller, C., Kiderlen, A., Emmendörffer, A., Wagner, H., and Lohmann-Matthes, M. 1991b. Application of purified polysaccharides from cell cultures of the plant *Echinacea purpurea* to mice mediates protection against systemic infections with *Listeria monocytogenes* and *Candida albicans*. *Int. J. Immunopharmacol.* **13**, 27–37.

Rogers, K.L., Grice, I.D., Mitchell, C.J., and Griffiths, L.R. 1998. High performance liquid chromatography determined alkamide levels in Australian-grown *Echinacea* spp. *Aust. J. Exp. Agric.* **38**, 403–408.

Schieffer, G. 2000. Validated HPLC method for caffeic-acid derivatives and alkylamides of *Echinacea* solid-dosage forms using a single extraction procedure. *J. Am. Nutraceutical Assoc.* **3**, 67–81.

Schieffer, G. and Kohn, M. 2002. HPLC assay of *Echinacea purpurea*/Goldenseal (*Hydrastis canadensis*) combination formulations for the phenolic acids, alkylamides, alkaloids. *J. Liq. Chromatogr. Rel. Technol.* **25**, 263–274.

Schmidt, U., Albrecht, M., and Schenk, N. 1990. Immunstimulans Senkt Häufigkeit Grippaler Infekt. Plazebokontrollierte Doppelblindstudie mit 646 Studenten der Kölner Universität. *Nat. Und Ganzheitsmedizin* **3**, 277–281.

Schulten, B., Bulitta, M., Ballering-Bühl, B., Köster, U., and Schäfer, M. 2001. Efficay of *Echinacea purpurea* in patients with a common cold. *Arzneim.-Forsch. (Drug Res.)* **51**, 563–568.

Schulthess, B.H., Giger, E., and Baumann, T.W. 1991. *Echinacea*: anatomy, phytochemical pattern, germination of the achene. *Planta Med.* **57**, 384–388.

Schulz, H., Pfeffer, S., Quilitzsch, R., Steuer, B., and Reif, K. 2002. Rapid and non-destructive determination of the echinacoside content in *Echinacea* roots by ATR-IR and NIR spectroscopy. *Planta Med.* **68**, 926–929.

Schumacher, A. and Friedberg, K. 1991. Untersuchungen Zur Wirkung von Echinacea angustifolia auf die Unspezifische Zelluläre Immunantwort der Maus. *Arzneim.-Forsch. (Drug Res.)* **41**, 141–147.

Schöneberger, D. 1992. The influence of immune stimulating effects of pressed juice from *Echinacea purpurea* on the course and severity of colds. *Forum Immunol.* **8**, 2–12.

Schwarz, E., Metzler, J., Diedrich, J., Freudenstein, J., Bode, C., and Bode, J. 2002. Oral administration of freshly expressed juice of *Echinacea purpurea* herbs fail to stimulate the nonspecific immune response in healthy young men: results of a double-blind, placebo-controlled crossover study. *J. Immunother.* **25**, 413–420.

See, D.M., Broumand, N., Sahl, L., and Tilles, J.G. 1997. *In vitro* effects of *Echinacea* and Ginseng on natural killer and antibody-dependent cell cytotoxicity in healthy subjects and chronic fatigue syndrome or acquired immunodeficiency syndrome patients. *Immunopharmacology* **35**, 229–235.

Sloley, B.D., Urichuk, L., Tywin, C., Coutts, R., Pang, P., and Shan, J. 2001. Comparison of chemical components and antioxidant capacity of different *Echinacea* species. *J. Pharm. Pharmacol.* **53**, 849–857.

South, E. and Exon, J. 2001. Multiple immune functions in rats fed *Echinacea* extracts. *Immunopharm. Immunotoxicol.* **23**, 411–421.

Speroni, E., Govoni, P., Guizzardi, S., Renzulli, C., and Guerra, M. 2002. Anti-inflammatory and cicatrizing activity of *Echinacea pallida Nutt.* rot extract. *J. Ethnopharmacol.* **79**, 265–272.

Steinmüller, C., Roesler, J., Grottrup, E., Franke, G., Wagner, H., and Lohmann-Matthes, M.L. 1993. Polysaccharides isolated from plant cell cultures of *Echinacea purpurea* enhance the resistance of immunosuppressed mice against systemic infections with *Candida albicans* and *Listeria monocytogenes*. *Int. J. Immunopharmacol.* **15**, 605–614.

Stotzem, C., Hungerland, U., and Mengs, U. 1992. Influence of *Echinacea purpurea* on the phagocytosis of human granulocytes. *Med. Sci. Res.* **20**, 719–720.

Stuart, D.L. and Wills, R.B.H. 2000a. Alkylamides and cichoric acid levels in plant sections of *Echinacea purpurea* during growth. *J. Herbs Spices Med. Plants* **7**, 91–101.

Stuart, D.L. and Wills, R.B.H. 2000b. Factors affecting the extraction of alkylamides and cichoric acid during ethanolic processing of *Echinacea purpurea* (L.) Moench. *Aust. J. Exp. Agric.* **40**, 873–877.

Sun, L., Currier, N., and Miller, S. 1999. The American Coneflower: a prophylactic role involving nonspecific immunity. *J. Altern. Complement. Med.* **5**, 437–446.

Sun, L., Rezaei, K., Temelli, F., and Ooraikul, B. 2002. Supercritical fluid extraction of alkylamides from *Echinacea angustifolia*. *J. Agric. Food Chem.* **50**, 3947–3953.

Tubaro, A., Tragni, E., Del Negro, P., Galli, C., and Loggia, R. 1987. Anti-inflammatory activity of a polysaccharidic fraction of *Echinacea angustifolia*. *J. Pharm. Pharmacol.* **39**, 567–569.

Turner, R., Riker, D., and Gangemi, J.D. 2000. Ineffectiveness of *Echinacea* for prevention of experimental rhinovirus colds. *Antimicrob. Agents Chemother.* **44**, 1708–1709.

Vömel, T. 1985. Der einflußeines pflanzlichen immunostimulans auf die phagozytose von erythrozyten durch das retikulohistozytäre stystem der isoliert perfundierten rattenleber. *Arzneim.-Forsch (Drug Res.)* **35**, 1437–1439.

Wacker, A. and Hilbig, W. 1978. Virus-inhibition by *Echinacea purpurea* (Author's Transl). *Planta Med.* **33**, 89–102.

Wagner, H. and Jurcic, K. 1991. Immunologic studies of plant combination preparations. *In-vitro* and *in-vivo* studies on the stimulation of phagocytosis. *Arzneim.-Forsch.* **41**, 1072–1076.

Wagner, H., Proksch, A., Riess-Maurer, I., Vollmar, A., Odenthal, S., Stuppner, H., Jurcic, K., Le Turdu, M., and Heur, Y.H. 1984. Immunostimulant action of polysaccharides (heteroglycans) from higher plants. Preliminary communication. *Arzneim.-Forsch.* **34**, 659–661.

Wagner, H., Proksch, A., Riess-Maurer, I., Vollmar, A., Odenthal, S., Stuppner, H., Jurcic, K., Le Turdu, M., and Fang, J. 1985. Immunostimulating action of polysaccharides (heteroglycans) from higher plants. *Arzneim.-Forsch.* **35**, 1069–1075.

Wagner, H., Stuppner, H., Schafer, W., and Zenk, M. 1988. Immunologically active polysaccharides of *Echinacea purpurea* cell cultures. *Phytochemistry* **27**, 119–126.

Wildfeuer, A. and Mayerhoer, D. 1994. *Echinacea Purpurea* und Eleutherococcus senticosus. *Arzneim.-Forsch. (Drug Res.)* **44**, 361–366.

Wills, R.B.H. and Stuart, D.L. 1998. Levels of active constituents in manufactured *Echinacea* products. *Chem. Aust.* **65**, 17–19.

Wills, R.B.H. and Stuart, D.L. 1999. Alkylamide and cichoric acid levels in *Echinacea purpurea* grown in Australia. *Food Chem.* **67**, 385–388.

Wills, R.B.H. and Stuart, D.L. 2000. Effect of handling and storage on alkylamide and cichoric acid levels in *Echinacea purpurea*. *J. Sci. Food Agric.* **80**, 1402–1406.

Wills, R.B.H., Bone, K., and Morgan, M. 2000. Herbal products: active constituents, modes of action and quality control. *Nutr. Res. Rev.* **13**, 47–77.

Wilson, D. 1998. Nutraceuticals: a natural choice for grain-based products. *Cereal Foods World* **43**, 718–719.

Wüstenberg, P., Köhler, G., and Stammwitz, U. 1999. Efficacy and mode of action of an immunomodulator herbal preparation containing *Echinacea*, wild indigo, white cedar. *Adv. Ther.* **16**, 51–70.

BIOACTIVE PEPTIDES AND PROTEINS

ANNE PIHLANTO AND HANNU KORHONEN

MTT Agrifood Research Finland
Food Research
31600 Jokioinen
Finland

I. INTRODUCTION

It is well documented that dietary proteins possess nutritional, functional and biological properties, and that these are often affected by the technological processes used in food manufacture and processing (Korhonen *et al.*, 1998a). Proteins may also be added as functional ingredients to foods to emulsify, to bind water or fat, to form foams or gels, and to alter the flavor, appearance and texture (Anantharaman and Finot, 1993). The role of proteins as physiologically active components in the diet has been

ADVANCES IN FOOD AND NUTRITION RESEARCH VOL 47
ISBN: 0-12-016447-7
DOI: 10.1016/S1043-4526(03)47004-6

increasingly acknowledged in recent years (Tomè and Debabbi, 1998; Walzem *et al.*, 2002). Such proteins or their precursors may occur naturally in raw food materials, exerting their physiological action directly or upon enzymatic hydrolysis *in vitro* or *in vivo*. For example, several dietary proteins can act as a source of biologically active peptides. These peptides are inactive within the sequence of the parent protein, and can be released during gastrointestinal (GI) digestion or food processing. Once liberated, the bioactive peptides may provide different functions *in vitro* or *in vivo*. At present, milk proteins are considered the most important source of bioactive peptides, although other animal as well as plant proteins, especially soybean also contain potential bioactive sequences (Clare and Swaisgood, 2000; Korhonen and Pihlanto-Leppälä, 2001; Meisel, 2001). In addition, it is well documented that a number of amino acids possess specific physiological properties, both beneficial and detrimental; for example, they participate in many biochemical pathways and are precursors of active metabolites. In addition to essential amino acids, the amino acids that are considered physiologically beneficial include arginine, glutamine, histidine, lysine, taurine, tyrosine and tryptophan (Marshall, 1994). The best sources of these amino acids are meat, eggs and dairy products.

This article reviews the current state of knowledge about the biological characteristics of the best known dietary proteins derived from various food sources. Also, the modern techniques available for isolation and enrichment of these proteins and their fractions are discussed, describing the current and potential fields of applications.

II. BIOACTIVE FOOD PROTEINS AND THEIR BIOLOGICAL FUNCTIONS

A. MILK PROTEINS

Normal bovine milk contains about 3.5% of protein, of which casein constitutes 80% and whey proteins 20%. The concentration changes significantly during lactation, especially during the first few days *post partum*, and the greatest change occurs in the whey protein fraction. It is believed that the natural function of milk proteins is to supply young mammals with the essential amino acids required for the development of muscular and other protein-containing tissues as well as with a number of biologically active proteins such as immunoglobulins, vitamin- and metal-binding proteins, and various protein hormones (for reviews see Fox and Flynn, 1992; Pakkanen and Aalto, 1997; Shah, 2000; Walzem *et al.*, 2002).

Bovine casein is further divided into α_{s1}-, α_{s2}-, β- and κ-casein, whereas human casein consists mainly of β-casein and a small fraction of κ-casein. The bovine whey protein fraction contains two main components, α-lactalbumin (α-la) and β-lactoglobulin (β-lg), and several minor proteins. The major whey proteins in human milk are IgA immunoglobulin (Ig), lactoferrin (LF) and α-la, whereas no indigenous β-lg is present (Goldman, 1993). The primary structures of most of the genetic variants of caseins and whey proteins are known. The secondary structures of the whey proteins have also been determined, and those of the caseins have been predicted from spectral studies. Caseins, although less ordered in structure and more flexible than the typical globular whey proteins, have significant amounts of secondary and tertiary structures (Sawyer and Holt, 1993; Swaisgood, 1993; Walstra *et al.*, 1999). A number of review articles and textbooks on the nutritional and biological properties of milk proteins have been published over the past 10 years (Mulvihill and Fox, 1994; Friedman, 1996; Korhonen *et al.*, 1998b; McIntosh *et al.*, 1998; Parodi, 1998; Tomè and Debabbi, 1998; Steijns, 2001). Table I presents a summary of the findings of recent research concerning the composition and main biological functions of the major bovine milk proteins.

1. Caseins

The current technologies used for large-scale fractionation of casein from milk are based on either acidic coagulation at an isoelectric pH of 4.6 or enzymatic hydrolysis of caseins with rennet (chymosin E.C. 3.4.23.4). The chemical compositions of both the casein preparations differ from each other, thus affecting their functional properties (Mulvihill, 1992). According to the present knowledge, caseins have no specific biological activity when occurring in their native form in mammary secretions. Those properties which lead to the formation of micelles by incorporating Ca^{2+} and PO_4^- ions in the mammary secretory cell, however, are physiologically significant. Casein micelles are primarily considered to have nutritional functions as they are good carriers of the above ions and, at the same time, an ample source of amino acids (Swaisgood, 1993; Walstra *et al.*, 1999).

Whole casein and individual casein fractions and their derivatives have been shown to modulate lymphocyte proliferation *in vitro*. Carr *et al.* (1990) found that α_{s1}-casein can enhance the mitogen-stimulated proliferation of murine splenic T-lymphocytes, when induced in *in vitro* cell culture at a concentration of 10^{-6} M. Wong *et al.* (1996b) showed that β-casein significantly enhances the mitogen-induced proliferation of ovine T- and B-lymphocytes in a dose-dependent manner, when added to *in vitro* cell culture. With κ-casein, on the other hand, the opposite effect was found, as κ-casein was suppressive for murine

TABLE I

CONCENTRATION AND SUGGESTED BIOLOGICAL FUNCTIONS OF MAJOR PROTEINS OF BOVINE COLOSTRUM AND MILK

Protein	Concentration (g/l)		Suggested or established biological functions	References
	Colostrum	Milk		
Caseins (α, β and κ)	26	28	Ion carrier (Ca, PO_4, Fe, Zn, Cu), precursor of bioactive peptides, immunomodulation	Walstra *et al.* (1999); Vegarud *et al.* (2000)
β-Lactoglobulin	8.0	3.3	Retinol carrier, binding fatty acids, potential antioxidant, precursor for bioactive peptides	Perez and Calvo (1995); Pihlanto-Leppälä (2001); Walzem *et al.* (2002)
α-Lactalbumin	3.0	1.2	Lactose synthesis in mammary gland, Ca carrier, immunomodulation, anticarcinogenic, precursor for bioactive peptides	Pihlanto-Leppälä (2001); Walzem *et al.* (2002)
Immunoglobulins	20–150	0.5–1.0	Specific immune protection (antibodies and complement system), potential precursor for bioactive peptides	Butler (1994); Korhonen *et al.* (2000a,b)
IgG1	46.4	0.60		
IgG2	2.9	0.06		
IgM	6.8	0.09		
IgA	5.4	0.08		
Glycomacropeptide	NA	1.2	Antimicrobial, antithrombotic, bifido genic, gastric regulation	Abd El-Salam *et al.* (1996); Brody (2000)
Lactoferrin	1.5	0.1	Antimicrobial, antioxidative, anticarcinogenic, anti-inflammatory, immunomodulation, iron transport, cell growth regulation, precursor for bioactive peptides	Schanbacher *et al.* (1998); Steijns and van Hooijdonk (2000); Walzem *et al.* (2002)

TABLE I (*continued*)

CONCENTRATION AND SUGGESTED BIOLOGICAL FUNCTIONS OF MAJOR PROTEINS OF BOVINE COLOSTRUM AND MILK

Protein	Concentration (g/l)		Suggested or established biological functions	References
	Colostrum	Milk		
Lactoperoxidase	0.02	0.03	Antimicrobial, synergistic effect with Igs and LF	Kussendrager and van Hooijdonk (2000)
Lysozyme	0.0004	0.0004	Antimicrobial, synergistic effect with Igs and LF	Shah (2000)
Serum albumin	1.3	0.3	Precursor for bioactive peptides, binding fatty acids	Walstra *et al.* (1999)
Proteose-peptones	NA	1.2	Potential mineral carrier	Walstra *et al.* (1999)
Growth factors			Cell growth stimulation and differentiation, intestinal cell protection and repair, wound healing, regulation of immune system	Pakkanen and Aalto (1997); Schanbacher *et al.* (1998); Xu (1998); Playford *et al.* (2000)
IGF-1	50–2000 μg/l	<10 μg/l		
IGF-2	200–600 μg/l	<10 μg/l		
TGF-β	20–40 mg/l	1–2 mg/l		
EGF	4–8 mg/l	<2 μg/l		
Complement components (C1–C9)			Antimicrobial, anti-inflammatory, pro-inflammatory	Korhonen *et al.* (2000a)

NA, not announced.

and rabbit lymphocyte proliferation induced by a range of T- and B-cell mitogens (Otani and Hata, 1995). The major immunosuppressive effect of κ-casein has been reported to be due to the glycomacropeptide (GMP) component (Otani *et al.*, 1992). Different-sized subfractions of GMP possess different modulatory capabilities, the low carbohydrate-containing fractions showing no suppressive activity whereas others with a higher sugar content being potent suppressants. In particular, those with a high *N*-acetylneuraminic acid content exhibit strong activity against T- but not against B-lymphocytes (Otani *et al.*, 1995). Digestion of GMP with different commercial enzymes affects its modulatory potential. In general, pepsin, chymotrypsin or neuraminidases tend to ablate the immunosuppressive potential, whereas trypsin, pancreatin or pronase have less effect or even enhance the degree of suppression in some enzyme-digested fractions (Otani and Monnai, 1995). Extensive research on κ-casein and its derivatives has demonstrated a wide range of immunomodulatory effects. While intact casein may only modulate the B-lymphocyte function, κ-casein and its subfractions and digestion products have the potential to affect T- or B-lymphocytes, or both, or have no effect. Immunoregulatory proteins and peptides in bovine milk have recently been reviewed by Cross and Gill (2000) and Gill *et al.* (2000).

2. *Whey proteins*

Apart from being a source of nitrogen, whey proteins act as carriers for ligands and trace elements and have various biological functions. Also, the major whey proteins, α-la and β-lg, are known to possess diverse functional properties which can be employed in different applications (Jost, 1993; Mulvihill and Fox, 1994; Kelly and McDonagh, 2000). In normal milk, the concentration of whey proteins ranges from 4 to 7 g/l, as compared to about 28 g of casein. Whey proteins are known to have high nutritional value due to their varied amino acid composition and good digestibility (de Wit, 1998). They are a very heterogeneous group of proteins that remain in the serum or whey after precipitation of the caseins with acid at pH 4.6 or with rennet. In the following, the main characteristics and suggested health effects of the major whey proteins are discussed in more detail.

a. Total whey proteins. Many of the whey proteins are claimed to possess physiological properties, most of which are related to the immune or digestive systems. Evidence from animal and cell culture studies suggests that the whey proteins have anticarcinogenic and immunomodulatory properties (Guimont *et al.*, 1997; McIntosh *et al.*, 1998). A whey protein diet has been shown to significantly inhibit the development of chemically induced colon tumors in mice (Bounous *et al.*, 1989). This finding has later been confirmed

and extended by McIntosh *et al.* (1995), who demonstrated a protective role for whey protein against the development of tumors in the GI tract. They showed that dietary whey protein and casein were more protective against the development of intestinal cancers in rats than was red meat or soybean protein. Cell culture studies have demonstrated that whey protein or whey protein components selectively inhibit cancer cell growth (Parodi, 1998). Whey protein, cultured with the oestrogen-responsive human breast cancer cell line and a prostate cancer cell line, significantly reduces cell growth. Bounous *et al.* (1991) proposed a possible mechanism for the anticancer effect. The authors suggested that the protective efficacy of dietary whey protein concentrate (WPC) could be due to whey proteins enhancing the tissue glutathione concentration, since whey protein is known to be rich in substrates for glutathione synthesis. Moreover, whey, especially β-lg, is a good source of cysteine, which is an essential amino acid for glutathione synthesis. The presence of high levels of glutathione in tissues has been suggested to suppress tumor development at various sites in the body, possibly by reducing free radical and oxidant-induced damage to chromosomal DNA. Furthermore, glutathione transferase enzymes catalyse the conjugation of potentially damaging chemical mutagens and carcinogens, which can then be eliminated from the body (Parodi, 1998). Bounous *et al.* (1989) reported that mice fed on diets containing WPC as a protein source had higher liver and heart tissue levels of glutathione than mice fed on diets containing casein or mouse chow. Similar results have been obtained by McIntosh *et al.* (1995), who found that liver glutathione concentration was highest in WPC- and casein-fed rats and lowest in soybean protein-fed rats. These studies indirectly support the role of whey proteins in enhancing tissue glutathione levels and thus providing a degree of protection against tumor development. In a recent review by Bounous (2000), case reports are presented which suggest an anti-tumor effect of a whey protein dietary supplement in patients suffering from urogenital cancers. In addition, results from a preliminary clinical trial carried out by Bounous *et al.* (1993) suggested that dietary whey proteins may have beneficial effects in human immuno-deficiency virus (HIV) infected patients. In another recent study, (Micke *et al.*, 2001) short-term (14 days) oral supplementation (45 g/day) with whey protein formulas increased plasma glutathione levels of glutathione-deficient patients with advanced HIV-infection. Further clinical studies are warranted to establish the efficacy of whey protein supplementation in the therapy of these fatal diseases.

Milk-derived whole whey proteins, whey protein hydrolysates and individual whey proteins have been shown to modulate lymphocyte functions *in vitro*. Whole whey protein was demonstrated to suppress bovine T-lymphocyte mitogenesis at a concentration of 1.1 ng/ml in cell culture (Torre and Oliver, 1989a,b). Wong *et al.* (1996a, 1998b) observed that bovine

lactoperoxidase (LP) suppressed ovine T-cell mitogenesis *in vitro*, although, similar to LF, it had no measurable effect on B-cell proliferation. In further studies Wong *et al.* (1998a) observed that β-lg significantly increased cell proliferation and the production of IgM in murine spleen cells. Both alkaline treatment and trypsin digestion of the β-lg preparation markedly reduced its effectiveness. Otani and Mizumoto (1998) reported that bovine and human α-la suppressed mitogen-induced proliferative responses of mouse spleen cells stimulated by T- and B-lymphocyte mitogens, and that α-la had cytotoxicity towards mouse spleen cells. It has been further reported that bovine milk IgG, but not serum IgG, inhibits pokeweed mitogen-induced antibody secretion by human peripheral blood mononuclear cells (Kulczycki *et al.*, 1987).

In contrast to the majority of reports which give characterised whey-derived proteins as predominantly immunosuppressive in *in vitro* culture, many *in vivo* studies suggest that whey proteins stimulate lymphocyte function following an *in vivo* exposure. Wong and Watson (1995) reported that mice fed on whey protein-enriched diets had significantly elevated spleen-derived T- and B-cell proliferative responses to mitogens, compared to animals fed on control (soy- or wheat-based) protein diets. According to studies by Debbabi *et al.* (1998), oral delivery of LF enhanced murine antigen-specific lymphocyte responses in both Peyer's patch and spleen cell extracts, indicating that dairy products have the potential to affect local as well as systemic lymphocyte function.

Monnai *et al.* (1998) indicated that dietary GMP has an immunosuppressing activity in mice. They observed enhanced proliferative response to T-lymphocytes, whereas no significant changes were noted for B-lymphocyte responses. These reports provide evidence that bovine whey proteins can modulate lymphocyte function, but there is clearly a need for further studies to establish whether these *in vivo* effects are manifested when the proteins are included in diets.

Furthermore, the antihypertensive effect of whey proteins has been revealed in recent studies. Wu *et al.* (1998) showed that a diet supplemented with whey minerals had a clear antihypertensive effect. The development of hypertension was markedly attenuated and the systolic blood pressure (SBP) was about 50 mmHg lower in spontaneously hypertensive rats (SHR) receiving a diet supplemented with whey than in the control SHR group. Blood pressures in the normotensive Wistar–Kyoto rats remained comparable during the whole study.

b. α-Lactalbumin. α-la is a subunit of lactose synthase, the modifier protein in lactose biosynthesis, and is a calcium-binding metalloprotein. α-la appears to be of major importance from a nutritional point of view, as it is

readily digestible and its amino acid composition meets the amino acid requirements of a newborn baby (Sawyer and Holt, 1993). All biological functions of α-la, have not yet been elucidated, but it has been suggested, that it possesses, e.g., immunomodulatory and anticarcinogenic properties (McIntosh *et al.*, 1998; Parodi, 1998). Studies by Håkansson *et al.* (1995, 1999) that indicate a multimeric α-la from human milk induced apoptosis in tumor cells and immature cells. Apoptosis-inducing activity of α-la was also shown to depend on the three-dimensional structure of the protein, which was different from the native form (Svensson *et al.*, 1999). Moreover, Matin *et al.* (2001) found that bovine, goat and human α-la displayed cytotoxicity towards mouse spleen cells and that the activity was significantly induced when the fractions were treated at acidic pH. Thus, these facts support that α-la achieves cytotoxic ability toward lymphocytes via formation of a multimeric state. Accordingly, it appears that α-la may play an important role in the intestinal tract of suckling mammals by restricting some cell populations unwelcome by mammals.

c. β-Lactoglobulin. The physicochemical and functional characteristics of β-lg are quite well known, but its biological function is not yet clear. Bovine β-lg is included in the lipocalin family and its ability to bind a variety of small hydrophobic molecules, such as retinol, lipids and fatty acids, is well documented. The globular structure of β-lg is stable against the acids and proteolytic enzymes present in the stomach. In this respect, β-lg is more resistant than α-la (Perez and Calvo, 1995). Yoshida *et al.* (1991) reported that bovine milk proteins, including β-lg, bind mutagenic heterocyclic amines, which have an effect on cell proliferation. This binding was higher at pH conditions above 7.4 and was lost at a pH less than 5.5. These interactions have led to the assumption that β-lg may be a transporter of small hydrophobic compounds (Perez and Calvo, 1995). Zsila *et al.* (2002) showed that all *trans*-retinoic acids bind to the hydrophobic internal cavity of β-lg. Below pH 7, retinoic acid starts to dissociate from its binding site, the ligand release is completely reversible upon neutralisation of the solution. This behaviour is explained by the conformational change of β-lg from open to closed conformation in the course of pH lowering. Intestinal receptors specific for β-lg and the protein's resistance to proteolytic activity in the stomach might indicate that it is involved in retinol transport from mother to neonate. However, little endogenous retinol is found bound to β-lg when it is first purified and the ligand most closely associated with the protein is palmitate. However, facilitation of vitamin uptake must remain a possibility. For example, β-lg might bind to a cell-surface receptor in such a way as to enhance the receptor's interaction with retinol (Knotopidis *et al.*, 2002). In addition,

the A and B variants of β-lg have been shown to exhibit different mitogenic activities *in vitro* (Moulti-Mati *et al.*, 1991). The potential immunomodulatory properties of β-lg have been demonstrated by Wong *et al.* (1998a) and discussed previously.

d. Lactoferrin

General. LF is an iron-binding glycoprotein which is closely related to serum transferrin, also occurring in milk but in a lesser amount. All mammals seem to be able to produce these iron-binding proteins in milk, although their concentrations vary between species. LF is synthesised in the mammary gland and in other exocrine glands and, consequently, is found in all external secretions—for example, in saliva, pancreatic fluid, tears, sweat, seminal and synovial fluids, as well as in leukocytes (Masson and Heremans, 1966). Human milk is particularly rich in LF, with concentrations ranging from <1 to 16 g/l in colostrum and being about 1 g/l in mature milk. In bovine colostrum, the LF amount ranges from 0.2 to 5 g/l and decreases to about 0.1 g/l in mature milk. Very high concentrations (up to 50 g/l) of LF are found in secretions of non-lactating human and bovine mammary glands. In mastitic milk, the concentration of LF increases many-fold due to the high amounts of LF being released from activated leukocytes (Bishop *et al.*, 1976; Korhonen, 1977; Hambraeus and Lönnerdal, 1994). The high affinity of LF for iron is a property that is linked to the majority of its proposed biological activities. Many non-iron-related functions, such as immunomodulatory and anticarcinogenic, have been described as well. Apart from its antimicrobial and antioxidative properties, LF is proposed to enhance the bioavailability of iron, stimulate the immune system and modulate the intestinal microflora (Lönnerdahl and Iyer, 1995; Nuijens *et al.*, 1996). In view of these functions, LF is considered to play an important role in the natural non-specific defence system of the body. Thus, based on the above, LF has been applied in infant formulas and has found increasing use in the area of functional foods, sports nutrition and health supplements (Steijns and van Hooijdonk, 2000).

Structure and biochemical properties. LF is composed of a single-chain polypeptide sequence of about 700 amino acids. Shared antigenic determinants have been demonstrated among human, bovine and pig LF. The molecular and spatial structure of human and bovine LF have been characterised in detail and also the genes for human and bovine LF have been cloned and sequenced (Spik *et al.*, 1994a; Baker *et al.*, 1998). The molecular weight (MW) of human LF is between 77,000 and 82,000 Da, depending on the attached carbohydrates, and the molecule contains 16 disulphide bonds. Bovine LF has a MW of about 80,000 Da and it differs from human LF with regard to a few amino acids and the glycan side-chain. The role of the glycans

has not been elucidated, but they may aid in the protection of LF against proteolytic enzymes. Each LF molecule can bind two ferric ions (Fe^{3+}) with the concomitant incorporation of a bicarbonate or carbonate ion. The affinity of LF to bind iron is very high, about 300 times higher than that of transferrin, which has the iron-transporting function in serum. The bound iron is strongly attached to the LF molecule even in an acidic medium of pH 3, but is dissociated in the presence of strong iron scavengers such as a citrate ion (Reiter, 1985). The rate of iron saturation of LF is relatively low in human milk, about 6–8%, as compared to 20–30% of bovine LF. In its natural state, LF has a salmon pink colour whose intensity depends on the degree of iron saturation. Iron-depleted LF with less than 5% saturation is called apo-LF, whereas the iron-saturated form is referred to as holo-LF. In addition to iron, LF also binds other metal ions like copper, cobalt, zinc and manganese (Steijns and van Hooijdonk, 2000). LF is positively charged and strongly binds different polyanions. However, at a low pH, e.g., in the stomach, LF is cleaved by pepsin into several polypeptides, some of which have strong antimicrobial properties. LF has a very high isoelectric point (pI), 9.4 and 9.5 for bovine and human LF, respectively.

Technological properties. The technological properties of bovine LF have been recently reviewed by Steijns and van Hooijdonk (2000). The thermal stability of LF has been mainly studied in model systems using buffered aqueous solutions or when added to milk. According to many experimental studies, the standard pasteurisation regimes (72°C/15 s) used in the dairy industry have practically no effect on the LF structure, antibacterial activity or bacterial interaction. Also, preheating at 70°C for 3 min followed by ultra high temperture (UHT) treatment (130°C/2 s) leads to loss of only 3% in residual iron-binding capacity. UHT treatment, however, abolishes both the ability of iron-saturated LF to bind to bacteria and the bacteriostatic activity of apo-LF (Paulsson *et al.*, 1993). A marginal loss of LF activity has been observed in spray drying of milk. Apo-LF denatures faster than holo-LF in the above heat treatments. LF seems to protect unsaturated fatty acids against oxidation and may contribute to the extension of the shelf-life of iron-enriched and high-fat dairy foods and plant-derived foods (Lindmark-Månsson and Åkesson, 2000; Steijns and van Hooijdonk, 2000).

Biological functions. The potential biological role of LF has been studied extensively over the last 30 years. Originally, the function of LF was considered essentially antimicrobial, but later, this glycoprotein has proven to be far more multifunctional. At present, the major known or speculated *in vivo* activities of LF may be summarised as follows (Hambraeus and Lönnerdal, 1994; Hutchens *et al.*, 1994; Nuijens *et al.*, 1996; Spik *et al.*, 1998; Baveye *et al.*, 1999):

(1) Defence against infections of the mammary gland and the GI tract (antimicrobial activity, modulation of the immune system).
(2) Nutritional effects (bioavailability of iron, source of amino acids).
(3) Mitogenic and trophic activities on the intestinal mucosa.

These activities will be discussed further below:

Antimicrobial effect. The *in vitro* antibacterial, antifungal and antiviral activities of LF are well demonstrated and have been reviewed in many excellent articles (Reiter, 1985; Hutchens *et al.*, 1994; Naidu and Arnold, 1997; Vorland, 1999). LF exerts its antimicrobial effects by various mechanisms, which can be divided into two main patterns:

(a) iron sequestering in order to produce iron deprivation, and
(b) binding of LF or its cleavage products to membrane structures of microbes so as to disrupt the functions and integrity of cell membranes.

The best known mechanism is mediated by binding iron from the environment to apo-LF. Iron is an essential factor for the growth of many microorganisms and an important factor for the virulence of many pathogenic bacteria. Deprivation of iron in the medium by LF has been demonstrated to lead to inhibition of growth of a variety of bacteria and yeasts *in vitro*, e.g., *Escherichia coli*, *Klebsiella*, *Salmonella*, *Proteus*, *Pseudomonas*, *Listeria*, *Bacillus*, *Streptococcus* and *Candida albicans*. The iron deprivation-related bacteriostatic effect is most pronounced with respect to *E. coli*, while some other bacterial strains, e.g., *Streptococcus lactis* and *Lactobacillus casei*, are unaffected by this mode of action. On the other hand, LF may have a direct bacteriostatic or bactericidal effect on Gram-negative bacteria by destabilising their outer membrane, which results in the liberation of lipopolysaccharide, (LPS) (Ellison *et al.*, 1988; Erdei *et al.*, 1994). Enhanced synergistic antibacterial action of LF is achieved in the presence of specific antibodies or lysozyme (LZM) (Spik *et al.*, 1978; Stephens *et al.*, 1980; Rainard, 1986; Ellison, 1994). Some iron-requiring pathogenic microorganisms have receptors for the uptake of LF and may exploit iron (bound in LF) for promoting their growth and pathogenicity. Such microorganisms include, *Helicobacter pylori*, *Neisseria sp.*, *Treponema* and *Shigella* sp. These bacteria may, thus, benefit from the inflammatory reaction of the host.

LF has been demonstrated to inhibit *in vitro* the multiplication of different viruses, such as human cytomegalovirus, HIV, herpes simplex viruses 1 and 2, influenza virus, human hepatitis C virus and human poliovirus type 1 (Vorland, 1999). Also, LF has been shown to prevent rotavirus infection in the human enterocyte-like cell-line HT-29 (Superti *et al.*, 1997). It is speculated that LF prevents the binding of viruses to the host cells by

interaction with cell-surface glycosaminoglycans and low-density lipoprotein receptors, which act as binding sites for some enveloped viruses.

Partial hydrolysis of the LF molecule by heat as well as by pepsin results in the formation of an antibacterial peptide referred to as lactoferricin, which exerts a much stronger antimicrobial effect than the intact molecule (Section III.A.5.).

There is an increasing evidence suggesting that innate LF is actively involved in the prevention of certain microbial infections *in vivo*. Also, orally administered bovine LF may be beneficial in the prevention and treatment of various microbial infections in humans and farm animals. As reviewed by Reiter (1985), LF has been shown to prevent udder infections in cows during the non-lactating period. Furthermore, LF-supplemented feeds have proven beneficial in lowering the incidence of scouring in newborn calves and piglets. Promising results have also been obtained in many animal model studies. Feeding of bovine LF or lactoferricin to pathogen-free mice has been found to be effective in suppressing the growth of various intestinal bacteria, e.g., clostridia, and bacterial translocation of *E. coli* from the intestine into other organs (Teraguchi *et al.*, 1995a,b). Zagulski *et al.* (1989) have shown that the feeding of relatively small amounts of LF can protect mice against a lethal dose of *E. coli* in an experimental infection.

Recently, Wada *et al.* (1999) have demonstrated that a daily oral dose of 10 mg of bovine LF for 3–4 weeks to *H. pylori*-infected mice significantly decreased the number of this bacterium colonising in the stomach. The authors suggested that the glycans present in LF may bind to the bacterial adhesins, thus interfering with the attachment of *H. pylori* to the epithelial cells. These findings are supported by another mouse model study (Dial *et al.*, 1998), which showed that oral administration of 4 mg of bovine LF per day for 3 weeks reduces gastric urease activity and *H. pylori* colonisation in the stomach.

The potential contribution of the ingested LF against microbial infections in humans still remains to be proven, although it was first suggested about 30 years ago by Bullen *et al.* (1972). Bottle-feeding of human infants with LF-supplemented infant formulas has been shown to reduce the number of coliform bacteria marginally and to increase the number of bifidobacteria in faeces similarly (Roberts *et al.*, 1992). There is direct evidence for the generation of lactoferricin in the human stomach after ingestion of bovine LF (Kuwata *et al.*, 1998). The significance of this finding remains to be elucidated further.

Modulation of the immune system and inflammatory response. There is solid evidence that LF modulates host defence systems by acting through multiple mechanisms, for example, by modulating the immune system and inflammatory responses (Spik *et al.*, 1994b; Baveye *et al.*, 1999;

Conneely, 2001). LF has the ability to bind to the surface of several types of immune cells, which suggests that it can modulate immune functions. Both stimulatory and inhibitory effects of LF on lymphocyte proliferation have been described in the literature. LF has been reported to induce *in vitro* maturation of T- and B-lymphocytes, to modulate the activity of natural killer cells and to enhance the phagocytic activity of neutrophils. In mice, bovine LF has been shown to induce both mucosal and systemic immune responses (Debbabi *et al.*, 1998). Cell-culture studies have demonstrated that LF and peptides derived from LF influence the production of various cytokines which regulate the immune and inflammatory responses of the body (Crouch *et al.*, 1992; Shinoda *et al.*, 1996).

The inflammatory response appears to be modulated by LF through a variety of mechanisms which still remains unclear. Baveye *et al.* (1999) and Vorland (1999) have reviewed recent studies on the subject, which will be described briefly in the following.

LF has been found to suppress the inflammatory response to bacterial endotoxin by binding bacterial LPS and preventing its interaction with and activation of leukocytes. LF also seems to be involved in reducing the formation of free oxygen radicals during inflammatory processes. In particular, LF appears to prevent peroxidation of cell-membrane lipids. Further, LF increases the cytotoxicity of natural killer cells *in vitro*, but the mode of action is not known. Human LF inhibits complement-mediated lysis of antibody-coated red blood cells and has been reported to have an anti-coagulant effect *in vitro*. The possible *in vivo* importance of these reactions, however, is not known. Moreover, it has been shown that LF is transported into the cell nucleus where it can bind DNA, suggesting that LF may regulate the phenotypic traits of the host.

Recent studies further suggest that LF may have a role in the development and progression of tumors (Tsuda *et al.*, 2002). Orally administered bovine LF has been found to inhibit the development of tumors in the colon, oesophagus and lung carcinogenesis in a rat or mouse model, but the mode of action remains to be resolved (Sekine *et al.*, 1997; Ushida *et al.*, 1999; Kuhara *et al.*, 2000). The anti-tumor activity may be mediated by the enhanced cytokine production or the activating effect on natural killer cells and be independent of the iron-saturation level.

It has also been suggested that LF exerts mitogenic and trophic effects in the GI tract (Hambraeus and Lönnerdal, 1994). The potential regulatory role of LF in the intestinal maturation of the infant, however, warrants further research, as the observed effects may have been of non-specific character.

Nutritional significance. As one of the major whey proteins in human milk and also relatively abundant in bovine colostrum, LF is of interest as a dietary source of amino acids as well as for the bioavailability of iron. LF has an

amino acid composition that indicates a high nutritional value, but this is perhaps not its main role, since it is known to be quite resistant to digestive enzymes (Spik *et al.*, 1994a). Although the amount of intact LF found in faeces constitutes only about 10% of the amount ingested, this is thought to be supportive of a physiological role for LF in the gut of the infant. LF, may thus exert its biological functions in an undigested and partially digested form in the GI tract. In infants and adults with a pepsin-secreting stomach, gastric digestion of lacteal LF probably releases antimicrobial peptides. These peptides may also be capable of binding to lymphocyte receptors, suggesting effects on the balance of microbial flora and regulation of the intestinal mucosal immune system. So far, clinical evidence to substantiate such functions is lacking. In addition, the role of LF in the absorption of iron from milk remains controversial. The high bioavailability of iron from human milk and the discovery of LF receptors on intestinal brush border membranes of many species provide a basis for the assumption that LF promotes iron absorption in breast-fed infants. However, animal model studies with human LF and bovine LF as well as clinical studies in infants fed a formula supplemented with bovine LF have failed to show any significant improvement in iron absorption (Lönnerdahl and Iyer, 1995). It has been suggested that the absorption capacity is affected by the highly species-specific receptors of LF, which may have an impact on the uptake of iron by endocytosis. Again, further research is needed on this specific issue.

Commercial applications. Over the last decade, bovine LF has been commercialised in various applications, e.g., in milk-based infant formulas, health supplements, functional foods and drinks, cosmetics, oral care products, chewing gums and feed supplements (Steijns, 2001). Such products are targeted at optimal iron delivery, mimicking human breast milk or boosting natural defense systems against infections. Also, LF could be exploited as a natural antioxidant due to its strong ability to bind iron, which is an important catalyst for free radical formation inside the cells.

(e). Lactoperoxidase. Lactoperoxidase (LP; EC1.11.1.7) is found in the mammary, salivary and lachrymal glands of mammals and in their respective secretions, e.g., in milk, saliva and tears. The biological significance of LP is related to the natural host defence system against invading microorganisms. In bovine lacteal secretions, LP is one of the indigenous antimicrobial agents. In the presence of H_2O_2, LP catalyses the oxidation of thiocyanate anions (SCN^-) and certain halides, and produces intermediate products with antimicrobial properties. This mechanism is generally referred to as the LP-system. The physicochemical properties of LP were recently reviewed by Kussendrager and van Hooijdonk (2000). Bovine LP consists of a single polypeptide chain containing 612 amino acid residues. Its amino acid

sequence is known and the MW is approximately 78,000 Da. LP is a basic protein having a high isoelectric point of 9.6. Bovine LP has been found resistant *in vitro* to acidity as low as pH 3 and to human gastric juice. Also, it is relatively heat-resistant and is only partially inactivated by short-time pasteurisation at 74°C. LP is, next to xanthine oxidase, the most abundant enzyme in bovine milk and colostrum, with concentrations ranging between 13–30 and 11–45 mg/ml, respectively (Korhonen, 1977; Carmen *et al.*, 1990; de Wit and van Hooijdonk, 1996).

The thiocyanate anion is widely distributed in animal tissues and secretions. Its concentrations in bovine serum and milk depend on the feeding regime of the animal. Plants belonging to the genus *Brassica*, e.g., cabbage, are particularly rich in SCN precursors (Reiter and Perraudin, 1991). Hydrogen peroxide may be generated in milk endogenously, e.g., by polymorphonuclear leukocytes in the process of phagocytosis (Korhonen and Reiter, 1983). Under aerobic conditions many lactobacilli, lactococci and streptococci may produce sufficient H_2O_2 to activate the LP system. H_2O_2 can also be provided by addition to the system in an aqueous or bound form, e.g., as sodium percarbonate or magnesium peroxide. Also, H_2O_2-producing enzymatic systems such as glucose/glucose oxidase and hypoxanthine/xanthine oxidase have proven effective means of generating H_2O_2 for the LP system (Reiter and Perraudin, 1991).

The mechanism of action of the LP-system has been a subject of intensive study ever since the discovery of the antibacterial activity of LP in the 1960s (Reiter and Oram, 1967). This complex mechanism is now fairly well characterised and has been reviewed in many articles (Reiter and Härnulv, 1984; Reiter, 1985; de Wit and van Hooijdonk, 1996; Kussendrager and van Hooijdonk, 2000). The short-lived oxidation products of SCN, which are responsible for the antimicrobial activity, have been identified as hypothiocyanate anions (OSCN) and hypothiocyanous acid (HOSCN). The antibacterial effect of the LP system is proportional to the SCN concentration present. The maximal effect is obtained at an equimolar concentration of SCN and H_2O_2. There is always enough of LP in raw milk to activate the system, while SCN and H_2O_2 are the limiting factors. For commercial applications, these compounds are provided in standardised portions so as to achieve optimal functioning conditions for the LP system. The antimicrobial action of the system is based on the oxidation of sulphydryl (SH) groups of microbial enzymes and other proteins in the cytoplasmatic membrane of sensitive organisms. As a result of structural damage of the membrane, potassium ions, amino acids and peptides are leaked into the medium, subsequently inhibiting the uptake of glucose and amino acids in the cell and impairing the synthesis of proteins, DNA and RNA (Reiter and Perraudin, 1991).

The antimicrobial activity of the LP-system has been established against a wide range of bacteria, viruses, yeasts and moulds (Korhonen, 1980; Reiter and Härnulv, 1984; Wolfson and Sumner, 1993; Stadhouders and Beumer, 1994). Gram-negative, catalase-positive bacteria, such as *Pseudomonas*, *Salmonella*, *Shigella* and coliform bacteria, are not only inhibited by the LP-system but, depending on the medium conditions, may be killed. Gram-positive, catalase-negative bacteria, such as *Streptococcus* and *Lactobacillus*, are generally inhibited but not killed by the activated LP-system. The difference in sensitivity can probably be explained by the differences in the cell-wall structure and barrier properties of these bacteria.

Since the 1970s, various industries have investigated the possibility of utilising the LP-system as a natural antimicrobial agent in a great number of diverse products. Reports on such research have been reviewed by Stadhouders and Beumer (1994) and de Wit and Hooijdonk (1996). Most of the applications concern the preservation of different foodstuffs, mainly dairy products, but raw fish and meat products have also been a subject of applied research. The efficiency of the LP-system in extending the shelf-life of raw milk is well established (Reiter *et al.*, 1976; Björck, 1978) and its applicability confirmed under practical conditions in field tests (Korhonen, 1980; Stadhouders and Beumer, 1994). In 1991, the Codex Alimentarius Committee authorised the use of the LP system as a temporary means of preserving raw milk when milk cannot be properly refrigerated. Since then, the FAO has carried out extensive field tests and introduced a technology transfer programme for the application of the LP-system for raw milk preservation in the developing countries (Lambert, 2001). Other areas of application with commercialised products include feedstuffs, oral-care products and cosmetics. These applications have been reviewed by van Hooijdonk *et al.* (2000).

f. Glycomacropeptide. The biological activity of bovine κ-casein GMP has received much attention in recent years. GMP, often also termed as caseinomacropeptide, is the C-terminal hydrophilic peptide released by the action of chymosin on κ-casein. This casein fraction is hydrolysed into *para*-κ-casein (residues 1–105), which remains with the curd, and GMP (residues 106–169), which is removed with the whey (van Hooijdonk *et al.*, 1984). GMP is, therefore, normally found in significant quantities (10–20% of total protein content) in the whey of rennet-coagulated cheese (Abd El-Salam *et al.*, 1996). Several large-scale methods have been developed for the isolation of GMP from cheese whey (Section IV.A). GMP contains no aromatic amino acids and, thus, is not visible at 280 nm, the common protein detection wavelength. The amino acid sequences of both κ-casein and GMP have been well defined (Fiat and Jolles, 1989). The basic GMP molecule has a MW of

8000 Da, but this varies according to the attached oligosaccharide content. GMP also contains sialic acid, which makes this molecule highly interesting biologically, as sialic acid is considered to play an important role in brain development. Sialic acid is a vital component of brain gangliosides, which play an essential role in the transmission and storage of information in the brain. Human and bovine GMP differ from each other with regard to their oligosaccharide content (Brody, 2000).

There are a number of physiological functions attributed to GMP, including:

(a) binding of cholera and *E. coli* enterotoxins,
(b) inhibition of bacterial and viral adhesion,
(c) suppression of gastric secretions,
(d) promotion of bifidobacterial growth, and
(e) modulation of immune system responses.

These potential activities are discussed in more detail in the following, based on excellent literature reviews by Abd El-Salam *et al.* (1996) and Brody (2000). GMP was noticed to be released in the stomach during milk protein digestion. Ledoux *et al.* (1999) showed that GMP appears in the jejunal effluents within the first 20 min after meal ingestion at a level varying from meal to meal. GMP release was observed in the stomach of healthy adults during milk and yoghurt digestion. Furthermore, short peptides derived from GMP digestion were also released and GMP was present for 8 h in the plasma of young children after milk or yoghurt ingestion (Chabance *et al.*, 1998). These results suggest that GMP may have physiological activity in humans, particularly in the digestion process. An interesting biological feature of GMP is that it is capable of binding enterotoxins, such as cholera toxin (Kawasaki *et al.*, 1992) and heat-labile enterotoxins LT-I and LT-II of *E. coli* (Isoda *et al.*, 1990). The latter researchers observed that oral administration of 1 mg per day of GMP could protect mice against diarrhoea caused by the toxins. These promising results have been reported in a published patent by Isoda *et al.* (1990) but they warrant further confirmation.

GMP has been implied to inhibit the adhesion of various bacteria and viruses to the intestinal epithelium or other biological surfaces. Neeser *et al.* (1994) demonstrated that GMP prevents the binding of cariogenic bacteria like *Streptococcus sobrinus* and *S. sanguis*, but not *Actinomyces viscous*, on saliva-coated hydroxyapatite beads. It has been further suggested (Schupbach *et al.*, 1996) that GMP reduces dental caries by changing the microbial flora of dental plaque from streptococci to less cariogenic *Actinomyces*. Kawasaki *et al.* (1993) observed that GMP, even in amounts as small as 80 ppm, inhibits hemagglutination by four strains of human influenza virus. Obviously, further

research is needed to establish the antimicrobial properties of bovine GMP before it can be considered as an effective ingredient for antimicrobial formulations.

Contradictory results have been reported about the potential physiological functions of GMP in the GI tract. Guilloteau *et al.* (1994) demonstrated that feeding GMP to preruminant calves resulted in a temporary inhibition of gastric secretion. Using a rat model, Beucher *et al.* (1994) found that feeding one GMP fraction stimulated the intestinal hormone cholecystokinin, which influences food intake. They suggested that glycosylation might affect the digestive function of GMP. According to studies by Yvon *et al.* (1994), both the peptide chain and the carbohydrate structure are important for stimulating gastric secretions. These studies also indicate that GMP acts by triggering receptors on the intestinal mucosa. By fixing directly on the luminal receptors, GMP could favor the release of cholecystokinin. In the same way, it could have a direct influence on other endocrine cells of the digestive tract like D- and G-cells, producing somatostatin and gastrin, respectively. A recent study by Pedersen *et al.* (2000) demonstrated that intraduodenal administration of enriched-GMP isolate stimulates exocrine pancreatic secretion in anaesthetized rats and it is likely due to the specific activity on cholecystokinin release from cells. In addition, Froetschel *et al.* (2001) observed that satiety associated with premeal loads of casein is related to changes in GI function of meal-fed animals and involves both opioid and cholecystokinin regulation. In contrast to animal model studies, the results of a recent human intervention study by Gustafson *et al.* (2001) showed that the daily oral intake of a beverage containing 20 g of GMP had no effect on the energy intake or weight of the adult subjects. Further research is, therefore, needed to establish the speculated effect of GMP as an appetite-controlling substance (Clare, 1998).

The role of GMP in stimulating the growth of bifidobacteria appears to be quite complex. According to Brody (2000), the currently available research data do not favor bovine GMP as a specific bifidus growth promoter.

The potential modulation of immune system responses by GMP is also discussed in the article by Brody (2000). A number of studies have shown that GMP stimulates the proliferation of normal human B-lymphocytes, but not of T-lymphocytes. This would indicate that GMP upregulates the humoral immune system with a subsequent increase in the production of IgA antibodies, in particular. Moreover, GMP appears to specifically affect the production of various cytokines. It is clear that further research is required in this field so as to elucidate the significance of GMP for the immune system.

The potential nutritional and health benefits of GMP have been advocated in a few articles (Steijns, 1996; Clare, 1998; LaBell, 1998). A number of patents related to the preparation and use of GMP to promote health or

nutrition have been granted, but no commercial breakthrough products based on GMP have been launched in the market, so far.

g. Immunoglobulins

Structure and functions. Immunoglobulins (Igs), also referred to as antibodies, are present in the milk and colostrum of all lactating species. In mammals, five major classes of Igs have been characterised: IgG, IgM, IgA, IgD and IgE. The basic structure of all immunoglobulins is similar. They are composed of two identical light chains (MW of each around 23 kDa) and two y-shaped identical heavy chains (MW of each 53 kDa). There are two types of light chains (κ and λ), differing in chain structure but having homologous amino acid sequences. The light chains contain a constant region (C_L) and variable region (V_L). The V_L-region determines the immunological specificity. The light chains are attached to the heavy chains by a disulphide bond and the two heavy chains are held together by disulphide bonds near a hinge region. The two identical antigen-binding sites (referred to as the F(ab)2 fragment) are formed by the N-terminal part of one heavy chain and the variable region of one light chain. The C-terminal end of the heavy chains is referred to as the Fc fragment. The complete Ig molecule has a MW that varies around 160 kDa. Monomeric IgM and IgA have a similar basic structure to IgG except for the addition of a C-terminal octapeptide to the heavy chains. IgA occurs as a monomer or dimer, the latter comprising two IgA molecules joined together by a J-chain and a secretory component. This complex is called secretory IgA (SIgA) and has a MW of about 380 kDa. IgM consists of five subunits, similar to monomeric IgA, which are linked together in a circular mode by disulphide bonds and a J-chain; the MW of pentameric IgM is approximately 900 kDa (Larson, 1992; Butler, 1994; Telemo and Hanson, 1996).

In addition to antigen-binding, all Igs exhibit one or more effector functions. While the F(ab)2 fragment binds to antigen, the other parts, mainly the Fc region, interact with other elements. The effector functions include binding of some Ig classes to leukocytes or to host tissues or to complement protein C1q. IgG binds specifically to bacteria and in this way augments the recognition and phagocytosis of bacteria by leukocytes. This process is generally referred to as opsonisation. Another important function of IgG is the activation of the complement-mediated bacteriolytic reactions, which contribute to the immune defence of the body. Igs also prevent the adhesion of microbes to surfaces, including intestinal epithelial linings, inhibit bacterial metabolism, agglutinate bacteria, and neutralise toxins and viruses. IgM antibodies, although produced in smaller amounts than IgG, are considerably more efficient than IgG with regard to most of the above activities, especially complement-mediated lysis. Bovine IgA, in contrast, does not fix complement or opsonise bacteria, but agglutinates antigens and

neutralises viruses and bacterial toxins. The main function of SIgA is to bind bacteria, preventing them from attaching to mucosal epithelial cells, which is an important first step in the initiation of most infections. The milk Igs have proven to exert a synergistic effect on the activity of non-specific antimicrobial factors, such as LF and LZM as well as LP (Butler, 1994; Korhonen, 1998; Korhonen *et al.*, 2000a).

Concentration in milk and colostrum. The concentration of different Ig classes in milk and colostrum varies considerably according to species, breed, age, stage of lactation, and health status, and is often different from that in blood. In human milk and colostrum, the IgA class comprises about 90% and in blood 15–20% of total Ig, whereas the IgG class is dominant in bovine milk, colostrum and blood (about 60–70% of total Ig).

In colostrum Igs make up 70–80% of the total protein content, whereas in milk they account for only 1–2% of the protein. The main change from colostrum to normal milk occurs in the first few milkings after parturition and continues at reduced rates for approximately 5–7 days. At the first milking *post partum*, the IgG concentration ranges from 15 to 180 g/l, the mean being approximately 60 g/l. Thereafter, the IgG concentration falls sharply to about 1 g/l at the 12th–14th milkings. Two IgG subclasses have been characterised in bovine milk, IgG_1 and IgG_2, of which IgG_1 accounts for about 50–80% of total Ig in the lacteal secretions. The average concentrations of IgG_2, IgM and IgA are relatively small as compared to IgG_1. In serum, both IgG subclasses are present at about equal concentrations (IgG_1 11.2 g/l, IgG_2 9.2 g/l), while IgA and IgM occur at concentrations of about 0.4 and 3.1 g/l, respectively. The transport of Igs from serum to milk is a selective process favouring homologous IgG in most species. Specific receptors are involved in the process enabling the characteristic concentration of Ig isotypes in milk and colostrum of different species (Larson, 1992; Levieux and Ollier, 1999).

Biological importance. It is generally accepted that the primary biological functions of Igs in the lacteal secretions are to give the offspring immunological protection against microbial pathogens and toxins and to protect the mammary gland against infections. Bovine colostral and milk antibodies represent the cow's immune response against a variety of microorganisms present in the cow's environment and feed. Thus, Igs contribute to the natural antimicrobial properties of milk. The bacteriostatic and bactericidal activity of bovine colostrum and milk against a great number of pathogenic and non-pathogenic microorganisms is well documented, and is attributed to specific antibodies in addition to other antimicrobial factors (Reiter, 1985; IDF, 1991; Korhonen *et al.*, 2000b; Korhonen, 2001). Newborn calves and pigs, which do not receive colostrum show a high mortality and poor weight gain during the first weeks of life. In many species, the absorption of Igs from the intestine is selective and receptor-mediated. In humans,

practically no absorption of Igs takes place, whereas in ruminants the absorption of Igs is non-selective during the first 12–36 h after birth of offspring. In contrast to human neonate, the ruminant offspring is born virtually without Igs, and the colostral Igs are, therefore, considered essential for survival. It is recommended that a newborn calf should be given a minimum of 2 l of first colostrum, equivalent to about 70–100 g of Ig, to protect the calf against scouring (Quigley and Drewry, 1998).

In human colostrum, SIgA is the predominant Ig class, and IgG is transported via the placenta to the circulation of the embryo. Many studies suggest that the Igs of human colostrum reduce the risk of GI infections of an infant (for reviews see Goldman, 1993; Telemo and Hanson, 1996; Lilius and Marnila, 2001). The sites of the immune protection are mainly restricted to the GI tract because milk Igs apparently are not absorbed in significant quantities from the infant gut. Secretory IgA antibodies appear to be particularly important during the first days *post partum*, when the infant's own mucosal IgA production is deficient. Also, milk IgA may contribute to the prevention of food allergies by blocking the passage of antigens through the GI surfaces of the infant (Telemo and Hanson, 1996).

Antibodies ingested by humans are normally degraded by proteases in the stomach and intestine into small peptides and amino acids, which are subsequently absorbed. SIgA is more resistant to proteolytic digestive enzymes in the GI tract than other Igs due to the secretory piece. SIgA (20–80%) present in human colostrum passes undegraded through the gut of the human infant (Goldman, 1993). Also, bovine milk Igs, which have been subjected to proteolytic conditions of the human intestine retain their immunological activity partially. Bovine colostral Igs are quite resistant to gastric acids but are degraded by proteases and are rather sensitive to trypsin, except for IgG_1, which is relatively resistant. Various studies have shown that 10–30% of orally administered bovine Igs can be recovered intact or immunologically active from the stool of human infants and adults (Roos *et al.*, 1995; Kelly *et al.*, 1997).

Technological properties. Milk Igs may have an adverse effect on various dairy processes. For example, the fermentation process may be disturbed by the antimicrobial properties of Igs. Retarded fermentation by dairy starters is noted in colostrum and mastitic milk, which contain increased amounts of Igs. Also, high Ig concentrations may adversely affect antibiotic residual tests based on microbial growth, causing false positive results. Immunoglobulins contribute to cream formation by agglutinating fat globules, a process which accelerates the ascent of cream to the surface. The agglutination property of Ig is, however, inactivated by pasteurisation and mechanical agitation (IDF, 1991).

The effects of processing and storage conditions on the stability of purified Ig or Ig concentrates have been the subject of many recent studies. Thermal treatment influences the stability of Ig activity in colostrum or milk during processing (Li-Chan *et al.*, 1995; Dominguez *et al.*, 1997; Mainer *et al.*, 1999). In ordinary high-temperature short-time (HTST, 72°C/15 s) or batch pasteurisation (63°C/30 min), only 0.5–10% of Ig activity is lost, whereas ultra-high temperature (UHT) treatment (138°C/4 s) and evaporation processing destroy the majority of the specific immune activity of milk (Li-Chan *et al.*, 1995). However, bovine IgG added to UHT milk has been shown to retain its specific immune activity for over 6 months (Fukumoto *et al.*, 1994a).

Commercial utilisation. Recent progress in the modern fractionation technologies, e.g., membrane separation techniques, has enabled large-scale isolation of Igs from bovine colostrum and milk for commercial purposes (Scammell, 2001). Subsequently, Ig concentrates derived from colostrum, cheese whey or blood serum have been developed and launched on the market for neonatal calf, lamb or piglet feeding. The efficacy of such colostrum replacers or supplements has been shown to vary, but those based on native colostral Igs have proved beneficial to the health of newborn calves (Nousiainen *et al.*, 1994; Mee and Mehra, 1995). The efficacy of colostral supplements can be improved by immunising cows with specific antigens derived from pathogenic microbes. Systemic immunisation of pregnant cows during the dry period produces colostrum with high concentrations of specific antibodies against the vaccine used (Saif *et al.*, 1984; Korhonen *et al.*, 1995). These antibodies can be enriched in an active form from colostrum by membrane separation and chromatographic techniques to make specific Ig concentrates. Such immune milk preparations have been found to be effective in the prevention or treatment of various enteric diseases of calves or piglets caused, e.g., by rotavirus (Schaller *et al.*, 1992), enterotoxigenic *E. coli* (Moon and Bunn, 1993) or *Cryptosporidium parvum* (Perryman *et al.*, 1999).

Apart from animal studies, a large number of clinical studies have been carried out since the 1970s to demonstrate the efficacy of immune milk preparations in the prophylaxis or therapy of human GI diseases. These studies have been reviewed in several articles (Facon *et al.*, 1993; Hammarström *et al.*, 1994; Ruiz, 1994; Davidson, 1996; Weiner *et al.*, 1999; Korhonen *et al.*, 2000b; Lilius and Marnila, 2001). Examples of immune milk trials carried out in humans are described in Table II. Clinical evidence obtained in most of these studies indicates that immune milk preparations are protective and, to some extent, also therapeutic against rotavirus infections in children (Ebina *et al.*, 1985; Davidson *et al.*, 1989; Mitra *et al.*, 1995; Sarker *et al.*, 1998). A protective or therapeutic effect of immune milk has also been demonstrated in humans against

TABLE II

EFFICACY OF BOVINE IMMUNE COLOSTRUM OR MILK AGAINST MICROBIAL INFECTIONS IN HUMANS

Microorganism used in immunization	Target disease	Treatment dose/period	Clinical effect	References
Escherichia coli	Diarrhoea	1 g Ig/kg BW/day for 10 days	Reduced symptoms and number of *E. coli* in feces of infected children	Mietens *et al.* (1979)
E. coli	Diarrhoea	5 g cw/day for 7 days	Prevented infection in adults after experimental challenge	Tacket *et al.* (1988)
E. coli ETEC colonization factor	Diarrhoea	3 doses[a] mw/day for 7 days	Prevented diarrhoea in adults after experimental challenge	Freedman *et al.* (1998)
E. coli (ETEC and EPEC strains)	Diarrhoea	20 g mw/day for 4 days	No reduction of symptoms and duration of diarrhoea in infected children	Casswall *et al.* (2000)
Helicobacter pylori	Gastritis	12 g cw/day for 21 days	Reduced chronic inflammation and number of *H. pylori* in gastric antrum of infected children	Oona *et al.* (1997)
H. pylori	Gastritis	1 g cw/day for 30 days	No eradication of infection in infants	Casswall *et al.* (1998)
Shigella flexneri	Dysenteria	30 g cw/day for 7 days	Prevented infection in adults after experimental challenge	Tacket *et al.* (1992)
Streptococcus mutans	Dental caries	4 g mw/day for 14 days	Reduced number of *S. mutans* in dental plaque of adults	Filler *et al.* (1991)

TABLE II (*continued*)

EFFICACY OF BOVINE IMMUNE COLOSTRUM OR MILK AGAINST MICROBIAL INFECTIONS IN HUMANS

Microorganism used in immunization	Target disease	Treatment dose/period	Clinical effect	References
S. mutans	Dental caries	3 rinses/day with 5% solution for 3 days	Reduced acidogenicity and number of *S. mutans* in dental plaque	Loimaranta *et al.* (1999)
Cryptosporidium parvum	Cryptosporidiosis	200–500 ml c/day for 10–21 days	Reduced or ceased diarrhoea	Tzipori *et al.* (1987)
C. parvum	Cryptosporidiosis	30 g cw/day for 5 days	Reduced diarrhoea and oocyst exretion in adults after experimental challenge	Okhuysen *et al.* (1998)
Rotavirus	Diarrhoea	20–50 ml c/day for 3 days	Prevented infection in healthy children	Ebina *et al.* (1985)
Rotavirus	Diarrhoea	50 ml c/day for 1 day	Prevented infection in healthy children	Davidson *et al.* (1989)
Rotavirus	Diarrhoea	300 ml c/day for 3 days	Shortened duration of diarrhoea in infected children	Mitra *et al.* (1995)
Rotavirus	Diarrhoea	10 g cw/day for 4 days	Shortened duration and decreased severity of diarrhoea in infected children	Sarker *et al.* (1998)

Ig, immunoglobulin; cw, colostral whey concentrate; mw, milk whey concentrate; c, colostrum; BW, body weight

[a]Size not indicated.

enteropathogenic or enterotoxigenic *E. coli* infections (Tacket *et al.*, 1988; Freedman *et al.*, 1998) and *Shigella flexneri* (Tacket *et al.*, 1992). Further, encouraging results have also been reported in the treatment of HIV-infected patients with immune bovine colostrum containing specific antibodies to *Cryptosporidium parvum* (Okhuysen *et al.*, 1998). This parasite often causes infections in individuals who are immunosuppressed due to HIV infection. Promising results were recorded in a clinical trial where children infected with *H. pylori* were treated with an immune milk preparation containing specific anti-*H. pylori* antibodies (Oona *et al.*, 1997). Bovine antibodies may also provide protection against dental caries caused by cariogenic streptococci. It has been shown that a colostrum-based immune milk concentrate has significant antimetabolic potential against mutans streptococci (Loimaranta *et al.*, 1997), actively inhibits *in vitro* the adherence of these bacteria to hydroxyapathite (Loimaranta *et al.*, 1998a) and supports the natural antimicrobial systems present in saliva (Loimaranta *et al.*, 1998b, 1999a). So far, only a couple of clinical human trials have been reported on the application of anti-caries immune milk preparations (Filler *et al.*, 1991; Loimaranta *et al.*, 1999b). The results obtained in those studies, however, were encouraging.

Although most of the controlled clinical trials with immune milk preparations have yielded good results, some of the field studies have failed to demonstrate the beneficial efficacy of such preparations in the prevention of diarrhoea in infants (Brunser *et al.*, 1992) as well as in the treatment of *H. pylori* infections (Casswall *et al.*, 1998) and *E. coli*-induced diarrhoea in children (Casswall *et al.*, 2000). These studies concluded that the daily dosage of immune milk used was probably not adequate or the antibodies contained in the preparations did not match with the antigenic structures of the bacteria causing infections in the children. These results suggest that immune milk products intended for field use should contain a mixture of antibodies against a number of different serotypes or against the common virulence factors of the pathogenic organism concerned.

Immune milk products with specific antibodies against rotavirus, *Clostridium difficile* or *E. coli* have been launched on the market in Australia and the United States. It has been suggested that such preparations could provide a potential alternative for, or a supplement to, antibiotics, especially in the case of treatment of antibiotic-resistant bacteria (Ruiz, 1994; Weiner *et al.*, 1999; Korhonen *et al.*, 2000b). The supplementation of infant formulas with specific antibodies has also been proposed, but no such product has been introduced on the market, so far (Goldman, 1989; Davidson, 1996).

h. Other proteins. Mammary secretions, especially colostrum, contain many growth factors which modulate: (1) growth and differentiation of a variety of cell types; (2) mammary development, and (3) probably also

neonatal development of the GI tract and other organ systems (Koldovsky, 1996; Pakkanen and Aalto, 1997; Parodi, 1998; Xu, 1998; Playford *et al.*, 2000). These non-nutrient components contribute, for example, to the specific stimulation of jejunal and skeletal muscle protein synthesis in colostrum-fed neonatal pigs and calves (Burrin *et al.*, 1995; Buhler *et al.*, 1998). Recent data suggest that feeding trace amounts of colostral growth factors augments intestinal absorptive capacity as well as protein and fat metabolism and exerts beneficial effects on the endocrine system of neonatal calves (Hammon and Blum, 1998; Rauprich *et al.*, 2000). Also, it has been suggested that the colostral growth factors stimulate brain and heart protein synthesis in colostrum-fed neonatal pigs (Burrin *et al.*, 1997).

Among the most abundant growth factors in bovine colostrum are the insulin-like growth factors IGF-I and IGF-II, which promote cell proliferation and differentiation. Bovine colostrum contains much higher concentrations of IGF-I than does human colostrum (500 μg/l compared with 20 μg/l), with lower concentrations in mature bovine milk (10 μg/l). IGF-I is an anabolic agent and is at least partly responsible for mediating the growth-promoting activity of the growth hormone. IGF-II is present in bovine colostrum and milk at concentrations of 200–600 μg/l and <10 μg/l, respectively. IGF-II has anabolic activity and has been shown to reduce the catabolic state in starved animals. The transforming growth factor-α (TGF-α) is present in human colostrum and milk at concentrations of 2.2–7.2 μg/l. It has been suggested that the major physiological role of TGF-α is to act as a mucosal-integrity peptide, maintaining normal epithelial function in the non-damaged mucosa (Playford *et al.*, 2000). TGF-β stimulates the proliferation of cells in the connective tissue and acts as a growth inhibitor of some other cell types like lymphocytes and epithelial cells. TGF-β and TGF-β-like molecules are present at high concentrations in both bovine colostrum (20–40 mg/l) and milk (1–2 mg/l). It has been suggested that TGF-β derived from colostrum or milk could be exploited in functional foods for infants or in therapies for specific intestinal diseases, such as Chrohn's disease (Donnet-Hughes *et al.*, 2000).

The epidermal growth factor (EGF) comprises a family of molecules which are found primarily in colostrum. In human colostrum, the EGF concentration is about 200 μg/l and ranges in milk from 30 to 50 μg/l. EGF is not found in significant amounts in bovine secretions. The potential applications of bovine colostrum or milk-derived growth factors have not yet been realised because their efficacy in humans remains to be proven. There is a rather close homology with regard to the amino acid composition between some of the human and bovine growth factors, and recent physiological studies support the view that certain bovine growth factors and hormones may contribute to human body functions (Parodi, 1998; Playford *et al.*, 2000). Moreover, recent human studies suggest that oral administration

of colostrum-based products containing active growth factors increases protein synthesis during and after physical exercise (Mero *et al.*, 1997) and prevent the side-effects of non-steroid anti-inflammatory drugs (NSAIDs) used in arthritis prophylaxis (Playford *et al.*, 1999).

Also other growth factors, cytokines and hormones are found in human and bovine colostrum, but their physiological significance remains obscure. These include the vascular endothelial growth factor, platelet derived growth factor, growth hormone and its releasing factor, insulin, prolactin, melatonin, granulocyte-, macrophage- and granulocyte/macrophage-colony stimulating factors, interferon-γ and interleukins -1β, -6 and 10, and the tumor necrosis factor-α (Hagiwara *et al.*, 2000; Playford *et al.*, 2000).

Bovine milk also contains binding proteins for vitamins B12, folic acid and riboflavin. It has been suggested that the folate-binding protein contributes to the absorption of folate in the intestines (Parodi, 1998).

Lysozyme (LZM) is a potent antibacterial enzyme acting against a range of bacteria, especially Gram-positive, but due to its low concentration in bovine milk (in contrast to human milk), LZM may not contribute significantly to the overall antimicrobial properties of cow's milk and colostrum. However, LZM is known to add to the antimicrobial activity of LF and specific antibodies (Reiter, 1985; IDF, 1991; Shah, 2000).

B. EGG PROTEINS

The protein content of a hen's egg is approximately 13% by weight, divided mainly between three parts: egg membranes, egg white and egg yolk. These contain about 4, 45 and 31% protein by weight, respectively. Egg is, thus, a rich source of proteins with different physicochemical and biological characteristics. The major portion of egg yolk proteins is in the form of lipoprotein, which is divided into the plasma and granule fractions. The Igs are found in egg yolk; their concentration in the whole egg is about 100 mg. The granule fractions contain a phosphoprotein, phosvitin, having a phosphorus content of about 10%. Phosvitin contains 54% of serine, which is exclusively present as esters of phosphoric acid. Under low ionic strength and acidic conditions, phosvitin becomes water soluble and available for complexing with different divalent cations. This is the reason why phosvitin acts as a carrier of Ca^{2+}, or Fe^{2+} (Sugino *et al.*, 1997). Egg white consists of more than 40 different kinds of proteins, many of which are still uncharacterised because of their low concentration. The major proteins in egg white are ovalbumin (54%), ovotransferrin (12%), ovomucoid (11%), ovoglobulins (10%), LZM (3.5%) and ovomucin (3%). The degree of glycosylation of the egg white proteins is high, more than 90%, which may range from only a few per cent in ovalbumin to about 50% in ovomucins. Egg white has multiple functionality, such as

gelation, emulsification, foaming, water-binding and heat coagulation, making it a highly desirable source of protein in many foods. Although no definite biological function has so far been found for the egg white proteins, they possess unique properties, such as antimicrobial, enzymatic, protease inhibitory, cell growth stimulatory, vitamin-binding and immunological effects. Of the many different types of proteins found in egg albumen, most appear to be antimicrobially active or have certain physiological functions to interfere with the growth and spread of invading bacteria. Thus, they may act as: (1) proteinase inhibitors, e.g., ovoinhibitor, ovomucoid, ovomacroglobulin and cystatine; (2) bacteriolytic enzymes, e.g., LZM; (3) vitamin-chelating proteins, e.g., avidin and ovoflavoprotein; (4) metal-chelating proteins, e.g., ovotransferrin, and (5) jelly-like proteins, e.g., ovomucin. Accordingly, the egg white proteins appear to function primarily as a defensive barrier (Tranter and Board, 1982; Ibrahim, 1997).

The egg white protein that has probably attracted the most attention is LZM, which is now being used as an antimicrobial agent as well as in various pharmaceutical compounds. LZM has also shown promise as a food preservative, being used to prevent blowing of cheese, and also has some potential for preserving meat by reducing pathogen levels. LZM is easy to separate from egg white commercially by using crystallisation or ion-exchange resins (Kijowski *et al.*, 1999). Baron *et al.* (1999) tested the inhibitory potency of different egg white proteins on the *Salmonella* and showed that ovotransferrin played the major role in inhibiting the *Salmonella* growth in egg white. The egg Igs (IgY) have generated interest since hens can be hyperimmunised to produce specific antibodies against pathogens, and the IgY yield per egg is high. IgYs can be enriched and isolated in a highly purified form using a serial filtration system or by ultracentrifugation combined with a chromatographic purification process (Li-Chan, 1999). IgY has already found use in immunoassay techniques, and may, in future, find applications as an ingredient of functional foods and feeds aimed at preventing or curing GI infections. Various animal studies have shown that hyperimmune IgY preparations can be effective against rotavirus (Ebina *et al.*, 1990; Sarker *et al.*, 2001). IgY specific to *Streptococcus mutans* can provide protection against pathogens causing dental caries, when applied in a mouth rinse containing about 1% immune preparation of which about 6% is IgY (Hatta *et al.*, 1997).

C. PLANT PROTEINS

Cereals are by far the most important staple food of mankind, providing the major portion of energy and protein and much of the other

nutrients needed. The protein content of the grains is relatively low, and the composition of the essential amino acids may be unbalanced depending on the amino acid composition. Gliadin and glutein are the wheat endosperm storage proteins and form approximately 85% of the protein content of wheat flour. Corn proteins consist of three distinct zein classes: α-, β- and γ-zein. α-Zein is the main component of the maize endosperm protein, accounting for 75–85% of the total zein. The biological activity of these cereal proteins has been attributed to specific peptide sequences, which are freed by enzymatic hydrolysis. Furthermore, wheat gliadin seems to pose the major problem in celiac disease; gliadin antibodies are commonly found in the immune complexes associated with this disease (Friedman, 1996).

The protein content of soybeans (50% w/w) is much higher than of cereal grains, three times richer than of eggs and 11 times richer than of milk. Soybean protein quality is comparable to that of meat and eggs. The use of soy for the prevention or treatment of chronic diseases has already been advocated for a number of years (Friedman and Brandon, 2001). Areas where beneficial effects have been shown or are expected include cardiovascular diseases, cancer, diabetes, osteoporosis, hypertension, GI disorders and renal disease. The active ingredients are fibres, isoflavones and proteins. Many scientific studies carried out both on animals and humans have demonstrated that a soy protein diet reduces the total and LDL cholesterol, an established risk factor for cardiovascular disease. The actual mechanism by which soy proteins lower blood lipid concentrations in humans remains to be elucidated (Messina *et al.*, 2002). In Japan, meat products and (fermented) drinks with soy proteins as active ingredients for reducing blood pressure have been granted Foods for Specified Health Uses (FOSHU) status. The Food and Drug Administration (FDA) of the United States concluded that the amount of soy protein associated with a reduction in cholesterol levels was 25 g or more per day. The high level of protein required has raised concerns among toxicologists, since the intake of phytochemicals with oestrogen-like activity will subsequently increase. In October 1999, the FDA permitted the use of claims such as: “25 g of soy protein a day, as part of a diet low in saturated fat and cholesterol, may reduce the risk of heart disease”.

The occurrence of sweet-tasting proteins, such as thaumatin, monellin, mabinlin and pentadin, in the pulp of fruits of various rain forest species has been reported. The sweet-tasting proteins have different molecular lengths (from 54 residues of brazzein to 207 residues of thaumatin), virtually no sequence homology and very little structural homology. Thaumatin, the most characterised sweet protein, is 100,000 times sweeter than sugar on a molar

basis and 3000 times on a weight basis. It is extremely soluble in water, but not in organic solvents. Even at pH values below 5.5, heat stability above 100°C has been reported with no loss in sweetness. It is also stable under pasteurisation conditions. These properties are probably due to the presence of 8 disulphide bonds. The onset of sweetness is relatively slow with a slight liquorice aftertaste. The safety of thaumatin has been proven for animals and humans and it is currently commercially available as a sweetener, flavor enhancer, additive to pharmaceuticals, chewing gum and animal feeds (Gibbs *et al.*, 1996).

Sweet-tasting proteins interact with the same receptor that binds small MW sweetener, the T1R2-T1R3 G-protein coupled receptor (Li *et al.*, 2002). The key groups on the protein surface responsible for the biological activity have not yet been identified with certainty for any of the known sweet proteins. Temussi (2002) postulated that sweet proteins, contrary to small ligands, do not bind to glutamate-like pocket but stabilize the free form of the receptor by attachment to a secondary binding site.

Two taste-modifying proteins were found from berries and fruits. They turn sour into sweet taste, for example, lemons tend to taste sweet after ingestion. The amino acid sequencing of these proteins has revealed that they contain interchain and intrachain disulphide bridges and 114–191 amino acid residues. Although the MW is similar, neither has any real homology been found nor are they homologous sweet proteins (Gibbs *et al.*, 1996).

D. MISCELLANEOUS PROTEINS

Tannins are polyphenolic compounds, widely distributed in plant-based foods, which may have harmful effects on animals, including humans. Furthermore, they are frequently associated with a bitter taste, but they also give rise to oral sensation of astringency. Several studies have shown that salivary proline-rich proteins can bind to the polyphenols and precipitate them, thereby effectively preventing them from becoming bioavailable and having any effect on the GI tract. This function has been associated to the basic salivary proline-rich proteins, which have no known biological functions (Lu and Bennick, 1998; Sarni-Manchado *et al.*, 1999; Charlton *et al.*, 2002). It, therefore, seems likely that the astringent sensation is a consequence of interactions between the basic proline rich proteins and polyphenols.

Antifreeze proteins (AFPs) are ice-binding proteins found in some organisms (such as fish, insects, plants and soil bacteria) that live at the temperature of their surroundings and encounter freezing conditions. AFPs help organisms to survive below 0°C by inhibiting ice growth. AFPs are structurally diverse, each is radically different from the others in its primary,

secondary and tertiary structures, but typically has multiple isoforms that vary in length or sequence at a few amino acid positions. The MW is also extremely wide, ranging from 2.5 kDa in some fish AFPs to 36 kDa for one of the AFPs of winter rye. Although the sequences and structures that contribute to the ice-binding sites are distinct, a pattern can be observed. Ice-binding sites are relatively flat surfaces and a significant portion of their surface is involved in ice contact. Furthermore, the ice-binding sites tend to be less polar and more hydrophobic than the other AFP surfaces (Jia and Davies, 2002).

AFPs protect against ice by several mechanisms. These not only include lowering the point at which ice-crystals grow (lowering the freezing point but not the melting point, the so-called thermal hysteresis effect), but also modification of ice crystallization, such that smaller crystals and crystals of different shapes are formed. AFPs appear to exert their effect by accumulating at the water–ice interface and thereby modifying crystal growth, with different AFPs apparently showing preference for different crystal planes (Barret, 2001). However, at the molecular level the mechanism of interaction of the different AFPs may be different. It was earlier proposed that the binding mechanism relied almost entirely on a hydrogen bond match between AFP and ice, it now seems probable that van der Waals and hydrophobic interactions make a significant contribution to the enthalpy of adsorption (Cheng and Merz, 1997; Chao *et al.*, 1997; Baardsnes and Davies, 2002).

The intake of AFPs in the diet is likely to be substantial in most northerly and temperate regions. Much of this intake is likely to be from edible plants, given their importance in the diet, but in some regions intake from fish will be significant. As far as can be ascertained, AFPs are consumed with no evidence of adverse health effects, either short or long term. Given the structural diversity of AFPs, one firm general conclusion that can be drawn from the history of consumption of AFPs is that their functional characteristics do not impart any toxicologically significant effect (Crevel *et al.*, 2002).

There is a great promise of application of AFPs in foods with their ability to depress solution freezing temperature and inhibit recrystallisation in freezing. One potential direct application is to inhibit recrystallisation of ice in dairy products such as ice creams and de-icing agents. Furthermore, they may also be very useful in chilled and frozen meat, where large ice crystals may form intracellularly resulting in drip and loss of nutrition during thawing. The use of AFPs in foods will most likely depend on the cost. At present, although commercial products of AFPs are sold, they are only suitable for research or special uses because of their high price (Li and Sun, 2002).

III. BIOACTIVE PEPTIDES DERIVED FROM FOOD PROTEINS

A. BIOLOGICAL ACTIVITIES AND STRUCTURES

Peptides with specific biological activity may be located within the amino acid sequence of a given protein. Enzymatic degradation of foodstuffs in the gut and in food processing releases short-chain peptide sequences from intact proteins, glycoproteins and lipoproteins. In some cases, peptides may act as regulatory compounds with a hormone-like activity, based on their amino acid composition and sequence. Bioactive peptides usually contain 3–20 amino acid residues per molecule. Although animal- as well as plant-proteins contain potential bioactive sequences, milk proteins currently form the main source of a range of biologically active peptides. In order to elicit a biological response, peptides must both cross the intestinal epithelium and enter the blood circulation, or bind directly to specific epithelial cell-surface receptor sites.

1. Peptides with opioid activity

a. General structures and occurrence in the sequence of proteins. Opioid peptides, such as enkephalins, are defined as peptides having both an affinity for an opiate receptor and opiate-like effects inhibited by naloxone. Typical opioid peptides originate from three precursor proteins: proopiomelanocortin (endorphins), proenkephalin (enkephalin) and prodynorphin (dynorphins) (Höllt, 1983). These peptides have the same N-terminal sequence, Tyr-Gly-Gly-Phe. Opioid peptides exert their activity by binding to particular receptors of the target cell. The individual receptors are responsible for specific physiological effects, e.g., the μ-receptor for emotional behaviour and suppression of intestinal motility, the σ-receptor for emotional behaviour, and the κ-receptor for sedation and food intake. Opioid peptides that are derived from a variety of precursor proteins are called ‘atypical’, since they carry various amino acid sequences at their N-terminal regions; only the N-terminal tyrosine is conserved. The N-terminal sequence of ‘atypical’ opioid peptides is Tyr-X-Phe or Tyr-X1-X2-Phe. The tyrosine residue at the N-terminal and the presence of another aromatic amino acid at the third or fourth position form an important structural motif that fits into the binding site of the opioid receptors (Paroli, 1988; Teschemacher *et al.*, 1994, 1997).

b. Milk protein-derived opioid peptides. The major exogenous opioid peptides, β-casomorphins, are fragments of the β-casein sequence 60–70 (Tyr-Pro-Phe-Pro-Gly-Pro-Ile-Pro-Asn-Ser-Leu) (Chiba and Yoshikawa, 1986; Paroli, 1988; Koch and Brantl, 1990; Teschemacher *et al.*, 1990, 1997).

The first found β-casomorphin was β-casomorphin-7, β-casein sequence 60–66 (Henschen *et al.*, 1979). Later, β-casomorphin-4, an amide derivative of β-casomorphin-4, morphiceptin, as well as 8-prolyl-β-casomorphin were identified from bovine casein digests using specific radioimmunoassays (Chang *et al.*, 1985). Stepwise hydrolysis from the C-terminal of the heptapeptide generates a series of hexa-, penta- and tetrapeptides with different activities, as shown in Table III. β-Casomorphins are found at analogous positions in sheep, water buffalo and human β-casein (Teschemacher *et al.*, 1997). These peptides have been shown to display opioid activity in an opiate receptor assay as well as in isolated organ assays. β-Casomorphins and morphiceptin have been found to behave like μ-type opioid agonists in opioid receptor binding studies and in isolated organ preparations (Brantl *et al.*, 1981; Chang *et al.*, 1982, 1985).

α-Casein-derived opioid peptides, called exorphins, are released following pepsin digestion of cow casein (Zioudrou *et al.*, 1979). α-Casein exorphins correspond to bovine α_{s1}-casein residues 90–96 and 90–95. These peptides and the synthetic fragment without the N-terminal arginine residue exhibit, although in moderate strength, typical opioid properties *in vitro*, i.e., binding to rat brain opioid receptors and inhibiting the contractions of electrically stimulated mouse *vas deferens* preparations. The α-casein exorphins have also been shown to be δ-selective receptor ligands (Loukas *et al.*, 1983, 1990). Kampa *et al.* (1996) found that a peptide with the sequence Tyr-Val-Pro-Phe-Pro from human α_{s1}-casein f(158-162) has a high affinity for all sub-types of the κ-opioid receptor.

Peptides isolated from a peptic and tryptic digest of bovine κ-casein display low but clear-cut opioid antagonist properties (Yoshikawa *et al.*, 1986; Chiba *et al.*, 1989). The first identified peptide which showed high affinity for opioid receptors in the radioreceptor assay was found to correspond to the methyl ester of residues 33–38 of κ-casein and was named casoxin-6. Afterwards various peptide derivatives have been synthesised and isolated from κ-casein which possess opioid antagonist activity (Teschemacher *et al.*, 1997). The opioid antagonist peptides can be expressed by the general formula X_A-Arom-X_B-Tyr-OCH_3 (Arom: aromatic residues). An amino acid in position X_A may affect the specificity of the antagonist peptide for an opioid receptor. Peptides having a basic residue, such as arginine, in the X_A-position have been found to show a preference for κ-receptors (Yoshikawa *et al.*, 1988).

Whey proteins contain opioid-like sequences, namely α-la (both bovine and human) f(50-53) and β-lg (bovine) f(102-105), in their primary structure. These peptides have been termed α- and β-lactorphins (Chiba and Yoshikawa, 1986). Studies by Antila *et al.* (1991) indicated that proteolysis of α-la with pepsin produces α-lactorphin, and that digestion of β-lg with

TABLE III
EXAMPLES OF MILK PROTEIN-DERIVED BIOACTIVE PEPTIDES

Precursor protein	Release protease	Fragment	Name	Bioactivity	References
Casein					
Total casein	Trypsin	α_{s1}-cn f(194-199)	α_{s1}-immunocasokinin	Immunomodulatory, ACE-inhibitory	Maruyama *et al.* (1987); Migliore-Samour *et al.* (1989)
	Synthesis	β-cn f(193-202)	β-casokinin-10	Immunomodulatory, ACE-inhibitory	Kayser and Meisel (1996); Meisel and Schlimme (1994)
	Trypsin	β-cn f(1-25)4P	Caseinophosphopeptide	Mineral-binding, immunomodulatory, cytomodulatory	Reynolds (1992); Hata *et al.* (1998, 1999); Meisel and Gunther (1998)
	Trypsin	α_{s1}-cn f(43-58)2P	Caseinophosphopeptide	Mineral-binding	Juilleart *et al.* (1989)
	Duodenum (human)	β-cn f(7-18)3P	Caseinophosphopeptide	Mineral-binding	Chabance *et al.* (1998)
	Trypsin	α_{s1}-cn f(23-34)	Casokinin	ACE-inhibitory	Maruyama *et al.* (1985)
	Trypsin	β-cn f(177-183)	Casokinin	ACE-inhibitory, cytomodulatory	Maruyama *et al.* (1987); Nagaune *et al.* (1989)

(*continued on next page*)

TABLE III (*continued*)
EXAMPLES OF MILK PROTEIN-DERIVED BIOACTIVE PEPTIDES

Precursor protein	Release protease	Fragment	Name	Bioactivity	References
β-Casein	Trypsin	β-cn f(60-66)	β-casomorphin-7	Opioid agonist, ACE-inhibitory, immunomodulatory, cytomodulatory	Teschemacher *et al.* (1997); Kayser and Meisel (1996); Meisel and Gunther (1998)
α_{s1}-Casein	Pepsin	α_{s1}-cn f(90-96)	α-casein exorphin	Opioid agonist	Loukas *et al.* (1983)
κ-Casein	Pepsin	κ-cn f(33-38)	Casoxin-6	Opioid antagonist	Chiba and Yoshikawa (1986)
	Trypsin	κ-cn f(25-34)	Casoxin-C	Opioid antagonist	Chiba *et al.* (1989)
α_{s1}-Casein	Chymosin	α_{s1}-cn f(1-23)	Isracidin	*In vivo* antimicrobial	Lahov and Regelson (1996)
αs_2-Casein	Trypsin	α_{s2}-cn f(165-203)	Casocidin-I	Antimicrobial	Zucht *et al.* (1995)
		α_{s2}-cn f(174-181)	Casokinin	ACE-inhibitory	Tauzin *et al.* (2002)
		α_{s2}-cn f(174-179)			
Glycomacropeptide	Trypsin	κ-cn f(106-116)	Casoplatelin	Antithrombotic	Fiat *et al.* (1989)
	Trypsin	κ-cn f(112-116)	Thrombin inhibitory peptide	Antithrombotic	Qian *et al.* (1995)
Human milk	Pepsin	κ-cn f(63-117)		Antimicrobial	Liepke *et al.* (2001)

TABLE III (*continued*)
EXAMPLES OF MILK PROTEIN-DERIVED BIOACTIVE PEPTIDES

Precursor protein	Release protease	Fragment	Name	Bioactivity	References
Whey protein					
α-Lactalbumin	Pepsin	α-la f(50-53)	α-lactorphin	Opioid agonist, ACE-inhibitory	Antila *et al.* (1991); Mullally *et al.* (1996)
α-Lactalbumin	Trypsin	α-la f(104-108)	Lactokinin	ACE-inhibitory	Pihlanto-Leppälä *et al.* (2000)
α-Lactalbumin	Trypsin/ chymotrypsin	α-la fragments		Antimicrobial	Pellegrini *et al.* (1999)
β-Lactoglobulin	Pepsin + trypsin	β-lg f(102-105)	β-lactorphin	Opioid agonist, ACE-inhibitory	Antila *et al.* (1991); Mullally *et al.* (1996)
β-Lactoglobulin	Trypsin	β-lg f(142-148)	Lactokinin	ACE-inhibitory	Mullally *et al.* (1997a,b)
β-Lactoglobulin	Trypsin	β-lg f(15-20), f(92-100)		Antimicrobial	Pellegrini *et al.* (2001)
Lactoferrin	Pepsin	Lf f(17-41)	Lactoferricin immunomodulatory	Antimicrobial	Bellamy *et al.* (1992)

pepsin and then with trypsin, or with trypsin and chymotrypsin, yields β-lactorphin. Furthermore, α-lactorphin exerted weak but consistent opioid activity in the guinea pig ileum and in connection with receptor-binding, whereas β-lactorphin, despite its similar receptor-binding affinity, had an apparent non-opioid stimulatory effect on the guinea pig ileum. These peptides have been found to show a very low affinity for opioid receptors and are μ-type receptor ligands (Paakkari *et al.*, 1994). Moreover, bovine blood serum albumin f(399-404), named serorphin, displays opioid activity (Tani *et al.*, 1994). Peptides with an affinity for opioid receptors have also been observed in an artificially methyl-esterified peptic digest of human LF. These peptides are named lactoferroxins and behave like opioid antagonist peptides, such as casoxins (Tani *et al.*, 1990).

c. Opioid peptides derived from other proteins. Apart from milk proteins, wheat gluten is the most well-known source of opioid peptides (exorphins). Pepsin–thermolysin and pepsin–trypsin–chymotrypsin digestion of wheat gluten produces Gly-Tyr-Tyr-Pro-Thr (exorphin A5), Tyr-Gly-Gly-Trp-Leu (exorphin B5) and Tyr-Pro-Ile-Ser-Leu (exorphin C). For example, the sequence of exorphin A5 is found 15 times in the primary structure of high MW gluten. The structure of these gluten exorphins differs considerably from any of the endogenous or exogenous peptides reported, only the N-terminal tyrosine is found. The gluten exorphins show an affinity for δ- and μ-receptors and decrease the tension of the guinea pig ileum *in vitro*. Whole gluten hydrolysates reveal similar activity (Fukudome and Yoshikawa, 1992, 1993).

Bovine haemoglobin, the protein from erythrocytes which occurs as a minor component in meat and meat products, is also a precursor of opioid peptides (haemorphins). These opioid peptides are released by pepsin digestion *in vitro* and may also be produced by macrophages. Moreover, haemorphins have been found to decrease the tension of the guinea pig ileum *in vitro* (Nyberg *et al.*, 1997).

2. *Peptides with angiotensin I-converting enzyme inhibition activity*

Angiotensin I-converting enzyme (ACE; peptidyldipeptide hydrolase, EC 3.4.15.1) is a key enzyme in the regulation of peripheral blood pressure. ACE has been classically associated with the renin-angiotensin system, which converts angiotensin I into a potent vasoconstrictor, angiotensin II. In the kinin–kallikrein system ACE also degrades vasodilative bradykinin and stimulates the release of aldosterone in the adrenal cortex. Consequently, ACE plays a major physiological role in the regulation of local levels of several endogenous bioactive peptides (Petrillo and Ondetti, 1982).

Exogenous ACE inhibitors having an antihypertensive effect *in vivo* were first discovered in snake venom (Ondetti *et al.*, 1971). Afterwards, various ACE-inhibitors have been found from enzymatic hydrolysates and related synthetic peptides of food proteins. These food proteins include such as bovine and human casein and whey, zein, gelatin, yeast and corn (Ariyoshi, 1993; Yamamoto, 1997; FitzGerald and Meisel, 2000; Pihlanto-Leppälä, 2001). So far, ACE-inhibitory peptides are the most commonly known group of bioactive peptides of food protein origin. Some examples of these peptides are presented in Table III.

ACE is an exopeptidase, which cleaves dipeptides from the C-terminal of various peptide substrates. It is an unusual zincmetallopeptidase, since it is activated by chloride and lacks a narrow *in vitro* substrate specificity (Ondetti and Cushman, 1984). Structure–activity correlations between different peptide inhibitors indicate that binding to ACE is strongly influenced by the C-terminal tripeptide sequence of the substrate. Although this substrate specificity is not clearly understood, ACE appears to prefer substrates or competitive inhibitors containing hydrophobic amino acid residues at each of the three C-terminal positions. Many of the known ACE-inhibitors contain proline, lysine or arginine as C-terminal amino acids. The presence of positively charged C-terminal lysine or arginine residues does not fit with the ACE-active site model proposed by Ondetti and Cushman (1984). Nevertheless, structure–activity data suggest that a positive charge on the arginine and lysine side-chain contributes substantially to the inhibitory potency. It is postulated that the mechanisms of ACE-inhibition involve inhibitor interaction with an anionic binding site, which is distinct from the catalytic site (Meisel, 1997). Accordingly, it is expected that the peptide structure adopted in a specific environment should contribute to the ACE-inhibitory potency.

a. Casokinins. Casokinin sequences have been found in all casein fractions, but α_{s1}- and β-caseins, in particular, are rich in ACE-inhibitory sequences. Maruyama and Suzuki (1982) showed that a tryptic hydrolysate of casein contains ACE-inhibitors. The active peptide inhibitors were purified and identified as α_{s1}-casein f(23-34). Later it was found that the peptides corresponding to β-casein f(177-183), α_{s1}-casein f(23-27) and, α_{s1}-casein f(194-199) had ACE-inhibitory activity (Maruyama *et al.*, 1985, 1987). In addition to trypsin hydrolysis, lactic acid fermentation also produces casokinins. Yamamoto *et al.* (1994) showed that peptides produced by *Lactobacillus* proteases had ACE-inhibitory activity. Furthermore, two ACE-inhibitory tripeptides, Val-Pro-Pro and Ile-Pro-Pro, were obtained during the fermentation of milk proteins with *Lactobacillus helveticus* and *Saccharomyces cerevisiae* (Nakamura *et al.*, 1995a).

b. Lactokinins. The first ACE-inhibitory peptides derived from whey proteins were synthetic peptides corresponding to the known bioactive sequence of β-lg (β-lactorphin and β-lactotensin) and α-la (α-lactorphin) (Mullally *et al.*, 1996). Our studies have indicated that the tripeptide Tyr-Gly-Leu (α-la f(50-52)) has ACE-inhibitory activity at about the same range as the α-lactorphin (Pihlanto-Leppälä *et al.*, 2000). Peptides originating from α-la f(99-110) may contribute considerably to the ACE-inhibitory activity of α-la hydrolysates, since we found ACE-inhibitory activity in α-la f(99-108), f(104-108) and f(105-110). The ACE-inhibitory activity of β-lg hydrolysates is a result of various peptides liberated from different regions of the β-lg chain. Trypsin, e.g., releases several peptides with moderate activity, namely β-lg f(22-25), (32-40) (81-83) and (142-148) (Mullally *et al.*, 1997a; Pihlanto-Leppälä *et al.*, 1999, 2000a). Several peptides have been isolated from whey protein digested with proteinase K, among them is a peptide corresponding to β-lg f(78-80) (β-lactosin) which showed the highest ACE-inhibitory activity (Abubakar *et al.*, 1998).

c. Other ACE-inhibitory peptides. A limited number of ACE-inhibitory peptides have additionally been identified from non-milk dietary proteins (Table IV). Oshima *et al.* (1979) reported ACE-inhibitory peptides obtained by digesting gelatine with bacterial collagenase among the first found bioactive peptides. They identified six potent ACE-inhibitors, which were tri-, hexa-, nona- and dodeca-peptides. Miyoshi *et al.* (1995) isolated ACE-inhibitory peptides from the corn endosperm protein γ-zein fraction hydrolysed by thermolysin. All of the identified ACE-inhibitory peptides were tripeptides (Leu-Arg-Pro, Leu-Ser-Pro and Leu-Gln-Pro), had a proline residue and exhibited a similar structure. Yano *et al.* (1996) found that a major component of maize protein, α-zein was almost completely hydrolysed into small peptides by digestion with thermolysin and most of the peptide fractions showed at least some ACE-inhibitory activity. Matsui *et al.* (1999) isolated 16 peptides, composed of 2–7 amino acid residues, from wheat germ hydrolysates. A tripeptide, Ile-Val-Tyr, was identified as a main contributor to the ACE-inhibition of the hydrolysates. Tryptic hydrolysates of zein and hordein protein have been found to contain peptides which have ACE-inhibitory activity and which, additionally, inhibit endopeptidase and crude proteinase from *Pseudomonas fluorescens* (ATCC 948) (Gobbetti *et al.*, 1997). A peptic digest of protein prepared from wakama (*Undaria pinnatifida*) contains four tetrapeptides with ACE-inhibitory properties (Suetsuna and Nakano, 2000). Furthermore, a concentrate of an aqueous extract of *Allium sativum* L. (garlic) has seven ACE-inhibitory dipeptides (Ser-Tyr, Gly-Tyr, Phe-Tyr, Asn-Tyr, Ser-Phe, Gly-Phe and Asn-Phe) (Suetsuna, 1998).

TABLE IV

BIOACTIVE PEPTIDES DERIVED FROM VARIOUS ANIMAL AND PLANT PROTEINS

Protein source	Treatment	Peptide sequence	Bioactivity		References
			ACE-inhibitory activity[a] IC_{50} μM	Effect on systolic blood pressure in SHR[b]	
Corn endosperm	Thermolysin	LRP LDP LQP	0.27 1.7 1.9	Hydrolysate cont. peptides decreases SBP p.o.[c]	Miyoshi *et al.* (1995)
Zein	Trypsin	SAYPGQITSN	7		Gobbetti *et al.* (1997)
Hordein	Trypsin	QVSLNSGYY	23		Gobbetti *et al.* (1997)
Wakame	Pepsin	YNKL	21	↓ 40–50 mmHg (5 mg/kg)	Suetsuna and Nakano (2000)
Maize, α-chain	Thermolysin	FNQ LF	41 68		Yano *et al.* (1996)
Wheat germ	Alkaline protease	IVY	0.48	↓ 19.2 mmHg (5 mg/kg) (MAP) i.v.[d]	Matsui *et al.* (1999, 2000)
Soy	Alcalase	Low molecular weight peptides	ND	↓ 38 mmHg (100 mg/kg) p.o.	Wu and Ding (2001)
Genetically modified soybean protein	Trypsin and chymotrypsin	RPLKPW	ND	↓ (5–10 mg/kg) p.o.	Matoba *et al.* (2001)
Allium sativum L (garlic)	Aqueous extract	SY FY	66.3 3.74	↓ (200 mg/kg) p.o.	Suetsuna (1998)

(*continued on next page*)

TABLE IV (*continued*)
BIOACTIVE PEPTIDES DERIVED FROM VARIOUS ANIMAL AND PLANT PROTEINS

Protein source	Treatment	Peptide sequence	Bioactivity		References
			ACE-inhibitory activity[a] IC_{50} μM	Effect on systolic blood pressure in SHR[b]	
Sardine muscle	Alkaline protease	KY	1.63		Matsufuji *et al.* (1994)
		AKK	3.13		
Bonito bowels	Autolysis	ARPY	320		Matsumura *et al.* (1993)
		VRP	2.2		
Indonesian dried-salted fish	Pepsin	VAWKL	31.97		Astawan *et al.* (1995)
		CWLPVY	22.2		
Porcine skeletal muscle	Thermolysin	TNP	207.4		Arihara *et al.* (2001)
		ITTNP	549	↓ 21 mmHg (1 mg/kg)	Nakashima *et al.* (2002)
Chicken	Thermolysin	IKW	0.21	↓ 50 mmHg (10 mg/kg)	Fujita *et al.* (2000)
		LKP	0.32	↓ 75 mmHg (10 mg/kg)	
Egg ovalbumin	Chymotrypsin	RADHPF	Vasodilatation >1 mM	↓ 10 mmHg (10 mg/kg)	Matoba *et al.* (1999)
	Pepsin	LW	6.8	↓ 45 mmHg (10 mg/kg)	Fujita *et al.* (2000)
		ERKIKVYL	1.2	0	

TABLE IV (*continued*)

BIOACTIVE PEPTIDES DERIVED FROM VARIOUS ANIMAL AND PLANT PROTEINS

Protein source	Treatment	Peptide sequence	Bioactivity		References
			ACE-inhibitory activity[a] IC_{50} μM	Effect on systolic blood pressure in SHR[b]	
	Pepsin	FRADHPFL		↓ 18 mmHg (20 mg/kg)	Fujita *et al.* (1995)
Buckwheat pollen		APVLQIKKTGSN	Immunomodulation		Liu *et al.* (1998)
Egg ovotransferrin	Trypsin and acid	OT f(109-200)	Antibacterial		Ibrahim *et al.* (1997, 2000)
Egg yolk phosvitin	Trypsin	ND	Calcium-binding		Jiang and Mine (2000)

SBP, systolic blood pressure; ND, not determined.

[a]IC_{50} μM = peptide concentration required to inhibit ACE (angiotensin converting enzyme) by 50%.

[b]Spontaneously hypertensive rat.

[c]Oral administration.

[d]MAP, mean arterial blood pressure; i.v., intravenous administration.

Twelve ACE-inhibitory peptides have been identified from sardine muscle hydrolysate, revealing that a dipeptide, Val-Tyr, acts as a key inhibitor (Matsufuji *et al.*, 1994). Of the identified ACE-inhibitory peptides, the tripeptides (Leu-Arg-Pro, Ile-Val-Tyr) and the dipeptide (Val-Tyr) show strong inhibitory activity. Moreover, two inhibitory peptides (myopentapeptides A and B) have been purified from a thermolysin digest of porcine skeletal muscle proteins. The sequences were found in the primary structure of the myosin heavy chain (Arihara *et al.*, 2001).

3. *Peptides with antithrombotic activity*

Certain functional similarities have been shown to exist between milk and blood coagulation, as well as sequence homologies between the fibrinogen γ-chain and κ-casein (Jolles and Caen, 1991). Hydrolysis of bovine κ-casein by chymosin constitutes the first stage of milk clotting. In this reaction, one bond (Phe_{105}–Met_{106}) of κ-casein is rapidly hydrolysed, leading to the release of an insoluble N-terminal fragment (*para*-κ-casein; residues 1–105) and a soluble C-terminal fragment (caseinomacropeptide, residues 106–169) from which a series of tryptic peptides active in platelet function has been characterised. These peptides are referred to as casoplatelins. Jolles *et al.* (1986) reported that a dodecapeptide, corresponding to bovine κ-casein residues 106–116 (Met-Ala-Ile-Pro-Pro-Lys-Lys-Asn-Gln-Asp-Lys), inhibits ADP-induced platelet aggregation and combines with the fibrinogen receptor of blood platelets, consequently preventing fibrinogen-binding with blood platelets, in a concentration-dependent manner. The two smaller tryptic peptides (κ-casein f(106-112) and f(113-116)) exert a much lower effect on platelet aggregation and do not inhibit fibrinogen-binding (Fiat *et al.*, 1989). Three peptides from bovine κ-casein have been found to exhibit antithrombotic activity in the guinea pig: namely, the caseinoglycopeptide f(106-169) and its split peptides, the dodecapeptide f(106-116) and the pentapeptide f(112-116), which is the least active peptide (Bal dit Sollier *et al.*, 1996). The C-terminal (residues 106–171) part of sheep κ-casein, called caseinoglycopeptide, inhibits thrombin- and collagen-induced platelet aggregation in a dose-dependent manner. Three peptides which completely inhibit thrombin-induced aggregation have been derived from an enzymatic hydrolysate of caseinoglycopeptide (residues 112–116, 163–171 and 165–171) (Qian *et al.*, 1995).

4. *Peptides with immunomodulating activities*

a. Bovine milk-derived immunopeptides. Milk protein-derived peptides are known to have an effect on the cells of the immune system, as well as on

downstream immunological responses and cellular functions. Casein hydrolysates modulate the immune function, with different modulatory effects attributable to varying enzyme digestion regimes. Pancreatin and trypsin digests of α_{s2}- and β-casein have been shown to significantly inhibit the proliferative responses of murine splenic lymphocytes and rabbit Peyer's patch cells, whereas digests derived from pepsin and chymotrypsin treatment have no effect when added to *in vitro* culture with mitogen-stimulated cells (Otani and Hata, 1995). Peptides derived from pepsin–trypsin hydrolysis of α_{s1}- and β-casein also significantly suppress mitogen-induced proliferation of human peripheral blood mononuclear cells *in vitro* (Kayser and Meisel, 1996). The C-terminal β-casein f(193-199), obtained by a chymosin–pepsin digest of bovine casein, has been found to directly stimulate the proliferation of rat lymphocytes *in vitro*, in the absence of mitogens or antigens (Coste *et al.*, 1992). Further, human milk hydrolysed by trypsin possesses immunostimulating activity (Jolles *et al.*, 1981). Parker *et al.* (1984) isolated a hexapeptide, Val-Glu-Pro-Ile-Pro-Tyr (β-casein f(54-59)) and found that it stimulates the phagocytosis of sheep's red blood cells by murine peritoneal macrophages in an *in vitro* assay system, as well as enhances the resistance of mice to *Klebsiella pneumoniae* infection when given intravenously. Tryptic hydrolysis of human and bovine casein results in two hexapeptides, i.e., Pro-Gly-Pro-Ile-Pro-Asn (β-casein f(63-68)) and Thr-Thr-Met-Pro-Leu-Trp (α_{s1}-casein f(194-199)), and two tripeptides Gly-Leu-Phe (human and bovine α-la) and Leu-Leu-Tyr (β-casein f(191-193)). All these peptides stimulate murine peritoneal macrophages at quite low doses. Two peptides (Val-Glu-Pro-Ile-Pro-Tyr and Gly-Leu-Phe) from human caseins have been shown to increase the phagocytosis of human and murine macrophages and protect mice against *K. pneumoniae* infection (Migliore-Samour *et al.*, 1989; Fiat *et al.*, 1993). These two peptides exert a significant effect on binding of senescent red blood cells to human monocytic-macrophagic cells and their subsequent phagocytosis in a dose-dependent manner (Gattegno *et al.*, 1988). Stimulation of phagocytosis by the two peptides is mediated by different receptor molecules. Gly-Leu-Phe-specific binding sites have been demonstrated on the two phagocytic human blood cells, monocytes and polymorphonuclear leucocytes, the latter presenting the most important binding capacity. In contrast, Val-Glu-Pro-Ile-Pro-Tyr specifically binds only to monocytes-macrophages (Jaziri *et al.*, 1992).

Two synthetic peptides mimicking milk protein-derived peptides have been shown to enhance the proliferation of human peripheral blood lymphocytes. These peptides are Tyr-Gly and Tyr-Gly-Gly, which correspond to fragments of bovine κ-casein and α-la. β-Casomorphin-7 and β-casokinin-10 show a suppression of lymphocyte proliferation at low concentrations, but reveal stimulation at higher concentrations.

Protein synthesis is enhanced with di- and tripeptides, while no marked effect has been found with β-casomorphin-7 and β-casokinin-10 (Kayser and Meisel, 1996). Sütas *et al.* (1996b) showed that casein hydrolysed by a probiotic *Lactobacillus* GG strain and digestive enzymes (pepsin and trypsin) generates compounds with both suppressive and enhancing effects on lymphocyte proliferation. The α_{s1}-casein fractions were found to be more suppressive on T-lymphocytes than the β-casein fractions. Several known immunostimulating peptides have been identified from these hydrolysates (Rokka *et al.*, 1997). Ganjam *et al.* (1997) reported that a yoghurt fraction, generated by membrane dialysis and filtration, demonstrated an antiproliferative effect on cultured mammalian intestinal cells. Cell division was not inhibited in response to a similarly produced milk fraction or in response to solutions of lactic acid, indicating that the bioactive peptides produced from milk during bacterial fermentation are the active compounds. α_{s1}-Casein residues 59–79, having a phosphoserine-rich region and isolated from a trypsin digest of α_{s1}-casein, have been found to display mitogenic activity and enhance immunoglobulin production in mouse spleen cells. Similar results have been obtained with β-casein f(1-25) containing a cluster sequence of phosphoserine residues, as some commercial mitogens are able to enhance cell proliferation in the presence of this peptide (Hata *et al.*, 1998).

Opioid receptors are present in human T-cell lymphocytes (Wybran *et al.*, 1979), which may provide a link between the central nervous system and the immune system. There is a considerable body of literature, which demonstrates a modulatory function of the immune system by opioids. Opioids alter the biochemical and proliferative properties of various cellular components of the immune system (Webster, 1998). β-Casomorphins affect the human mucosal immune system, possibly via the opiate receptor, since the opiate receptor antagonist, naloxone, reverses the activity (Elitsur *et al.*, 1991). β-Casomorphins and α-casein exorphins inhibit the cell proliferation of human prostate cell lines by a mechanism partly involving opioid receptors (Kampa *et al.*, 1997).

b. Other immunopeptides. A proline-rich polypeptide (PRP) isolated from ovine colostral whey has been found to possess regulatory properties that stimulate or suppress immune responses. A nonapeptide fragment (Val-Glu-Ser-Tyr-Val-Pro-Leu-Phe-Pro) has been isolated from a chymotryptic digest of ovine PRP. The corresponding synthetic peptide and its C-terminal penta- and hexapeptides show immunoregulatory activities in mice, similar to native PRP, including enhancement of splenic antibody responses to foreign erythrocyte antigens when administered 3 h before immunisation (Janusz *et al.*, 1987). Further, ovine PRP has been shown to induce cytokine

production by murine macrophages as well as growth and differentiation of resting B-lymphocytes (Julius *et al.*, 1988; Blach-Olszewska and Janusz, 1997).

A tetrapeptide, Thr-Lys-Pro-Arg, called tuftisin, is derived from endopeptidase and leukokininase cleavage of the heavy chain F_c region of IgG. Human Ig-derived tuftisin has a variety of immunoregulatory effects, such as stimulation of leukocyte chemotaxis and phagocyte motility, enhancement of phagocyte oxidative metabolism and antigen processing, and increase in monocyte- and NK cell-mediated tumor cell cytotoxicity (Werner *et al.*, 1986). The immunoregulatory role of bovine Ig-derived tuftisin has not been determined, but the high Ig content of bovine milk and colostrum would suggest that tuftisin could be of importance for the neonate offspring.

Sulphated glycopeptides in ovomucin, chalazae and yolk membrane have been obtained via tryptic hydrolysis of hen egg proteins. These glycopeptides activate *in vitro* mice macrophages. They enhance macrophage proliferation, as well as production of interleukin-1 and hydrogen peroxide from the cells (Tanizaki *et al.*, 1997). The glycosidic residues contain *N*-acetylgalactosamine, galactose and *N*-acetylneuraminic acid. Glycosidic and sulphate residues play a key role in interactions with macrophage components. Oryzatensin, a peptide isolated from a tryptic hydrolysate of rye proteins, probably from the albumin fraction, stimulates *in vitro* phagocytosis of human polymorphonuclear leukocytes and also the production of superoxide anions by human leukocytes. Furthermore, oryzatensin and its fragments cause smooth muscle contraction by stimulating the production of histamine and a substance with prostaglandin-like activity (Takahashi *et al.*, 1996). Trypsin hydrolysis of soybean protein releases a peptide with the sequence His-Cys-Gln-Arg-Pro-Arg. This peptide has been found to stimulate phagocytosis *in vitro* as well as the production of tumor necrosis factor in mice (Yoshikawa *et al.*, 1993).

There is a growing awareness that oligopeptides play an important role as signalling molecules in plants. Phytosulphokine-α, a sulphated pentapeptide derived from rice (*Oryza sativa* L.) and asparagus cells, is involved in plant cell proliferation mediated by specific membrane-associated binding. Furthermore, novel signal transduction pathways have been found that activate genes responsible for cell proliferation in plants (Matsubayashi *et al.*, 1997; Matsubayashi and Sakagami, 1999).

Pronase-treated hen egg white ovomucin contains two highly glycosylated peptide fragments which have an anti-tumor effect. In mice, these fragments have been found to cure the treated tumor directly and entirely and to inhibit the growth of a distant one indirectly and slightly. An increase in immunosuppressive acid protein in serum suggests a slight activation of the immune system (Watanabe *et al.*, 1998).

5. *Peptides with antimicrobial activity*

The natural antimicrobial activity of milk is mainly associated with whey proteins, primarily with LF (see Section II.A.d). Early reports associating LF with the prevention of microbial growth attributed this function to its ability to bind and sequester iron, and to deprive microorganisms of this essential nutrient (Bullen *et al.*, 1972; Sanchez *et al.*, 1992). However, hydrolysis of LF by pepsin produces hydrolysates in which the antimicrobial potency is higher than in undigested LF. The iron-binding capacity of the hydrolysates is lost, but the antimicrobial activity is not affected by the addition of iron. These results, thus, indicate that the antibacterial activity of these LF hydrolysates is not dependent on iron. Peptides with a low MW generated by pepsin cleavage or by heat treatment at an acidic pH of LF have been found to show broad-spectrum antimicrobial activity *in vitro* (Tomita *et al.*, 1991; Saito *et al.*, 1994). A bactericidal domain has now been isolated and identified. All the identified antimicrobial peptides, called lactoferricins, are cationic and originate from the N-terminal of the molecule (Bellamy *et al.*, 1992b; Tomita *et al.*, 1994; Dionysius and Milne, 1997; Hoek *et al.*, 1997). These peptides have antimicrobial activity against various Gram-positive and -negative bacteria, yeast and filamentous fungi (Bellamy *et al.*, 1992a; Kang *et al.*, 1996). Shin *et al.* (1998) observed that lactoferricin B killed four clinical isolates of enterohaemorrhagic *E. coli* O157:H7 within 3 h of concentrations above 10 μg/ml. The pool of fragments obtained after LF was digested with trypsin, in which the amount of intact protein was less than 1% by weight, retained its antiviral activity toward herpes simplex virus Type I. This indicates that the inhibition of the viral infection is not exclusively linked to native bovine LF. The main antiviral activity was found to be associated with the N-lobe (f1-280) and C-lobe (f345-689) of LF (Siciliano *et al.*, 1999).

An antibacterial peptide has also been purified from bovine milk hydrolysed by serine proteases. This peptide was identified as α_{s2}-casein f(165-203), casocidin-I, and it inhibits the growth of *E. coli* and *Staphylococcus carnosus* (Zucht *et al.*, 1995). α-La and β-lg hydrolysed by pepsin and trypsin have been shown to lower the metabolic activity of a recombinant *E. coli* strain (Pihlanto-Leppälä *et al.*, 1999a). Pellegrini *et al.* (1999, 2001) reported that tryptic or chymotryptic digestions of α-la and β-lg yielded several bactericidal polypeptide fragments. The polypeptides were mostly active against Gram-positive bacteria while Gram-negative bacteria were only poorly susceptible to the bactericidal action of the polypeptides. *Bacillus subtilis* was the most susceptible bacterial strain to the action of these peptides. In addition to milk-derived peptides, an antimicrobial peptide has been identified from hen ovotransferrin (Ibrahim *et al.*, 2000). This peptide is

a cationic fragment and consists of 92 amino acid located within the 109–200 sequence of the N-lobe. Further, this peptide showed strong bactericidal activity against *S. aureus* and *E. coli* strains.

All the identified LF peptides are distinct from the iron-binding site of the molecule, which indicates that the bactericidal mechanism is independent of iron chelation. No sequence similarities exist between LF peptides and any other antimicrobial peptides. However, like various other antimicrobial peptides that display membrane-disruptive properties, LF peptides contain a high proportion of basic amino acid residues. Similar to LF-derived peptides, casocidin-I is also a cationic peptide, and 10 of its 39 amino acid residues are basic (Zucht *et al.*, 1995). The contribution of basic amino acids to antibacterial potency has been further illustrated by the observation that the antimicrobial activity is lost in shorter peptides from lactoferricin, where all basic amino acids are substituted by glutamic acid (Kang *et al.*, 1996). The cationic peptides are known to form ion channels in artificial membranes and are thought to exert their lethal effect by disrupting essential cell-membrane functions (Jack *et al.*, 1998). Electron microscopy studies have revealed that lactoferricin B induces a profound change in cell ultrastructural features and causes substantial damage in bacteria and fungi (Yamauchi *et al.*, 1993). Bellamy *et al.* (1993) showed that lactoferricin B binds rapidly to the surface of *E.coli* and *B. subtilis*. The rate of binding is consistent with the rate of killing caused by this peptide. The binding rate is reduced in the presence of Mg^{2+} and Ca^{2+}, indicating that ionic interactions have an important role in the cell-binding event. The results obtained by Kang *et al.* (1996) suggest that the 11-residue peptide of lactoferricin B is involved in the interaction with the bacterial phospholipid membranes. Transmission electron microscopy studies show that lactoferricin B acts on the cell surface and affects the cytoplasmic contents (Shin *et al.*, 1998). Furthermore, lactoferricin B has been found to disrupt the outer membrane of Gram-negative bacteria by releasing LPS (Yamauchi *et al.*, 1993). The ovotransferrin antimicrobial peptide is capable of killing Gram-negative bacteria by crossing the outer membrane by a self-promoted uptake and cause damage to the biological function of cytoplasmic membrane (Ibrahim *et al.*, 2000). These results indicate that a disruption of normal membrane permeability is at least partly responsible for the antibacterial mechanism of cationic peptides.

6. *Peptides with mineral-binding properties*

The occurrence of bioactive substances in milk which influence mineral metabolism was first documented by Mellander (1950) when he reported that

casein-derived phosphorylated peptides enhance vitamin D-independent bone calcification in rachitic infants. Caseins are known to contain phosphorylated regions. The extent of phosphorylation is dependent on the casein type: α_{s2}-casein contains 10–13 phosphate groups, α_{s1}-casein contains 7–9, β-casein contains 5, and κ-casein has only one phosphate group per mole of casein for the common genetic variants (Mercier, 1981). These confer to the proteins the ability to chelate calcium, which is related to their level of phosphorylation; thus αs2 > αs1 > β > κ. Further studies on the primary structure have indicated that the phosphorylated residues are not evenly spread throughout the protein chain, but are often clustered with three or more common cluster sequences, such as Ser(P)-Ser(P)-Ser(P)-Glu-Glu-. Phosphate groups appear as monoesters of the two hydroxyl amino acids, serine and threonine. These phosphorylated fragments are believed to play a crucial role in protecting the milk gland against calcification by controlling the calcium phosphate precipitation. Furthermore, the fragments help to create thermodynamically stable casein micelles, supersaturated with calcium and phosphate, and thus contribute to the stability of milk during heat processing (for reviews see West, 1986 and Swaisgood, 1993).

The unique properties of caseinophosphopeptides (CPP) have led to much interest in the isolation of CPP fractions and individual peptides. By cleavage of casein with enzymes such as trypsin and alcalase, several CPPs have been identified *in vitro*. For example, α_{s1}-casein f(43-58), f(59-79), f(43-79), α_{s2}-casein f(1-24) and f(46-70) and β-casein f(1-28), f(2-28), f(1-25), f(33-48) have been isolated from the tryptic hydrolysate of whole casein (Juilleart *et al.*, 1989; Adamson and Reynolds, 1995; Gagnaire *et al.*, 1996). The highly anionic character of these peptides renders them resistant to further proteolytic attack. The calcium-chelating activity of CPP-fragments *in vitro* has been attributed to the role of component phosphoserine residues (polar acidic domain) in stabilising the colloidal calcium phosphate of casein micelles. De-phosphorylated peptides do not bind minerals (Sato *et al.*, 1986; Berrocal *et al.*, 1989). Further evidence of the role of phosphoserine residues in mineral binding is illustrated by the observation that chemical phosphorylation of α_{s1}- and β-casein increases the binding capacity and stability of these proteins in the presence of Ca^{2+} (Yoshikawa *et al.*, 1981). The proportion of phosphopeptides interacting with colloidal calcium phosphate correlates with their relative content of phosphoserine residues (Gagnaire *et al.*, 1996).

The Ca^{2+} binding constant of CPPs is reported to be within 10^2–10^3 M^{-1} (Sato *et al.*, 1983, 1991; Berrocal *et al.*, 1989; Meisel *et al.*, 1991). Several studies have shown that the α_s-casein-derived peptide fractions have greater binding capacity of Ca^{2+} than the β-casein peptide fractions at a high total

calcium concentration. At a low total calcium concentration, the binding patterns are similar (Park and Allen, 1998; Park *et al.*, 1998). This is probably due in part to the reduced number of phosphoseryl residues in β-casein. Other side-chains, such as glutamic and aspartic acids, may also contribute to metal binding.

In addition, peptides binding different minerals have been found in whey proteins, i.e., from β-lg, α-la and LF. Since these proteins are not phosphorylated, the minerals seem to bind through other binding sites than caseins. Seventeen (17) different peptides have been identified by hydrolysis of β-lg with thermolysin using two different concentrations of calcium. Also, peptides from α-la and LF using trypsin, chymotrypsin or pepsin have been reported. Studies with β-lg and α-la peptides have shown a higher affinity for iron than the native proteins (Vegarud *et al.*, 2000).

7. Peptides with other bioactivities

Several peptides derived from various food proteins exhibit ileum-contracting activity. Yamauchi (1992) reported that two peptides derived from serum albumin and β-lg induce the contraction of the guinea pig ileum longitudinal muscle when the test is completed without electric stimulation in the absence of an agonist. The peptides, referred to as "peptides acting on smooth muscle", contain serum albumin f(208-216) (albutensin A) and β-lg f(146-149) (β-lactotensin). Digestion of β-lg with chymotrypsin has been found to produce β-lactotensin, whose effect in the guinea pig ileum is similar to that of β-lactorphin (Pihlanto-Leppälä *et al.*, 1997). Takahashi *et al.* (1994) isolated a peptide, oryzatensin, showing ileum-contracting and immunostimulating activity, from the tryptic digest of rice-soluble protein. The ileal contracting was biphasic and the rapid contraction was mediated through a histamine release and the slow one by a prostaglandin E_2-like substance. Furthermore, this peptide showed affinity for C3a receptors (Takahashi *et al.*, 1996).

Some of the peptides derived from milk proteins have more than one functional role, e.g., peptides from the sequence 60–70 of β-casein show immunostimulatory, opioid and ACE-inhibitory activities. This sequence has been defined as a strategic zone (Migliore-Samour and Jolles, 1988). The sequence is protected from proteolysis because of its high hydrophobicity and the presence of proline residues.

In addition to the strategic zone, some other multifunctional peptides can be liberated from milk proteins. Peptide inhibitors of ACE may also have immune-system stimulatory activity. ACE catalyses the inactivation of bradykinin, which is able to stimulate macrophages to enhance lymphocyte migration and increase the secretion of lymphokines

(Paegelow and Werner, 1986). It has further been reported that α_{s1}-casein f(194-199) has immunomodulatory and ACE-inhibitory activity (Maruyama *et al.*, 1987; Migliore-Samour *et al.*, 1989). Also, the opioid peptides α- and β-lactorphin have been found to exhibit ACE-inhibitory activity (Chiba and Yoshikawa, 1986; Mullally *et al.*, 1996; Nurminen *et al.*, 2000). An opioid antagonist peptide derived from κ-casein, casoxin C, has activity towards C3a receptors and shows phagocyte-stimulating activity (Takahashi *et al.*, 1994, 1997). The complement C3a is cleaved from C3 upon activation of the complement system; this is an important inflammatory mediator in host defense (Hugli, 1989). A human α_{s1}-casein-derived peptide, casoxin D (Tyr-Val-Pro-Phe-Pro-Pro-Phe), possesses various kinds of activity, such as opioid antagonist, ileum-contracting and ACE-inhibitory activities (Yoshikawa *et al.*, 1994). As phosphopeptides also have immunomodulatory properties (Hata *et al.*, 1998, 1999), it can be concluded that many of the known bioactive peptides have more than one functional property, at least *in vitro*.

Recent studies have shown that antioxidative peptides can be released from casein. Casein-derived peptides have been shown to have free radical scavenging activity to inhibit enzymatic and non-enzymatic lipid peroxidation (Suetsuna *et al.*, 2000; Rival *et al.*, 2001a,b). Nagaoka *et al.* (2001) identified a novel hypocholesterolemic peptide (Ile-Ile-Ala-Glu-Lys) from the tryptic hydrolysate of β-lg. This peptide suppressed *in vitro* cholesterol absorption by Caco-2 cells and elicited hypocholesterolemic activity *in vivo* in rats upon oral administration of the peptide solution. In the test group, total cholesterol levels in serum were significantly lower, whereas HDL cholesterol concentration and the atherogenic index were significantly higher than in the control groups fed a casein tryptic hydrolysate or β-sitosterol containing diet. The mechanism of the hypocholesterolemic effect remains to be elucidated.

In addition to biological activities, peptides play an important role in the development of flavor in protein-rich foods, such as cheese, meat, sausage and fermented soy products. Hydrolysed vegetable proteins contain savoury flavor which is assumed to be caused by a high content of free amino acids, low MW peptides, salt and organic acids. Savoury peptides contain high molar contents of glutamic acid and hydrophilic amino acid residues (Arai *et al.*, 1972; Aaslyng *et al.*, 1998). Noguchi *et al.* (1975) reported that, for example, acid peptides Glu-Asp-Glu, Asp-Glu-Ser and Ser-Glu-Glu found in fish protein hydrolysate, had savoury properties similar to those of sodium glutamate. Comparison of the taste profiles of different wheat gluten enzymatic hydrolysate revealed that acid-deaminated wheat gluten elicited an intense glutamate-like taste. From the hydrolysate four pyroglutamyl peptides were identified:

pGlu-Pro-Ser-, pGlu-Pro, pGlu-Pro-Glu and pGlu-Pro-Gln. Apparently, these peptides were formed by cyclization of the N-terminal glutamine residues during the preparation of the hydrolysates, and were, at least, partly responsible for the glutamate-like taste (Schlichtherle–Cerny and Amadó, 2002).

On the other hand, peptides have also been described as being responsible for the undesirable bitter tastes of cheese and enzymatically hydrolysed fish, soybean and corn proteins (Saha and Hayashi, 2001). The bitter taste is due to the formation of low MW peptides, consisting of 2–23 amino acid residues or in the molecular range 500–3000 Da, composed mainly of hydrophobic amino acids. The bitter peptides are not a major component; they account for only 5–10% of the weight of the hydrolysate. Trypsin, for example, produces a casein hydrolysate in which most of the bitter taste can be ascribed to only one peptide, β-casein f(203-209) (Gly-Pro-Phe-Pro-Ile-Ile-Val) (Matoba *et al.*, 1970). In cheese, lactococcal proteinases and rennet are responsible for the formation of bitter peptides from caseins in Cheddar cheese. Bitter peptides can be degraded to non-bitter peptides and amino acids by peptidases; accordingly, the overall bitterness intensity depends on the rate of formation and degradation of the bitter peptides. β-casein f(193-209) is an important bitter peptide which can be found in Gouda and Cheddar cheese. The bitter recognition level was reported to be 0.35 mg/ml (Smit *et al.*, 2000; Soeryapranata *et al.*, 2002). Seki *et al.* (1996) hydrolysed 12 different food proteins by *B. licheniformis* alkaline proteases to peptides of average chain length 2.26–4.02. Hydrophobic amino acids situated in the interior of protein molecules were exposed by fragmentation and the peptides containing hydrophobic amino acid residues were found in aqueous solution. The peptides from casein showed the highest hydrophobicity and most bitter taste. Several bitter-tasting hydrophobic peptides with 3–6 amino acid residues were isolated from soybean protein hydrolysed by alcalase. The peptides were predominantly composed of hydrophobic amino acids and with leucine, valine or tyrosine at the C-terminal part (Kukman *et al.*, 1995). Henriksen and Stahnke (1997) performed sensory and chromatographic evaluation of water-soluble fractions from dried sausages. They found that bitterness was dependent on the level of hydrophobic amino acids present in these fractions. A number of approaches have been tried to remove the bitterness of protein hydrolysates, for example, by extraction of bitter peptides with organic solvents, hydrophobic interaction chromatography or activated carbon. Bio-based methods include further hydrolysis of bitter peptides with enzymes such as aminopeptidase, alkaline/neutral protease and carboxypeptidase, condensation of bitter peptides using protease, and use of *Lactobacillus* as a de-bittering starter adjunct (Stevenson *et al.*, 1998; Saha and Hayashi, 2001).

B. POTENTIAL PHYSIOLOGICAL IMPORTANCE

1. Liberation and fate of peptides in vivo

To exert their physiological effects *in vivo*, bioactive peptides must be released during intestinal digestion and then reach their target sites at the luminal side of the intestinal tract or, after absorption, in the peripheral organs. Figure 1 presents a scheme of the intestinal assimilation of protein and the routes of bioactive peptide liberation.

The GI tract of humans contain a number of enzymes involved in the hydrolysis of proteins and peptides and they are located in a number of sites. It is important to recognize that peptidase enzymes never occur alone. Throughout the GI tract, there is always a mixture of peptidases working synergistic. The main event in the intraluminal digestion of proteins consists of cleavage of polypeptides by pancreatic proteases, such as trypsin,

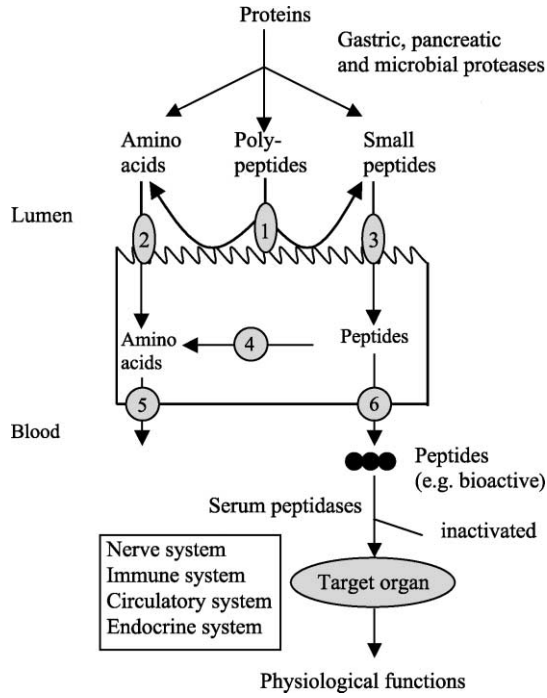


FIG. 1 Digestion and absorption of proteins in the small intestine. (1) Brush-border peptidases, (2) brush-border amino acid transport systems, (3) brush-border peptide transport systems, (4) cytoplasmic peptidases, (5) basolateral amino acid transport systems, (6) basolateral peptide transport systems.

chymotrypsin, elastase and carboxypeptidase. Furthermore, the microorganisms of the colon produce large numbers of peptidase enzymes in considerable quantities that participate in protein digestion. As a result of intraluminal digestion, the brush-border membrane of the enterocyte is faced by a mixture of oligopeptides and free amino acids. The brush-border has to clear the products of intraluminal protein digestion, essentially by means of two mechanisms: brush-border hydrolysis of oligopeptides with subsequent transport of the resulting free amino acids, and membrane translocation of small peptides with subsequent hydrolysis of these peptides by cytosolic peptidases. There are a large number of peptidases found in the brush-border membrane of the enterocyte, mainly belonging to four classes: endopeptidases, aminopeptidases, carboxypeptidase and dipeptidases (Gardner, 1984 and Woodley, 1994). Studies in which bovine milk proteins were incubated under conditions imitating GI digestion have demonstrated the release of, e.g., β-casomorphins, α-casein exorphins and casoxins (Zioudrou *et al.*, 1979; Petrilli *et al.*, 1984; Chang *et al.*, 1985; Yoshikawa *et al.*, 1986). In addition, the contents of the small intestine have been examined both in animal and in human studies after ingestion of milk proteins. Moreover, several studies have already provided evidence for the liberation of β-casomorphins, CPPs and immunostimulatory peptides from casein into the intestinal lumen of mammals after ingestion of milk or a diet containing casein (Naito *et al.*, 1972; Sato *et al.*, 1983; Meisel, 1986; Meisel and Frister, 1989; Scanff *et al.*, 1992). An antimicrobial peptide, lactoferricin B, has been detected in the gastric content of rats fed bovine LF. This finding indicates that active peptides of LF can be generated by gastric pepsin digestion *in vivo* (Tomita *et al.*, 1994).

The small intestine is the principal site of protein absorption. Within the small intestine, there are regional variations in the absorptive capacities for protein digestion products. Additionally, the end-products, amino acids and peptides, are absorbed by different mechanisms. The ability of the small intestine to absorb amino acids and peptides varies significantly due to several factors. These variations are seen during development, pregnancy and lactation, and also in response to diseases, intestinal secretion, and the quantity and quality of the diet. The results using electrophysiological methods, during the 1970s and 1980s have suggested the existence of a peptide transport system in the intestinal epithelium by which peptides would be actively transported through the apical membrane under a H^+ gradient (Canapathy and Leibach, 1985; Hoshi, 1986). However, this transport mechanism carries only di- and tripeptides (Daniel *et al.*, 1992). The peptide transporter protein has been cloned from the intestine and the results confirmed the specificity of this transporter (Fei *et al.*, 1994). Oligopeptides with more than four residues are hardly, if at all, recognized by this

transporter system. Three different transport routes, namely paracellular, fluid-phase and adsorptive transcytosis, may participate in oligopeptide transport across the intestinal epithelium (Burton *et al.*, 1992; Pappenheimer *et al.*, 1994; Shimizu *et al.*, 1997). The contribution of each route must be different among the peptides, depending on the molecular size and other structural properties such as hydrophobicity.

Di- and tripeptides, such as immunopeptides and several ACE-inhibitors, may pass across the intestine in quantitatively significant amounts to reach peripheral target sites. After absorption in the intestinal tract, serum peptidases can further hydrolyse the peptide bonds. Resistance to peptidase degradation may, in fact, be a prerequisite for a physiological effect following oral ingestion and/or the intravenous infusion of biologically active peptides/hydrolysates. The absorption and degradation of natural β-casomorphins and their analogues have been studied intensively. Natural β-casomorphins have been demonstrated to resist gastric and pancreatic proteolytic enzymes based on their high proline content (Brantl *et al.*, 1979; Henschen *et al.*, 1979). Studies have shown no intact transepithelial passage of β-casomorphins, however, which were rapidly degraded by the intestinal or blood enzymes (Kerchner and Geary, 1983; Tomé *et al.*, 1987; Mahé *et al.*, 1989; Read *et al.*, 1990). Nevertheless, β-casomorphin immunoreactive material was found in the plasma of newborn calves, dogs and human infants after ingestion of bovine milk (Umbach *et al.*, 1988; Singh *et al.*, 1989; Storm, 1990), as well as in the brain stem of the human infant (Pasi *et al.*, 1993). Indirect evidence suggests the presence of β-casomorphins in the intestinal contents of humans after milk ingestion, whereas milk-derived opioid peptides do not seem to permeate into the cardiovascular compartment in more than negligible amounts in adult mammals (Svedberg *et al.*, 1985). Koch *et al.* (1988, 1994) detected β-casomorphin immunoreactive material in the plasma of pregnant women and in the plasma after parturition. On the other hand, Teschemacher *et al.* (1986) did not find β-casomorphins in human plasma after ingestion of milk, since the enzymatic degradation of peptides in the intestinal wall and in the blood appeared to prevent it. Accordingly, β-casomorphin peptides are likely to be destroyed before crossing the intestinal lining and reaching opioid receptors. In addition, two antithrombotic peptides derived from κ-casein have been detected in the plasma of newborn infants after ingestion of a cow's milk-based formula or human milk (Chabance *et al.*, 1995).

2. *Effects on the nervous system*

Opioid peptides can be considered as compounds having possible effects on the nervous system. The existence of μ, κ and δ opioid receptors in the central

nervous system is well documented. As opioid receptor ligands, these peptides can be expected to behave like other opioids, i.e., to act as agonists or antagonists, to bind to receptors and to elicit effects in all cells or tissues where opioids are known to be active. Natural β-casomorphins show a preferential affinity for the μ-receptors (Teschemacher *et al.*, 1994). In rats β-casomorphins have been found to cause analgesia (Brantl *et al.*, 1981; Grecksch *et al.*, 1981; Widy-Tyskiewicz and Czlonkowski, 1989), apnea (Hedner and Hedner, 1987) as well as changes in the sleep of neonatal rats (Taira *et al.*, 1990). Naloxone pre-treatment reverses these effects, suggesting that opioid μ-receptors are involved. Blass and Blom (1996) demonstrated that the behavior of infant rats is sensitive to elevations in central β-casomorphin concentration and that the effectiveness against pain is mediated through central opioid pathways. These effects have been demonstrated by intracerebral, intraperitoneal or intraventicular injections. On the other hand, it has been found that oral milk infusion also causes analgesia reversed by naloxone in rats (Blass and Fitzgerald, 1988). A casein hydrolysate containing a decapeptide as its active component has been shown to reveal anxiolytic-like activity *in vivo*, both in animal and human studies (Lefranc, 2002).

3. *Effects on the GI tract*

Opiates are reported to influence GI function in two ways: first, they affect smooth muscle, which reduces the transit time, and second, they affect the intestinal transport of electrolytes, which explains their antisecretory properties (Wüster *et al.*, 1981).

It has been shown that casomorphins inhibit intestinal motility in isolated segments of the rat ileum (Allescher *et al.*, 1994). In healthy human volunteers, morphiceptin has been shown to delay the GI transit time (Schulte-Frohlinde *et al.*, 1994). Furthermore, an *in vivo* study showed that luminal administration of morphiceptin had a significant antisecretory effect at micromolar concentrations in rats (Erll *et al.*, 1994). The enhancement of net water and electrolyte absorption by β-casomorphin in the intestine leads to antidiarrhoeal action (Daniel *et al.*, 1990, 1991). This action seems to depend on the transfer of intact peptides from the luminal to the blood side of the tissue where the opioid receptors are located. The action is prevented by the hydrolysis of natural peptides. Tomé *et al.* (1988) proposed that a β-casomorphin analogue acts through a neuromediated mechanism, since it is inhibited by a neurotoxin. This is consistent with earlier findings showing that opiate receptors are not present on the enterocyte membrane of the rabbit intestine, but are mainly located in the submucosal and myenteric plexuses (Binder *et al.*, 1984).

4. *Antihypertensive effect*

The inhibition of ACE, located in different tissues (e.g., plasma, lung, kidney, heart, skeletal muscle, pancreas, brain) may influence various regulatory systems (Ondetti and Cushman, 1984). ACE plays a pivotal role in two independent humoral systems that affect blood pressure, since it is responsible for the generation of a vasopressor agent, angiotensin II, and for the inactivation of a vasodepressor agent, bradykinin. Many specific ACE-inhibitors have been developed for use as a potent, orally administered antihypertensive drug (Wyvratt and Patchett, 1985).

A number of studies have been carried out on the antihypertensive effect of ACE-inhibitory peptides in SHR (Table V). For example, intraperitoneal or oral administration of casein hydrolysates and oral administration of ACE-inhibitory peptides (α_{s1}-casein f(23-34), f(194-199) or β-casein f(177-183)) decreased blood pressure in SHR but not in normotensive rats (Yamamoto *et al.*, 1994). Moreover, cheese whey digested with proteinase K had a depressive effect on SBP, with the highest antihypertensive activity being found with the tripeptide Ile-Pro-Ala derived from β-lactoglobulin (Abubakar *et al.*, 1998). Yamamoto *et al.* (1999) observed a strong antihypertensive effect in SHR after oral administration of whey from a yoghurt-like product where a dipeptide (Tyr-Pro) was formed upon fermentation with a *L. helveticus* CPN4 strain. However, the ACE-inhibitory activity of this peptide was not strong. Nurminen *et al.* (2000) demonstrated that subcutaneous administration of a synthetic tetrapeptide, α-lactorphin (Tyr-Gly-Leu-Phe), dose-dependently lowered the systolic and diastolic blood pressure in SHR as well as in normotensive Wistar–Kyoto rats. The antihypertensive effect of a Japanese commercial fermented milk has been demonstrated both in SHR and in mildly hypertensive humans (Hata *et al.*, 1996; Takano, 1998). This product contains two ACE-inhibitory tripeptides (Val-Pro-Pro and Ile-Pro-Pro) which are formed from β-casein and κ-casein by fermentation of milk with *L. helveticus* and *S. cerevisiae*. Nakamura *et al.* (1995b) found that oral administration of 5 ml of above-mentioned fermented milk/kg of body weight (BW) significantly decreased SBP in SHR, and the peptides showed dose-dependent activity up to a dosage of 5 mg/kg BW. Neither the peptides alone nor the fermented milk changed the SBP of normotensive rats. Masuda *et al.* (1996) detected these tripeptides in the aorta of SHR after oral administration of the same fermented milk. Furthermore, the ACE activity in fractions from the aorta was lower in the rats given fermented milk than in the control group. The tripeptides were thus absorbed directly, reached the abdominal aorta, inhibited ACE and displayed antihypertensive activity. A placebo-controlled human study showed that the blood pressure of mildly hypertensive subjects decreased significantly

TABLE V

SELECTED MILK PROTEIN-DERIVED PEPTIDES WITH ANTIHYPERTENSIVE ACTIVITY

Peptide	Origin	Preparation	ACE-inhibitory activity (μM)[a] IC_{50} μM	Antihypertensive oral dose in SHR[b] (mg/kg)	Maximal decrease in SBP,[c] (mean ± SEM) (mmHg)	References
TTNPLW	α_{s1}-cn f(194-199)	Casein + trypsin	16	100	14 ± 4	Karaki
FFVAPFPEVFGK	α_{s1}-cn f(23-34)		59	100	34 ± 13	*et al.* (1990)
AVPYPQR	β-cn f(177-183)		15	100	10 ± 1	
YKVPQL	α_{s1}-cn f(104-109)	Casein +	22	1	12 ± 5	Maeno
KVLPVPQ	β-cn f(169-175)	*L. helveticus* proteinase	1000	2	32 ± 6	*et al.* (1996)
VPP	β-cn f(74-76), f(84-86)	*L. helveticus* fermentation	9	5	20 ± 2	Nakamura *et al.* (1995a,b)
IPP	κ-cn f(108-110)		5	5	18 ± 4	
YP	α-, β- and κ-cn	*L. helveticus* CPN4 fermentation	720	10	32 ± 7	Yamamoto *et al.* (1999)
YGLF	α-la f(50-53)	α-la + pepsin	733	0.1	23 ± 4	Nurminen *et al.* (2000)
IPA	β-lg f(78-80)	Whey + proteinase K	141	8	31	Abubakar *et al.* (1998)
RPKHPIKHQ	α_{s1}-cn f(1-9)	Gouda cheese	13	6.1–7.5	9 ± 5	Saito *et al.* (2000)

[a] IC_{50} μM = peptide concentration required to inhibit ACE (angiotensin converting enzyme) by 50%.
[b] Spontaneously hypertensive rat.
[c] Systolic blood pressure.

between 4 and 8 weeks after daily ingestion of 95 ml of the above-mentioned fermented milk (Hata *et al.*, 1996). In the placebo group, no major changes in blood pressure were observed. The antihypertensive effect *in vivo* of milk-derived peptides has been supported by recent studies of Sipola *et al.* (2001, 2002a). A long-term (up to 12 weeks) intake of a *L. helveticus* fermented milk containing the tripeptides Val-Pro-Pro and Ile-Pro-Pro attenuated significantly the development of hypertension in young SHR, whereas skim milk intake did not affect the blood pressure. The effect was detectable after 6 weeks of treatment. Furthermore, Seppo *et al.* (2002) demonstrated that a daily ingestion of 150 ml of this fermented milk for 8 weeks decreased the blood pressure in slightly hypertensive human subjects.

The results obtained in above studies suggest that the ACE-inhibitory peptides are absorbed from the digestive tract, that they inhibit endogenous ACE activity and that they decrease the blood pressure. The importance of food proteins for cardiovascular function may, therefore, not only be that they support the maintenance of the blood vessel walls but also that they inhibit ACE activity and help to maintain normal blood pressure. Besides ACE inhibition, other mechanisms may also be involved in the blood pressure-lowering action of various peptides. For example, the blood pressure-lowering mechanism of α-lactorphin is not ACE inhibition, but rather appears to be due to an interaction with opioid receptors, since the response can be antagonised by pre-treatment with naloxone (Nurminen *et al.*, 2000; Sipola *et al.*, 2002b). Several studies have demonstrated the cardiovascular effect of endogenous opioids (Feuerstein and Siren, 1987; Widy-Tyszkiewicz and Czlonkowski, 1991; Wang and Ingenito, 1994; Chu *et al.*, 1999). It is difficult to clarify the mechanism of this phenomenon because of the numerous opioid peptides and receptor subtypes, and is, therefore, at least partly unknown.

5. Effect on the defense mechanism

The systems involved in the defense mechanism of the body are both varied and complex. Investigating the role of biologically active peptides has proved to be a very promising line of research. The main focus is on two peptide groups, namely on immunomodulatory (stimulating the immune response) and antimicrobial (inhibiting pathogenic microbes) peptides.

The immunomodulatory effect of many peptides has been demonstrated *in vitro* in a number of studies (Migliore-Samour *et al.*, 1989; Fiat *et al.*, 1993; Kayser and Meisel, 1996; Sütas *et al.*, 1996a,b). Immunomodulatory milk peptides affect both the immune system and the cell proliferation responses. With regard to *in vivo* effects, there is only a very limited amount of

information available. Parker *et al.* (1984) observed increased resistance to *K. pneumoniae* in rats treated intravenously with a hexapeptide derived from human β-casein. The Tyr-Gly and Tyr-Gly-Gly peptides, potentially derived from κ-casein and α-la, have been found to be active in a dialysed leukocyte extract from normal donors. These peptides modulate the lymphokines production *in vitro* and enhance dermal skin test responses *in vivo* when given intracutaneously with a tetanus toxoid antigen. Encouraging results have been obtained after a bi-weekly treatment of 93 patients with an AIDS-related syndrome; the patients showed a significantly reduced tendency to progress to a clinically relevant endpoint or to AIDS (Hadden, 1991).

Various antimicrobial peptides have been shown to inhibit *in vitro* the growth of many pathogenic and non-pathogenic microbes. In particular, lactoferricin, a peptide derived from LF by pepsin digestion, has been found to display antimicrobial activity *in vitro* against both Gram-positive and Gram-negative microorganisms (Bellamy *et al.*, 1992a,b; Jones *et al.*, 1994). α_{s1}-Casein f(1-23), isracidin, obtained by chymosin hydrolysis, has been shown to protect mice against *S. aureus* and *C. albicans* at concentrations comparable with known antibiotics. Field trials have indicated that the injection of isracidin into the udder gives protection against mastitis in sheep and cows (Lahov and Regelson, 1996). Moreover, bactericidal peptides may assist in protecting against microbial challenge, especially in the neonatal intestinal tract, and thus support the non-immune defense of the gut (Jelen, 1992). These results have been obtained by parenteral administration of the peptides, but for the moment there are no studies available to demonstrate the antimicrobial effect when the peptides are given orally.

6. *Effect on mineral absorption*

Several studies have been performed during the last two decades on CPPs which may function as carriers for different minerals, especially calcium. Published data on the effect of CPP/casein on mineral solubility and absorption are inconsistent, partly due to the diversity of the experimental approaches. Most of the findings in the literature that deal with the mineral absorption-stimulating effect of CPP are based on *in vitro*, *in situ*, cell culture or single meal studies. Majority of the studies have been done with rats and have provided considerable evidence for the potential effect of casein-derived phosphopeptides to improve mineral absorption. This potential is not limited to calcium but is also valid for zinc and iron, and possibly other elements that have not been investigated so far (FitzGerald, 1998). Furthermore, CPPs have been shown to have anticariogenic properties, based on their ability to localise amorphous phosphate in dental plaque (Reynolds, 1998).

Data obtained by *in situ* loop techniques demonstrate that CPPs increase the intestinal Ca^{2+} absorption (Mykkänen and Wasserman, 1980; Lee *et al.*, 1980, 1983; Kitts and Yuan, 1992; Kitts *et al.*, 1992). In the rat pup, not only calcium but also zinc absorption was improved after gastric intubations of Ca-containing CPP in the presence of phytate. No effect was found when a Na-containing CPP preparation or casein or whey protein was added (Hansen *et al.*, 1996). In balance studies with weaning rats, CPP supplementation has not shown any influence on intestinal Ca^{2+} absorption (Pointillart and Guéguen, 1989; Yuan and Kitts, 1991). Saito *et al.* (1998) demonstrated that CPP supplementation enhanced Ca absorption under conditions of marginal dietary calcium. Accordingly, they suggested that the Ca content of the diet might be an important factor to determine the effect of CPP on Ca absorption. However, studies that have failed to find an effect of CPP on Ca absorption have used both high and low levels of dietary Ca (Kopra *et al.*, 1992; Tsuchita *et al.*, 1993). Bennett *et al.* (2000) showed that Ca absorption was enhanced by high-casein meals in rats, but at high-dietary casein intakes the Ca absorption efficiency was reduced, probably either due to adaptation in the active trans-cellular Ca transport or acceleration in the rate of gastric emptying. Tsuchita *et al.* (2001) recently indicated that the addition of CPP to Ca-fortified milk could increase Ca absorption in young male rats. In contrast, no effect of extrinsic CPP on Ca absorption was apparent when the animals were given unfortified milk. Increasing the amount of soluble Ca is most probably the key mechanism to augmenting Ca absorption from the small intestine. Therefore, it is effective to move CPP simultaneously with Ca from the stomach to the small intestine, where the interaction between CPP and Ca would take place. Good availability of Ca has been shown when all dietary Ca is bound to CPP in advance (Tsuchita *et al.*, 1996).

There is controversy also in reports from studies on human subjects. For example, Hansen *et al.* (1997) found that Ca absorption from high- or low-phytate meals was not significantly influenced by the addition of CPP in healthy adult subjects. Heaney *et al.* (1994) reported that CPP administration was associated with better absorption of co-ingested Ca by postmenopausal women with low basal absorptive performance. This finding suggests that CPP supplementation is particularly useful for persons whose basal absorptive performance is low.

It has been further reported that not only the calcium metabolism but also other minerals and other aspects of mineral status may be influenced by CPPs. Ait-Oukhatar *et al.* (1997) found that in young iron-deficient rats, CPP-bound iron had a positive effect on some parameters of iron status and metabolism, such as mean cell volume, haemoglobulin and haematocrit, and a negative effect on some parameters, such as urine iron. Other parameters,

like iron absorption of red blood cells, were not affected. The authors concluded that binding iron to CPP seemed to improve its bioavailability, a finding recently confirmed by Peres *et al.* (1999) and Ait-Oukhatar *et al.* (2000). In addition, Chaud *et al.* (2002) showed that iron from iron–peptide complex was transferred to the blood in a dose-dependent manner, and the serum iron levels were significantly higher than in a similar group of rats treated with iron sulfate or free peptide with iron sulfate. These results suggest the iron–peptide complex is a potential compound for use as an iron source in biological situations.

CPPs have been shown to stabilise amorphous calcium phosphate (ACP), and may, thus, be used to localise ACP in dental plaque, maintaining a state of supersaturation with respect to tooth enamels, reducing demineralization and enhancing remineralization (Reynolds, 1998). Studies by Rose (2000a,b) have shown that CPP–ACP binds well to dental plaque, providing a large calcium reservoir which is likely to restrict mineral loss during a cariogenic episode and to provide a potential source of calcium for subsequent remineralisation. Overall, once in place, the CPP–ACP will restrict the progress of caries. Another interesting property associated with CPPs is their potential to enhance mucosal immunity. In a recent study, Otani *et al.* (2000) reported that oral administration of a commercial caseinphosphopeptide preparation enhanced the intestinal IgA levels in piglets.

IV. TECHNOLOGICAL PROCESSES FOR THE PRODUCTION OF BIOACTIVE PROTEINS AND PEPTIDES

A. FRACTIONATION TECHNIQUES OF BIOACTIVE PROTEINS

Most of the biologically active proteins occur naturally in relatively low concentrations and may, therefore, not be potent enough to produce beneficial effects *in vivo*, as expected. Also, traditional isolation techniques, such as extraction with organic solvents or coagulation with strong acids or alkalines, may denature the desirable proteins, thus making them inactive. Consequently, it has been necessary to develop appropriate novel technologies for the isolation and enrichment of bioactive proteins in an active form. The advent of membrane separation techniques in the 1970s has contributed to the commercial production of whole-whey protein products, e.g., WPC with protein contents of 30–80%. Basic membrane separation processes, such as reverse osmosis, ultrafiltration (UF) and diafiltration, are now industrially applied to the manufacture of ordinary whey powder and WPCs. The development of industrial-scale gel filtration and ion-exchange

chromatography techniques has made it possible to manufacture whey protein isolates (WPI) with protein contents of 90–95%. These well-established technologies have been reviewed in several articles (Jelen, 1992; Morr and Ha, 1993; Rosenberg, 1995; Timmer and van der Horst, 1998). A more recent technique, nanofiltration, allows the selective separation of salts and ions from whey. By means of this technique, it has become possible to manufacture industrially demineralised and highly fractionated whey protein ingredients. The chemical composition and functionality of whey protein preparations are largely affected by the method used in the process (Kinsella and Whitehead, 1989; Mangino, 1992; Mulvihill, 1992; Kilara, 1994; Korhonen *et al.*, 1998a). Due to the inconsistent functionality of the WPCs and WPIs, they have found only a limited range of applications, mainly in the dairy, bakery and meat industries (Kelly and McDonagh, 2000).

Techniques for isolating individual whey proteins have now progressed from laboratory-scale to large-scale processing. There is, however, a need to improve the chemical purity of the commercial protein products available. Different combinations of heat precipitation and UF using selective membranes have been applied for the fractionation of β-lg and α-la in enriched or purified form (Pearce, 1983; Maubois and Ollivier, 1997; Maubois, 2000). In this process, α-la undergoes isoelectric precipitation at pH 4.2 and at 55–65°C due to the dissociation of calcium ions and hydrophobic interactions. Other minor whey proteins also precipitate under these conditions, while β-lg remains soluble and can be separated, concentrated by membrane methods, and finally dried. The purity of the α-la and β-lg preparations manufactured by these techniques ranged from 50 to 80% and 60 to 99%, respectively (Timmer and van der Horst, 1998). In addition to selective membrane separation, ion-exchange chromatography using basic silica and polystyrene anion resins has been employed successfully for the fractionation of β-lg from whey in a pilot-scale process (Outinen *et al.*, 1996). Other novel methods developed for the separation of the above major whey proteins are based on enzymatic hydrolysis of whey proteins by pepsin and concentration of β-lg, which is resistant to pepsin, by UF (Kinekawa and Kitabatake, 1996; Konrad *et al.*, 2000; Sannier *et al.*, 2000). Other recent techniques developed for the separation of whey proteins are based on radial flow chromatography columns, i.e., the Sepralac TM-process (Ahmed, 1998), on ion-charged chromatographic membranes and beads in columns (Timmer and van der Horst, 1998), and on anion-exchange binding material (de Jongh *et al.*, 2001).

There is considerable commercial interest currently in the isolation of biologically active minor whey proteins, such as LF, LP, Ig and GMP. Over the past decade, a number of patented pilot- or industrial-scale methods have been developed for the purification or enrichment of these compounds

from colostral or cheese whey (Mulvihill and Fox, 1994; de Wit and Hoojdonk, 1996; Maubois and Ollivier, 1997). Large-scale production techniques based on cation-exchange resins followed by gel filtration or UF have been developed for the isolation of LF and LP from cheese whey (Burling, 1994; Uchida *et al.*, 1996). Chiu and Etzel (1997) successfully used a microporous membrane containing immobilised sulfonic acid moieties to fractionate LP and LF from cheese whey. Recovery rates were 73 ± 6% for LP and 50 ± 5 for LF. The membrane system was more rapid, smaller and used a higher flow rate than traditional bead-based systems. Also, several chromatographic or membrane separation methods have been devised for the isolation of GMP (Kawasaki *et al.*, 1994; Outinen *et al.*, 1995; Abd El-Salam *et al.*, 1996; Maubois, 2000). Similar techniques have been applied for the separation of immunoglobulins from colostral or cheese whey (Stott and Lucas, 1989; Fukumoto *et al.*, 1994a,b; Korhonen *et al.*, 1998b). All the above-mentioned proteins are nowadays commercially available as separate ingredients and have found applications in specific products, such as infant formulas, clinical diets, colostral supplements and toothpaste, and as a preservative of raw milk (Horton, 1995; Kelly and McDonagh, 2000; Steijns, 2001).

The recent introduction on the market of a new category of health-promoting functional food has created a need to develop appropriate novel technologies in order to optimise the desired beneficial properties of bioactive ingredients. To this end, novel techniques, such as high hydrostatic pressure, supercritical fluid extraction, microencapsulation and pulsed electric field techniques may provide feasible options in the future (Korhonen, 2002).

B. ENRICHMENT OF SPECIFIC PEPTIDE FRACTIONS

So far, the most common way to produce bioactive peptides has been enzymatic digestion using different scales and techniques. Hydrolysis can be performed by conventional batch hydrolysis or by continuous hydrolysis using UF membranes. The traditional batch method has several disadvantages, such as the relatively high cost of the enzymes and their inefficiency compared to a continuous process, as noted in numerous studies (Mannheim and Cheryan, 1990; Chiang *et al.*, 1995). UF membrane reactors have been shown to improve the efficiency of enzyme-catalysed bioconversion, to increase product yields, and to be easily scaled-up. Furthermore, UF membrane reactors yield a consistently uniform product with desired molecular mass characteristics (Mehaia and Cheryan, 1990). UF steps using low molecular mass cut-off membranes may be useful to separate small peptides from high molecular mass residues and remaining enzymes (Figure 2).

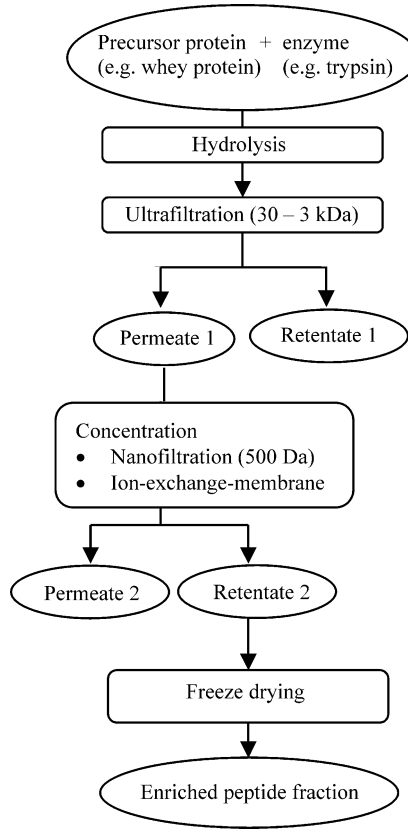


FIG. 2 Flow diagram for production and separation of bioactive peptides from food proteins obtained by enzymatic hydrolysis.

Pancreatic enzymes, preferably trypsin, have been used for the chemical characterisation and identification of many known bioactive peptides. For example, ACE-inhibitory peptides as well as CPPs are most commonly produced by trypsin (Maruyama and Suzuki, 1982; Berrocal *et al.*, 1989). On the other hand, other enzymes and different enzyme combinations of proteinases, including alcalase, chymotrypsin, pancreatin and pepsin, as well as enzymes from bacterial and fungal sources have also been utilised to generate bioactive peptides. Higher yields of CPPs and, particularly, higher amounts of α_{s1}-casein f(59-79) in the hydrolysate have been obtained with casein micelles successively digested with pepsin and trypsin

than from acid-precipitated casein and casein micelles by tryptic digestion alone (Ono *et al.*, 1998). Microbial enzymes have also been successfully used to generate ACE-inhibitory peptides (Yamamoto *et al.*, 1994; Maeno *et al.*, 1996). Murakami and Hirata (2000) produced ACE-inhibitory peptides from corn protein hydrolysed by thermolysin in an aqueous two-phase system.

After hydrolysis the peptides in the hydrolysates can be fractionated and enriched using different methods. For example, following tryptic digestion, CPPs are isolated by isoelectric precipitation of residual casein followed by scalable methods such as selective precipitation (Adamson and Reynolds, 1995) or UF (Reynolds, 1992). However, these methods produce products of low purity. Adamson and Reynolds (1995) succeeded in producing high-purity CPP by applying selective precipitation of CPPs using 100 mM $CaCl_2$ and 50% (v/v) ethanol. The most selective procedures for isolating CPPs involve chromatography, but these methods produce a fairly low CPP yield (Berrocal *et al.*, 1989; Juilleart *et al.*, 1989). Ellegård *et al.* (1999) have developed a process-scale isolation method of high-purity CPPs using acid precipitation, diafiltration and anion-exchange chromatography.

UF membranes have been successfully used to enrich specific peptide fractions (Visser *et al.*, 1989; Turgeon and Gauthier, 1990; Vreeman *et al.*, 1994; D'Alvise *et al.*, 2000). For example, an UF membrane reactor has been applied for the continuous extraction of permeates enriched with bioactive fragments in order to generate antithrombotic peptides (Bouhallab *et al.*, 1992). Pihlanto-Leppälä *et al.* (1996) applied selective UF membranes (30 and 1 kDa) for enrichment of opioid peptides, α-lactorphin and β-lactorphin, from pepsin-hydrolysed α-la, and pepsin and trypsin hydrolysed β-lg, respectively. Bordenave *et al.* (1999) demonstrated that α-lactorphin was successfully generated with continuous hydrolysis of goat whey in an UF reactor. Righetti *et al.* (1997) proposed a multicompartment enzyme reactor operating under an electric field, for the continuous hydrolysis of milk proteins. This set-up allows for continuous harvesting of some biologically active peptides, e.g., phosphopeptides and precursors of casomorphins, in a pure form using trypsin to digest β-casein. Membranes consisting of negatively charged materials have been used to desalt whey hydrolysates (Wijers *et al.*, 1998) and to enrich cationic peptides with antibacterial properties from cheese whey (Recio and Visser, 1999). Negatively charged CPPs can be isolated by anion-exchange membranes (Recio *et al.*, 2000). These techniques provide new possibilities for enriching peptides with low molecular masses and are easily up-scaled to gram or even kilogram quantities.

C. LIBERATION OF BIOACTIVE PEPTIDES DURING FOOD PROCESSING

Bioactive peptides may also be liberated during the manufacture of milk products. Hydrolysed milk proteins used for hypoallergenic infant formulas, for clinical application and as food ingredients, for example, consist exclusively of peptides (van Beresteijn *et al.*, 1994). Proteases from food itself, such as plasmin in milk, can hydrolyse proteins during food processing and storage. Bacterial starter cultures contain several proteolytic enzymes that are responsible for the breakdown of protein into peptides and amino acids. During fermentation, various long oligopeptides are liberated by degradation of caseins, which could be precursors of peptides with biological activity when cleaved by other enzymes. Intracellular peptidases of lactic acid bacteria in fermented milk products will most likely contribute to the further degradation after lysis (Thomas and Pritchard, 1987; Poolman *et al.*, 1995). The specificities of known peptidases suggest that all peptide bonds in caseins can be partially cleaved. The formation of casomorphins in fermented milk products is unlikely, since the used lactic acid bacteria all contain a X-prolyl-dipeptidyl-aminopeptidease (Law and Haandrikman, 1997). Table VI enlists experimental studies where the release of bioactive peptides has been observed upon fermentation of milk, whey or casein fractions using different live proteolytic microorganisms or proteolytic enzymes derived from such microorganisms.

Various studies have been reported on casomorphins, ACE-inhibitory peptides and phosphopeptides found in fermented milk products (Table VII). Cheese contains phosphopeptides as natural constituents (Roudot-Algaron *et al.*, 1994; Singh *et al.*, 1997), and secondary proteolysis during cheese ripening leads to the formation of other bioactive peptides, such as those with ACE-inhibitory activity (Meisel *et al.*, 1997; Smacchi and Gobbetti, 1998; Ryhänen *et al.*, 2001). Muehlenkamp and Warthesen (1996) either found no β-casomorphins at all in commercial cheese products or their concentration in the cheese extract was below 2 μg/ml. They further noted that the enzymatic degradation of β-casomorphins was influenced by a combination of pH and salt concentration at the cheese ripening temperature. Therefore, if formed in cheese, β-casomorphins may be degraded under conditions similar to Cheddar cheese ripening. Precursors of β-casomorphins, on the other hand, have been identified in Parmesan cheese (Addeo *et al.*, 1992). Matar and Goulet (1996) detected β-casomorphin-4 in milk fermented with *L. helveticus* L89 deficient in X-prolyl-dipeptidyl-aminopeptidase. Pihlanto-Leppälä *et al.* (1998) demonstrated that commercial lactic acid starters were not able to produce ACE-inhibitory peptides from whey or casein proteins, but further proteolysis with digestive enzymes produced ACE-inhibitory activity. In an

TABLE VI

RELEASE OF BIOACTIVE PEPTIDES FROM MILK PROTEINS BY VARIOUS MICROORGANISMS AND MICROBIAL ENZYMES

Substrate	Microorganisms used	Precursor protein	Peptide sequence	Bioactivity	References
Milk	*Lactobacillus helveticus*, *Saccharomyces cerevisiae*	β-cn, κ-cn	VPP, IPP	ACE-inhibitory, antihypertensive	Nakamura *et al.* (1995a); Takano (1998)
	Lactobacillus helveticus	x	x	Immunostimulatory	Matar *et al.* (1996)
	Lactobacillus GG enzymes + pepsin and trypsin	β-cn, α_{s1}-cn	YPFP AVPYPQR TTMPLW	Opioid, ACE-inhibitory, immunostimulatory	Rokka *et al.* (1997)
	Lactobacillus helveticus CPN 4	Whey proteins	YP	ACE-inhibitory	Yamamoto *et al.* (1999)
	Lactobacillus delbrueckii subsp. *bulgaricus* SS1, *Lactococcus lactis* subsp. *cremoris* FT4	β-cn, κ-cn	Many fragments	ACE-inhibitory	Gobbetti *et al.* (2000)
Whey	*Kluyveromyces marxianus* var. *marxianus*	β-lg	YLLF	ACE-inhibitory	Belem *et al.* (1999)
	Tritirachium album derived proteinase K	β-lg	IPA	Antihypertensive	Abubakar *et al.* (1998)
Casein	*Lactobacillus helveticus* CP90 proteinase	β-cn	KVLPVP (E)	ACE-inhibitory	Maeno *et al.* (1996)
Casein fractions	*Lactobacillus* GG	α_{s1}-cn, β-cn, κ-cn	x	Immunomodulatory	Sütas *et al.* (1996a,b)

x, precursor protein or active peptides not identified.

TABLE VII
BIOACTIVE PEPTIDES IDENTIFIED IN FERMENTED FOODS

Product	Examples of identified bioactive peptides	Bioactivity	References
Italian cheeses varieties: Mozzarella, Crescenza, Italico, Gorgonzola	β-cn f(58-72)	ACE-inhibitory	Smacchi and Gobbetti (1998)
Finnish cheeses varieties: Edam, Emmental, Turunmaa, Cheddar	x	ACE-inhibitory	Korhonen and Pihlanto-Leppälä (2001)
Festivo	α_{s1}-cn f(1-9), f(1-7), f(1-6)	ACE-inhibitory	Ryhänen *et al.* (2001)
Australian cheese varieties: Cheddar, Edam, Swiss, Feta, Camembert, Blue vein	x	Immunomodulatory, ACE-inhibitory, antiamnesic, opioid agonist	Dionysius *et al.* (2000)
Gouda cheese	α_{s1}-cn f(1-9), β-cn f(60-68)	ACE-inhibitory	Saito *et al.* (2000)
Parmigiano-Reggiano cheese	β-cn f(8-16), f(58-77), α_{s2}-cn f(83-33)	Phosphopeptides, precursor of β-casomorphin	Addeo *et al.* (1992)
Cheddar cheese	α_{s1}- and β-casein fragments	Several phosphopeptides	Singh *et al.* (1997)
Enzyme modified cheese	β-cn f(60-66)	Opioid activity, ACE-inhibitory	Haileselassie *et al.* (1999)
Sour milk	β-cn f(176-188)	Precursor of ACE-inhibitory	Kahala *et al.* (1993)
Sour milk	β-cn f(74-76, f(84-86), κ-cn f(108-111)	Antihypertensive	Nakamura *et al.* (1995a,b)
Yoghurt	x	Weak ACE-inhibitory	Meisel *et al.* (1997)
Soy sauce	x	ACE-inhibitory	Okamoto *et al.* (1995)

x, active peptides not identified.

earlier study (Kahala *et al.*, 1993), an ACE-inhibitory peptide (f176-188) was identified from a Finnish commercial fermented milk product.

D. BIOACTIVE PEPTIDES AS INGREDIENTS

In addition to fermented milk products, different hydrolysates containing bioactive peptides as active substances have been developed. Scientific data showing the biological activities of various peptides is growing fast and there has been a growing interest in using milk protein-derived bioactive peptides for application within the food industry. Large-scale production of the peptides will depend on the development of feasible technologies suitable for isolation and purification of the desired compounds from the mixture of various peptides likely to be produced in the hydrolysis step, as well as on overcoming the problem of low recovery from the raw material feedstock. Casein-derived peptides, which can be manufactured on industrial scale, have already been considered for interesting applications. According to present knowledge CPPs, ACE-inhibitory and immunomodulatory peptides are the preferred bioactive peptides for application in foodstuffs to provide health benefits to customers. Some examples of ingredients containing bioactive peptides and their possible applications are described below. Those products include CPPs, antihypertensive and anxiolytic peptides. Table VIII provides examples of commercial dairy products and ingredients which contain various bioactive peptides.

The divalent mineral-binding effect of CPPs can be put in use in applications where one wants to increase the availability for absorption of these minerals in the gut. Drinks with calcium and iron are examples for commercial uses of CPPs; examples can be found especially in the Japanese market. Products for children that incorporate calcium or milk minerals and CPPs in sweets or cookies are found in the South Asian market. As mineral accretion is high during early childhood, incorporation of CPPs provides good solubility and availability for absorption of calcium or zinc and thus is worth considering for infant nutrition. Other possible uses are in calcium-enriched dairy products and natural calcium supplements. In addition, dental applications are obvious, since complexes of calcium, CPPs and phosphate may reduce caries in a dose-dependent fashion.

In recent years, a few fermented dairy products with naturally occurring antihypertensive peptides have been launched in both the Japanese and Finnish market. The Japanese sour milk product "Calpis"™ is made by inoculating skim milk with a starter containing *L. helveticus* and *S. cerevisiae*. The fermented drink is rich in the peptides Val-Pro-Pro and Ile-Pro-Pro, which have proven to lower blood pressure both in animal model studies and in clinical trials with hypertensive humans (Takano 2002).

TABLE VIII

COMMERCIAL DAIRY PRODUCTS/INGREDIENTS WITH HEALTH CLAIMS BASED ON BIOACTIVE PEPTIDES

Brand name	Type of product	Claimed functional bioactive peptides	Health claims	Manufacturer	References
Calpis	Sour milk	β-casein, κ-casein, Val-Pro-Pro, Ile-Pro-Pro	Reduction of blood pressure	Calpis Co., Japan	Hata *et al.* (1996); Takano (2002)
Evolus	Calcium-enriched fermented milk drink	β-casein, Val-Pro-Pro, Ile-Pro-Pro	Reduction of blood pressure	Valio Oy, Finland	Seppo *et al.* (2002, 2003)
Bio Zate	Hydrolysed whey protein isolate	β-lactoglobulin, f (142-148)	Reduction of blood pressure	Davisco Foods International Inc., USA	Klink (2002)
Prodiet F200	Flavoured milk drink Confectionery Capsules	α_{s1}-casein f (91-100), Tyr-Leu-Gly-Tyr-Leu-Glu-Gln-Leu-Leu-Arg	Reduction of stress effects	Ingredia, France	Lefranc (2002)
Festivo	Fermented low-fat hard cheese	α_{s1}-casein f (1-9), α_{s1}-casein f (1-7), α_{s1}-casein f (1-6)	No health claim as yet	MTT Agrifood Research Finland	Ryhänen *et al.* (2001)

Another beverage containing antihypertensive dodecapeptide was recently developed in Japan (Sugai, 1998). The peptide was obtained from tryptic hydrolysates of milk casein and the antihypertensive effect was studied in animals and humans. Those studies suggested the usefulness of the peptide as an ingredient of physiologically functional foods to prevent hypertension. Furthermore, the preventive effect of the peptide against cardiovascular diseases was shown stroke-prone SHR. The product, "Casein DP", was approved by the Ministry of Health and Welfare as a FOSHU.

The Finnish fermented milk drink "Evolus" is fermented with a *L. helveticus* strain and contains the same tripeptides as "Calpis". The "Evolus" drink has been demonstrated to exert a significant reduction in blood pressure of mildly hypertensive human subjects upon daily intake of 150 ml during a 21-week intervention period (Seppo *et al.*, 2003). A fermented low-fat hard cheese "Festivo" was developed in Finland (Ryhänen *et al.*, 2001) with probiotic lactic acid bacteria and was found to produce during maturation, high amounts of ACE-inhibitory peptides derived from α_{s1}-casein. The peptides emerged at the age of three months and their level remained rather stable at least for six months.

A whey protein hydrolysate "BioZate", containing ACE-inhibitory peptide was recently developed by Davisco Foods International Inc. The effect on blood pressure was studied with 30 unmedicated, non-smoking, borderline hypertensive men and women, and daily dose was 20 g. The results indicated that there was a significant drop in both systolic and diastolic blood pressure after 1-week treatment, which persisted throughout the study of 6 weeks. The application of this product is varied and flexible. In addition to the bioactive peptides, it has functional properties such as emulsification and foaming (Klink, 2002).

Ingredia, a French dairy company, has developed "Prodiet F 200", a milk protein hydrolysate that contains a bioactive peptide with relaxing properties. The patented product has an anti-stress effect proven by several clinical studies and does not cause the classical side effects of anxiolytics. Food supplements, chocolate and animal feed are examples of its applications (Lefranc, 2002).

V. SAFETY IMPLICATIONS

The development of health-promoting foods is likely to entail increasing use of different protein sources known to contain bioactive components. They may be natural constituents of plant or animal origin, or genetically modified or transferred from another source. It is likely that, in the future, more and more traditional food products will appear on the market, containing

added protein fractions derived from different sources; e.g., yoghurt may be spiked with bioactive proteins or peptides derived from plants. Such food products might cause allergic reactions to plant-protein sensitive persons.

On the other hand, a few amino acid derivatives that are formed during food processing, such as lysinoalanine, D-amino acids and biogenic amines, may cause undesirable metabolic or even toxic events in the body (Taylor, 1986; Haláz *et al.*, 1994; Finot, 1997). Many dietary proteins may naturally pose as potential allergens, and protein-derived allergenic properties have, in fact, been mentioned as possible side-effects of genetically engineered foodstuffs (Taylor and Lehner, 1996; Pastorello, 1997; Wal, 1998; Taylor and Hefle, 2001). The development and application of novel processing and isolation techniques aimed at minimizing such health risks will play a crucial role in this respect. Also, there is a need to develop sensitive analytical methods for the detection of potential protein-derived risk factors in novel foodstuffs.

VI. RESEARCH NEEDS

The occurrence of many natural bioactive proteins or their precursors in animal and plant proteins is now well established. There are, however, a great number of scientific and technological issues to be solved before these substances can be optimally exploited for human nutrition and health. At present, there is a great need to develop novel technologies, e.g., chromatographic and membrane separation techniques, by means of which active peptide fractions can be produced and enriched from different protein sources. Further, it is important to study the technological properties of the active peptide fractions and to develop model foods, which contain these peptides and retain their activity for a guaranteed period. To this end, the potential interactions of peptides with carbohydrates and lipids as well as the influence of the processing conditions (especially heating) on peptide activity and bioavailability should be further investigated.

Particularly, the possible formation of toxic, allergenic or carcinogenic substances, such as acrylamide or biogenic amines, warrants intensive research. In this respect, modern methods need to be developed to study the safety of foodstuffs containing biologically active peptides. In addition to focus on technological aspects, molecular studies are needed to assess the mechanisms by which the bioactive proteins and peptides exert their activities. This research area is currently considered as the most challenging, due to the understanding that most of the known bioactive proteins or peptides are not absorbed as such from the GI tract into the blood circulation. Their effect is, therefore, likely to be mediated directly in the gut lumen or through

receptors on the intestinal cell wall. In this respect, the target functions of the components concerned are of utmost importance. It is anticipated that in the near future such targets will be related to various lifestyle-related disease groups, such as cardiovascular diseases, cancers, osteoporosis, stress and obesity. In particular, physiologically active peptides derived from milk or other dietary proteins offer a promising approach to prevent, control and even treat such disease conditions through a regulated diet. The most important future research needs related to bioactive proteins and peptides are summarised hereunder:

- Screening for potential bioactivity among minor proteins of milk, egg, vegetables, cereals, and fruits.
- Development of novel fractionation and purification methods for bioactive proteins and their hydrolysates.
- Study of the effects of conventional and novel processing technologies on the bioactivity of the proteins.
- Study of interactions of bioactive proteins/peptides/amino acids with other food components during processing and effects of these interactions on bioactivity.
- Study of the technological functionality of bioactive proteins, e.g., LF, immunoglobulins, egg proteins and bioactive peptides.
- Basic research on the transgenic production of bioactive proteins and the potential side-effects, e.g., allergenicity and toxicity of such proteins.
- Evaluation of the efficacy of bioactive proteins in animal model and human clinical studies *per se* and in food systems.

REFERENCES

Aaslyng, M.D., Martens, M., Poll, L., Nielsen, P.M., Flyge, H., and Larsen, L.M. 1998. Chemical and sensory characterization of hydrolyzed vegetable protein, a savory flavoring. *J. Agric. Food Chem.* **46**, 481–489.

Abd El-Salam, M.H., El-Shibiny, S., and Buchheim, W. 1996. Characteristics and potential uses of the casein macropeptide. *Int. Dairy J.* **6**, 327–341.

Abubakar, A., Saito, T., Kitazawa, H., Kawai, Y., and Itoh, T. 1998. Structural analysis of new antihypertensive peptides derived from cheese whey protein by proteinase K digestion. *J. Dairy Sci.* **81**, 3131–3138.

Adamson, N.J. and Reynolds, E.C. 1995. Characterization of tryptic casein phosphopeptides prepared under industrially relevant conditions. *Biotechnol. Bioengng*. **45**, 196–204.

Addeo, F., Chianes, L., Salzano, A., Sacchi, R., Cappuccio, U., Ferranti, P., and Malorni, A. 1992. Characterization of the 12% trichloroacetic acid-insoluble oligopeptides of Parmigiano-Reggiano cheese. *J. Dairy Res.* **59**, 401–411.

Ahmed, S., Mozaffar, Z., Saxena, V., and Miranda, Q.R. 1998. Sepralac™ process for the separation of whey proteins. *In* "Whey", Proc. Second Int. Whey Conference, Chicago, USA, 27–29 October 1997, pp. 100, IDF Special Issue 9804.

Ait-Oukhatar, N., Bouhallab, S., Bureau, F., Arhan, P., Maubois, J.-L., Drosdowsky, M.A., and Bougle, D.L. 1997. Bioavailability of caseinophosphopeptide bound iron in young rat. *J. Nutr. Biochem.* **8**, 190–194.

Ait-Oukhatar, N., Bouhallab, S., Bureau, F., Arhan, P., Maubois, J.-L., and Bougle, D.L. 2000. *In vitro* digestion of caseinophosphopeptides–iron complex. *J. Dairy Res.* **67**, 125–129.

Allescher, H.D., Piller, C., Brantl, V., and Schusdziarra, V. 1994. Effect of bovine β-casomorphin-4-amide on enteric nerve pathways of the rat ileum. *In* "β-Casomorphins and Related Peptides: Recent Developments" (V. Brantl and H. Teschemacher, eds), pp. 150–154. VCH, Weinheim.

Anantharaman, K. and Finot, P.A. 1993. Nutritional aspects of foods in relation to technology. *Food Rev. Int.* **9**, 629–655.

Antila, P., Paakkari, I., Järvinen, A., Mattila, M.J., Laukkanen, M., Pihlanto-Leppälä, A., Mäntsälä, P., and Hellman, J. 1991. Opioid peptides derived from *in vitro* proteolysis of bovine whey proteins. *Int. Dairy J.* **1**, 215–229.

Arai, S., Yamashita, M., and Fujimaki, M. 1972. Glutamyl oligopeptides as factors responsible for tastes of a proteinase modified soybean protein. *Agric. Biol. Chem.* **36**, 1253–1256.

Arihara, K., Nakashima, Y., Mukai, T., Ishikawa, S., and Itoh, M. 2001. Peptide inhibitors for angiotensin I-converting enzyme from enzymatic hydrolysates of porcine skeletal muscle proteins. *Meat Sci.* **57**, 319–324.

Ariyoshi, Y. 1993. Angiotensin-converting enzyme inhibitors derived from food proteins. *Trends Food Sci. Technol.* **4**, 139–144.

Astawan, M., Wahyuni, M., Yasuhara, T., Yamada, K., Tadokoro, T., and Maekawa, A. 1995. Effects of angiotensin I-converting enzyme inhibitory substances derived from Indonesia dried-salted fish on blood pressure of rats. *Biosci. Biotechnol. Biochem.* **59**, 425–429.

Baardsnes, J. and Davies, P.L. 2002. Contribution of hydrophobic residues to ice binding by fish type III antifreeze protein. *Biochim. Biophys. Acta* **1601**, 49–54.

Baker, E.N., Anderson, B.F., Baker, H.M., Mac Gillivray, R.T.A., Moore, S.A., Peterson, N.A., Shewry, S.C., and Tweedie, J.W. 1998. Three-dimensional structure of lactoferrin. Implications for function, including comparisons with transferrin. *In* Advances in Experimental Medicine and Biology (G. Spik, D. Legrand, J. Mazurier, A. Pierce, and J.-P. Perraudin, eds), Vol. 443, pp. 1–21. Plenum Press, New York.

Bal dit Sollier, C., Drouet, L., Pignaud, G., Chevallier, C., Caen, J., Fiat, A.M., Izquierdo, C., and Jolles, P. 1996. Effect of kappa-casein split peptides on platelet aggregation and on thrombus formation in the guinea-pig. *Thromb. Res.* **81**, 427–437.

Baron, F., Fauvel, S., and Gautier, M. 1999. Behaviour of *Salmonella enteritidis* in industrial egg white: egg naturally contains factors inhibitory to *Salmonella* growth. *In* "Egg Nutrition and Biotechnology" (J.S. Sim, S. Nakai, and W. Guenter, eds), pp. 417–430. CABI Publishing, Oxon, UK.

Barret, J. 2001. Thermal hysteresis of proteins. *Int. J. Biochem. Cell B* **33**, 105–107.

Baveye, S., Elass, E., Mazurier, J., Spik, G., and Legrand, D. 1999. Lactoferrin: a multifunctional glycoprotein involved in the modulation of the inflammatory process. *Clin. Chem. Lab. Med.* **37**, 281–286.

Belem, M.A.F., Gibbs, B.F., and Lee, B.H. 1999. Proposing sequences for peptides derived from whey fermentation with potential bioactive sites. *J. Dairy Sci.* **82**, 486–493.

Bellamy, W.R., Takase, M., Wakabayashi, H., Kawase, K., and Tomita, M. 1992a. Antibacterial spectrum of lactoferricin B, a potent bactericidal peptide derived from the N-terminal of bovine lactoferrin. *J. Appl. Bacteriol.* **73**, 472–479.

Bellamy, W.R., Takase, M., Yamauchi, K., Wakabayashi, H., Kawase, K., and Tomita, M. 1992b. Identification of the bactericidal domain of lactoferrin. *Biochim. Biophys. Acta* **1121**, 130–136.

Bellamy, W.R., Wakabayashi, H., Takase, M., Kawase, K., Shimamura, S., and Tomita, M. 1993. Role of cell binding in the antibacterial mechanism of lactoferricin B. *J. Appl. Bacteriol.* **75**, 478–484.

Bennett, T., Desmond, A., Harrington, M., McDonagh, D., FitzGerald, R., Flynn, A., and Cashman, K.D. 2000. The effect of high intakes of casein and casein phosphopeptide on calcium absorption in the rat. *Br. J. Nutr.* **83**, 673–680.

Berrocal, R., Chanton, S., Juilleart, M.A., Pavillard, B., Scherz, J.-C., and Jost, R. 1989. Tryptic phosphopeptides from whole casein. II. Physicochemical properties related to the solubilization of calcium. *J. Dairy Res.* **56**, 335–341.

Beucher, S., Levenez, F., Yvon, M., and Corring, T. 1994. Effect of caseinomacropeptide (CMP) on cholecystokinin (CCK) release in rat. *Reprod. Nutr. Dev.* **34**, 613–614.

Binder, H.J., Laurenson, J.P., and Doppins, J.W. 1984. Role of opiate receptors in regulation of enkephalin stimulation of active sodium and chloride absorption. *Am. J. Physiol.* **247**, 432–436.

Bishop, J.G., Schanbacher, F.L., Ferguson, L.C., and Smith, L. 1976. *In vitro* growth inhibition of mastitis causing coliform bacteria by apo-lactoferrin and reversal of inhibition by citrate and high concentrations of apo-lactoferrin. *Infect. Immun.* **14**, 911–918.

Björck, L. 1978. Antibacterial effect of the lactoperoxidase system on psychrotrophic bacteria in milk. *J. Dairy Res.* **45**, 109–118.

Blach-Olszewska, Z. and Janusz, M. 1997. Stimulatory effect of ovine colostrinine (a proline-rich polypeptide) on interferons and tumor necrosis factor production by murine resident peritoneal cells. *Arch. Immunol. Ther. Exp.* **45**, 43–47.

Blass, E.M. and Blom, J. 1996. β-Casomorphin causes hypoalgesia in 10-day-old rats: evidence for central mediation. *Pediatr. Res.* **39**, 199–203.

Blass, E.M. and Fitzgerald, E. 1988. Milk-induced analgesia and comforting in 10-day-old rats: opioid mediation. *Pharmacol. Biochem. Behav.* **29**, 9–13.

Bordenave, S., Sannier, F., Ricart, G., and Piot, J.M. 1999. Continuous hydrolysis of goat whey in an ultrafiltration reactor: generation of alpha-lactorphin. *Prep. Biochem. Biotechnol.* **29**, 189–202.

Bouhallab, S., Molle, D., and Leonil, J. 1992. Tryptic hydrolysis of caseinomacropeptide in membrane reactor: preparation of bioactive peptides. *Biotechnol. Lett.* **14**, 805–810.

Bounous, G. 2000. Whey protein concentrate (WPC) and glutathione modulation in cancer treatment. *Anticancer Res.* **20**, 4785–4792.

Bounous, G., Batist, G., and Gold, P. 1989. Immunoenhancing property of dietary whey protein in mice, role of glutathione. *Clin. Investig. Med.* **12**, 154–161.

Bounous, G., Batist, G., and Gold, P. 1991. Whey proteins in cancer prevention. *Cancer Lett.* **57**, 91–94.

Bounous, G., Baruchel, S., Falutz, J., and Gold, P. 1993. Whey proteins as a food supplement in HIV-seropositive individuals. *Clin. Investig. Med.* **16**, 204–209.

Brantl, V., Teschemacher, H., Henschen, A., and Lottspeich, F. 1979. Novel opioid peptides derived from casein (β-casomorphins) I. Isolation from bovine casein peptone. *Hoppe-Seyler's Z. Physiol. Chem.* **360**, 1211–1216.

Brantl, V., Bläsig, J., and Henschen, A. 1981. Opioid activities of β-casomorphins. *Life Sci.* **28**, 1903–1909.

Brody, E.P. 2000. Biological activities of bovine glycomacropeptide. *Br. J. Nutr.* **84**, S39–S46.

Brunser, O., Espinoza, J., Figueroa, G., Araya, M., Spencer, E., Hilpert, H., Link-Amster, H., and Brussow, H. 1992. Field trial of an infant formula containing anti-rotavirus and anti-*Escherichia coli* milk antibodies from hyperimmunized cows. *J. Pediatr. Gastroenterol. Nutr.* **15**, 63–72.

Buhler, C., Hammon, H., Rossi, G.L., and Blum, J.W. 1998. Small intestinal morphology in eight-day-old calves fed colostrum for different durations or only milk replacer and treated with long-R3-insulin-like growth factor I and growth hormone. *J. Anim. Sci.* **76**, 758–765.

Bullen, J.J., Rogers, H.J., and Leigh, L. 1972. Iron-binding proteins in milk and resistance to *Escherichia coli* infections in infants. *Br. Med. J.* **1**, 69–75.

Burling, H. 1994. Isolation of bioactive components from cheese whey. *Scand. Dairy Inf.* **8**, 54–56.

Burrin, D.G., Davis, T.A., Ebner, S., Schoknecht, P.A., Fiorotto, M.L., Reeds, P.J., and McAvoy, S. 1995. Nutrient-independent and nutrient-dependent factors stimulate protein synthesis in colostrum-fed newborn pigs. *Pediatr. Res.* **37**, 593–599.

Burrin, D.G., Davis, T.A., Ebner, S., Schoknecht, P.A., Fiorotto, M.L., and Reeds, P.J. 1997. Colostrum enhances the nutritional stimulation of vital organ protein synthesis in neonatal pigs. *J. Nutr.* **127**, 1284–1289.

Burton, P.S., Conradi, R.A., Hilgers, A.R., Ho, N.F.H., and Maggiora, L.L. 1992. The relationship between peptide structure and transport across epithelial cell monolayers. *J. Control Release* **19**, 87–98.

Butler, J.E. 1994. Passive immunity and immunoglobulin diversity. *In* "Indigenous Antimicrobial Agents of Milk—Recent Developments", IDF Special Issue 9404, pp. 14–50.

Canapathy, V. and Leibach, F.H. 1985. Is intestinal peptide transport energized by a proton gradient? *Am. J. Physiol.* **249**, 153–160.

Carmen, M., Hernandez, M., van Markwijk, B.W., and Vreeman, H.J. 1990. Isolation and properties of lactoperoxidase from bovine milk. *Neth. Milk Dairy J.* **44**, 213–221.

Carr, R.I., Webster, D., Sadi, D., Williams, H., and Walsh, N. 1990. Immunomodulation by opioids from dietary casein. *Ann. NY Acad. Sci.* **574**, 374–376.

Casswall, T.H., Sarker, S.A., Albert, M.J., Fuchs, G.J., Bergström, M., Björck, L., and Hammarström, L. 1998. Treatment of *Helicobacter pylori* infection in infants in rural Bangladesh with oral immunoglobulins from hyperimmune bovine colostrum. *Aliment. Pharmacol. Ther.* **12**, 563–568.

Casswall, T.H., Sarker, S.A., Faruque, S.M., Weintraub, A., Albert, M.J., Fuchs, G.J., Alam, N.H., Dahlström, A.K., Link, H., Brussow, H., and Hammarström, L. 2000. Treatment of enterotoxigenic and enteropathogenic *Escherichia coli*-induced diarrhoea in children with bovine immunoglobulin milk concentrate from hyperimmunized cows: a double-blind, placebo-controlled, clinical trial. *Scand. J. Gastroenterol.* **35**, 711–718.

Chabance, B., Jollés, P., Izquierdo, C., Mazoyer, E., Francoual, C., Drouet, L., and Fiat, A.-M. 1995. Characterization of an antithrombotic peptide from κ-casein in newborn plasma after milk ingestion. *Br. J. Nutr.* **73**, 583–590.

Chabance, B., Marteau, P., Rambaud, J.C., Migliore-Samour, D., Boynard, M., Perrotin, P., Guillet, R., Jollés, P., and Fiat, A.-M. 1998. Casein peptide release and passage to the blood in humans during digestion of milk or yogurt. *Biochimie* **80**, 155–165.

Chang, K.-J., Cuatrecasas, P., Wei, E.T., and Chang, J.K. 1982. Analgesic activity of intracerebroventricular administration of morphiceptin and β-casomorphins: correlation with the morphine (u) receptor binding affinity. *Life Sci.* **30**, 1547–1551.

Chang, K.-J., Su, Y.F., Brent, D.A., and Chang, J.K. 1985. Isolation of a specific μ-opiate receptor peptide, morphiceptin from an enzymatic digest of milk proteins. *J. Biol. Chem.* **260**, 9706–9712.

Chao, H., Houston, M.E., Hodges, R.S., Kay, C.M., Sykes, B.D., Loewen, M.C., Davies, P.L., and Sonnichsen, F.D. 1997. A diminished role for hydrogen bonds in antifreeze protein binding to ice. *Biochemistry* **36**, 14652–14660.

Charlton, A.J., Baxter, N.J., Khan, M.L., Moir, A.J.G., Haslam, E., Davies, A.P., and Williamson, M.P. 2002. Polyphenol/peptide binding and precipitation. *J. Agric. Food Chem.* **50**, 1593–1601.

Chaud, M.V., Izumi, C., Nahaal, Z., Shuhama, T., De Lourdes, M., Bianchi, P., and De Freitas, O. 2002. Iron derivatives from casein hydrolysates as a potential source in the treatment of iron deficiency. *J. Agric. Food Chem.* **50**, 847–877.

Cheng, A. and Merz, K.M. 1997. Ice-binding mechanism of winter flounder antifreeze proteins. *Biophys. J.* **73**, 2851–2873.

Chiang, W.E., Cordle, C.T., and Thomas, R.L. 1995. Casein hydrolysate produced using a formed-in-place membrane reactor. *J. Food Sci.* **60**, 1349–1352.

Chiba, H. and Yoshikawa, M. 1986. Biologically functional peptides from food proteins: new opioid peptides from milk proteins. *In* "Protein Tailoring for Food and Medical Uses" (R.E. Feeney and J.R. Whitaker, eds), pp. 123–153. Marcel Dekker, New York.

Chiba, H., Tani, F., and Yoshikawa, M. 1989. Opioid antagonist peptides derived from κ-casein. *J. Dairy Res.* **56**, 363–366.

Chiu, C.K. and Etzel, M.R. 1997. Fractionation of lactoperoxidase and lactoferrin from bovine whey using a cation exchange membrane. *J. Food Sci.* **62**(5), 996–1000.

Chu, X., Xu, N., Li, P., Mao, L., and Wang, J.Q. 1999. Inhibition of cardiovascular activity following microinjection of novel opioid-like neuropeptide nociceptin (orphanin FQ) into the rat rostral ventrolateral medulla. *Brain Res.* **829**, 134–142.

Clare, R. 1998. The benefits of CMP. *Dairy Ind. Int.* **63**, 29–31.

Clare, D.A. and Swaisgood, H.E. 2000. Bioactive milk peptides: a prospectus. *J. Dairy Sci.* **83**, 1187–1195.

Conneely, O.M. 2001. Antiinflammatory activities of lactoferrin. *J. Am. Coll. Nutr.* **20**, S389–S395.

Coste, M., Rochet, V., Leonil, J., Mollé, D., Bouhallab, S., and Tomé, D. 1992. Identification of C-terminal peptides of bovine β-casein that enhance proliferation of rat lymphocytes. *Immunol. Lett.* **33**, 41–46.

Crevel, R.W.R., Fedyk, J.K., and Spurgeon, M.J. 2002. Antifreeze proteins: characteristics, occurrence and human exposure. *Food Chem. Toxicol.* **40**, 899–903.

Cross, M.L. and Gill, H.S. 2000. Immunomodulatory properties of milk. *Br. J. Nutr.* **84**, S81–S89.

Crouch, S.P., Slater, K.J., and Fletcher, J. 1992. Regulation of cytokine release from mononuclear cells by the iron-binding protein lactoferrin. *Blood* **80**, 235–240.

D'Alvise, N., Lesueur-Lambert, C., Fertin, B., Dhulster, P., and Guillochon, D. 2000. Hydrolysis and large scale ultrafiltration study of alfalfa protein concentrate enzymatic hydrolysate. *Enzyme Microb. Technol.* **27**, 286–294.

Daniel, H., Wessendorf, A., Vohwinkel, M., and Brantl, V. 1990. Effect of D-Ala^{2,4},Tyr^{5}-β-casomorphin-5-amide on gastrointestinal functions. *In* "β-Casomorphins and Related Peptides" (F. Nyberg and V. Brantl, eds), pp. 95–104. Fyris-Tryck AB, Uppsala.

Daniel, H., Vohwinkel, M., and Rehner, G. 1991. Effect of casein and β-casomorphins on gastrointestinal motility in rats. *J. Nutr.* **120**, 252–257.

Daniel, H., Morse, E.L., and Adibi, S.A. 1992. Determinants of substrate affinity for the oligopeptide/H^+ symporter in the renal brush border membrane. *J. Biol. Chem.* **267**, 9565–9573.

Davidson, G.P. 1996. Passive protection against diarrhoeal disease. *J. Pediatr. Gastroenterol. Nutr.* **23**, 207–212.

Davidson, G.P., Daniels, E., Nunan, H., Moore, A.G., Whyte, P.B.D., Franklin, K., McCloud, P.I., and Moore, D.J. 1989. Passive immunisation of children with bovine colostrum containing antibodies to human rotavirus. *Lancet* **2**, 709–712.

de Jongh, H.H., Groneveld, T., and de Groot, J. 2001. Mild isolation procedure discloses new protein structural properties of beta-lactoglobulin. *J. Dairy Sci.* **84**, 562–571.

de Wit, J.N. 1998. Nutritional and functional characteristics of whey proteins in food products. *J. Dairy Sci.* **81**, 597–608.

de Wit, J.N. and van Hooijdonk, A.C.M. 1996. Structure, functions and applications of lactoperoxidase in natural antimicrobial systems. *Neth. Milk Dairy J.* **50**, 227–244.

Debbabi, H., Dubarry, M., Rautureau, M., and Tomé, D. 1998. Bovine lactoferrin induces both mucosal and systemic immune responses in mice. *J. Dairy Res.* **65**, 283–293.

Dial, E.J., Hall, L.R., Serna, H., Romero, J.J., Fox, J.G., and Lichtenberger, L.M. 1998. Antibiotic properties of bovine lactoferrin on *Helicobacter pylori*. *Dig. Dis. Sci.* **43**, 2750–2756.

Dionysius, D.A. and Milne, J.M. 1997. Antibacterial peptides of bovine lactoferrin: purification and characterization. *J. Dairy Sci.* **80**, 667–674.

Dionysius, D.A., Marscke, R.J., Wood, A.J., Milne, J., Beattie, T.R., Jiang, H., Treloar, T., Alewood, P.F., and Grieve, P.A. 2000. Identification of physiologically functional peptides in dairy products. *Aust. J. Dairy Technol*. **55**, 103.

Dominguez, E., Perez, M.D., and Calvo, M. 1997. Effect of heat treatment on the antigen-binding activity of anti-peroxidase immunoglobulins in bovine colostrum. *J. Dairy Sci.* **80**, 3182–3187.

Donnet-Hughes, A., Duc, N., Serrant, P., Vidal, K., and Schiffrin, E.J. 2000. Bioactive molecules in milk and their role in health and disease: the role of transforming growth factor-β. *Immunol. Cell Biol.* **78**, 74–79.

Döring, F., Will, J., Amasheh, S., Clauss, W., Ahlbrecht, H., and Daniel, H. 1998. Minimal molecular determinants of substrates for recognition by the intestinal peptide transporter. *J. Biochem. Tokyo* **273**, 23211–23218.

Ebina, T., Sato, A., Umezu, K., Ishida, N., Ohyama, S., Oizumi, A., Aikawa, K., Katagiri, S., Katsushima, N., Imai, A., Kitaoka, S., Suzuki, H., and Konno, T. 1985. Prevention of rotavirus infection by oral administration of colostrum containing antihumanrotavirus antibody. *Med. Microbiol. Immunol.* **174**, 177–185.

Ebina, T., Tsukada, K., Umezu, K., Nose, M., Tsuda, K., Hatta, H., Kim, M., and Yamamoto, T. 1990. Gastroenteritis in suckling mice caused by human rotavirus can be prevented with egg yolk immunoglobulin (IgY) and treated with a protein-bound polysaccharide preparation (PSK). *Microbiol. Immunol.* **34**, 617–629.

Elitsur, Y. and Luk, G.D. 1991. Beta-casomorphin (BCM) and human colonic lamina propria lymphocyte proliferation. *Clin. Exp. Immunol.* **85**, 493–497.

Ellegård, K.H., Gammelgård-Larsen, C., Sorensen, E.S., and Fedosov, S. 1999. Process scale chromatographic isolation, characterization and identification of tryptic bioactive casein phosphopeptides. *Int. Dairy J.* **9**, 639–652.

Ellison, R.T. 1994. The effects of lactoferricin on Gram-negative bacteria. *Adv. Exp. Med. Biol.* **357**, 71–90.

Ellison, R.T. III, Giehl, T.J., and LaForce, F.M. 1988. Damage of the outer membrane of enteric Gram-negative bacteria by lactoferrin and transferrin. *Infect. Immun.* **56**, 2774–2781.

Erdei, J., Forsgren, A., and Naidu, A.S. 1994. Lactoferrin binds to porins OmpF and OmpC in *Escherichia coli*. *Infect. Immun.* **62**, 1236–1240.

Erll, G., Hahn, A., Brantl, V., and Daniel, H. 1994. β-Casomorphins and intestinal net fluid transport *in vivo*. *In* "β-Casomorphins and Related Peptides: Recent Developments" (V. Brantl and H. Teschemacher, eds), pp. 143–149. VCH, Weinheim.

Facon, M., Skura, B.J., and Nakai, S. 1993. Potential immunological supplementation of foods. *Food Agric. Immunol.* **5**, 85–91.

Fei, Y.-J., Kanai, Y., Nussberger, S., Canapathy, V., Leibach, F., Romero, M.F., Singh, S.K., Boron, W.F., and Hediger, M.A. 1994. Expression cloning of a mammalian proton-coupled oligopeptide transporter. *Nature* **368**, 563–566.

Feuerstein, G. and Siren, A.-L. 1987. The opioid peptides. A role in hypertension. *Hypertension* **9**, 561–565.

Fiat, A.-M. and Jollès, P. 1989. Caseins of various origins and biologically active casein peptides and oligosaccharides: structural and physiological aspects. *Mol. Cell. Biochem.* **87**, 5–30.

Fiat, A.-M., Levy-Toledano, S., Caen, J.P., and Jollès, P. 1989. Biologically active peptides of casein and lactoferrin implicated in platelet function. *J. Dairy Res.* **56**, 351–355.

Fiat, A.-M., Migliore-Samour, D., Jollès, P., Drouet, L., Collier, C., and Caen, J. 1993. Biologically active peptides from milk proteins with emphasis on two examples concerning antithrombotic and immunomodulating activities. *J. Dairy Sci.* **76**, 301–310.

Filler, S.J., Gregory, R.L., Michalek, S.M., Katz, J., and McGhee, J.R. 1991. Effect of immune bovine milk on *Streptococcus mutans* in human dental plaque. *Arch. Oral Biol.* **36**, 41–47.

Finot, P.A. 1997. Effects of processing and storage on the nutritional value of food proteins. *In* "Food Proteins and Their Applications" (S. Damodaran and A. Paraf, eds), pp. 551–577. Marcel Dekker Inc., New York.

FitzGerald, R.J. 1998. Potential uses of caseinophosphopeptides. *Int. Dairy J.* **8**, 451–457.

FitzGerald, R.J. and Meisel, H. 2000. Milk protein-derived peptide inhibitors of angiotensin-I-converting enzyme. *Br. J. Nutr.* **84**, S33–S37.

Fox, P.F. and Flynn, A. 1992. Biological properties of milk proteins. *In* "Advanced Dairy Chemistry" (P. Fox, ed.), Vol. 1, pp. 255–284, Elsevier Applied Science, Essex.

Freedman, D.J., Tacket, C.O., Delehanty, A., Maneval, D.R., Nataro, J., and Crabb, J.H. 1998. Milk immunoglobulin with specific activity against purified colonization factor antigens can protect against oral challenge with enterotoxigenic *Escherichia coli*. *J. Infect. Dis.* **177**, 662–667.

Friedman, M. 1996. Nutritional value of food proteins from different food resources. *J. Agric. Food Chem.* **44**, 6–29.

Friedman, M. and Brandon, D.L. 2001. Nutritional and health benefits of soy proteins. *J. Agric. Food Chem.* **49**, 1069–1086.

Froetschel, M.A., Azain, M.J., Edwards, G.L., Barb, C.R., and Amos, H.E. 2001. Opioid and cholecystokinin antagonists alleviate gastric inhibition of food intake by premeal loads of casein in meal-fed rats. *J. Nutr.* **131**, 3270–3276.

Fujita, H., Usui, H., Kurahashi, K., and Yoshikawa, M. 1995. Isolation and characterization of ovokinin, a Bradykinin B_1 agonist peptide derived from ovalbumin. *Peptides* **46**, 780–790.

Fujita, H., Yokoyama, K., and Yoshikawa, M. 2000. Classification and antihypertensive activity of angiotensin I-converting enzyme inhibitory peptides derived from food proteins. *J. Food Sci.* **65**, 564–569.

Fukudome, S. and Yoshikawa, M. 1992. Opioid peptides derived from wheat gluten, their isolation and characterization. *FEBS Lett.* **296**, 107–111.

Fukudome, S. and Yoshikawa, M. 1993. A novel opioid peptide derived from wheat gluten. *FEBS Lett.* **316**, 17–19.

Fukumoto, L.R., Skura, B.J., and Nakai, S. 1994a. Stability of membrane-sterilized bovine immunoglobulins aseptically added to UHT milk. *J. Food Sci.* **59**, 757–759.

Fukumoto, L.R., Li-Chan, E., Kwan, L., and Nakai, S. 1994b. Isolation of immunoglobulins from cheese whey using ultrafiltration and immobilized metal affinity chromatography. *Food Res. Int.* **27**, 335–348.

Gagnaire, V., Pierre, A., Molle, D., and Leonil, J. 1996. Phosphopeptides interacting with colloidal calcium phosphate isolated by tryptic hydrolysis of bovine casein micelles. *J. Dairy Res.* **63**, 405–422.

Ganjam, L.S., Thornton, W.H. Jr., Marshall, R.T., and MacDonald, R.S. 1997. Antiproliferative effects of yogurt fractions obtained by membrane dialysis on cultured mammalian intestinal cells. *J. Dairy Sci.* **80**, 2325–2329.

Gardner, M.L. 1984. Intestinal assimilation of intact peptides and proteins from the diet—a neglected field? *Biol. Rev.* **59**, 289–331.

Gattegno, L., Migliore-Samour, D., Saffar, L., and Jollés, P. 1988. Enhancement of phagocytic activity of human monocytic macrophagic cells by immunostimulating peptides from human casein. *Immunol. Lett.* **18**, 27–32.

Gibbs, B.F., Alli, I., and Mulligan, C. 1996. Sweet and taste-modifying proteins: a review. *Nutr. Res.* **16**, 1619–1630.

Gill, H.S., Doull, F., Rutherfurd, K.J., and Cross, M.L. 2000. Immunoregulatory peptides in bovine milk. *Br. J. Nutr.* **84**, S111–S117.

Gobbetti, M., Smacchi, E., Corsetti, A., and Bellucci, M. 1997. Inhibition of proteolytic enzymes from *Pseudomonas fluorescens* ATCC 948 and angiotensin I-converting enzyme by peptides from zein, hordein and gluten hydrolysates. *J. Food Protect.* **60**, 499–504.

Gobbetti, M., Ferranti, P., Smacchi, E., Goffredi, F., and Addeo, F. 2000. Production of angiotensin-I-converting-enzyme-inhibitory peptides in fermented milks started by *Lactobacillus delbrueckii subsp. bulgaricus* SS1 and *Lactococcus lactis subsp. cremoris* FT4. *Appl. Environ. Microbiol.* **66**(9), 3898–3904.

Goldman, A.S. 1989. Immunologic supplementation of cow's milk formulations. *IDF Bull.* **244**, 38–42.

Goldman, A.S. 1993. The immune system of human milk: antimicrobial, antiinflammatory and immunomodulating properties. *Pediatr. Inf. Dis. J.* **12**, 664–671.

Grecksch, G., Schweigert, C., and Matthies, H. 1981. Evidence for analgesic activity of β-casomorphin in rats. *Neurosci. Lett.* **27**, 325–328.

Guilloteau, P., Le Huerou-Luron, I., Chayviaille, J.A., Toullec, R., Legeas, M., Bernard, C., Roger, L., and Mendy, F. 1994. Effect of caseinomacropeptide (CMP) on gastric secretion and plasma gut regulatory peptides in preruminant calves. *Reprod. Nutr. Dev.* **34**, 612–613.

Guimont, C., Marchall, E., Girardet, J.M., and Linden, G. 1997. Biologically active factors in bovine milk and dairy byproducts: influence on cell culture. *Crit. Rev. Food Sci. Nutr.* **37**, 393–410.

Gustafson, D.R., McMahon, D.J., Morrey, J., and Nan, R. 2001. Appetite is not influenced by a unique milk peptide: caseinomacropeptide (CMP). *Appetite* **36**, 157–163.

Hadden, J.W. 1991. Immunotherapy of human immunodeficiency virus infection. *Trends Pharmacol. Sci.* **12**, 107–111.

Hagiwara, K., Kataoka, S., Yamanaka, H., Kirisawa, R., and Iwai, H. 2000. Detection of cytokines in bovine colostrum. *Vet. Immunol. Immunopathol.* **76**, 183–190.

Haileselassie, S.S., Lee, B.H., and Gibbs, B.F. 1999. Purification and identification of potentially bioactive peptides from enzyme-modified cheese. *J. Dairy Sci.* **82**(8), 1612–1617.

Håkansson, A., Zhivotovsky, B., Orrenius, S., Sabharwal, H., and Svanborg, C. 1995. Apoptosis induced by a human milk protein. *Proc. Natl Acad. Sci. USA* **92**, 8064–8068.

Håkansson, A., Andreasson, J., Zhivotovsky, B., Karpman, D., Orrenius, S., and Svanborg, C. 1999. Multimeric alpha-lactalbumin from human milk induces apoptosis through a direct effect on cell nuclei. *Exp. Cell Res.* **246**, 451–460.

Haláz, A., Baráth, A., Simo-Sarkadi, L., and Holzapfel, W. 1994. Biogenic amines and their production by microorganisms in food. *Trends Food Sci. Technol.* **5**, 42–49.

Hambraeus, L. and Lönnerdal, B. 1994. The physiological role of lactoferrin. *In* "Indigenous Antimicrobial Agents of Milk—Recent Developments", IDF Special Issue 9404, pp. 97–107.

Hammarström, L., Gardulf, A., Hammarström, V., Janson, A., Lindberg, K., and Smith, C.I. 1994. Systemic and topical immunoglobulin treatment in immunocompromised patients. *Immunol. Rev.* **139**, 43–70.

Hammon, H.M. and Blum, J.W. 1998. Metabolic and endocrine traits of neonatal calves are influenced by feeding colostrum for different durations or only milk replacer. *J. Nutr.* **128**, 624–632.

Hansen, M., Sandström, B., and Lönnerdal, B. 1996. The effect of casein phosphopeptides on zinc and calcium absorption from high phytate infant diets assessed in rat pups and caco-2 cells. *Pediatr. Res.* **40**, 547–552.

Hansen, M., Sandström, B., Jensen, M., and Sørensen, S.S. 1997. Casein phosphopeptides improve zinc and calcium absorption from rice-based but not from whole-grain infant cereal. *J. Pediatr. Gastroenterol. Nutr.* **24**, 56–62.

Hata, Y., Yamamoto, M., Ohni, H., Nakajima, K., Nakamura, Y., and Takano, T. 1996. A placebo-controlled study of the effect of sour milk on blood pressure in hypertensive subjects. *Am. J. Clin. Nutr.* **64**, 767–771.

Hata, I., Higasiyama, S., and Otani, H. 1998. Identification of a phosphopeptide in bovine αs1-casein digest as a factor influencing proliferation and immunoglobulin production in lymphocyte cultures. *J. Dairy Res.* **65**, 569–578.

Hata, I., Ueda, J., and Otani, H. 1999. Immunostimulatory action of a commercially available casein phosphopeptide preparation, CPP-III, in cell cultures. *Milchwissenschaft* **54**, 3–7.

Hatta, H., Tsuda, K., Ozeki, M., Kim, M., Yamamoto, T., Otake, S., Hirasawa, M., Katz, J., Childers, N.K., and Michalek, S.M. 1997. Passive immunization against dental plaque formation in humans: effect of a mouth rinse containing egg yolk antibodies (IgY) specific to *Streptococcus mutans*. *Caries Res.* **31**, 268–274.

Heaney, R.P., Saito, Y., and Orimo, H. 1994. Effect of casein phosphopeptides on absorbability of co-ingested calcium in normal and postmenopausal women. *J. Bone Miner. Metab.* **12**, 77–81.

Hedner, J. and Hedner, T. 1987. Beta-casomorphins induce apnea and irregular breathing in adult rats and newborn rabbits. *Life Sci.* **41**, 2303–2312.

Henriksen, A.P. and Stahnke, L.H. 1997. Sensory and chromatographic evaluations of water soluble fractions from dried sausages. *J. Agric. Food Chem.* **45**, 2679–2684.

Henschen, A., Lottspeich, F., Brantl, V., and Teschemacher, H. 1979. Novel opioid peptides derived from casein (β-casomorphins). *Hoppe-Seyler's Z. Physiol. Chem.* **360**, 1217–1224.

Hoek, K.S., Milne, J.M., Grieve, P.A., Dionysius, D.A., and Smith, R. 1997. Antibacterial activity of bovine lactoferrin-derived peptides. *Antimicrob. Agents Chemother.* **41**, 54–59.

Horton, B.S. 1995. Commercial utilization of minor milk components in the health and food industries. *J. Dairy Sci.* **78**, 2584–2589.

Hoshi, T. 1986. Proton-coupled transport dipeptides across renal and intestinal brush border membranes. *In* "Ion Gradient-Coupled Transport" (F. Alvardo and C.H. van Os, eds), pp. 183–191. Elsevier Science, New York.

Hugli, T.E. 1989. Structure and function of C3a anaphylatoxin. *Curr. Top. Microbiol. Immunol.* **153**, 181–208.

Hutchens, T.W., Rumball, S.V., and Lönnerdal, B. (eds) 1994. *In* "Lactoferrin: structure and function", Adv. Exp. Med. Biol., 357, pp. 1–298.

Höllt, V. 1983. Multiple endogenous opioid peptides. *Trends Neurosci.* **6**, 24–26.

Ibrahim, H.R. 1997. Insights into the structure–function relationships of ovalbumin, ovotransferrin, and lysozyme. *In* "Hen Eggs: Their Basic and Applied Science" (T. Yamamoto, L.R. Juneja, H. Hatta, and M. Kim, eds), pp. 37–56. CRC Press, Boca Raton.

Ibrahim, H.R., Sugimoto, Y., and Aoki, T. 2000. Ovotransferrin antimicrobial peptide (OTAP-92) kills bacteria through a membrane damage mechanism. *Biochim. Biophys. Acta* **1523**, 196–205.

IDF, 1991. Significance of the indigenous antimicrobial agents of milk to the dairy industry. *IDF Bull.* **264**, 2–19.

Isoda, H., Kawasaki, Y., Tanimoto, M., Dosako, S., and Idota, T. 1990. Use of compounds containing or binding sialic acid to neutralize bacterial toxins. European Patent No. 385112.

Jack, R.W., Bierbaum, G., and Sahl, H.-G. 1998. "Lantibiotics and Related Peptides." Springer-Verlag, Berlin.

Janusz, M., Wieczorek, Z., Spiegel, K., Kubik, A., Szewczuk, Z., Siemion, I., and Lisowski, J. 1987. Immunoregulatory properties of synthetic peptides, fragments of a proline-rich polypeptide (PRP) from ovine colostrum. *Mol. Immunol.* **24**, 1029–1031.

Jaziri, M., Migliore-Samour, D., Casabianca-Pignede, M.R., Keddad, K., Morgat, J.L., and Jolles, P. 1992. Specific binding sites on human phagocytic blood cells for Gly-Leu-Phe and Val-Glu-Pro-Ile-Pro-Tyr, immunostimulating peptides from human milk proteins. *Biochim. Biophys. Acta* **1160**, 251–261.

Jelen, P. 1992. Pressure-driven membrane processes: principles and definitions. *In* "New Applications of Membrane Processes", IDF Special Issue 9201, pp. 7–14.

Jia, Z. and Davies, P.L. 2002. Antifreeze proteins. An unusual receptor–ligand interaction. *Trends Biochem. Sci.* **27**, 101–106.

Jiang, B. and Mine, Y. 2000. Preparation of novel functional oligophosphopeptides from hen yolk phosvitin. *J. Agric. Food Chem.* **48**, 990–994.

Jolles, P. and Caen, J.P. 1991. Parallels between milk clotting and blood clotting, opportunities for milk-derived products. *Trends Food Sci. Technol.* **2**, 42–43.

Jolles, P., Parker, F., Floc'h, F., Migliore, D., Alliel, P., Zerial, A., and Werner, G.G. 1981. Immunostimulating substances from human casein. *J. Pharmacol.* **3**, 363–369.

Jolles, P., Lévy-Toledano, S., Fiat, A.-M., Soria, G., Gillessen, D., Thomaidis, A., Dunn, F.W., and Caen, J.P. 1986. Analogy between fibrinogen and casein. Effect of an undecapeptide isolated from κ-casein on platelet function. *Eur. J. Pharmacol.* **158**, 379–382.

Jones, E.M., Smart, A., Bloomberg, G., Burgess, L., and Millar, M.R. 1994. Lactoferricin, a new antimicrobial peptide. *J. Appl. Bacteriol.* **77**, 208–214.

Jost, R. 1993. Functional characteristics of dairy proteins. *Trends Food Sci. Technol.* **4**, 283–288.

Juilleart, M.A., Berrocal, R., Chanton, S., Scherz, J.-C., and Jost, R. 1989. Tryptic phosphopeptides from whole casein. 1. Preparation and analysis by fast protein liquid chromatography. *J. Dairy Res.* **56**, 603–611.

Julius, M.H., Janusz, M., and Lisowski, J. 1988. A colostral protein that induces the growth and differentiation of resting B lymphocytes. *J. Immunol.* **140**, 1366–1371.

Kahala, M., Pahkala, E., and Pihlanto-Leppälä, A. 1993. Peptides in fermented Finnish milk products. *Agric. Sci. Finland* **2**, 379–386.

Kampa, M., Loukas, S., Hatzoglou, A., Martin, P., Martin, P.-M., and Castanas, E. 1996. Identification of a novel opioid peptide (Tyr-Val-Pro-Phe-Pro) derived from human αs1-casein (αs1-casomorphin and αs1-casomorphin amide). *Biochem. J.* **319**, 903–908.

Kampa, M., Bakogeorgou, E., Hatzoglou, A., Damianaki, A., Martin, P.-M., and Castanas, E. 1997. Opioid alkaloids and casomorphin peptides decrease the proliferation of prostatic cancer cell lines (LNCaP, PC3 and DU145) through a partial interaction with opioid receptors. *Eur. J. Pharmacol.* **335**, 255–265.

Kang, J.H., Lee, M.K., Kim, K.L., and Hahm, K.-S. 1996. Structure–biological activity relationship of 11-residue highly basic peptide segment of bovine lactoferrin. *Int. J. Peptide Protein Res.* **48**, 357–363.

Karaki, H., Doi, K., Sugano, S., Uchiwa, H., Sugai, R., Murakami, U., and Takemoto, S. 1990. Antihypertensive effect of tryptic hydrolysate of milk casein in spontaneously hypertensive rats. *Comp. Biochem. Physiol.* **96C**, 367–371.

Kawasaki, Y., Isoda, H., Tanimoto, M., Dosako, S., Idota, T., and Ahiko, K. 1992. Inhibition by lactoferrin and κ-casein glycomacropeptide of binding of cholera toxin to its receptor. *Biosci. Biotech. Biochem.* **56**, 195–198.

Kawasaki, Y., Isoda, K., Shinmoto, H., Tanimoto, M., Dosako, S., Idota, T., and Nakajima, I. 1993. Inhibition by κ-casein glycomacropeptide and lactoferrin of influenza virus hemaglutination. *Biosci. Biotech. Biochem.* **57**, 1214–1215.

Kawasaki, Y., Dosako, S., Shimatani, M., and Idota, T. 1994. Process for producing κ-casein glycomacropeptides. United States Patent 5,280,107.

Kayser, H. and Meisel, H. 1996. Stimulation of human peripheral blood lymphocytes by bioactive peptides derived from bovine milk proteins. *FEBS Lett.* **383**, 18–20.

Kelly, P.M. and McDonagh, D. 2000. Innovative dairy ingredients. *World Food Ingred.* October/November, 24–32.

Kelly, C.P., Chetman, S., Keates, S., Bostwick, E., Roush, A.M., Castagliolo, I., Lamont, J.T., and Pothoulakis, C. 1997. Survival of anti-*Clostridium difficile* bovine immunoglobulin concentrate in the human gastrointestinal tract. *Antimicrob. Agents Chemother.* **41**, 236–241.

Kerchner, G.A. and Geary, L.E. 1983. Studies on the transport of enkephalin-like oligopeptides in rat intestinal mucosa. *J. Pharmacol. Exp. Ther.* **226**, 33–38.

Kijowski, J., Lesnierowski, G., and Fabisz-Kijowska, A. 1999. Lysozyme polymer formation and functionality of residuals after lysozyme extraction. *In* "Egg Nutrition and Biotechnology" (J.S. Sim, S. Nakai, and W. Guenter, eds), pp. 269–285. CABI Publishing, Wallingford, UK.

Kilara, A. 1994. Whey protein functionality. *In* "Protein Functionality in Food Systems" (N. Hettiarachchy and G.R. Ziegler, eds), pp. 325–355. Marcel Dekker, Inc., New York.

Kinekawa, Y.-I. and Kitabatake, N. 1996. Purification of β-lactoglobulin from whey protein concentrate by pepsin treatment. *J. Dairy Sci* **79**, 350–358.

Kinsella, J.E. and Whitehead, D.M. 1989. Proteins in whey: chemical, physical, functional properties. *Adv. Food Nutr. Res.* **33**, 343–438.

Kitts, D.K. and Yuan, Y.V. 1992. Caseinophosphopeptides and calcium bioavailability. *Trends Food Sci. Technol.* **3**, 31–35.

Kitts, D.K., Yuan, Y.V., Nagasawa, T., and Moriyama, Y. 1992. Effect of casein, casein phosphopeptide and calcium intake on ileal 45-Ca disappearance and temporal systolic blood pressure in spontaneously hypertensive rats. *Br. J. Nutr.* **68**, 765–781.

Klink, L. 2002. Milk-derived bioactives. *The World of Food Ingredients* September, 28–35.

Knotopidis, G., Holt, C., and Sawyer, L. 2002. The ligand binding site of bovine β-lactoglobulin: evidence for a function. *J. Mol. Biol.* **318**, 1043–1055.

Koch, G. and Brantl, V. 1990. Binding of β-casomorphins to opioid receptors. *In* "β-Casomorphins and Related Peptides" (F. Nyberg and V. Brantl, eds), pp. 43–52. Fyris-Tryck AB, Uppsala.

Koch, G., Wiedemann, K., Drebes, E., Zimmerman, W., Link, G., and Teschemacher, H. 1988. Human β-casomorphin-8 immunoreactive material in the plasma of women during pregnancy and after delivery. *Regul. Peptides* **20**, 107–117.

Koch, G., Lange, E., Link, G., Bödeker, R.H., and Teschemacher, H. 1994. Analysis of clinical data and plasma levels of immunoreactive β-casomorphin-8 in pregnant women. *In* "β-Casomorphins and Related Peptides: Recent Developments" (V. Brantl and H. Teschemacher, eds), pp. 227–239. VCH, Weinheim.

Koldovsky, O. 1996. The potential physiological significance of milk-borne hormonally active substances for the neonate. *J. Mammary Gland Biol. Neoplasia.* **1**, 317–323.

Konrad, G., Lieske, B., and Faber, W. 2000. A large-scale isolation of native β-lactoglobulin: characterization of physicochemical properties and comparison with other methods. *Int. Dairy J.* **10**, 713–721.

Kopra, N., Scholz-Ahrens, K.-E., and Barth, C. 1992. Effect of casein phosphopeptides on utilization of calcium in vitamin D-replete and vitamin D-deficient rats. *Milchwissenschaft* **47**, 488–493.

Korhonen, H. 1977. Antimicrobial factors in bovine colostrum. *J. Sci. Agric. Soc. Finland* **49**, 434–447.

Korhonen, H. 1980. A new method for preserving raw milk—the lactoperoxidase antibacterial system. *World Animal Rev.* **35**, 23–29.

Korhonen, H. 1998. Colostrum immunoglobulins and the complement system—potential ingredients of functional foods. *IDF Bull.* **336**, 36–40.

Korhonen, H. 2001. Antibacterial and antiviral activities of whey proteins. In "The Importance of Whey and Whey Components in Food and Nutrition", Proc. 3rd Int. Whey Conference, Munich, pp. 303–321. B. Behr's Verlag GmbH & Co., Hamburg.

Korhonen, H. 2002. Technology options for new nutritional concepts. *Int. J. Dairy Technol.* **55**, 79–88.

Korhonen, H. and Pihlanto-Leppälä, A. 2001. Milk protein-derived bioactive peptides—novel opportunities for health promotion. *IDF Bull.* **363**, 17–26.

Korhonen, H.J. and Reiter, B. 1983. Production of H_2O_2 by bovine blood and milk polymorphonuclear leucocytes. *Acta Microbiol. Pol.* **32**, 53–64.

Korhonen, H., Syväoja, E.-L., Ahola-Luttila, H., Sivelä, S., Kopola, S., Husu, J., and Kosunen, T.U. 1995. Bactericidal effect of bovine normal and immune serum, colostrum and milk against *Helicobacter pylori*. *J. Appl. Bacteriol.* **78**, 655–662.

Korhonen, H., Pihlanto-Leppälä, A., Rantamäki, P., and Tupasela, T. 1998a. Impact of processing on bioactive proteins and peptides. *Trends Food Sci. Technol.* **9**, 307–319.

Korhonen, H., Pihlanto-Leppälä, A., Rantamäki, P., and Tupasela, T. 1998b. The functional and biological properties of whey proteins: prospects for the development of functional foods. *Agr. Food Sci. Finland* **7**, 283–296.

Korhonen, H., Marnila, P., and Gill, H.S. 2000a. Milk immunoglobulins and complement factors. *Br. J. Nutr* **84**, S75–S80.

Korhonen, H., Marnila, P., and Gill, H.S. 2000b. Bovine milk antibodies for health. *Br. J. Nutr* **84**, S135–S146.

Kuhara, T., Iigo, M., Itoh, T., Ushida, Y., Sekine, K., Terada, N., Okamura, H., and Tsuda, H. 2000. Orally administered lactoferrin exerts an antimetastatic effect and enhances production of IL-18 in the intestinal epithelium. *Nutr. Cancer* **38**(2), 192–199.

Kukman, I.L., Zelenik-Blatnik, M., and Abram, V. 1995. Isolation of low-molecular-mass hydrophobic bitter peptides in soybean protein hydrolysates by reversed-phase high-performance liquid chromatography. *J. Chromatogr.* **704**, 113–120.

Kulczycki, A. Jr, Nash, G.S., Bertovich, M.J., Burack, H.D., and MacDermott, R.P. 1987. Bovine milk IgG, but not serum IgG, inhibits pokeweed mitogen-induced antibody secretion by human peripheral blood mononuclear cells. *J. Clin. Immunol.* **7**, 37–45.

Kussendrager, K.D. and van Hooijdonk, A.C. 2000. Lactoperoxidase: physico-chemical properties, occurrence, mechanism of action and applications. *Br. J. Nutr.* **84**, S19–S25.

Kuwata, H., Yip, T.T., Tomita, M., and Hutchens, T.W. 1998. Direct evidence of the generation in human stomach of an antimicrobial peptide domain (lactoferricin) from ingested lactoferrin. *Biochim. Biophys. Acta* **1429**, 129–141.

LaBell, F. 1998. Health-enhancing whey proteins. *Prepared Foods* **167**, 143.

Lahov, E. and Regelson, W. 1996. Antibacterial and immunostimulating casein-derived substances from milk: caseicidin, isracidin peptides. *Food Chem. Toxicol.* **34**, 131–145.

Lambert, J.C. 2001. Global lactoperoxidase programme. *IDF Bull.* **365**, 19–20.

Larson, B.L. 1992. Immunoglobulins of the mammary secretions. *In* "Advanced Dairy Chemistry 1: Proteins" (P.F. Fox, ed.), pp. 231–254. Elsevier Science Publishers, London.

Law, J. and Haandrikman, A. 1997. Proteolytic enzymes of lactic acid bacteria. *Int. Dairy J.* **7**, 1–11.

Ledoux, N., Mahe, S., Duarry, M., Bourras, M., Benamouzig, R., and Tome, D. 1999. Intraluminal immunoreactive caseinomacropeptide after milk protein ingestion in humans. *Nahrung* **43**, 196–200.

Lee, Y.S., Noguchi, T., and Naito, H. 1980. Phosphopeptides and soluble calcium in the small intestine of rats given casein diet. *Br. J. Nutr.* **43**, 457–467.

Lee, Y.S., Noguchi, T., and Naito, H. 1983. Intestinal absorption of calcium in rats given diets containing casein or amino acid mixture; the role of casein phosphopeptides. *Br. J. Nutr.* **49**, 67–76.

Lefranc, C. 2002. Prodiet^{TM} F200: an innovative stress-relieving ingredient. *Agro-Food-Industry Hi-Tech.* July/August, 9–11.

Levieux, D. and Ollier, A. 1999. Bovine immunoglobulin G, beta-lactoglobulin, alpha-lactalbumin and serum albumin in colostrum and milk during the early post partum period. *J. Dairy Res.* **66**, 421–430.

Li, B. and Sun, D.W. 2002. Novel methods for rapid freezing and thawing of foods—a review. *J. Food Engin.* **54**, 175–182.

Li, X., Staszewski, L., Xu, H., Durick, K., Zoller, M., and Adler, E. 2002. Human receptors for sweet and umami taste. *Proc. Natl Acad. Sci. USA* **99**, 4692–4696.

Li-Chan, E.C.Y. 1999. Applications of egg immunoglobulins in immunoaffinity chromatography. *In* "Egg Nutrition and Biotechnology" (J.S. Sim, S. Nakai, and W. Guenter, eds), pp. 323–340. CABI Publishing, Wallingford, UK.

Li-Chan, E., Kummer, A., Losso, J.N., Kitts, D.D., and Nakai, S. 1995. Stability of bovine immunoglobulins to thermal treatment and processing. *Food Res. Int.* **28**, 9–16.

Liepke, C., Zucht, H., Forssmann, W., and Ständker, L. 2001. Purification of novel peptide antibiotics from human milk. *J. Chromatogr.* **752**, 369–377.

Lilius, E.-M. and Marnila, P. 2001. The role of colostral antibodies in prevention of microbial infections. *Curr. Opin. Infect. Dis.* **14**, 295–300.

Lindmark-Månsson, H. and Åkesson, B. 2000. Antioxidative factors in milk. *Br. J. Nutr.* **84**, S103–S110.

Liu, J., Wang, S., Qi, J., Wang, X., and Song, Y. 1998. The immunostimulatory effect of bioactive peptide from pollen on murine and human lymphocytes. *Mech. Ageing Dev.* **104**, 125–132.

Loimaranta, V., Tenovuo, J., Virtanen, S., Marnila, P., Syväoja, E., Tupasela, T., and Korhonen, H. 1997. Generation of bovine immune colostrum against *Streptococcus mutans* and *Streptococcus sobrinus* and its effect on glucose uptake and extracellullar polysaccharide formation by mutans streptococci. *Vaccine* **15**, 1261–1268.

Loimaranta, V., Carlen, A., Olsson, J., Tenovuo, J., Syväoja, E., and Korhonen, H. 1998a. Concentrated bovine colostral whey proteins from *Streptococcus mutans*/*Strep. sobrinus* immunised cows inhibit the adherence of *Strep. mutans* and promote the aggregation of mutans streptococci. *J. Dairy Res.* **65**, 599–607.

Loimaranta, V., Tenovuo, J., and Korhonen, H. 1998b. Combined inhibitory effect of bovine immune whey and peroxidase-generated hypothiocyanate against glucose uptake by *Streptococcus mutans*. *Oral Microbiol. Immunol.* **13**, 378–381.

Loimaranta, V., Nuutila, J., Marnila, P., Tenovuo, J., Korhonen, H., and Lilius, E. 1999a. Colostral proteins from cows immunised with *Streptococcus mutans*/*Strep. sobrinus* support the phagocytosis and killing of mutans streptococci by human leukocytes. *J. Med. Microbiol.* **48**, 1–10.

Loimaranta, V., Laine, M., Söderling, E., Vasara, E., Rokka, S., Marnila, P., Korhonen, H., Tossavainen, O., and Tenovuo, J. 1999b. Effects of bovine immune- and non-immune whey preparations on the composition and pH response of human dental plaque. *Eur. J. Oral Sci.* **107**, 244–250.

Lönnerdahl, B. and Iyer, S. 1995. Lactoferrin: molecular structure and biological function. *Ann. Rev. Nutr.* **15**, 93–110.

Loukas, S., Zioudrou, C., Streaty, R.A., and Klee, W.A. 1983. Opioid activities and structures of α-casein-derived exorphins. *Biochemistry* **22**, 4567–4573.

Loukas, S., Panetsos, F., Donga, E., and Zioudrou, C. 1990. Selective δ-antagonist peptides, analogs of a α-casein exorphin, as probes for the opioid receptor. *In* "β-Casomorphins and Related Peptides" (F. Nyberg and V. Brantl, eds), pp. 65–75. Fyris-Tryck AB, Uppsala.

Lu, Y. and Bennick, A. 1998. Interaction of tannin with human salivary prolin-rich proteins. *Arch. Oral Biol.* **43**, 717–728.

Maeno, M., Yamamoto, N., and Takano, T. 1996. Isolation of an antihypertensive peptide from casein hydrolysate produced by a proteinase from *Lactobacillus helveticus* CP790. *J. Dairy Sci.* **79**, 1316–1321.

Mahé, S., Tome, D., Dumontier, A.-M., and Desjeux, J.-F. 1989. Absorption of intact β-casomorphins (β-cm) in rabbit ileum *in vitro*. *Reprod. Nutr. Dev.* **29**, 725–732.

Mainer, G., Dominguez, E., Randrup, M., Sanchez, L., and Calvo, M. 1999. Effect of heat treatment on anti-rotavirus activity of bovine immune milk. *J. Dairy Res.* **66**, 131–137.

Mangino, M.E. 1992. Properties of whey protein concentrates. *In* "Whey and Lactose Processing" (J.G. Zadow, ed.), pp. 231–270. Elsevier Applied Science, Cambridge.

Mannheim, A. and Cheryan, M. 1990. Continuous hydrolysis of milk protein in a membrane reactor. *J. Food Sci.* **55**, 381–385.

Marshall, W.E. 1994. Amino acids, peptides, proteins. *In* "Functional Foods" (I. Goldberg, ed.), pp. 242–260. Chapman & Hall, New York.

Maruyama, S. and Suzuki, H. 1982. A peptide inhibitor of angiotensin I converting enzyme in the tryptic hydrolysate of casein. *Agric. Biol. Chem. Tokyo* **46**, 1393–1394.

Maruyama, S., Nakagomi, K., Tomizuka, N., and Suzuki, H. 1985. Angiotensin I-converting enzyme inhibitor derived from an enzymatic hydrolysate of casein. II. Isolation and bradykinin-potentiating activity on the uterus and the ileum of rats. *Agric. Biol. Chem. Tokyo* **49**, 1405–1409.

Maruyama, S., Mitachi, H., Tanaka, H., Tomizuka, N., and Suzuki, H. 1987. Studies on the active site and antihypertensive activity of angiotensin I-converting enzyme inhibitors derived from casein. *Agr. Biol. Chem. Tokyo* **51**, 1581–1586.

Masson, P.L. and Heremans, J.F. 1966. Studies on lactoferrin, the iron-binding protein of secretions. *Prot. Biol. Fluids* **14**, 115–142.

Masuda, O., Nakamura, Y., and Takano, T. 1996. Antihypertensive peptides are present in aorta after oral administration of sour milk containing these peptides to spontaneously hypertensive rats. *J. Nutr.* **126**, 3063–3068.

Matar, C. and Goulet, J. 1996. β-Casomorphin-4 from milk fermented by a mutant of *Lactobacillus helveticus*. *Int. Dairy J.* **6**, 383–397.

Matar, C., Amiot, J., Savoie, L., and Goulet, J. 1996. The effect of milk fermentation by *Lactobacillus helveticus* on the release of peptides during *in vitro* digestion. *J. Dairy Sci.* **79**, 971–979.

Matin, M.A., Mizumoto, G., and Otani, H. 2001. Cytotoxic properties of α-lactalbumins toward mammalian cells. *Milchwissenschaft* **56**, 430–433.

Matoba, T., Hayashi, R., and Hata, T. 1970. Isolation of bitter peptides in tryptic hydrolyzates of casein and their chemical structures. *Agric. Biol. Chem.* **34**, 1235–1243.

Matoba, N., Usui, H., Fujita, H., and Yoshikawa, M. 1999. A novel anti-hypertensive peptide derived from ovalbumin induces nitric oxide-mediated vasorelaxation in an isolated SHR mesenteric artery. *FEBS Lett.* **452**, 181–184.

Matoba, N., Yamada, Y., Usui, H., Nakagiri, R., and Yoshikawa, M. 2001. Designing potent derivatives of ovokinin(2-7), an anti-hypertensive peptide derived from ovalbumin. *Biosci. Biotechnol. Biochem.* **65**(3), 736–739.

Matsubayashi, Y. and Sakagami, Y. 1999. Characterization of specific binding sites for a mitogenic sulfated peptide, phytosulfokine-α, in the plasma-membrane fraction derived from *Oryza sativa* L. *Eur. J. Biochem.* **262**, 666–671.

Matsubayashi, Y., Takagi, L., and Sakagami, Y. 1997. Phytosulfokine-α, sulfated pentapeptide, stimulates the proliferation of rice cells by means of specific high- and low-affinity binding sites. *Proc. Natl Acad. Sci. USA* **94**, 13357–13362.

Matsufuji, H., Matsui, T., Seki, E., Osajima, K., Nakashima, M., and Osajima, Y. 1994. Angiotensin I-converting enzyme inhibitory peptides in an alkaline protease hydrolysate derived from sardine muscle. *Biosci. Biotechnol. Biochem.* **57**, 922–925.

Matsui, T., Li, C.H., and Osajima, Y. 1999. Preparation and characterization of novel bioactive peptides responsible for angiotensin I-converting enzyme inhibition from wheat germ. *J. Peptide Sci.* **5**, 289–297.

Matsumura, N., Fujii, M., Takeda, Y., Sugita, K., and Shimizu, T. 1993. Angiotensin I-converting enzyme inhibitory peptides derived from bonito bowels autolysate. *Biosci. Biotechnol. Biochem.* **57**, 695–697.

Maubois, J.L. (ed.) 2000. New applications of membrane technology in the dairy industry. Special issue, Le Lait , **80** (1), 1–208.

Maubois, J.L. and Ollivier, G. 1997. Extraction of milk proteins. *In* "Food Proteins and Their Applications" (S. Damodaran and A. Paraf, eds), pp. 579–595. Marcel Dekker, Inc., New York.

McIntosh, G.H., Regester, G.O., Le Leu, R.K., Royle, P.J., and Smithers, G.W. 1995. Dairy proteins protect against dimethylhydrazine-induced intestinal cancer in rats. *J. Nutr.* **125**, 809–816.

McIntosh, G.H., Royle, P.J., Le Leu, R.K., Regester, G.O., Johnson, M.A., Grinsted, R.L., Kenward, R.S., and Smithers, G.W. 1998. Whey proteins as functional ingredients? *Int. Dairy J.* **8**, 425–434.

Mee, J.F. and Mehra, R. 1995. Efficacy of colostrum substitutes and supplements in farm animals. *Agro-Food-Industry Hi-Tech* **6**, 31–35.

Mehaia, M.A. and Cheryan, M. 1990. Membrane bioreactors: enzyme processes. *In* "Biotechnology and Food Process Engineering" (H.G. Schwartzberg and M.A. Rao, eds), pp. 67–126. Marcel Dekker, Inc., New York.

Meisel, H. 1986. Chemical characterization and opioid activity of an exorphin isolated from *in vivo* digests of casein. *FEBS Lett.* **196**, 223–227.

Meisel, H. 1997. Biochemical properties of bioactive peptides derived from milk proteins: potential nutraceuticals for food and pharmaceutical applications. *Livest. Prod. Sci.* **50**, 125–138.

Meisel, H. 2001. Bioactive peptides from milk proteins: a perspective for consumers and producers. *Aust. J. Dairy Technol.* **56**, 83–92.

Meisel, H. and Frister, H. 1989. Chemical characterization of bioactive peptides from *in vivo* digestion of casein. *J. Dairy Res.* **56**, 343–349.

Meisel, H. and Gunther, S. 1998. Food proteins as precursors of peptides modulating human cell activity. *Nahrung* **42**, 175–176.

Meisel, H. and Schlimme, E. 1994. Inhibitors of angiotensin converting enzyme derived from bovine casein (casokinins). *In* "β-Casomorphins and Related Peptides: Recent Developments" (V. Brantl and H. Teschemacher, eds), pp. 27–33. VCH, Weinheim.

Meisel, H., Behrens, S., and Schlimme, E. 1991. Calcium-bindungsstudien an Phosphopeptidfraktionen aus der *in vitro*-Proteolyse von Casein. *Kieler Milchwirt. Forschungsber.* **43**, 199–212.

Meisel, H., Goepfert, A., and Günther, S. 1997. ACE-inhibitory activities in milk products. *Milchwissenschaft* **52**, 307–311.

Mellander, O. 1950. The physiological importance of the casein phosphopeptide calcium salts II. Peroral calcium dosage of infants. *Acta Soc. Med. Uppsala* **55**, 247–255.

Mercier, J.-C. 1981. Phosphorylation of caseins, present evidence for an amino acid triplet code postranslationally recognised by specific kinases. *Biochimie* **63**, 1–17.

Messina, M., Gardner, C., and Barnes, S. 2002. Gaining insight into the health of soy but a long way still to go: commentary on the fourth international symposium on the role of soy in preventing and treating chronic disease. *J. Nutr.* **132**, 547S–551S.

Mero, A., Miikkulainen, H., Riski, J., Pakkanen, R., Aalto, J., and Takala, T. 1997. Effects of bovine colostrum supplementation on serum IGF-I, IgG, hormone, saliva IgA during training. *J. Appl. Physiol.* **83**, 1144–1151.

Micke, P., Beeh, K.M., Schlaak, J.F., and Buhl, R. 2001. Oral supplementation with whey proteins increases plasma glutathione levels of HIV-infected patients. *Eur. J. Clin. Invest.* **31**, 171–178.

Mietens, C., Keinhorst, H., Hilpert, H., Gerber, H., Amster, H., and Pahud, J.J. 1979. Treatment of infantile *E. coli* gastroenteritis with specific bovine anti-*E. coli* milk immunoglobulins. *Eur. J. Pediatr.* **132**, 239–252.

Migliore-Samour, D. and Jolles, P. 1988. Casein, prohormone with an immunomodulating role for the newborn. *Experientia* **44**, 188–193.

Migliore-Samour, D., Floc'h, F., and Jolles, P. 1989. Biologically active casein peptides implicated in immunomodulation. *J. Dairy Res.* **56**, 357–362.

Mitra, A.K., Mahalanabis, D., Unicomb, L., Eeckels, R., and Tzipori, S. 1995. Hyperimmune cow colostrum reduces diarrhoea due to rotavirus: a double-blind, controlled clinical trial. *Acta Paediatr.* **84**, 996–1001.

Miyoshi, S., Kaneko, T., Ishikawa, H., Tanaka, H., and Maruyama, S. 1995. Production of peptides from corn endosperm proteins by some proteases. *Ann. NY Acad. Sci.* **750**, 731.

Monnai, M., Horimoto, Y., and Otani, H. 1998. Immunomodificatory effect of dietary bovine κ-caseinoglycopeptide on serum antibody levels and proliferative responses of lymphocytes in mice. *Milchwissenschaft* **53**, 129–132.

Moon, H.W. and Bunn, T.O. 1993. Vaccines for preventing enterotoxigenic *Escherichia coli* infections in farm animals. *Vaccine* **11**, 213–220.

Morr, C.V. and Ha, E.Y.V. 1993. Whey protein concentrates and isolates: processing and functional properties. *CRC Crit. Rev. Food Sci. Nutr.* **6**, 431–476.

Moulti-Mati, F., Mati, A., Capiaumont, J., Belleville, F., Linden, G., and Nabet, P. 1991. Role de la β-lactoglobuline dans l'activité proliférative du lactosérum. *Lait* **71**, 543–553.

Muehlenkamp, M.R. and Warthesen, J.J. 1996. β-Casomorphins: analysis in cheese and susceptibility to proteolytic enzymes from *Lactococcus lactis* ssp. *cremoris*. *J. Dairy Sci.* **79**, 20–26.

Mullally, M.M., Meisel, H., and FitzGerald, R.J. 1996. Synthetic peptides corresponding to β-lactalbumin and β-lactoglobulin sequences with angiotensin-I-converting enzyme inhibitory activity. *Biol. Chem. Hoppe-Seyler* **377**, 259–260.

Mullally, M.M., Meisel, H., and FitzGerald, R.J. 1997a. Identification of a novel angiotensin-I-converting enzyme inhibitory peptide corresponding to a tryptic fragment of bovine β-lactoglobulin. *FEBS Lett.* **402**, 99–101.

Mullally, M.M., Meisel, H., and FitzGerald, R.J. 1997b. Angiotensin-I-converting enzyme inhibitory activities of gastric and pancreatic proteinase digests of whey proteins. *Int. Dairy J.* **7**, 299–303.

Mulvihill, D.M. 1992. Production, functional properties and utilization of milk protein products. *In* "Advanced Dairy Chemistry, Proteins" (P.F. Fox, ed.), Vol. 1. pp. 369–404. Elsevier Science Publishers, London.

Mulvihill, D.M. and Fox, P.F. 1994. Developments in the production of milk proteins. *In* "New and Developing Sources of Food Proteins" (B.J.F. Hudson, ed.), pp. 1–30. Chapman & Hall, London.

Murakami, Y. and Hirata, A. 2000. Novel process for enzymatic hydrolysis of proteins in an aqueous two-phase system for the production of peptide mixture. *Prep. Biochem. Biotechnol.* **30**, 31–37.

Mykkänen, H.M. and Wasserman, R.H. 1980. Enhanced absorption of calcium by casein phosphopeptides in rachitis and normal chicks. *J. Nutr.* **110**, 2141–2148.

Nagaoka, S., Futamura, Y., Miwa, K., Awano, T., Yamauchi, K., Kanamaru, Y., Tadashi, K., and Kuwata, T. 2001. Identification of novel hypocholesterolemic peptides derived from bovine milk β-lactoglobulin. *Biochem. Biophys. Res. Commun.* **281**, 11–17.

Nagaune, S., Azuma, N., Ishino, Y., Mori, H., Kaminogawa, S., and Yamauchi, K. 1989. DNA-synthesis stimulating peptide from bovine β-casein. *Agric. Biol. Chem.* **12**, 3275–3278.

Naidu, A.S. and Arnold, R.R. 1997. Influence of lactoferrin on host–microbe interactions. *In* "Lactoferrin: Interactions and Biological Functions" (T.W. Hutchens and B. Lönnerdahl, eds), pp. 259–275. Humana Press, Totowa, NJ, USA.

Naito, H., Kawakami, A., and Inamura, T. 1972. *In vivo* formation of phosphopeptide with calcium-binding property in the small intestinal tract of the rat fed on casein. *Agric. Biol. Chem. Tokyo* **36**, 409–415.

Nakamura, Y., Yamamoto, M., Sakai, K., Okubo, A., Yamazaki, S., and Takano, T. 1995a. Purification and characterization of angiotensin I-converting enzyme inhibitors from sour milk. *J. Dairy Sci.* **78**, 777–783.

Nakamura, Y., Yamamoto, N., Sakai, K., and Takano, T. 1995b. Antihypertensive effect of sour milk and peptides isolated from it that are inhibitors to angiotensin I-converting enzyme. *J. Dairy Sci.* **78**, 1253–1257.

Nakashima, Y., Arihara, K., Sasaki, A., Mio, H., Ishikawa, S., and Itoh, M. 2002. Antihypertensive activities of peptide derived from porcine skeletal muscle myosin in spontaneously hypertensive rats. *J. Food Sci.* **67**, 434–437.

Neeser, J.R., Golliard, M., Woltz, A., Rouvet, M., Dillmann, M.L., and Guggenheim, B. 1994. *In vitro* modulation of oral bacterial adhesion to saliva-coated hydroxyapatite beads by milk casein derivatives. *Oral Microbiol. Immunol.* **9**, 193–201.

Noguchi, M., Arai, S., Yamashita, M., Kato, H., and Fujimaki, M. 1975. Isolation and identification of acidic oligopeptides occurring in a flavor potentiating fraction from a fish protein hydrolysate. *J. Agric. Food Chem.* **23**, 49–53.

Nousiainen, J., Korhonen, H., Syväoja, E., Savolainen, S., Saloniemi, H., and Jalonen, H. 1994. The effect of colostral immunoglobulin supplement on the passive immunity, growth and health of neonatal calves. *Agric. Sci. Finl.* **3**, 421–427.

Nuijens, J.H., van Berkel, P.H., and Schanbacher, F.L. 1996. Structure and biological actions of lactoferrin. *J. Mamm. Gland Biol.* **1**, 285–295.

Nurminen, M.-L., Sipola, M., Kaarto, H., Pihlanto-Leppälä, A., Piilola, K., Korpela, R., Tossavainen, O., Korhonen, H., and Vapaatalo, H. 2000. α-Lactorphin lowers blood pressure via radiotelemetry in normotensive and spontaneously hypertensive rats. *Life Sci.* **66**, 1535–1543.

Nyberg, F., Sanderson, K., and Glämsta, E.-L. 1997. The hemorphins: a new class of opioid peptides derived from the blood protein hemoglobin. *Biopolymers* **43**, 147–156.

Okamoto, A., Hanagata, H., Matsumoto, E., Kawamura, Y., Koizumi, Y., and Yanagida, F. 1995. Angiotensin I converting enzyme inhibitory activities of various fermented foods. *Biosci. Biotechnol. Biochem.* **59**, 1147–1149.

Okhuysen, P.C., Chappell, C.L., Crabb, J., Valdez, L.M., Douglass, E.T., and DuPont, H.L. 1998. Prophylactic effect of bovine anti-*Cryptosporidium* hyperimmune colostrum immunoglobulin in healthy volunteers challenged with *Cryptosporidium parvum*. *Clin. Infect. Dis.* **26**, 1324–1329.

Ondetti, M.A. and Cushman, D.W. 1984. Angiotensin-converting enzyme inhibitors: biochemical properties and biological actions. *CRC Crit. Rev. Biochem. Mol. Biol.* **16**, 381–411.

Ondetti, M.A., Willimans, N.J., Sabo, E.F., Plusces, J., Weaver, E.R., and Kocy, O. 1971. Angiotensin-converting enzyme inhibitors from the venom of *Bothrops jararaca*. Isolation, elucidation of structure and synthesis. *Biochemistry* **19**, 4033–4039.

Ono, T., Takagi, Y., and Kunishi, I. 1998. Casein phosphopeptides from casein micelles by successive digestion with pepsin and trypsin. *Biosci. Biotechnol. Biochem.* **62**, 16–21.

Oona, M., Rägo, T., Maaroos, H., Mickelsaar, M., Lõivukene, K., Salminen, S., and Korhonen, H. 1997. *Helicobacter pylori* in children with abdominal complaints: has immune bovine colostrum some influence on gastritis? *Alpe Adria Microbiol. J.* **6**, 49–57.

Oshima, G., Shimabukuro, H., and Nagasawa, K. 1979. Peptide inhibitors of angiotensin I-converting enzyme in digests of gelatin by bacterial collagenase. *Biochim. Biophys. Acta* **566**, 128–137.

Otani, H. and Hata, I. 1995. Inhibition of proliferative responses of mouse spleen lymphocytes and rabbit Peyer's patch cells by bovine milk caseins and their digests. *J. Dairy Res.* **62**, 339–348.

Otani, H. and Mizumoto, G. 1998. Suppressive effect of lysozymes and alpha-lactalbumins on mitogen-induced proliferative responses of mouse lymphocytes. *Anim. Sci. Technol.* **69**, 1029–1039.

Otani, H. and Monnai, M. 1995. Induction of an interleukin-1 receptor antagonist-like component produced from mouse spleen cells by bovine kappa-caseinoglycopeptide. *Biosci. Biotechnol. Biochem.* **59**, 1166–1168.

Otani, H., Monnai, M., and Hosono, A. 1992. Bovine κ-casein as inhibitor of the proliferation of mouse splenocytes induced by lipopolysaccharide stimulation. *Milchwissenschaft* **47**, 512–515.

Otani, H., Monnai, M., Kawasaki, Y., Kawakami, H., and Tanimoto, M. 1995. Inhibition of mitogen-induced proliferative responses of lymphocytes by bovine κ-caseinoglycopeptides having different carbohydrate chains. *J. Dairy Res.* **62**, 349–357.

Otani, H., Kihara, Y., and Park, M. 2000. The immunoenhancing property of dietary casein phosphopeptide preparation in mice. *Food Agric. Immunol.* **12**, 165–173.

Outinen, M., Tossavainen, O., Syväoja, E.-L., and Korhonen, H. 1995. Chromatographic isolation of κ-casein macropeptide from cheese whey with a strong basic anion exchange resin. *Milchwissenschaft* **50**, 570–574.

Outinen, M., Tossavainen, O., Tupasela, T., Koskela, P., Koskinen, H., Rantamäki, P., Syväoja, E.-L., Antila, P., and Kankare, V. 1996. Fractionation of proteins from whey with different pilot scale processes. *Lebensm. Wiss. u.-Technol.* **29**, 411–417.

Paakkari, I., Järvinen, A., Antila, P., Mattila, M.J., and Pihlanto-Leppälä, A. 1994. Opioid effects of the milk whey-protein derived peptides α- and β-lactorphin. *In* "β-Casomorphins and Related Peptides. Recent Development" (V. Brantl and H. Teschemacher, eds), pp. 33–37. VCH, Weinheim.

Paegelow, I. and Werner, H. 1986. Immunomodulation by some oligopeptides. *Methods Find. Exp. Clin. Pharmacol.* **8**, 91–95.

Pakkanen, R. and Aalto, J. 1997. Growth factors and antimicrobial factors of bovine colostrum. *Int. Dairy J.* **7**, 285–297.

Pappenheimer, J.R., Dahl, C.E., Karnovsky, M.L., and Maggio, J.E. 1994. Intestinal absorption and excretion of octapeptides composed of D-amino acids. *Proc. Natl Acad. Sci. USA.* **91**, 1942–1945.

Park, O. and Allen, J.C. 1998. Preparation of phosphopeptides derived from α_s-casein and β-casein using immobilized glutamic acid-specific endopeptidase and characterization of their calcium binding. *J. Dairy Sci.* **81**, 2858–2865.

Park, O., Swaisgood, H.E., and Allen, J.C. 1998. Calcium binding of phosphopeptides derived from hydrolysis of α_s-casein or β-casein using immobilized trypsin. *J. Dairy Sci.* **81**, 2850–2857.

Parker, F., Migliore-Samour, D., Floc'h, F., Zerial, A., Werner, G.H., Jollés, J., Casaretto, M., Zahn, H., and Jollés, P. 1984. Immunostimulating hexapeptide from human casein, amino acid sequence, synthesis and biological properties. *Eur. J. Biochem.* **145**, 677–682.

Parodi, P.W. 1998. A role for milk proteins in cancer prevention. *Aust. J. Dairy Technol.* **53**, 37–47.

Paroli, E. 1988. Opioid peptides from food (the exorphins). *World Rev. Nutr. Diet.* **55**, 58–97.

Pasi, A., Mahler, H., Lansel, N., Bernasconi, C., and Messiha, F.S. 1993. Beta-casomorphin-immunoreactivity in the brain stem of the human infant. *Res. Commun. Chem. Pathol. Pharm.* **80**, 305–322.

Pastorello, E.A. 1997. Study of Nutritional Factors in Food Allergies and Food Intolerances. European Commission, Brussels, ISBN 92-827-9554-3.

Paulsson, M.A., Svensson, U., Kishore, A.R., and Naidu, A.S. 1993. Thermal behaviour of bovine lactoferrin in water and its relation to bacterial interaction and antibacterial activity. *J. Dairy Sci.* **76**, 3711–3720.

Pearce, R.J. 1983. Thermal separation of β-lactoglobulin and β-lactalbumin in bovine cheddar cheese whey. *Aust. J. Dairy Technol.* **38**, 144–149.

Pedersen, N.L.-R., Nagain-Domaine, C., Mahe, S., Chariot, J., Roze, C., and Tome, D. 2000. Caseinomacropeptide specifically stimulates exocrine pancreatic secretion in anesthetized rat. *Peptides* **21**, 1527–1535.

Pellegrini, A., Thomas, U., Bramaz, N., Huniziker, P., and von Fellenberg, R. 1999. Isolation and identification of three bactericidal domains in the bovine α-lactalbumin molecule. *Biochim. Biophys. Acta* **1426**, 4369–4448.

Pellegrini, A., Dettling, C., Thomas, U., and Hunziker, P. 2001. Isolation and characterization of four bactericidal domains in the bovine α-lactoglobulin. *Biochim. Biophys. Acta* **1526**, 131–140.

Peres, J.M., Bouhallab, S., Bureau, F., Neuville, D., Maubois, J.L., Devroede, G., Arhan, P., and Bougle, D. 1999. Mechanisms of absorption of caseinophosphopeptide bound iron. *J. Nutr. Biochem.* **10**, 215–222.

Perez, M.D. and Calvo, M. 1995. Interaction of β-lactoglobulin with retinol and fatty acids and its role as a possible biological function for this protein: a review. *J. Dairy Sci.* **78**, 978–988.

Perryman, L.E., Kapil, S.J., Jones, M.L., and Hunt, E.L. 1999. Protection of calves against cryptosporidiosis with immune bovine colostrum induced by a *Cryptosporidium parvum* recombinant protein. *Vaccine* **17**, 2142–2149.

Petrilli, P., Picone, D., Caporale, C., Auricchio, S., and Marino, G. 1984. Does casomorphin have a functional role? *FEBS Lett.* **169**, 1346–1350.

Petrillo, E.W. and Ondetti, M.A. 1982. Angiotensin-converting enzyme inhibitors, medical chemistry and biological actions. *Med. Res. Rev.* **2**, 1–41.

Pihlanto-Leppälä, A. 2001. Bioactive peptides derived from bovine whey proteins: opioid and ace-inhibitory peptides. *Trends Food Sci. Technol.* **11**, 347–356.

Pihlanto-Leppälä, A., Koskinen, P., Paakkari, I., Tupasela, T., and Korhonen, H. 1996. Opioid whey protein peptides obtained by membrane filtration. *IDF Bull.* **311**, 36–38.

Pihlanto-Leppälä, A., Paakkari, I., Rinta-Koski, M., and Antila, P. 1997. Bioactive peptide derived from *in vitro* proteolysis of bovine beta-lactoglobulin and its effect on smooth muscle. *J. Dairy Res.* **64**, 149–155.

Pihlanto-Leppälä, A., Rokka, T., and Korhonen, H. 1998. Angiotensin I converting enzyme inhibitory peptides derived from bovine milk proteins. *Int. Dairy J.* **8**, 325–331.

Pihlanto-Leppälä, A., Marnila, P., Hubert, L., Rokka, T., Korhonen, H., and Karp, M. 1999. The effect of alpha-lactalbumin and beta-lactoglobulin hydrolysates on the metabolic activity of *Escherichia coli* JM103. *J. Appl. Microbiol.* **87**, 540–545.

Pihlanto-Leppälä, A., Koskinen, P., Piilola, K., Tupasela, T., and Korhonen, H. 2000. Angiotensin I-converting enzyme inhibitory properties of whey protein digests: concentration and characterization of active peptides. *J. Dairy Res.* **67**, 53–64.

Playford, R.J., Floyd, D.N., Macdonald, C.E., Calnan, D.P., Adenekan, R.O., Johnson, W., Goodlad, R.A., and Marchbank, T. 1999. Bovine colostrum is a health food supplement which prevents NSAID induced gut damage. *Gut* **44**, 653–658.

Playford, R.J., Macdonald, C.E., and Johnson, W.S. 2000. Colostrum and milk-derived peptide growth factors for the treatment of gastrointestinal disorders. *Am. J. Clin. Nutr.* **72**, 5–14.

Pointillart, A. and Guéguen, L. 1989. Absence d'effet de l'incorporation d'un phosphopeptide du lait sur l'utilisation cu calcium et du phosphore chez le jeune porc. *Reprod. Nutr. Dev.* **29**, 447–486.

Poolman, B., Kunji, E.R.S., Hagting, A., Juillard, V., and Konings, W.N. 1995. The proteolytic pathway of *Lactococcus lactis*. *J. Appl. Bacteriol.* **79**, 65S–75S.

Qian, Z.Y., Jolles, P., Migliore-Samour, D., and Fiat, A.M. 1995. Isolation and characterization of sheep lactoferrin, an inhibitor of platelet aggregation and comparison with human lactoferrin. *Biochim. Biophys. Acta* **1243**, 25–32.

Quigley, J.D. and Drewry, J.J. 1998. Nutrient and immunity transfer from cow to calf pre- and postcalving. *J. Dairy Sci.* **81**, 2779–2790.

Rainard, P. 1986. Bacteriostatic activity of bovine milk lactoferrin against mastitic bacteria. *Vet. Microbiol.* **11**, 387–392.

Rauprich, A.B., Hammon, H.M., and Blum, J.W. 2000. Effects of feeding colostrum and a formula with nutrient contents as colostrum on metabolic and endocrine traits in neonatal calves. *Biol. Neonate* **78**, 53–64.

Read, L.C., Lord, A.P.D., Brantl, V., and Koch, G.H. 1990. Absorption of β-casomorphins from autoperfused lamb and piglet small intestine. *Am. J. Physiol.* **259**, G443–G452.

Recio, I. and Visser, S. 1999. Two ion-exchange methods for the isolation of antibacterial peptides from lactoferrin—*in situ* enzymatic hydrolysis on an ion-exchange membrane. *J. Chromatogr.* **831**, 191–201.

Recio, I., Floris, R., and Visser, S. 2000. Bioactive peptides from food proteins: a new isolation method. *Agro-Food-Ind. Hi-Tech* **2**, 9–11.

Reiter, B. 1985. Protective proteins in milk—biological significance and exploitation. *IDF Bull.* **191**, 1–35.

Reiter, B. and Härnulv, G. 1984. Lactoperoxidase antibacterial system: natural occurrence, biological functions and practical applications. *J. Food Protect.* **47**, 724–732.

Reiter, B. and Oram, J.D. 1967. Bacterial inhibitors in milk and other biological fluids. *Nature* **216**, 328–330.

Reiter, B. and Perraudin, J.P. 1991. Lactoperoxidase: biological functions. *In* "Peroxydases in Chemistry and Biology", pp. 143–180. CRC Press, Boca Raton.

Reiter, B., Marshall, V.M.E., Björck, L., and Rosen, C.G. 1976. Nonspecific bactericidal activity of the lactoperoxidase-thiocyanate-hydrogen peroxide system of milk against *Escherichia coli* and some Gram-negative pathogens. *Infect. Immun.* **13**, 800–807.

Reynolds, E.C. 1992. Production of phosphopeptides from casein. World Patent Application WO 92/18526.

Reynolds, E.C. 1998. Anticariogenic complexes of amorphous calcium phosphate stabilized by casein phosphopeptides: a review. *Spec. Care Dent.* **18**, 8–16.

Righetti, P.G., Nembri, F., Bossi, A., and Mortarino, M. 1997. Continuous enzymatic hydrolysis of beta-casein and isoelectric collection of some of the biologically active peptides in an electric field. *Biotechnol. Prog.* **13**, 258–264.

Rival, S.G., Boeriu, C.G., and Wichers, H.J. 2001a. Caseins and casein hydrolysates. 2. Antioxidative properties and relevance to lipoxygenase inhibition. *J. Agric. Food Chem.* **49**, 295–302.

Rival, S.G., Fornaroli, S., Boeriu, C.G., and Wichers, H.J. 2001b. Caseins and casein hydrolysates. 1. Lipoxygenase inhibitory properties. *J. Agric. Food Chem.* **49**, 287–294.

Roberts, A.K., Chierici, R., Sawatzki, G., Hill, M.J., Volpato, S., and Vigi, V. 1992. Supplementation of an adapted formula with bovine lactoferrin: 1. Effect on the infant faecal flora. *Acta Paediatr.* **81**, 119–124.

Rokka, T., Syväoja, E.-L., Tuominen, J., and Korhonen, H. 1997. Release of bioactive peptides by enzymatic proteolysis of *Lactobacillus* GG fermented UHT-milk. *Milchwissenschaft* **52**, 675–678.

Roos, N., Mahé, S., Benamouzig, R., Sick, H., Rautureau, J., and Tomé, D. 1995. 15-N-labeled immunoglobulins from bovine colostrum are partially resistant to digestion in human intestine. *J. Nutr.* **125**, 1238–1244.

Rose, R.K. 2000a. Binding characteristics of *Streptococcus mutans* for calcium and casein phosphopeptide. *Caries Res.* **34**, 427–431.

Rose, R.K. 2000b. Effects of an anticariogenic casein phosphopeptide on calcium diffusion in streptococcal model dental plaques. *Arch. Oral Biol.* **45**, 569–575.

Rosenberg, M. 1995. Current and future applications for membrane process in the dairy industry. *Trends Food Sci. Technol.* **6**, 12–19.

Roudot-Algaron, F., LeBars, D., Kerhoas, L., Einhorn, J., and Gripon, J.C. 1994. Phosphopeptides from Comté cheese: nature and origin. *J. Food Sci.* **59**, 544–547.

Ruiz, L.P. 1994. Antibodies from milk for the prevention and treatment of diarrhoeal disease. *In* "Indigenous antimicrobial agents of milk—Recent developments", IDF Special Issue 9404, pp. 108–121.

Ryhänen, E.-L., Pihlanto-Leppälä, A., and Pahkala, E. 2001. A new type of ripened, low-fat cheese with bioactive properties. *Int. Dairy J.* **11**, 441–447.

Saha, B.C. and Hayashi, K. 2001. Debittering of protein hydrolyzates. *Biotechnol. Adv.* **19**, 355–370.

Saif, L.J., Smith, K.L., Landmeier, B.J., Bohl, E.H., Theil, K.W., and Todhunter, D.A. 1984. Immune response of pregnant cows to bovine rotavirus immunization. *Am. J. Vet. Res.* **45**, 49–58.

Saito, H., Takase, M., Tamura, Y., Shimamura, S., and Tomita, M. 1994. Physicochemical and antibacterial properties of lactoferrin and its hydrolysate produced by heat treatment at acidic pH. *Adv. Exp. Med. Biol.* **357**, 219–226.

Saito, Y., Lee, Y.S., and Kimura, S. 1998. Minimum effective dose of casein phosphopeptides (CPP) for enhancement of calcium absorption in growing rats. *Int. J. Vitamin. Nutr. Res.* **68**, 335–340.

Saito, T., Nakamura, T., Kitazawa, H., Kawai, Y., and Itoh, T. 2000. Isolation and structural analysis of antihypertensive peptides that exist naturally in Gouda cheese. *J. Dairy Sci.* **83**, 1434–1440.

Sanchez, L., Calvo, M., and Brock, J.H. 1992. Biological role of lactoferrin. *Arch. Dis. Child.* **67**, 657–661.

Sannier, F., Bordenave, S., and Piot, J.M. 2000. Purification of goat beta-lactoglobulin from whey by an ultrafiltration membrane enzymic reactor. *J. Dairy Res.* **67**, 43–51.

Sarker, S.A., Casswall, T.H., Mahalanabis, D., Alam, N.H., Albert, N.J., Brüssow, H., Fuchs, G.J., and Hammarström, L. 1998. Successful treatment of rotavirus diarrhea in children with immunoglobulin from immunised bovine colostrum. *Pediatr. Infect. Dis. J.* **17**, 1149–1154.

Sarker, S.A., Casswall, T.H., Juneja, L.R., Hoq, E., Hossain, I., Fuchs, G.J., and Hammarström, L. 2001. Randomized, placebo-controlled, clinical trial of hyperimmunized chicken egg yolk immunoglobulin in children with rotavirus diarrhea. *J. Pediatr. Gastroenterol. Nutr.* **32**, 19–25.

Sarni-Manchado, P., Cheynier, V., and Moutounet, M. 1999. Interactions of grape seed tannins with salivary proteins. *J. Agric. Food Chem.* **47**, 42–47.

Sato, R., Noguchi, T., and Naito, H. 1983. The necessity for the phosphate portion of casein molecules to enhance Ca absorption from the small intestine. *Agric. Biol. Chem. Tokyo* **47**, 2415–2417.

Sato, R., Noguchi, T., and Naito, H. 1986. Casein phosphopeptide (CPP) enhances calcium absorption from the ligated segment of rat small intestine. *J. Nutr. Sci. Vitaminol.* **32**, 67–76.

Sato, R., Shindo, M., Gunshin, H., Noguchi, T., and Naito, H. 1991. Characterisation of phosphopeptide derived from bovine β-casein: an inhibitor to intra-intestinal precipitation of calcium phosphate. *Biochim. Biophys. Acta* **1077**, 413–415.

Sawyer, L. and Holt, C. 1993. The secondary structure of milk proteins and their biological function. *J. Dairy Sci.* **76**, 3062–3078.

Scammell, A.W. 2001. Production and uses of colostrum. *Aust. J. Dairy Technol.* **56**(2), 74–82.

Scanff, P., Yvon, M., Thirouin, S., and Pélissier, J.-P. 1992. Characterization and kinetics of gastric emptying of peptides derived from milk proteins in the preruminant calf. *J. Dairy Res.* **59**, 437–447.

Schaller, J.P., Saif, L.J., Cordle, C.T., Candler, E. Jr, Winship, T.R., and Smith, K.L. 1992. Prevention of human rotavirus-induced diarrhea in gnotobiotic piglets using bovine antibody. *J. Infect. Dis.* **165**, 623–630.

Schanbacher, F.L., Talhouk, R.S., Murray, F.A., Gherman, L.I., and Willet, L.B. 1998. Milk-borne bioactive peptides. *Int. Dairy J.* **8**, 393–403.

Schlichtherle-Cerny, H. and Amadó, R. 2002. Analysis of taste-active compounds in an enzymatic hydrolysate of deamidated wheat gluten. *J. Agric. Food Chem.* **50**, 1515–1522.

Schulte-Frohlinde, E., Schmid, R., Brantl, V., and Schusdziarra, V. 1994. Effect of bovine β-casomorphin-4-amide on gastrointestinal transit and pancreatic endocrine function in man. *In* "β-Casomorphins and Related Peptides: Recent Developments" (V. Brantl and H. Teschemacher, eds), pp. 155–160. VCH, Weinheim.

Schupbach, P., Neeser, J.R., Golliard, M., Rouvet, M., and Guggenheim, B. 1996. Incorporation of caseinoglycomacropeptide and caseinophosphopeptide into the salivary pellicle inhibits adherence of mutans streptococci. *J. Dent. Res.* **75**, 1779–1788.

Seki, E., Osajima, K., Matsufuji, H., Matsui, T., and Osajima, Y. 1996. Angiotensin I-converting enzyme inhibitory activity of short chain peptides derived from various food proteins. *J. Jpn. Soc. Food Sci. Technol.* **43**, 839–840.

Sekine, K., Ushida, Y., Kuhara, T., Iigo, M., Baba-toriyama, H., Moore, M.A., Murakoshi, M., Satomi, Y.M., Nishino, H., Kakizoe, T., and Tsuda, H. 1997. Inhibition of initiation and early stage development of aberrant crypt foci and enhanced natural killer activity in male rats administered bovine lactoferrin concomitantly with azoxymethane. *Cancer Lett.* **121**, 211–216.

Seppo, L., Kerojoki, O., Suomalainen, T., and Korpela, R. 2002. The effect of a *Lactobacillus helveticus* LBK-16 H fermented milk on hypertension—a pilot study on humans. *Milchwissenschaft* **57**, 124–127.

Seppo, L., Jauhiainen, T., Poussa, T., and Korpela, R. 2003. A fermented milk high in bioactive peptides has a blood-pressure lowering effect in hypertensive subjects. *Am. J. Clin. Nutr.* **77**, 326–330.

Shah, N.P. 2000. Effects of milk-derived bioactives: an overview. *Br. J. Nutr.* **84**, S3–S10.

Shimizu, M., Tsunogai, M., and Arai, S. 1997. Transepithelial transport of oligopeptides in the human intestinal cell, Caco-2. *Peptides* **18**, 681–687.

Shin, K., Yamauchi, K., Teraguchi, S., Hayasawa, H., Tomita, M., Otsuka, Y., and Yamazaki, S. 1998. Antibacterial activity of bovine and its peptides against enterohaemorrhagic *Escherichia coli* O157, H7. *Lett. Appl. Microbiol.* **26**, 407–411.

Shinoda, I., Takase, M., Fukuwatari, Y., Shimamura, S., Koller, M., and Konig, W. 1996. Effects of lactoferrin and lactoferricin on the release of interleukin 8 from human polymorphonuclear leukocytes. *Biosci. Biotechnol. Biochem.* **60**, 521–523.

Siciliano, R., Rega, B., Marchetti, M., Seganti, L., Antonini, G., and Valenti, P. 1999. Bovine lactoferrin peptidic fractions involved in inhibition of herpes simplex virus type I infection. *Biochem. Biophys. Res. Commun.* **264**, 19–23.

Singh, T.K., Fox, P.F., and Healy, A. 1997. Isolation and identification of further peptides in the diafiltration retentate of the water-soluble fraction of Cheddar cheese. *J. Dairy Res.* **64**, 433–443.

Singh, M., Rosen, C.L., Chang, K.J., and Haddad, G.G. 1989. Plasma beta-casomorphin-7 immunoreactive peptide increases after milk intake in newborn but not in adult dogs. *Pediatr. Res.* **26**, 34–38.

Sipola, M., Finckenberg, P., Santisteban, J., Korpela, R., Vapaatalo, H., and Nurminen, M.-L. 2001. Long-term intake of milk peptides attenuates development of hypertension in spontaneously hypertensive rats. *J. Physiol. Pharmacol.* **52**, 745–754.

Sipola, M., Finckenberg, P., Korpela, R., Vapaatalo, H., and Nurminen, M.-L. 2002a. Effect of long-term intake of milk products on blood pressure in hypertensive rats. *J. Dairy Res.* **69**, 103–111.

Sipola, M., Finckenberg, P., Vapaatalo, H., Pihlanto-Leppälä, A., Korhonen, H., Korpela, R., and Nurminen, M.-L. 2002b. α-Lactorphin and β-lactorphin improve arterial function in spontaneously hypertensive rats. *Life Sci.* **71**, 1245–1253.

Smacchi, E. and Gobbetti, M. 1998. Peptides from several Italian cheeses inhibitory to proteolytic enzymes of lactic acid bacteria, *Pseudomonas fluorescens* ATCC 948 and to the angiotensin I-converting enzyme. *Enzyme Microb. Technol.* **22**, 687–694.

Smit, G., Verheul, A., van Kranenburg, R., Ayad, E., Siezen, R., and Engels, W. 2000. Cheese flavor development by enzymatic conversions of peptides and amino acids. *Food Res. Int.* **33**, 153–160.

Soeryapranata, E., Powers, J.R., Fajarrini, F., Weller, K.M., Hill, H.H., and Siems, W.F. 2002. Relationship between MALDI-TOF analysis of β-cn f193-209 concentration and sensory evaluation of bitterness intensity of aged cheddar cheese. *J. Agric. Food Chem.* **50**, 4900–4905.

Spik, G., Cheron, A., Montreuil, J., and Dolby, J.M. 1978. Bacteriostasis of a milk-sensitive strain of *Escherichia coli* by immunoglobulins and iron-binding proteins in association. *Immunology* **35**, 663–671.

Spik, G., Coddeville, B., Mazurier, J., Bourne, Y., Cambillaut, C., and Montreuil, J. 1994a. Primary and three-dimensional structure of lactotransferrin (lactoferrin) glycans. *Adv. Exp. Med. Biol.* **357**, 21–32.

Spik, G., Legrand, D., Leveugle, B., Mazurier, J., Mikogami, T., Montreuil, J., Pierce, A., and Rochard, E. 1994b. Characterization of two kinds of lactotransferrin (lactoferrin) receptors on different target cells. *Adv. Exp. Med. Biol.* **357**, 13–19.

Spik, G., Legrand, D., Mazurier, J., Pierce, A., and Perraudin, J.-P. (eds) 1998. In "Advances in lactoferrin research", Advances in Experimental Medicine and Biology , Vol. 443, pp. 1–350. Plenum Press, New York.

Stadhouders, J. and Beumer, R.R. 1994. Actual and potential applications of the natural antimicrobial agents of milk in the dairy industry. *In* "Indigenous Antimicrobial Agents of milk—Recent Developments", pp. 175–197, IDF Special Issue **9404**.

Steijns, J. 1996. Dietary proteins as the source of new health promoting bio-active peptides with special attention to glutamine peptide. *Food Technol. Eur.* **3**, 80–84.

Steijns, J.M. 2001. Milk ingredients as nutraceuticals. *Int. J. Dairy Technol.* **54**, 81–88.

Steijns, J.M. and van Hooijdonk, A.C. 2000. Occurrence, structure, biochemical properties and technological characteristics of lactoferrin. *Br. J. Nutr.* **84**, S11–S17.

Stephens, S., Dolby, J.M., Montreuil, J., and Spik, G. 1980. Differences in inhibition of the growth of commensal and enteropathogenic strains of *Escherichia coli* by lactotransferrin and secretory immunoglobulin A isolated from human milk. *Immunology* **41**, 597–603.

Stevenson, D.E., Ofman, D.J., Morgan, K.R., and Stanley, R.A. 1998. Protease-catalyzed condensation of peptides as a potential means to reduce the bitter taste of hydrophobic peptides found in protein hydrolysates. *Enzyme Microb. Technol.* **22**, 100–110.

Storm, W. 1990. Human milk feeding and neonatal jaundice. *Kinderkrankenschwester* **9**, 52–53.

Stott, G.H. and Lucas, D.O. 1989. Immunologically active whey fraction and recovery process. US Patent No. 4,834,974.

Suetsuna, K. 1998. Isolation and characterization of angiotensin I converting enzyme inhibitor dipeptides derived from *Allium sativum* L (garlic). *J. Nutr. Biochem.* **9**, 415–419.

Suetsuna, K. and Nakano, T. 2000. Identification of an antihypertensive peptide from peptic digest of wakame (*Undaria pinnatifida*). *J. Nutr. Biochem.* **11**, 450–454.

Suetsuna, K., Ukeda, H., and Ochi, H. 2000. Isolation and characterization of free radical scavenging activities peptides derived from casein. *J. Nutr. Biochem.* **11**, 128–131.

Sugai, R. 1998. ACE inhibitors and functional foods. *IDF Bull.* **336**, 17–20.

Sugino, H., Nitoda, T., and Juneja, L.R. 1997. General chemical composition of hen eggs. *In* "Hen Eggs: Their Basic and Applied Sciences" (T. Yamamoto, L.R. Juneja, H. Hatta, and M. Kim, eds), pp. 13–24. CRC Press, Boca Raton.

Superti, F., Ammendolia, M.G., Valenti, P., and Seganti, L. 1997. Antirotaviral activity of milk proteins: lactoferrin prevents rotavirus infection in the enterocyte-like cell line HT-29. *Med. Microbiol. Immunol.* **186**, 83–91.

Sütas, Y., Hurme, M., and Isolauri, E. 1996a. Down-regulation of anti-CD3 antibody-induced IL-4 production by bovine caseins hydrolysed with *Lactobacillus* GG-derived enzymes. *Scand. J. Immunol.* **43**, 687–689.

Sütas, Y., Soppi, E., Korhonen, H., Syväoja, E.-L., Saxelin, M., Rokka, T., and Isolauri, E. 1996b. Suppression of lymphocyte proliferation *in vitro* by bovine caseins hydrolyzed with *Lactobacillus casei* GG-derived enzymes. *J. Allergy Clin. Immunol.* **98**, 216–224.

Svedberg, J., de Haas, J., Leimenstoll, G., Paul, F., and Teschemacher, H. 1985. Demonstration of beta-casomorphin immunoreactive materials in *in vitro* digests of bovine milk and in small intestine contents after bovine milk ingestion in adult humans. *Peptides* **6**, 825–830.

Svensson, M., Sabharwal, H., Hakansson, A., Mossberg, A.K., Lipniunas, P., Leffler, H., Svanborg, C., and Linse, S. 1999. Molecular characterization of alpha-lactalbumin folding variants that induce apoptosis in tumor cells. *J. Biol. Chem.* **274**, 6388–6396.

Swaisgood, H.E. 1993. Review and update of casein chemistry. *J. Dairy Sci.* **76**, 3054–3061.

Tacket, C.O., Losonsky, G., Link, H., Hoang, Y., Guesry, P., Hilpert, H., and Levine, M.M. 1988. Protection by milk immunoglobulin concentrate against oral challenge with enterotoxigenic *Escherichia coli*. *N. Engl. J. Med.* **318**, 1240–1243.

Tacket, C.O., Binion, S.B., Bostwick, E., Losonsky, G., Roy, M.J., and Edelman, R. 1992. Efficacy of bovine milk immunoglobulin concentrate in preventing illness after *Shigella flexneri* challenge. *Am. J. Trop. Med. Hyg.* **47**, 276–283.

Taira, T., Hilakivi, J., Aalto, J., and Hilakivi, I. 1990. Effect of beta-casomorphin on neonatal sleep in rats. *Peptides* **11**, 1–4.

Takahashi, M., Moriguchi, S., Yoshikawa, M., and Sasaki, R. 1994. Isolation and characterization of oryzatensin, a novel bioactive peptide with ileum-contracting and immunomodulating activities derived from rice albumin. *Biochem. Mol. Biol. Int.* **33**, 1151–1158.

Takahashi, M., Moriguchi, S., Ikeno, M., Kono, S., Ohata, K., Usui, H., Kurahashi, K., Sasaki, R., and Yoshikawa, M. 1996. Studies on the ileum-contracting mechanisms and identification as a complement C3a receptor antagonist of oryzatensin, a bioactive peptide derived from rice albumin. *Peptides* **17**, 5–12.

Takahashi, M., Moriguchi, S., Suganuma, H., Shiota, A., Tani, F., Usui, H., Kurahashi, K., Sasaki, R., and Yoshikawa, M. 1997. Identification of casoxin C, an ileum-contracting peptide derived from bovine κ-casein, as an agonist for C3a receptors. *Peptides* **18**, 329–336.

Takano, T. 1998. Milk derived peptides and hypertension reduction. *Int. Dairy J.* **8**, 375–381.

Takano, T. 2002. Anti-hypertensive activity of fermented dairy products containing biogenic peptides. *Antonie van Leeuwenhoek* **82**, 333–340.

Tani, F., Iio, K., Chiba, H., and Yoshikawa, M. 1990. Isolation and characterization of opioid antagonist peptides derived from human lactoferrin. *Agric. Biol. Chem. Tokyo* **54**, 1803–1810.

Tani, F., Shiota, A., Chiba, H., and Yoshikawa, M. 1994. Serorphin, an opioid peptide derived from bovine serum albumin. *In* "β-Casomorphins and Related Peptides: Recent Developments" (V. Brantl and H. Teschemacher, eds), pp. 49–53. VCH, Weinheim.

Tanizaki, H., Tanaka, H., Iwata, H., and Kato, A. 1997. Activation of macrophages by sulfated glycopeptides in ovomucin, yolk membrane, chalazae in chicken eggs. *Biosci. Biotechnol. Biochem.* **61**, 1883–1889.

Tauzin, J., Milco, L., and Gaillard, J.-L. 2002. Angiotensin-I-converting enzyme inhibitory peptides from tryptic hydrolysate of bovine α_{s2}-casein. *FEBS Lett.* **531**, 369–374.

Taylor, S.L. 1986. Histamine food poisoning: toxicology and clinical aspects. *Crit. Rev. Toxicol.* **17**, 91–128.

Taylor, S.L. and Hefle, S.L. 2001. Will genetically modified foods be allergenic? *J. Allergy Clin. Immunol.* **107**, 765–771.

Taylor, S.L. and Lehner, S.B. 1996. Principles and characteristics of food allergens. *Crit. Rev. Food Sci. Nutr.* **36S**, 91–118.

Telemo, E. and Hanson, L.A. 1996. Antibodies in milk. *J. Mamm. Gland Biol.* **1**, 243–249.

Temussi, P.A. 2002. Why are sweet proteins sweet? Interactions of brazzein, menellin and thaumatin with the T1R2-T1R3 receptor. *FEBS Lett.* **526**, 1–4.

Teraguchi, S., Shin, K., Ogata, T., Kingaku, M., Kaino, A., Miyauchi, H., Fukuwatari, Y., and Shimamura, S. 1995a. Orally administered bovine lactoferrin inhibits bacterial translocation in mice fed bovine milk. *Appl. Environ. Microb.* **61**, 4131–4134.

Teraguchi, S., Shin, K., Ozawa, K., Nakamura, S., Fukuwatari, Y., Tsuyuki, S., Namihira, H., and Shimamura, S. 1995b. Bacteriostatic effect of orally administered bovine lactoferrin on proliferation of *Clostridium* species in the gut of mice fed bovine milk. *Appl. Environ. Microb.* **61**, 501–506.

Teschemacher, H., Umbach, M., Hamel, U., Praetorius, K., Ahnert-Hilger, G., Brantl, V., Lottspeich, F., and Henschen, A. 1986. No evidence for the presence of β-casomorphins in human plasma after ingestion of cow's milk or milk products. *J. Dairy Res.* **53**, 135–138.

Teschemacher, H., Brantl, V., Henschen, A., and Lottspeich, F. 1990. β-Casomorphins—β-casein fragments with opioid activity: detection and structure. *In* "β-Casomorphins and Related Peptides" (F. Nyberg and V. Brantl, eds), pp. 9–14. Fyris-Tryck AB, Uppsala.

Teschemacher, H., Koch, G., and Brantl, V. 1994. Milk protein derived atypical opioid peptides and related compounds with opioid antagonist activity (Review). *In* "β-Casomorphins and Related Peptides: Recent Developments" (V. Brantl and H. Teschemacher, eds), pp. 3–17. VCH, Weinheim.

Teschemacher, H., Koch, G., and Brantl, V. 1997. Milk protein-derived opioid receptor ligands. *Biopolymers* **43**, 99–117.

Thomas, T.D. and Pritchard, G.G. 1987. Proteolytic enzymes of dairy starter cultures. *FEMS Microbiol. Rev.* **46**, 245–268.

Timmer, J.M.K. and van der Horst, H.C. 1998. Whey processing and separation technology: state-of-the-art and new developments. *In* "Whey", Proc. Second Int. Whey Conference, Chicago, USA, pp. 40–65, IDF Special Issue 9804.

Tomita, M., Bellamy, W.R., Takase, M., Yamauchi, K., Wakabayashi, H., and Kawase, K. 1991. Potent antibacterial peptides generated by pepsin digestion of bovine lactoferrin. *J. Dairy Sci.* **74**, 4137–4142.

Tomita, M., Takase, M., Bellamy, W.R., and Shimamura, S. 1994. A review, the active peptide of lactoferrin. *Acta Paediatr. Jpn.* **36**, 585–591.

Tomè, D. and Debabbi, H. 1998. Physiological effects of milk protein components. *Int. Dairy J.* **8**, 383–392.

Tomé, D., Dumontier, A.M., Hautefeuille, M., and Desjeux, J.F. 1987. Opiate activity and transepithelial passage of intact beta-casomorphins in rabbit ileum. *Am. J. Physiol.* **253**, G737–G744.

Tomé, D., Ben Mansour, A., Hautefeuille, M., Dumontier, A.M., and Desjeux, J.F. 1988. Neuromediated action of beta-casomorphins on ion transport in rabbit ileum. *Reprod. Nutr. Dev.* **28**, 909–918.

Torre, P.M. and Oliver, S.P. 1989a. Inhibition of bovine peripheral blood mononuclear cell blastogenesis by fractionated mammary secretion. *Comp. Biochem. Physiol. B* **92**, 157–165.

Torre, P.M. and Oliver, S.P. 1989b. Suppression of mitogenic response of peripheral blood mononuclear cells by bovine mammary secretions. *J. Dairy Sci.* **72**, 219–227.

Tranter, H.S. and Board, R.G. 1982. The inhibition of vegetative cell outgrowth and division from spores of *Bacillus cereus* T by hen egg albumen. *J. Appl. Bacteriol.* **52**, 67–73.

Tsuchita, H., Sekiguchi, I., Kuwata, T., Igarashi, C., and Ezawa, I. 1993. The effect of casein phosphopeptides on calcium utilization in young ovariectomized rats. *Z. Ernährungswiss.* **32**, 121–130.

Tsuchita, H., Goto, T., Shimizu, T., Yonehara, Y., and Kuwata, T. 1996. Dietary casein phosphopeptides prevent bone loss in aged ovariectomized rats. *J. Nutr.* **126**, 86–93.

Tsuchita, H., Suzuki, T., and Kuwata, T. 2001. The effect of casein phosphopeptides on calcium absorption from calcium-fortified milk in growing rats. *Br. J. Nutr.* **85**, 5–10.

Tsuda, H., Sekine, K., Fujita, K.I., and Iigo, M. 2002. Cancer prevention by bovine lactoferrin and underlying mechanisms—a review of experimental and clinical studies. *Biochem. Cell Biol.* **80**, 131–136.

Turgeon, S.L. and Gauthier, S.F. 1990. Whey peptide fractions obtained with a two-step ultrafiltration process: production and characterization. *J. Food Sci.* **55**, 6–110.

Tzipori, S., Robertson, D., Cooper, D.A., and White, L. 1987. Chronic cryptosporidial diarrhoea and hyperimmune cow colostrum. *Lancet* **2**, 344–345.

Uchida, T., Sato, K., Kawasaki, Y., and Dosako, S. 1996. Separation of lactoperoxidase, secretory component and lactoferrin from milk or whey with a cation exchange resin. US Patent No. 5,516,675.

Umbach, M., Teschemacher, H., Praetorius, K., Hirchhäuser, H., and Bostedt, H. 1988. Demonstration of a β-casomorphin immunoreactive material in the plasma of newborn calves after milk intake. *Regulat. Peptides* **12**, 223–230.

Ushida, Y., Sekine, K., Kuhara, T., Takasuka, N., Iigo, M., Maeda, M., and Tsuda, H. 1999. Possible chemopreventive effects of bovine lactoferrin on esophagus and lung carcinogenesis in the rat. *Jpn. J. Cancer Res.* **90**, 262–267.

van Beresteijn, E.C.H., Peeters, R.A., Kaper, J., Meijer, R.J.G.M., Robben, A.J.P.M., and Schmidt, D.G. 1994. Molecular mass distribution, immunological properties and nutritive value of whey protein hydrolysates. *J. Food Protect.* **57**, 619–625.

van Hooijdonk, A.C., Kussendrager, K.D., and Steijns, J.M. 2000. *In vivo* antimicrobial and antiviral activity of components in bovine milk and colostrum involved in non-specific defence. *Br. J. Nutr.* **84**, S127–S134.

van Hooijdonk, A.C.M., Olieman, C., and Hagedoorn, H.G. 1984. Kinetics of the chymosin-catalyzed proteolysis of κ-casein in milk. *Neth. Milk Dairy J.* **37**, 207–222.

Vegarud, G.E., Langsrud, T., and Svenning, C. 2000. Mineral-binding milk proteins and peptides; occurrence, biochemical and technological characteristics. *Br. J. Nutr.* **84**, 91–98.

Visser, S., Noorman, H.J., Slangen, C.J., and Rollema, H.S. 1989. Action of plasmin on bovine β-casein in a membrane reactor. *J. Dairy Res.* **56**, 323–333.

Vorland, L.H. 1999. Lactoferrin: a multifunctional glycoprotein. *Acta Pathol. Microbiol. Immunol. Scand.* **107**, 971–981.

Vreeman, H.J., Both, P., and Slangen, C.J. 1994. Rapid procedure for isolating the bitter carboxyl-terminal fragment 193-209 of β-casein on preparative scale. *Neth. Milk Dairy J.* **48**, 63–70.

Wada, T., Aiba, Y., Shimizu, K., Takagi, A., Miwa, T., and Koga, Y. 1999. The therapeutic effect of bovine lactoferrin in the host infected with *Helicobacter pylori*. *Scand. J. Gastroenterol.* **34**, 238–243.

Wal, J.M. 1998. Strategies for assessment and identification of allergenicity in (novel) foods. *Int. Dairy J.* **8**, 413–423.

Walstra, P., Geurts, T.J., Noomen, A., Jellema, A., and van Boekel, M.A.J.S. (eds) 1999. In "Milk", Dairy Technology: Principles of Milk Properties and Processes , pp. 3–170. Marcel Dekker, Inc., New York.

Walzem, R.L., Dillard, C.J., and German, J.B. 2002. Whey components: millennia of evolution create functionalities for mammalian nutrition: what we know and what we may be overlooking. *Crit. Rev. Food Sci. Nutr.* **42**, 353–375.

Wang, J.Q. and Ingenito, A.J. 1994. Cardiovascular effects of microinjection of dynorphin-A(1-8) into the hippocampus in conscious, spontaneously hypertensive and normotensive Wistar-Kyoto rats. *Clin. Exp. Hypertens.* **16**, 229–243.

Watanabe, K., Tsuge, Y., Shimoyamada, M., Ogama, N., and Ebina, T. 1998. Antitumor effects of pronase treated fragments, glycopeptides, form ovomucin in hen egg white in a double grafted tumor system. *J. Agric. Food Chem.* **46**, 3033–3038.

Webster, N.R. 1998. Opioids and immune system. *Br. J. Anesth.* **81**, 835–836.

Weiner, C., Pan, Q., Hurtig, M., Boren, T., Bostwick, E., and Hammarström, L. 1999. Passive immunity against human pathogens using bovine antibodies. *Clin. Exp. Immunol.* **116**, 193–205.

Werner, G.H., Floc'h, F., Migliore-Samour, D., and Jolles, P. 1986. Immunomodulating peptides. *Experientia* **42**, 521–531.

West, D.W. 1986. Structure and function of the phosphorylated residues of casein. *J. Dairy Res.* **53**, 333–352.

Widy-Tyskiewicz, E. and Czlonkowski, A. 1989. Analgesic activity of morphiceptin, β-casomorphin-4, deltakephalin in normotensive Wistar-Glaxo and spontaneously hypertensive rats. *Peptides* **10**, 539–544.

Widy-Tyszkiewicz, E. and Czlonkowski, A. 1991. Normotensive Wistar rats differ from spontaneously hypertensive and renal hypertensive rats in their cardiovascular responses to opioid agonists. *Clin. Exp. Pharmacol. P* **18**, 797–806.

Wijers, M.C., Pouliot, Y., Gauthier, S.F., Pouliot, M., and Nadeau, L. 1998. Use of nanofiltration membranes for the desalting of peptide fractions from whey protein enzymatic hydrolysates. *Le Lait* **78**, 621–632.

Wolfson, L.M. and Sumner, S.S. 1993. Antimicrobial activity of the lactoperoxidase system. A review. *J. Food Protect.* **56**, 887–892.

Wong, C.W. and Watson, D.L. 1995. Immunomodulatory effects of dietary whey proteins in mice. *J. Dairy Res.* **62**, 359–368.

Wong, C.W., Regester, G.O., Francis, G.L., and Watson, D.L. 1996a. Immunomodulatory activities of whey fraction in efferent prefemoral lymph of sheep. *J. Dairy Res.* **63**, 257–267.

Wong, C.W., Seow, H.F., Liu, A.H., Husband, A.J., Smithers, G.W., and Watson, D.L. 1996b. Modulation of immune responses by bovine β-casein. *Immunol. Cell Biol.* **74**, 323–329.

Wong, C.W., Middleton, N., Montgomery, M., Dey, M., and Carr, R.I. 1998a. Immunostimulation of murine spleen sells by materials associated with bovine milk protein fractions. *J. Dairy Sci.* **81**, 1825–1832.

Wong, C.W., Watson, D.L., Regester, G.O., and Smithers, G.W. 1998b. Comparison of immunomodulatory properties of purified lactoferrin, lactoperoxidase and β-casein in sheep. *J. Dairy Res.* **65**, 697–701.

Woodley, J.F. 1994. Enzymatic barriers for GI peptide and protein delivery. *Crit. Rev. Ther. Drug* **11**, 61–95.

Wu, J. and Ding, X. 2001. Hypotensive and physiological effect of angiotensin converting enzyme inhibitory peptides derived from soy protein on spontaneously hypertensive rats. *J. Agric. Food Chem.* **49**, 501–506.

Wu, X., Tolvanen, J.P., Hutri-Kähönen, N., Kähönen, M., Mäkynen, H., Korpela, R., Ruskoaho, H., Karjala, K., and Porsti, I. 1998. Comparison of the effects of supplementation with whey mineral and potassium on arterial tone in experimental hypertension. *Cardiovasc. Res.* **40**, 364–374.

Wüster, M., Schulz, R., and Herz, A. 1981. Multiple opiate receptors in peripheral tissue preparations. *Biochem. Pharmacol.* **30**, 1883–1887.

Wybran, J., Appelboom, T., Famaey, J.-P., and Govaerts, A. 1979. Suggestive evidence for receptors for morphine and methionine-enkelphalin on normal human blood lymphocytes. *J. Immunol.* **123**, 1068–1070.

Wyvratt, M.J. and Patchett, A.A. 1985. Recent developments in the design of angiotensin-converting enzyme inhibitors. *Med. Res. Rev.* **5**, 485–531.

Xu, R.-J. 1998. Bioactive peptides in milk and their biological and health implications. *Food Rev. Int.* **14**, 1–16.

Yamamoto, M. 1997. Antihypertensive peptides derived from food proteins. *Biopolymers* **43**, 129–134.

Yamamoto, M., Akino, A., and Takano, T. 1994. Antihypertensive effect of the peptides derived from casein by an extracellular proteinase from *Lactobacillus helveticus* CP790. *J. Dairy Sci.* **77**, 917–922.

Yamamoto, N., Maeno, M., and Takano, T. 1999. Purification and characterization of an antihypertensive peptide from a yoghurt-like product fermented by *Lactobacillus helveticus* CPN4. *J. Dairy Sci.* **82**, 1388–1393.

Yamauchi, K. 1992. Biologically functional proteins of milk and peptides derived from milk proteins. *IDF Bull.* **272**, 51–58.

Yamauchi, K., Tomita, M., Giehl, T.J., and Ellison, R.T. 1993. Antibacterial activity of lactoferrin and a pepsin-derived lactoferrin peptide fragment. *Infect. Immun.* **61**, 719–728.

Yano, S., Suzuki, K., and Funatsu, G. 1996. Isolation from α-zein of thermolysin peptides with angiotensin I-converting enzyme inhibitory activity. *Biosci. Biotechnol. Biochem.* **60**, 661–663.

Yoshida, S., Ye-Xiuyun, and Nishiumi, T. 1991. The binding ability of α-lactalbumin and β-lactoglobulin to mutagenic heterocyclic amines. *J. Dairy Sci.* **74**, 3741–3745.

Yoshikawa, M., Sasaki, R., and Chiba, H. 1981. Effect of chemical phosphorylation of bovine casein components on the properties related to casein micelle formation. *Agric. Biol. Chem. Tokyo* **45**, 909–914.

Yoshikawa, M., Tani, F., Ashikaga, T., Yoshimura, T., and Chiba, H. 1986a. Purification and characterization of an opioid antagonist from a peptide digest of bovine κ-casein. *Agric. Biol. Chem. Tokyo* **50**, 2951–2954.

Yoshikawa, M., Tani, F., Yoshimura, T., and Chiba, H. 1986b. Opioid peptides from milk proteins. *Agric. Biol. Chem. Tokyo* **50**, 2419–2421.

Yoshikawa, M., Tani, F., and Chiba, H. 1988. Structure activity relationship of opioid antagonist peptides derived from milk proteins. *In* "Peptide Chemistry" (T. Shiba, ed.), pp. 473–476. Protein Research Foundation, Osaka.

Yoshikawa, M., Kishi, K., Takahashi, M., Watanabe, A., Miyamura, T., Yamazaki, M., and Chiba, H. 1993. Immunostimulating peptide derived from soybean protein. *Ann. NY Acad. Sci.* **685**, 375–376.

Yoshikawa, M., Tani, F., Shiota, A., Usui, H., Kurahashi, K., and Chiba, H. 1994. Casoxin D, an opioid antagonist/ileum-contracting/vasorelaxing peptide derived from human α_{s1}-casein. *In* "β-Casomorphins and Related Peptides: Recent Developments" (V. Brantl and H. Teschemacher, eds), pp. 43–48. VCH, Weinheim.

Yuan, Y.V. and Kitts, D.D. 1991. Confirmation of calcium absorption and femoral utilization in spontaneously hypertensive rats fed casein phosphopeptide supplemented diets. *Nutr. Res.* **11**, 1257–1272.

Yvon, M., Beucher, S., Guilloteau, P., Le Huerou-Luron, I., and Corring, T. 1994. Effects of caseinomacropeptide (CMP) on digestion regulation. *Reprod. Nutr. Dev.* **34**, 527–537.

Zagulski, T., Lipinski, P., Zagulska, A., Broniek, S., and Jarzabek, Z. 1989. Lactoferrin can protect mice against a lethal dose of *Escherichia coli* in experimental infection *in vivo*. *Br. J. Exp. Pathol.* **70**, 697–704.

Zioudrou, C., Streaty, R.A., and Klee, W.A. 1979. Opioid peptides derived from food proteins. *J. Biochem. Tokyo* **254**, 2446–2449.

Zsila, F., Bikádi, Z., and Simony, M. 2002. Retinoic acid binding properties of the lipocalin member β-lactoglobulin studied by circular dichroism, electronic absorption spectroscopy and molecular modeling methods. *Biochem. Pharmacol.* **64**, 1651–1660.

Zucht, H., Raida, M., Adermann, K., Mägert, H.-J., and Forssmann, W. 1995. Casocidin-I: a casein-α_{s2} derived peptide exhibits antibacterial activity. *FEBS Lett.* **372**, 185–188.

PLANT PRODUCTS WITH HYPOCHOLESTEROLEMIC POTENTIALS

PULOK K. MUKHERJEE

Department of Pharmaceutical Technology
Natural Product Studies Laboratory
Jadavpur University
Kolkata 700032
India

ADVANCES IN FOOD AND NUTRITION RESEARCH VOL 47
ISSN: 1043-4526
DOI: 10.1016/S1043-4526(03)47005-8

I. INTRODUCTION

Cholesterol levels have become the source of health concerns, even though cholesterol is one of the most valuable substances in the human body. Over the past few years, the amount of cholesterol information and dietary advice bombarding the public has grown exponentially. Atherosclerosis is a complex multi-cellular process involving oxidation of cholesterol and the intracellular accumulation of oxidized cholesterol. This accumulation causes a cascade of inflammatory processes, resulting in an unstable atherosclerotic plaque that ultimately bursts, causing myocardial infraction. Botanical dietary supplements (herbs) can ameliorate this process and help to prevent cardiovascular disease at different steps in the process (Herber, 2001). Epidemiological and experimental evidence indicates that elevated plasma cholesterol carries a high risk of atherosclerotic diseases (AD). Risk begins to increase with cholesterol levels above 4 mmol/l, and then increases up to 6–6.5 mmol/l; persons with cholesterol levels persistently above 6–6.5 mmol/l require energetic management to bring down their lipid levels to the optimal range. The advantages of bringing down lipids to satisfactory levels have been confirmed by several experimental, animal and interventional studies indicating lowered morbidity and mortality in AD commensurate with reduction of serum cholesterol and/or improvement in high density lipoprotein (HDL)-cholesterol (Ginsberg, 1990; Havel and Kane, 1995; Tripathi, 1995; Mary *et al.*, 2000). An excessive concentration of lipids in plasma may alter the lipoprotein metabolism and lead to hypercholesterolemia, hypertriglyceridemia or both (Ginsberg, 1990;

Havel and Kane, 1995). Circulating plasma lipoproteins, known as HDL, low density lipoprotein (LDL) and very low density lipoprotein (VLDL) or chylomicrons, can be elevated by environmental causes, such as diet, or by inherited genetic defects in the synthesis or degradation of these compounds. Dyslipidemia or hypercholesterolemia is characterized by a combination of abnormalities in the plasma levels of triglycerides and HDL. Cholesterol, with or without elevated LDL cholesterol levels, affects many persons with premature coronary artery diseases (CAD). However, both qualitative and quantitative abnormalities in circulating triglyceride-rich lipoproteins (TRLPs) may particularly be a key factor in the development of CAD (Miek, 1999; Henry and Ginsberg, 2002). The lipoproteins play an essential role in the transport of endogenous lipids from the liver to the non-hepatic tissues through an endogenous pathway, while peripheral and hepatic chylomicrons transport dietary lipids from the intestine to the peripheral and hepatic tissues through an exogenous pathway (Figure 1).

A. CURRENTLY AVAILABLE THERAPY FOR HYPERLIPIDEMIA

Hypercholesterolemia and hyperlipidemia can be associated with a variety of clinical conditions as well as poor dietary practices. The proper management of hypercholesterolemia and hyperlipidemia is considered appropriate to prevent consequences that might develop including CAD and other conditions associated with extremely high levels of circulating triglycerides (Anonymous, 2000). For the management of hypercholesterolemia and hyperlipidemia, several drug therapies are available which are explained later (Rang *et al.*, 1999):

1. Bile acid sequestrates are anion-exchange resins, which sequester bile acid in the intestine. Cholestyramine and colestipol are the most commonly used in this category, which by this mechanism prevents bile acid re-absorption and causes decreased absorption of exogenous cholesterol and increased metabolism of endogenous cholesterol into bile acid in the liver by preventing enterohepatic recirculation. This leads to an increased expression of LDL receptors in liver and causes increased removal of LDL from blood and reduces the LDL cholesterol in the plasma.
2. Nicotinic acid inhibits hepatic triglyceride production and VLDL secretion, which lowers the plasma level of LDL and increases HDL. Nicotinic acid is mostly used to treat elevated LDL and VLDL by decreasing VLDL synthesis.
3. HMG (β-hydroxy-β-methylglutaryl)-CoA reductase is a rate-limiting enzyme, which catalyzes the conversion of HMG-CoA to mevalonic acid.

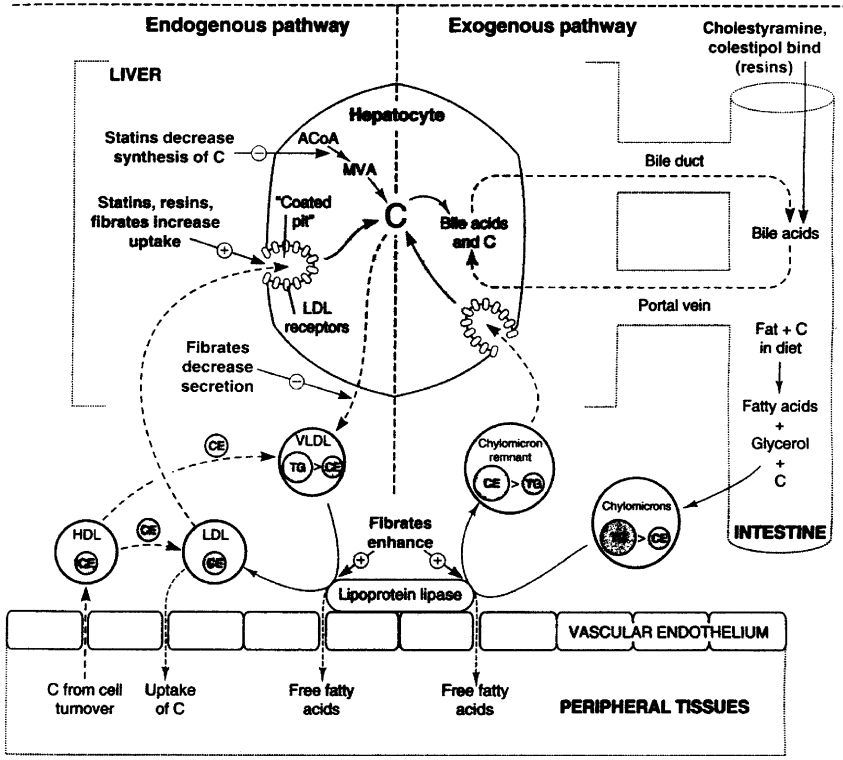


FIG. 1 Schematic diagram of cholesterol transport in the tissues, with sites of action of the main drug affecting lipoprotein metabolism (C = cholesterol; CE = cholesteryl ester; TG = triglycerides; MVA = mevalonate; HMG-CoA reductase = 3-hydroxy-3-methyl-glutaryl-CoA reductase; VLDL = very low density lipoproteins; LDL = low density lipoproteins; HDL = high density lipoproteins). (Reprinted from Rang *et al.* (1999), with permission from Elsevier Science.)

The HMG-CoA reductase inhibitors (Statins like simvastatin, lovastatin, pravastatin, fluvastatin, etc.) inhibit the enzyme and thereby decrease the hepatic cholesterol synthesis and increase the synthesis of LDL receptors causing increased clearance of LDL and a reduced concentration of LDL cholesterol in plasma. HMG-CoA reductase inhibitors are used to treat elevated LDL which also causes a small reduction in plasma triglycerides and an increase in HDL cholesterol.

4. Fibrates like gemfibrozil, clofibrate, ciprofibrate, etc. are fibric acid derivatives which stimulate lipoprotein lipase, an enzyme that breaks down lipids in lipoproteins and causes an increase in the hydrolysis of

triglycerides in chylomicrons and VLDL particles, thus liberating free fatty acids for storage in fat or metabolism in striated muscles. They also reduce hepatic VLDL and increase hepatic LDL uptake. Thus, they decrease VLDL synthesis and are used to treat elevated triglycerides and elevated lipoproteins by stimulating lipoprotein lipase.

5. Probucol lowers the concentration of both LDL and HDL in the plasma. It inhibits the oxidation of cholesterol thereby lowering the development of atherosclerosis.

Besides drugs, dietary factors play a key role in the development of cardiovascular disease. Epidemiological studies have shown that diets rich in fruits, herbs and spices are associated with a low risk of cardiovascular disease. Several dietary and nutritional herbs can be used to facilitate the metabolism of cholesterol. Potential therapeutic foods include garlic, artichoke, wheat germ, alfalfa sprouts, buckwheat, watercress, rice polishings, apple, celery, and cherries. Fresh juices such as carrot and pineapple with honey, liquid chlorophyll, parsley, alfalfa and spinach, beet and celery are also beneficial. Several reports on the use of dietary fibers and other food products have proved the efficacy of natural products of plant origin for the treatment of hypercholesterolemia (Agarwal and Chauhan, 1988; Glore *et al.*, 1994; Anderson, 1995; Navab *et al.*, 1996). Details on the various aspects of the cholesterol-lowering efficacy of the phytoconstituents as well as the individual plant products are explained in the subsequent sections.

II. PHYTOCONSTITUENTS WITH HYPOCHOLESTEROLEMIC POTENTIALS

Compounds with different structures but with the same therapeutic activity isolated from different plant species act as active moieties for the treatment of particular diseases. Some of these compounds have been abandoned due to toxicity but these compounds apparently do not cause serious adverse effects. Some of these active principles originate from edible plants and their inclusion in the diet would undoubtedly be of some value because of their hypocholesterolemic potential. Several phytoconstituents including inulin, pectin, gugglu lipids, flavonoids, ginkoloids, saponins, tannins, and others obtained from various plant sources have proven hypolipidemic potentials as has been further explained in Tables I and II. The chemical structures of a few potential phytoconstituents with hypolipidemic activity are shown in Figure 2. It is hoped that as new additions are made to the list of these active compounds causing only minimum untoward side effects, these naturally

TABLE I

SOME HYPOCHOLESTEROLEMIC, ANTI-ATHEROSCLEROTIC AND ANTI-THROMBOTIC NATURAL PRODUCTS

Constituents	Source	Activity	References
β-Carotene	Carrots, etc.	Hypocholesterolemic	Fuhrman *et al.* (1997)
Lycopene	Tomatoes, etc.	Hypocholesterolemic	Fuhrman *et al.* (1997)
Indole-3-carbinol	Brassica species	Hypocholesterolemic	Peluso and Schneeman (1994)
β-Sitosterol and sitostanol	Many plants	Hypocholesterolemic	Gylling and Miettinen (1996)
Saponin	Fenugreek, *Bupleurum chinense*, *Gyrostemma pentaphyllum*, etc.	Hypocholesterolemic	Miettinen *et al.* (1995)
Soybean protein	Soybean	Hypocholesterolemic	Mokady (1992)
Dietary fiber	Beans, *Hordeum vulgare*, *Juglans nigra*, etc.	Hypocholesterolemic	Story *et al.* (1984)
Mevinolin	*Aspergillus terreus*	Hypocholesterolemic	Booyens *et al.* (1984)
Polysaccharide	Fungi	Hypocholesterolemic	Kiho *et al.* (1996)
Sulfur compounds	Onion and garlic	Anti-atherosclerotic and anti-thrombotic	Wang and Ng (1999)
Flavonoids	Many plants	Anti-thrombotic	Wang and Ng (1999)
HMG-CoA reductase inhibitors	*Monasceus purpuries*; also from fungi and synthetic source	Anti-atherosclerotic and anti-thrombotic	Wang and Ng (1999)
Tocotrienols	Palm oil	Anti-thrombotic	Wang and Ng (1999)
Heparin	*Hipendula ulmaria*	Anti-thrombotic	Wang and Ng (1999)

Reprinted from Wang and Ng (1999), copyright (1999) with permission from Elsevier Science.

TABLE II

PHYTOCONSTITUENTS WITH HYPOLIPIDEMIC ACTIVITY

Name of the phytoconstituents	Family	Name of the plant	References
Inulin	Compositeae	*Chicorium intybus* L.	Roberfrodid (1999)
	Asteraceae	*Arctium lappa* L.	Iwakami *et al.* (1992)
Pectin, citrus flavonoids	Rutaceae	*Citrus lemon* L.	Carper (1988); Potter (1995); Bruneton (1995)
	Moraceae	*Oputia tuna mill Oputi ficus-indica*	Der Marderosian and Beutler (2002)
Gugglu lipids	Burseracaea	*Commiphora mukul*	Der Marderosian and Beutler (2002)
Gums	Fabaceae	*Cyamopsis tetragonolobus* (L). Taub	Robbers *et al.* (1996); Todd *et al.* (1990); Der Marderosian and Beutler (2002)
	Sterculiaceae	*Sterculia urens* Roxb.	Der Marderosian and Beutler (2002)
Flavonoids, polyphenolic compounds, isoflavones	Rosaceae	*Crataegus oxyacantha* L., *C. laevigata (Poir.) DC*, *C. monogyna Jacquin*	He (1990); Chen *et al.* (1995); Rajendran *et al.* (1996)
	Euphorbiaceae	*Emblica officinalis* Gaertin	Thakur and Mandal (1984); Thakur (1985); Mathur *et al.* (1996); Jacob *et al.* (1988)
	Ginkgoaceae	*Ginkgo biloba* L.	DeSmet *et al.* (1997)
	Leguminosae	*Glycine max*	Barnes (1998); Der Marderosian and Beutler (2002)
	Asteraceae	*Arctium lappa* L.	Sun (1992)
	Gramineae	*Avena sativa* L.	Anderson *et al.* (1984); Der Marderosian and Beutler (2002)
	Combretaceae	*Terminalia arjuna*, *T. bellirica*, *T. chebula*	Chevallier (1996); Thakur *et al.* (1988)
	Vitaceae	*Vitis vinifera V. labrusca V. rotundifera*	Der Marderosian and Beutler (2002)
	Zingiberaceae	*Zingiber officinale* Roscoe	Kiuchi *et al.* (1992)
Guar gum	Compositae	*Silybum marianum* L.	Der Marderosian and Beutler (2002)

(*continued on next page*)

TABLE II (*continued*)
PHYTOCONSTITUENTS WITH HYPOLIPIDEMIC ACTIVITY

Name of the phytoconstituents	Family	Name of the plant	References
	Rutaceae	*Citrus lemon* L.	Carper, 1988; Potter (1995); Bruneton (1995)
Alkaloid—Rhynchophylline	Rubiaceae	*Uncaria tomentosa* DC	Jones (1994); Hemingway and Phillipson (1974))
Fibers	Polyporaceae	*Grifola frondosa*	Kabir *et al.* (1987); Kubo and Nanba (1996); Kubo and Nanba (1997)
	Juglandaceae	*Juglans nigra*	Zambon *et al.* (2000); Potter (1995)
	Germineae	*Hordeum vulgare* L.	Lupton *et al.* (1994)
Saponins	Umbelliferae	*Bupleurum chinense* DC	Bone (1996); Der Marderosian and Beutler (2002)
	Cucurbitaceae	*Gynostemma pentaphyllum* (Thunb.) Makino	Der Marderosian and Beutler (2002)
	Leguminosea	*Medicago sativa* L.	Malinow *et al.* (1977,1978,1981); Cohen *et al.* (1990)
	Liliaceae	*Ruscus aculeatus* L.	Capelli and Nicora (1988)
	Araliaceae	*Panax quinquefolius* L. *Panax ginseng* C.A. mayer	Chen (1996)
Sterols	Combretaceae	*Terminalia arjuna, T. bellirica, T. chebula*	Chevallier (1996); Thakur *et al.* (1988)
	Leguminosea	*Medicago sativa* L.	Cohen *et al.* (1990)
Fixed oil and volatile oil	Euphorbiaceae	*Emblica officinalis*	Mathur *et al.* (1996); Jacob *et al.* (1988)
Essential fatty acids	Lamiaceae	*Perilla frutescens* (L) Britt.	Der Marderosian and Beutler (2002)
	Portulacaceae	*Portulaca oleracea* L	Reid (1986)
	Linaceae	*Linum usitatissimum* L.	Robinson (1991)
	Onagraceae	*Oenothera biennis* L.	Horrobin and Manku (1983); Puolakka and Makarainen (1985)
	Oleaceae	*Olea europaea*	Carper (1988); Aviram (1996)

TABLE II (*continued*)
PHYTOCONSTITUENTS WITH HYPOLIPIDEMIC ACTIVITY

Name of the phytoconstituents	Family	Name of the plant	References
Lycophene	Solanaceae	Ripe fruit of tomatoes	Der Marderosian and Beutler (2002)
Sulfur-containing principles	Liliacea	*Allium cepa*	Augusti and Mathew (1974); Lata *et al.* (1991); Der Marderosian and Beutler (2002)
Konjac mannan (Glucomannan)	Araceae	*Amorphophallus konjac* Koch	Der Marderosian and Beutler (2002)
Mono and oligosaccharides	Gramineae	*Avena sativa* L.	Anderson *et al.* (1984); Der Marderosian and Beutler (2002)
Triterpenes	Umbelliferae	*Bupleurum chinense* DC	Bone (1996); Der Marderosian and Beutler (2002)
Psyllium mucilloid (Metamucil)	Plantaginaceae	*Plantago lanceolata* L.	Greaves *et al.* (2000)
Stevia's glycosides (Rebaudioside A) Steviobioside	Asteraceae	*Stevia rebaudiana* Bertoni	Der Marderosian and Beutler (2002)

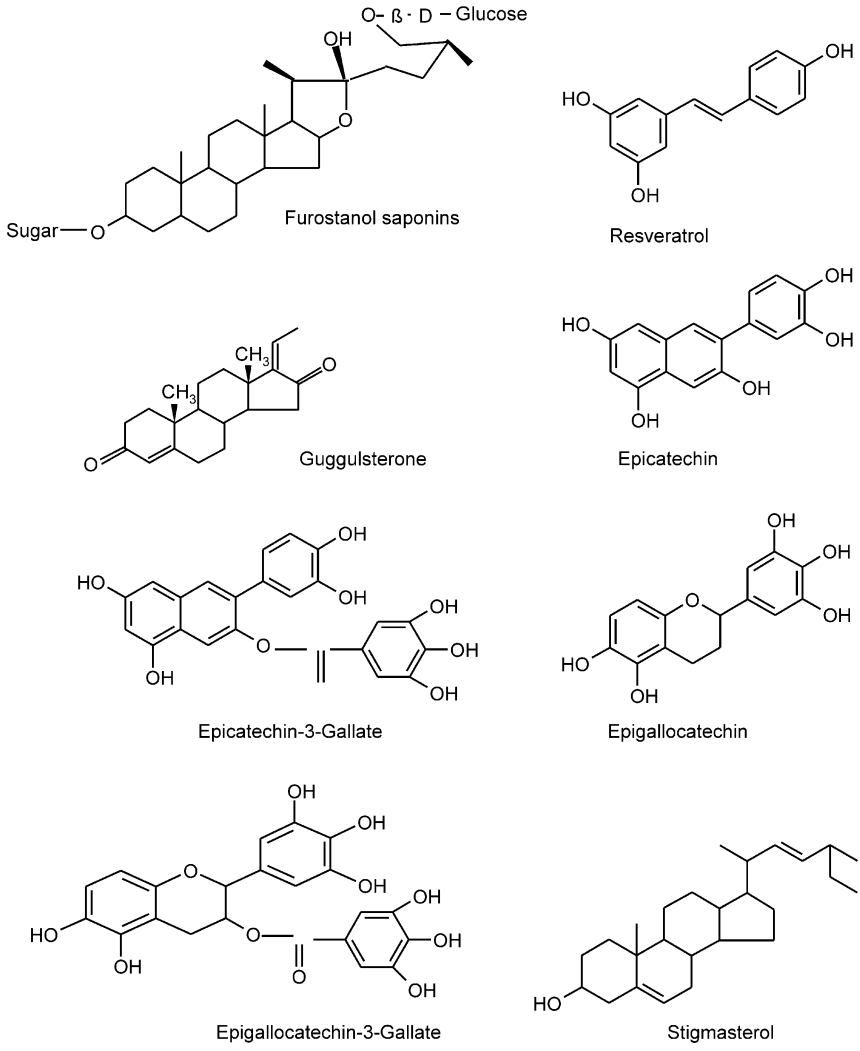
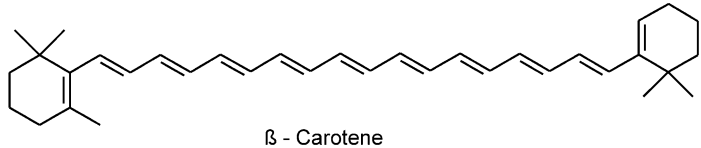
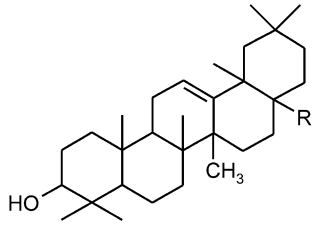


FIG. 2 Some hypocholesterolemic phytoconstituents.

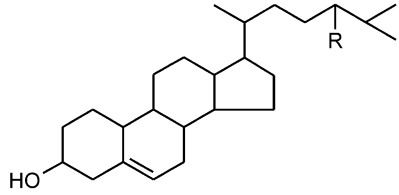
occurring components will definitely help in the development of therapeutically potent hypocholesterolemic drugs in the future and will also expedite the discovery of such drugs. Alternatively, several of these plants may be useful as nutraceutical ingredients. Several groups of plant constituents possessing significant therapeutic potentials as hypocholesterolemic agents are delineated in the following section.



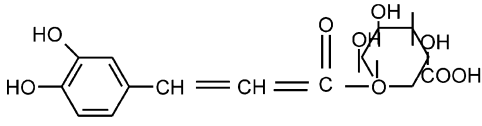
ß - Carotene



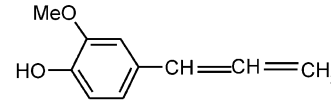
ß - Amyrin
Oleanolic acid (R = COOH)



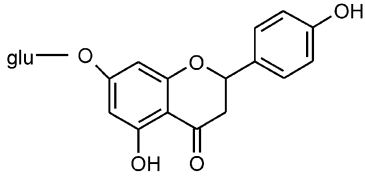
Sitosterol (R = Et), Campesterol (R = Me)



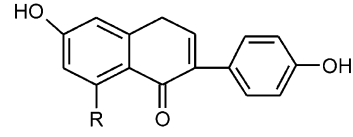
Chlorogenic acid



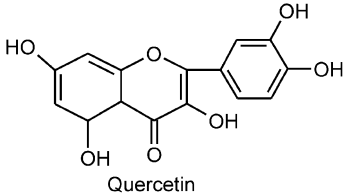
Eugenol



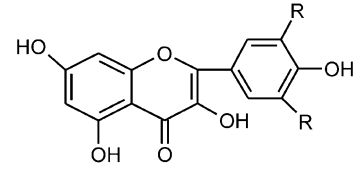
Naringin



Daidzein (R = H)
Genistein (R = OH)



Quercetin



Kaempferol (R = H)
Myricetin (R = OH)

FIG. 2 (*continued*)

A. FIBERS

Hordeum vulgare, *Juglans vigra*, *Amorphophallus konjac*, and *Grifolia frondosa* are rich in dietary fibers. Dietary fiber, especially soluble, viscous fibers effectively decrease serum cholesterol and LDL cholesterol concentrations (Anderson, 1995). Most soluble or viscous fibers have hypocholesterolemic effects (Glore *et al.*, 1994; Anderson, 1995). In general, these soluble fibers, such as psyllium, oat bran, guar and pectin, decrease serum cholesterol and LDL cholesterol concentrations without affecting serum triglycerides. LDL has a central role in the pathogenesis of atherosclerosis (Navab *et al.*, 1996). Oxidation of LDL in the sub-endothelial space of arteries sets the stage for macrophage uptake of LDL, foam cell development, and ultimately, fatty streak and atheroma formation (Navab *et al.*, 1996). Decreasing the LDL cholesterol concentrations is one of the most effective means of decreasing the risk for coronary heart disease (CHD). Often, consumption of these soluble fibers is accompanied by distinct reductions in serum HDL cholesterol concentrations (Glore *et al.*, 1994; Anderson, 1995). The cholesterol-lowering effects of soluble or viscous fibers relate to their gel-forming properties (Navab *et al.*, 1996). Soluble fibers such as psyllium and oat bran appear to exert their principal effects on cholesterol metabolism through decreases in bile acid absorption. These soluble fibers bind bile acids in the small intestine, alter micelle formation and decrease their absorption in the small intestine. Consequently, more bile acids are excreted with the feces. Acting somewhat like bile acid binding resins, these soluble fibers deplete the bile salt pool and divert cholesterol synthesis from lipoprotein precursors to bile acid synthesis (Glore *et al.*, 1994; Navab *et al.*, 1996). Fiber comes in many forms, including oats (*Avena sativa*), guar gum, and pectin. Recently, the U.S. Food & Drug Administration (FDA) made history by ruling in the first federally sanctioned health claim for manufactured foods that oat-rich cereals and other foods are permitted to advertise their products as "cholesterol lowering." Beta glucan, a viscous gel in the soluble fiber of oats, surrounds cholesterol-rich bile acids and limits their re-absorption by blood, shuttling them off into fecal excretion. Less bile is consequently returned to the liver which causes the liver to secrete more bile acids, the production of which uses up more cholesterol from the blood.

Guar gum, similar to pectin, is well documented regarding its ability to lower cholesterol levels. However, its principal drawback is its extreme viscosity, making foods containing guar gum very unpalatable. Non-viscous forms have been created via partial enzymatic hydrolysis of the gum's polysaccharide component, but there is some controversy regarding whether these processing techniques actually inhibit the guar gum's lipid-lowering

effects by reducing its digestibility rather than inhibiting bile acid re-absorption.

Chitosan is a form of fiber that absorbs dietary fat in the gut and can also inhibit LDL cholesterol while boosting desirable HDL cholesterol levels. The mechanism behind its action may be explained by chitosan's ability to bind both bile acids and phospholipids, reducing their absorption from the intestines and increasing fecal excretion of cholesterol. Chitosan has been shown to significantly lower plasma cholesterol and reduce the development of atherosclerotic plaques. Other fiber sources from food including kherjri beans (*Prosopsis cinceria*), prepalbanti (*Ficus religiosa*), barbum (*Ficus glomerata*) and teent (*Capparis decidua*) comprise cellulose, lignin, hemicelluloses, teent and pectin. These fiber sources affected the total lipid, cholesterol, triglycerides and phospholipids of the liver. Teent exerted the most conspicuous hypocholesterolemic effect probably by increasing the fecal excretion of cholesterol and bile salts (Agarwal and Chauhan, 1988).

B. PLANT STEROLS

The plant kingdom contains a number of sterols that differ from cholesterol by having ethyl or methyl groups or unsaturation in the side-chain. The predominant ones are sitosterol, stigmasterol, and campesterol. The most prominent is β-sitosterol, which differs from cholesterol in that it has an ethyl group at C-24 of the side-chain. Phytosterols belonging to the family of 4-desmesmethylsterols include β-sitosterol, campesterol and stigmasterol that are found mostly in plant oils such as soybean, canola, and sunflower. Phytosterols are natural components of plants and they play an important role in the cell membrane, analogous to that of cholesterol in animals. The 4-desmesmethysterols noted are the major phytosterols and comprise roughly 50% of the sterols in the western diet, with the remainder being cholesterol (Subbiah, 1971). β-Sitosterol is a natural extract from soybeans; sitostanol is a hydrogenated phytosterol found in extract from pine wood pulp.

The esterification of either of the sterols enhances its solubility in a hydrophilic environment. This modification makes the phytosterol a more active ingredient when used to fortify edible products that lower serum cholesterol. The cholesterol-lowering action of β-sitosterol was first reported more than 40 years ago (Farquhar *et al.*, 1956). The differences between the plant sterols and the cholesterol include the addition of an ethyl group to the aliphatic side-chain of both sterols. Saturation of the double bond in the second ring of β-sitosterol yields sitostanol. The presence of the ethyl group on the side-chain increases the hydrophobicity of both phytosterols such that it is greater than the hydrophobicity of the base molecule, cholesterol. This increase in hydrophobicity is expected to decrease the absorption of

these phytosterols into the body (Heinemann *et al.*, 1991, 1993). The low solubility of both native β-sitosterol and sitostanol in aqueous solutions leads to the use of the fatty acid esterified forms of these compounds, thus enabling their incorporation into food products. The esterification of the phytosterols is an important advance because it allowed more consistent and enhanced delivery of the sterols to the small intestine, the major site of cholesterol absorption (Mattson *et al.*, 1982). Delivery to the site of cholesterol absorption also enhanced its activity to inhibit absorption of the sterol.

It was found that the addition of sitosterol to the diet of cholesterol-fed chickens or rabbits lowered cholesterol levels in both species and inhibited atherogenesis in the latter (Ling and Jones, 1995). Sitosterol or mixtures of soy sterols were studied extensively as cholesterol-lowering agents (Compassi *et al.*, 1997). The mode of action appears to involve inhibition of cholesterol absorption, although the plant sterols themselves are absorbed very poorly (Tilvis and Miettinen, 1986). The mechanism of inhibition of cholesterol absorption is believed to be through crystallization and co-precipitation. Ingestion of 1 g of sitosterol reduced the absorption of cholesterol by 42% in a meal containing 500 mg of cholesterol (Mattson *et al.*, 1982). The decrease in plasma cholesterol is probably due to an increase in LDL receptor activity. However, the decline in plasma cholesterol is relatively lesser than the decrease in absorption, presumably because of a compensatory increase in cholesterol synthesis. Sitostanol, a saturated sitosterol derivative, reduced the intestinal absorption of cholesterol and serum cholesterol more effectively than sitosterol at doses below those of sitosterol (Heinemann *et al.*, 1986). In a recent study, sitostanol was interesterified with margarine, and the resultant product (1.9–2.6 g sitosterol per day) exhibited a hypocholesterolemic effect in a population with mild hypercholesterolemia (Miettinen *et al.*, 1995). The sitostanol was not absorbed and did not appear to interfere with the absorption of fat-soluble vitamins.

The use of plant sterols—β-sitosterol and sitostanol in consumer products to decrease cholesterol is supported by numerous clinical studies that document their efficacy in lowering mild hyperlipidemia (Jones *et al.*, 1998; Hallikainen and Uusitupa, 1999). Although the normal diet contains plant sterols that range from 160 to 360 mg/day, a 5- to 10-fold increase is required to exert a cholesterol-lowering effect. Consumer products with increased amounts of phytosterols that exceed the content found in the diet have been made available to the consumer. In evaluating the efficacy of including sitostanol ester in margarine as a dietary supplement for children with familial hypercholesterolemia (FH), it was found that serum total cholesterol (TC), intermediate density lipoprotein-cholesterol and LDL-cholesterol levels fell while the HDL-cholesterol/LDL-cholesterol ratio was elevated.

The proportions of delta 8-cholesterol and desmosterol in the serum rose while those of cholestanol, campesterol and sitosterol dropped, implying a decreased absorption of cholesterol and a compensatory increase in its synthesis. High basal precursor sterol proportions were predictive of a large decrement in titer of LDL cholesterol. It appeared that partial substitution of normal dietary lipid consumption with sitostanol was a safe and effective therapeutic measure for children with FH (Lees *et al.*, 1977; Wang and Ng, 1999). The effect of a small amount of sitosterol, sitostanol and sitostanol esters dissolved in rapeseed oil on serum lipids and cholesterol metabolism in patients with primary hypercholesterolemia and various apolipoprotein E phenotypes on a rapeseed oil diet showed a diminution in TC and LDL-cholesterol levels in the serum (Gylling and Miettinen, 1994).

Squalene, a sterol precursor also found in plant products, was originally suggested to have a cholesterol-lowering effect, but earlier studies in animals showed that it had no positive influence on atherosclerosis (Ling and Jones, 1995). Sitosterols and squalene are present in both monounsaturated and polyunsaturated vegetable oils and thus may be responsible for some of the variable cholesterol-lowering effects observed in studies using these products. Other cholesterol-lowering alcohols in rice bran oils include esters of triterpene alcohols that inhibit hepatic cholesterol esterase and tocotrienols that inhibit HMG Co-A reductase (Rukmini and Raghuram, 1991); however, there is conflicting evidence as to whether rice bran oil decreases plasma cholesterol levels in humans (Lichtenstein *et al.*, 1994).

Gugulipid, the oleoresin form of *Commiphora mukul*, is a mixture of diterpenes, sterols, steroids, esters and higher alcohols. The active ingredients responsible for the well known hypolipidemic effects of the plant are the guggulsterones, specifically guggulsterone E and guggulsterone Z. Fenusterols, commonly known as furostanol saponins from fenugreek seeds, are found to possess hypolipidemic activity. The principal furostanol saponin in fenugreek is diosgenin. Diosgenin has been proven to have various effects on cholesterol metabolism, specifically in lowering LDL plasma cholesterol levels. Other furostanols in fenugreek include gitogenin, tigogenin, smilagenin, sarsasaponin, etc. In light of the effect which furostanol saponins exert on appetite stimulation and hormone release, fenugreek seeds are traditionally used in appetite stimulation where the resulting increase in food intake ultimately triggers anabolism. Animal model studies have revealed that the furostanol saponin fraction is responsible for the anabolic effects of fenugreek seeds. In addition, the furostanol classes of plant steroids have pronounced immunostimulating and luteinizing hormone (LH) releasing properties. LH in turn signals the pituitary gland to produce more testosterone and high-testosterone levels have been correlated with improved athletic performance. The mechanism of hormone

releasing action of furostanols is linked to the conversion of cholesterol into active hormones, resulting in the simultaneous reduction of plasma cholesterol levels.

C. β-CAROTENE AND LYCOPENE

Dietary carotenoids are a family of about 600 fat-soluble plant pigments that serve as natural colors. The carotenoids, which have been studied most in this regard, are β-carotene, lycopene, lutein and zeaxanthanin; the former two belong to the carotene class, whereas the latter two belong to the xanthophylls. Xanthophylls are more polar than the carotenes as they contain one hydroxyl group (Johnson, 2002). β-Carotene is the most widely studied carotenoid and is one of the major carotenoids in our diet. It has been reported that dietary supplementation with lycopene or carotenoids like β-carotene cause a decline in plasma LDL cholesterol levels (Fuhrman *et al.*, 1997). Cholesterol synthesis from [3H]-acetate but not that from [14C] mevalonate in the macrophage cell line J-774A.1 has been reported to be suppressed by β-carotene or lycopene (10 μM) and the HMG-CoA reductase inhibitor, fluvastatin (10 μg/ml). The activity of the macrophage LDL receptor is enhanced by these three compounds. Thus, the hypocholesterolemic effect of β-carotene and lycopene is probably associated with the inhibition of HMG CoA reductase activity (Peluso and Schneeman, 1994).

D. FLAVONOIDS

Flavonoids might represent another beneficial group of naturally occurring hypolipidemic compounds. Flavonoids are widely distributed in the vegetable plant kingdom and exhibit distinctive pharmacological properties. Chemically flavonoids are the derivatives of 2-phenyl-1-benzopyran-4-one with varied chemical structures that are present in fruits, vegetables, nuts, and seeds (Kuhnau, 1976; Cook and Samman, 1996). The flavonoids can be widely classified into different categories like flavonols, flavones, catechins, flavanones and appear to have intensive biological properties that promote human health and help to reduce the risk of disease. Flavonoids act as antioxidants, protect LDL cholesterol from oxidation, inhibit platelet aggregation, and act as anti-inflammatory and anti-tumor agents (Smith and Yang, 1994; Cook and Samman, 1996; Manach *et al.*, 1996). Oxidative modification of LDL cholesterol is thought to play a key role during atherosclerosis. Plants such as *Camellia chinensis*, citrus fruits, *Ginkgo biloba*, soya beans, etc. contain large amounts of these polyphenolic compounds and exhibit various biological properties. These polyphenolic

compounds can inhibit oxidation of LDL cholesterol. Cells of the arterial wall including macrophages, smooth muscle cells, and endothelial cells can oxidize or otherwise modify LDL (Heinecke, 1997; Russell, 1999). Modified LDL can be a ligand for receptor-mediated processes leading to significant accumulation of cholesteryl esters (CE) in macrophages and smooth muscle cells (Heinecke, 1997; Russell, 1999). These CE-rich cells, known as foam cells, are the hallmark of the early atherosclerotic lesion. Flavonoids are usually present within the subendothelial space of the arterial wall in concentrations sufficient to protect lipoproteins such as LDL from oxidation. There is some evidence to suggest that flavonoids can be incorporated into lipoproteins within the liver or intestine and subsequently be transported within the lipoprotein particle (Fuhrman *et al.*, 1995; Kerry and Abbey, 1998). Flavonoids have been shown to inhibit the oxidation of LDL *in vitro* and, furthermore, the addition of the flavonoids, quercetin and catechin, to the diet have been shown to reduce LDL oxidation *ex vivo* in rats and was found to decrease the atherosclerotic lesion area in apoE-deficient mice (de Whalley *et al.*, 1990; Hayek *et al.*, 1997; da Silva *et al.*, 1998; Fremont *et al.*, 1998; Borradaile *et al.*, 1999). The mechanisms whereby flavonoids inhibit LDL oxidation are unclear. They may protect α-tocopherol in LDL from oxidation, possibly by being preferentially getting oxidized themselves, or they may reduce the formation or release of free radicals. Flavonoids can react with superoxide anions (Kuhnau, 1976), hydroxyl radicals (Husain *et al.*, 1987) and lipid peroxy radicals (Torel *et al.*, 1986). These compounds may also act by chelating iron, which is thought to catalyze processes leading to the formation of free radicals (Afanas'ev *et al.*, 1989; Morel *et al.*, 1993). A number of reports have suggested that these compounds may also influence atherogenesis through an effect on lipid and lipoprotein metabolism (Sharma, 1979; Basarkar and Nath, 1983; Choi *et al.*, 1991a,b; Jahromi *et al.*, 1993; Anthony *et al.*, 1997a; Yotsumoto *et al.*, 1997; Wilcox *et al.*, 1998; Borradaile *et al.*, 1999). In addition to direct oxidant scavenging, flavonoids may inhibit the enzymes involved in generating pro-oxidant molecules. For example, flavonoids have been shown to inhibit the generation or release of free radicals derived from lipoxygenase (LOX) (Huang *et al.*, 1997). It has been suggested that LOX is involved in the early events of atherosclerosis by inducing plasma LDL oxidation in the subendothelial space of the arterial wall (Huang *et al.*, 1997). While the majority of research focused on the anti-oxidant roles of flavonoids, studies in rats have shown that the flavonoids such as quercetin (Fuhrman *et al.*, 1995), hesperidin (Collins *et al.*, 1997), marsupin (Jahromi *et al.*, 1993), pterosupin (Jahromi *et al.*, 1993), liquiritigenin (Jahromi *et al.*, 1993), biochanin A (Anthony *et al.*, 1997), formononetin (Anthony *et al.*, 1997), and pratensein

(Anthony *et al.*, 1997) cause significant reductions in serum TC and triglyceride (TG). In non-human primates, dietary genistein, the isoflavone significantly reduces plasma LDL and VLDL cholesterol levels (Anthony *et al.*, 1997).

1. *Citrus flavonoids*

Naringin (naringenin-7-rhamnoglucoside), the bitter principle of grapefruit (*Citrus paradisi*) is found in the juice, flower, and rind of the fruit. Naringin and other naringenin glycosides can be found in a variety of other sources including propolis (Nagy *et al.*, 1985) and *Prunus davidiana* (Husain *et al.*, 1987). *Monotes engleri* contains a prenylated form of naringenin (6-(1,1-dimethylallyl)naringenin) (Seo *et al.*, 1997). The chemical name of naringenin is 2,3-dihydro-5,7-dihydroxy-2-(4-hydroxyphenyl)-4H-1-benzopyran-4-one. Naringenin is derived from the hydrolysis of the glycone forms of this flavanone, such as naringin or narirutin (Prasad, 1999). Another predominant flavonoid present in citrus fruit is hesperidin. Naringin exhibits anti-atherogenic property by inhibiting lipid peroxidation at 465–565 mM (Laughton *et al.*, 1991; Saija *et al.*, 1995), auto-oxidation of rat cerebral membranes at 322 mM (Saija *et al.*, 1995), LOXs and cyclooxygenases at $>$200 mM (Corvazier and Maclouf, 1985; Laughton *et al.*, 1991) and myeloperoxidase at 150 mM (Divi and Doerge, 1996). It also inhibits thromboxane B2 production at 175–200 mM (Corvazier and Maclouf, 1985) and platelet aggregation at 90–500 mM (Landolfi *et al.*, 1984; Corvazier and Maclouf, 1985). It has a significant effect on lipid metabolism by reducing apo-B secretion from hepatocytes at 50–100 mM (Wilcox *et al.*, 1998; Borradaile *et al.*, 1999) and inhibits acyl-CoA: cholesterol acyltransferase at 50–100 mM (Wilcox *et al.*, 1998).

E. TEA POLYPHENOLICS

Tea is a rich source of polyphenolics; green tea leaves consist specifically of catechins and black tea leaves contain theaflavins (catechin dimers). When rats were fed green tea polyphenols, blood cholesterol concentrations declined in hypercholesterolemic animals (Dreosti, 1996). These effects may be explained by the capacity of green tea catechins and gallate esters to reduce intestinal cholesterol absorption, lower blood coagulability, and inhibit proliferation of human aortic smooth muscle cells. It has been reported that LDL-cholesterol oxidation is inhibited by exposure to tea flavonoids, specifically the catechins from green tea leaves or theaflavins from black tea leaves (Dreosti, 1996). Catechin, epigallocatechin gallate provided the maximum protection and was more protective than vitamin E, whereas

the theaflavins exerted even stronger inhibitory effects than the catechins. In human epidemiological studies, green tea consumption was inversely associated with serum levels of TC and LDL-cholesterol, but had no significant correlation with levels of HDL-cholesterol or triglycerides (Kono *et al.*, 1996). Catechins are considered to lower cholesterol by a mechanism that suppresses cholesterol absorption in the intestine (Fukuyo *et al.*, 1986; Chisaka *et al.*, 1988; Ikeda *et al.*, 1992; Ishikawa *et al.*, 1997; Matsumoto *et al.*, 1998; Valsa *et al.*, 1998). In studies of animals fed high-fat or high-cholesterol diets, tea catechins reduced serum cholesterol and/or lipid levels in rats (Muramatsu *et al.*, 1986; Valsa *et al.*, 1995) mice (Matsuda *et al.*, 1986), and hamsters (Chan *et al.*, 1999), and increased fecal excretion of neutral steroids and bile acids in hamsters (Chan *et al.*, 1999). In rats fed a normal diet, a dose of 10 mg/kg of catechin produced the maximum reduction in plasma cholesterol and the maximum increase in excretion of fecal neutral steroids and bile acids (Valsa *et al.*, 1995). Thus tea polyphenols play an effective role in lowering elevated cholesterol levels.

F. SAPONIN

Among their diverse biological activities, saponins possess effective hypocholesterolemic action (Price *et al.*, 1992). Saponin–cholesterol interaction is an important part of the hypocholesterolemic action of alfalfa but interactions of bile acids with other components of alfalfa might be equally important. Alfalfa plant and sprout saponin have been found to be effective in binding significant amounts of cholesterol (Wang and Ng, 1999).

G. SOYBEAN PROTEIN

The cholesterol-lowering effects of soy protein have been recognized for more than 90 years (Anderson *et al.*, 1995a,b). It has been reported that soybean protein induced a reduction in serum apo A-1 with the relative concentration of HDL-cholesterol remaining at a higher level (Forsythe *et al.*, 1980). It has been observed that plant protein (50% from soybean meal and 25% each from corn and wheat) lowered plasma cholesterol levels in young male pigs compared with animal proteins (90% from casein and 10% from lactalbumin) (Forsythe *et al.*, 1980). Carroll *et al.* (1978) observed that plasma cholesterol levels were lower when soybean protein was included in the diet of young, healthy normo-lipidemic women. Studies over the past 20 years have shown that the daily consumption of 30–60 g of soy protein contributes to a decrease in total and LDL cholesterol level between 10 and 20% in individuals with elevated serum cholesterol (Caroll, 1991). An intake of 30–60 g of isolated soy protein in muffins, breads,

cookies and other commonly eaten bakery items effectively lowers raised cholesterol (Potter *et al.*, 1993). Mokady (1992) reported that rats fed a diet containing 10% protein derived from soy protein, wheat gluten, or wheat gluten supplemented with lysine and threonine possessed lower serum cholesterol and triglyceride levels with no LDL and a higher level of HDL.

H. PLANT INDOLES

Indole 3-carbinol induced a lowering of serum cholesterol levels and the serum LDL-cholesterol/VLDL cholesterol ratio (Mokady, 1992).

I. UNSATURATED FATTY ACIDS

Booyens *et al.* (1984) reported that unnatural dietary *trans* and *cis* unsaturated fatty acid isomers are a definite risk factor in the etiology of coronary disease, despite an early report about the hypocholesterolemic activity of unsaturated fatty acids from plant origin.

J. RESVERATROL

Resveratrol (*trans*-3,4′,5-trihydroxystilbene) is a phytopolyphenol isolated from the seeds and skins of grapes. In plants, resveratrol functions as a phytoalexin that protects against fungal infection (Hain *et al.*, 1990). Because of its high concentration in grape skin, significant amounts of resveratrol are present in wine. Recent studies indicate that resveratrol can block the process of multi-step carcinogenesis, namely, tumor initiation, promotion and progression. Resveratrol can also reduce the risk of cardiovascular disease in man. The molecular mechanisms of resveratrol in chemoprevention of cancer and cardiovascular disease are interesting and are under intensive investigation. *In vivo*, *ex vivo* and many animal experiments have shown that resveratrol possesses many biological attributes that favor protection against atherosclerosis, including anti-oxidant activity, modulation of hepatic apo-lipoprotein and lipid synthesis, inhibition of platelet aggregation (Soleas *et al.*, 1997). It has been suggested that it plays a role in the prevention of heart disease, as it inhibits platelet aggregation, alters eicosanoid synthesis and modulates lipid and lipoprotein metabolism (Soleas *et al.*, 1997). Platelets have a critical role in initiating the chemical signals that set in motion complex cellular events, resulting in atherosclerosis. Platelets adhere to the endothelial surface of arteries and trigger luminal occlusion leading to acute CHD (Renaud *et al.*, 1992). Gehm *et al.* (1997) reported a new facet of resveratrol, where, on the basis

of its structural similarity with the synthetic estrogen–diethylstilbosterol, they hypothesized that it might be a phytoestrogen (Gehm *et al.*, 1997).

K. PROPIONATE

A metabolic product of fiber fermentation, propionate may mediate some of the hypocholesterolemic effects of certain soluble plant fibers. In cholesterol-fed rats, propionate decreases serum cholesterol and liver triglyceride level where no changes in hepatic histology in response to propionate intake have been detected (Wang and Ng, 1999).

L. MEVINOLIN

Mevinolin produced by the fungus *Aspergillus tereus*, competitively inhibits HMG-Co-A reductase, a key enzyme in the cholesterol biosynthetic pathway, and thereby lowers cholesterol level (Wang and Ng, 1999).

M. FUNGAL POLYSACCHARIDES

Polysaccharides CS-F30, from cultural mycelia of *Cordyceps sinensis* decreased plasma cholesterol in mice (Jones *et al.*, 1998). The glucuronoxylomannan from *Tremella fuciformis* fruiting bodies brought about reduction in the plasma cholesterol level in mice (Wang and Ng, 1999).

N. ALGAL EXTRACT

An extract of Spirulina, unicellular filamentous blue-green algae for human consumption has been reported to have potential hypocholesterolemic activity (Chamorro *et al.*, 1996).

O. RICE BRAN OIL

Rice bran oil is made from the pericarp and germ of the *Orzya sativa* seed. It constitutes 10% of the rough rice grain and 18–22% oil. Pharmacological properties of rice bran oil and its active component, oryzanol, have been established for varied therapeutic potential including hypolipidemic, neuroendocrinological, gastroenterological and dermatological effects (Suzuki and Oshima, 1970). Rice bran oil and its varied components have a demonstrated ability to improve the plasma lipid pattern of rodents, rabbits, non-human primates and humans reducing total plasma cholesterol and triglyceride concentrations, and increasing the HDL cholesterol level (Cicero and Gaddi, 2001).

P. SULFUR-CONTAINING COMPOUNDS

Allium cepa and *Allium sativum* are rich in naturally occurring sulfur-containing compounds which influence plasma cholesterol and atherosclerosis. Garlic oil was in the pharmacopeia of the Babylonians and other ancient people (Kritchevsky, 1991). Garlic contains a number of compounds, but the most active are diallyl disulfide (DADS) and its mono *S*-oxide (allicin). Allicin was identified initially as the active compound responsible for anti-atherosclerotic effect. It has also been observed that water-soluble organosulfur compounds, especially *S*-allyl cysteine (SAC), present in aged garlic extract (AGE) and DADS, present in garlic oil are also potent inhibitors of cholesterol synthesis. Garlic oil or garlic has been shown to be hypolipidemic in humans, with a recent meta-analysis suggesting that one-half clove of garlic per day lowered serum cholesterol by approximately 9%. The same amount of garlic was shown to reduce cholesterol levels and severity of atherosclerosis in cholesterol-fed rabbits. The mechanism of hypocholesterolemia may be the inhibition of cholesterol synthesis (Yu-Yan and Liu, 2001). Abramovitz *et al.* (1999) investigated the effect of allicin as an active component of garlic on the formation of fatty streaks in the aorta and lipid profile in mice. While no significant differences were observed between the blood lipid profiles, the microscopic evaluation of the formation of fatty streaks in the aortic sinus showed that values for mice in the allicin treated groups were significantly lower by nearly 50% (Abramovitz *et al.*, 1999).

III. HERBS USEFUL IN HYPERCHOLESTEROLEMIA

Herbs are generally considered as a safe way to strengthen and tone the human body system. Herbs may be used as dried extracts or tinctures (alcohol extracts) or in any other dosage form, singly or in combination, as noted (Mukherjee, 2001). Currently available hypolipidemic drugs like gemfibrozil, bezafibrate, lovastatin, and nicotinic acid are not totally safe particularly when used for prolonged periods. Viewed in this context, the hypolipidemic potentials of medicinal plants need critical study. Several plant species have been reported to possess anti-hypercholesterolemic action. For example, garlic (*A. sativum*) has the effect of reducing cholesterol. It is most effective when included in the diet in the raw form, or taken in capsules. Herbs that support the claim to be used for their hypocholesterolemic effects include *Cicer arietinium*, *Commiphora mukul*, *Curcuma longa*, *Emblica officinalis*, *Inula racemosa*, *Terminalia* species of plants like *Terminalia arjuna*, *Terminalia chebula*, *Trigonella foenum graecum*, milk thistle (*Silybum marianum*), dandelion root (*Taraxacum officinale*), burdock root

(*Arctium lappa*), blue flag (*Iris versicolor*), greater celandine (*Chelidonium majus*), blue vervain (*Verbena bastata*), and hawthorn berries (*Crataegus oxyanthoides*). These herbs help to lower high blood pressure, promote cholesterol metabolism and suppress cholesterol synthesis as well as strengthening connective tissue and cardiac muscle. Ginger (*Zingier offiicinalis*), Alfalfa (*Medicago sativa*) and several other herbs have been shown to lower cholesterol levels (Sharma, 1997; Wang and Ng, 1999; Mukherjee, 2002). The hypolipidemic potentials of the various plant species has been further explained in Table III. Details on several plants including their general habits, pharmacognositical, pharmacological and clinical properties with hypolipidemic potential are discussed in the next section.

A. *PHYLLANTHUS NIRURI*

Phyllanthus niruri (Family—Euphorbiaceae) is a small herb distributed throughout the tropical and subtropical regions of both hemispheres. This plant is of medicinal importance for numerous ailments like dysentery, influenza, and diabetes and possesses diuretic, anti-hepatotoxic and anti-viral effects (Chopra *et al.*, 1986; Qian, 1996). The lipid-lowering activity of *P. niruri* has been studied in Triton-induced and cholesterol-fed hyperlipemic rats. Serum lipids were lowered by *P. niruri* extract orally fed (250 mg/kg b.w.) to the Triton WR-1339 induced hyperlipemic rats. Chronic feeding of this plant (100 mg/kg) in animals simultaneously fed with cholesterol (25 mg/kg) for 30 days caused lowering of the lipids and apoprotein levels of VLDL and LDL in experimental animals. The anti-hypercholesterolemic effect of this plant may be mediated through the inhibition of hepatic cholesterol biosynthesis, increased excretion of fecal bile acids, and enhanced plasma lecithin: cholesterol acyl transferase activity (Khanna *et al.*, 2002). *P. niruri* enhances the excretion of bile acids through feces and this contributes to the regress of cholestersteosis in liver damage. Disorders of lipid metabolism are associated with peroxidative degradation of membrane lipids and, like picroliv, this may also act as an anti-oxidant to inhibit lipid peroxidative liver damage (Chander *et al.*, 1988).

B. *CORAINDRUM SATIVUM*

Almost all the spices exhibit a wide range of physiological and pharmacological effects (Beena *et al.*, 1995, 1996) and thus are useful as domestic remedies for many of the human disorders (Nadkarni and Nadkarni, 1976; Chopra *et al.*, 1986). *Coraindrum sativum* popularly known as coriander seed is a very commonly used spice in Indian cuisines. The biochemical effects of this seed on lipid parameters in 1,2-dimethyl hydrazine (DMH) induced colon

TABLE III
PLANTS POSSESSING HYPOLIPIDEMIC POTENTIALS

Name of the plant	Family	Plant parts used	References
Achyranthus aspera	Amaranthaceae	Leaves, roots	Khanna *et al.* (1992)
Aegle marmelos	Rutaceae	Fruits, root, bark, leaves, flowers	Sharma and Dwivedi (1997)
Agave vera	Amaryllidaceae	Roots, leaves, gum	Sharma and Dwivedi (1997)
Alchemilla xanthochlora	Rosaceae	Leaf	Filipek (1992); Der Marderosian and Beutler (2002)
Allium cepa	Liliacea	Under ground bulb	Augusti and Mathew (1974); Lata *et al.* (1991); Der Marderosian and Beutler (2002)
Allium stivum	Liliacea	Bulb, oil	Sharma and Dwivedi (1997)
Aloe barbadensis	Liliacea	Leaves	Sharma and Dwivedi (1997)
Amorphophallus konjac Koch.	Araceae	Flour	Der Marderosian and Beutler (2002)
Apocynum venetum	Apocynaceae	Leaves, roots	Kim *et al.* (2000)
Arctium lappa L.	Asteraceae	Seeds, roots, leaves	Iwakami *et al.* (1992)
Avena sativa L.	Gramineae	Inner husk of the grain	Anderson *et al.* (1984); Der Marderosian and Beutler (2002)
Azadirachta indica	Meliaceae	Leaf	Mukherjee *et al.* (1995)
Bambusa arundunaceae	Gramineae	Leaves	Sharma and Dwivedi (1997)
Bidens pilosa	Asteraceae	Leaves	Dimo *et al.* (2001)
Bosswellia serrata	Burserraceae	Gum	Sharma and Dwivedi (1997)
Brassica varcapitata	Cruciferae	Oil	Sharma and Dwivedi (1997)
Bupleurum chinense DC	Umbelliferae	Roots	Bone (1996); Der Marderosian and Beutler (2002)
Cajanus cajan	Fabaceae	Seeds	Sharma and Dwivedi (1997)
Camellia sinensis L. *Kuntze* (*Green tea*)	Theaceae	Leaves	Yokozawa and Dong (1997); Tijburg *et al.* (1997)
Lycopersicon esculentum	Solanaceae	Ripe fruit of tomatoes	Der Marderosian and Beutler (2002)
Capparis deciduas	Capparaceae	Leaves, fruits, stem	Sharma and Dwivedi (1997)
Capsicum frutescens	Solanaceae	Fruits	Sharma and Dwivedi (1997)
Carthamus tinctorius L.	Compositae	Flower	Der Marderosian and Beutler (2002)

TABLE III (*continued*)

PLANTS POSSESSING HYPOLIPIDEMIC POTENTIALS

Name of the plant	Family	Plant parts used	References
Carum capaticum	Umbeliferae	Fruits, roots	Sharma and Dwivedi (1997)
Celastrus paniculatus	Celastraceae	Seed oil, bark, root, fruit	Sharma and Dwivedi (1997)
Cicer arietinum	Papillionaceae	Seeds, leaves	Sharma and Dwivedi (1997)
Cichorium intybus L.	Compositae	Root	Roberfrodid (1999)
Citrus lemon L.	Rutaceae	Fruit	Carper (1988); Bruneton (1995)
Cimicifuga racemosa (L.) *Nutt.*	Ranunculaceae	Root and rhizomes	Greaves *et al.* (2000)
Commiphora mukul	Burseracaea	Exudates	Der Marderosian and Beutler (2002)
Crataegus oxyacantha L. *C. laevigata* (Poir.) *C. monogyna* Jacquin	Rosaceae	Flowers, leaves, fruits	Chen *et al.* (1995); Rajendran *et al.* (1996)
Curcuma amada	Zinziberaceae	Rhizome	Sharma and Dwivedi (1997)
Curcuma longa	Zinziberaceae	Rhizome	Sharma and Dwivedi (1997)
Cyamopsis tetragonolobus (L.) Taub.	Fabaceae	Indian cluster bean	Todd *et al.* (1990); Der Marderosian and Beutler (2002)
Cynara scolymus L.	Compositae	Leaf	Gebhardt (1998); Anonymous (1999)
Emblica officinalis Gaertin.	Euphorbiaceae	Fruit	Thakur and Mandal (1984); Thakur (1985); Jacob *et al.* (1988); Mathur *et al.* (1996)
Emblica ribes	Myrsinaceae	Seeds, fruit, bark	Sharma and Dwivedi (1997)
Eleutherococcus senticosus Maxim	Araliaceae	Root and leaf	Der Marderosian and Beutler (2002)
Eugenia caryophyllata Thunb.	Myrtaceae	Buds	Der Marderosian and Beutler (2002)
Forsythia suspense (Thunb)	Oleaceae	Ripe fruit	Iwakami *et al.* (1992)
Ganoderma lucidum Karst.	Polyporaceae	Whole plant	Lininger (1998)
Ginkgo biloba L.	Ginkgoaceae	Leaves	DeSmet *et al.* (1997)
Glycine max	Leguminosae	Beans	Barnes (1998); Der Marderosian and Beutler (2002)
Grifola frondosa	Polyporaceae	Whole mushroom	Kabir *et al.* (1987); Kubo and Nanba (1996, 1997)

(*continued on next page*)

TABLE III (*continued*)
PLANTS POSSESSING HYPOLIPIDEMIC POTENTIALS

Name of the plant	Family	Plant parts used	References
Gynostemma pentaphyllum (Thunb.) Makino	Cucurbitaceae	Whole plant	Der Marderosian and Beutler (2002)
Hordeum vulgare L.	Gramineae	Barely grain flour	Lupton *et al.* (1994)
Juglans regia L.	Juglandaceae	Fruit	Chi (1982); Potter (1995)
Juglans nigra	Juglandaceae	Nuts	Der Marderosian and Beutler (2002)
Juniperus communis	Cupressacea	Berries and fruits	Tunon *et al.* (1995)
Linum usitatissimum L.	Linaceae	Seed	Robinson (1991)
Medicago sativa L.	Leguminosea	Leaves and sprouts	Malinow *et al.* (1977, 1978, 1981); Cohen *et al.* (1990)
Momordica charantia L.	Cucurbitaceae	Fruits, leaves and seeds	Der Marderosian and Beutler (2002)
Monascus purpureus		Fermented cooked rice	Der Marderosian and Beutler (2002)
Moringa oleifera	Moringaceae	Leaves	Ghasi *et al.* (2000)
Mucuna pruriens	Papilionacea	Roots, seeds, fruit	Sharma and Dwivedi (1997)
Musa sapientum	Musaceae	Fruits, root, stem, flower, leaves	Sharma and Dwivedi (1997)
Napeta hidostama	Labiatae	Whole plant	Sharma and Dwivedi (1997)
Oenothera biennis L.	Onagraceae	Seeds	Horrobin and Manku (1983); Puolakka and Makarainen (1985)
Olea europaea	Oleaceae	Fruit	Carper (1988); Aviram (1996)
Oputia tuna Mill *Oputi ficus*—Indica		Raw plant	Der Marderosian and Beutler (2002)
Oryza sativa	Gramineae	Rice bran oil	Seetharamaiah and Chandrasekharan (1989)
Panax quinquefolius L. *Panax ginseng* C.A.	Araliaceae	Root	Chen *et al.* (1995)
Paullinia cupana	Sapindaceae	Seeds	Bydlowski *et al.* (1991)
Persea Americana	Lauraceae	Fruit	Der Marderosian and Beutler (2002)
Perilla frutesceus (L.)	Lamiaceae	Seeds	Der Marderosian and Beutler (2002)
Phaseolus aureus	Fabaceae	Seed	Sharma and Dwivedi (1997)

TABLE III (*continued*)

PLANTS POSSESSING HYPOLIPIDEMIC POTENTIALS

Name of the plant	Family	Plant parts used	References
Phaseolus mungo	Fabaceae	Seed	Sharma and Dwivedi (1997)
Phyllanthus niruri	Euphorbiaceae	Whole plant	Khanna *et al.* (2002)
Picrorhiza kurroa	Scrophulariaceae	Root	Sharma and Dwivedi (1997)
Piper nigram	Piperaceae	Leaves	Sharma and Dwivedi (1997)
Polygonum multiflorum	Polygonaceae	Root, stem and leaf	Chevallier (1996); Reid (1986)
Portulaca oleracea L	Portulacaceae	Whole herb	Low and Rodd (1994); Reid (1986)
Pterocarpus marsupium	Papalionaceae	Gum, leaves	Sharma and Dwivedi (1997)
Plantago lanceolata L.	Plantaginaceae	Seeds	Greaves *et al.* (2000)
Ruscus aculeatus L.	Liliaceae	Root	Capelli and Nicora (1988)
Ruta graveolens L.	Rutaceae	Aerial parts	Chevallier (1996); Duke (1989)
Saussuraea lappa	Asteraceae	Root	Sharma and Dwivedi (1997)
Silybum marianum L.	Compositae	Seeds	Der Marderosian and Beutler (2002)
Stevia rebaudiana Bertoni.	Asteraceae	Leaf	Der Marderosian and Beutler (2002)
Tanacetum parthenium	Asteraceae	Leaves	Der Marderosian and Beutler (2002)
Terminalia arjuna Terminalia bellirica Terminalia chebula	Combretaceae	Bark and fruit	Chevallier (1996)
Thevatia nerifolia	Apocynaceae	Leaves	Sharma and Dwivedi (1997)
Tinospora cordifolia	Menispermaceae	Root	Der Marderosian and Beutler (2002)
Trachyspermum ammi L.	Apiaceae	Fruit	Srivastava (1988)
Trichosanthes dioca	Cucurbitacea	Fruit powder	Sharma and Dwivedi (1997)
Trigonella foenumgraecum	Leguminosae	Leaves, seeds	Sharma and Dwivedi (1997)
Tussilago farfara L.	Compositae	Buds	Hwang *et al.* (1987)
Uncaria tomentosa DC	Rubiaceae	Bark	Cohen *et al.* (1990); Hemingway and Phillipson (1974)
Vitis vinifera V. labrusca V. rotundifera	Vitaceae	Grapes and leaves	Der Marderosian and Beutler (2002)
Zingiber officinale Roscoe	Zingiberaceae	Rhizome	Kiuchi *et al.* (1992)

cancer in rats has been reported (Chitra and Leelamma, 2000). The level of cholesterol decreased in the coriander-fed group compared with the DMH control group at the end of the 30-week period. Cholesterol and its metabolites, namely the secondary bile acids stimulate the proliferative activity of the colonic epithelium resulting in tumor promotion (Takano *et al.*, 1981). The observed decrease in the level of cholesterol in the experimental group may be due to lower cholesterogenesis (Petrovich *et al.*, 1984) as well as increased excretion of sterols and their metabolites. The cholesterol/phospholipids ratio is closely related to membrane fluidity. The lower ratio of cholesterol/phospholipids in the spice-fed group is closely associated with membrane stability. A change in the concentration of cholesterol will greatly affect the fluidity of the membrane and thereby can bring about abnormal changes in the membrane properties and function. The spice also prevents changes in the ratio of cholesterol/phospholipid, thereby maintaining the membrane fluidity, integrity and function. Thus, the inclusion of this spice in the daily diet plays a significant role in the protection of colon against chemical carcinogenesis (Marsch *et al.*, 1976; Chitra and Leelamma, 2000).

C. *MORINGA OLIEFERA*

Moringa oliefera Lam (English-horse radish plant or drumstick tree) commonly known as 'Sajna' is a very well known botanical used in traditional medicine in India. It is native to South Asia, but grows in tropical Africa and Latin America. The leaves are eaten commonly as a food in India (Ramachandran *et al.*, 1980; Pal *et al.*, 1996). A crude extract of the leaf of *M. oliefera* Lam has been shown to possess hypocholesterolemic activity (Ghasi *et al.*, 2000). A dose of 1 mg/g extract when co-administered with a high-fat diet, daily for a period of 30 days, had a cholesterol-reducing effect in the serum, liver, and kidneys, compared to the high-fat-fed group. The presence of the phytosterol, β-sitosterol, has been established in the leaf of this plant which is presumably the bioactive component that lowers the plasma concentration of LDL and exerts the cholesterol-lowering property (Ghasi *et al.*, 2000).

D. *APOCYNUM VENETUM*

Apocynum venetum L. known as Luobuma in China and Rafuma in Japan has been used for a long time in traditional Chinese medicine (TCM) for the treatment and prevention of hypertension, bronchitis, common cold and other ailments. The therapeutic potential of the leaves of this plant as an anti-hypertensive, anti-aging and anti-hyperlipidemic has been reported (Muramatsu *et al.*, 1986; Ma and Chen, 1989). This when used as a

tea product drink is useful as an anti-hypercholesterolemic and anti-atherosclerotic (Matsuda *et al.*, 1986; Muramatsu *et al.*, 1986). Luobuma extracts decreased the serum TC and LDL-cholesterol levels and the atherogenic index, as well as the hepatic TC level in hypercholesterolemic rats, but they increased the HDL-cholesterol level. Clinical report on the Luobuma extract indicates that it decreases TC and triglyceride levels of patients with hyperlipidemia (Ma and Chen, 1989).

E. *CICER ARIETINUM*

This plant belongs to the papillionaceae family and commonly known as *Channa* in Hindi and Bengal gram in English. It is rich in amino acids like cystene, lysine, methionine, tryptophan, arginine and glutamic acid. *C. arietinum* has been found to have highly significant hypocholesterolemic action in rats and rabbits. It lowered both ester and free cholesterol contents of beta-lipoproteins. The hypocholesterolemic property was detected in lipid extracts of the plant as well as in the defatted portion, the former being more potent in this respect. Its lipid-lowering action could possibly be attributed to inhibition of the synthesis of cholesterol in the liver, and increased catabolism and excretion of cholesterol end products in the feces (Mathur *et al.*, 1968a,b). In patients fed a high-fat diet, supplementation of *C. arietinum* has been reported to lower serum lipids significantly. The hypocholesterolemic effect was associated with a statistically significant increase in 24-h fecal excretion of total bile acids without any significant increase in neutral sterols (Mathur *et al.*, 1968c). *C. arietinum* has been observed to have a very significant anti-artherogenic effect in rabbits. The atherosclerotic process is delayed in onset and is also less severe in degree in rabbits treated with *C. arietinum*. It has also been reported that the cholesterol content of the aortic wall in the control animals was significantly higher in the cholesterol-fed rabbits in comparison to *C. arietinum* (Mathur *et al.*, 1968b). In albino rats having egg-yolk induced hypercholesterolemia, supplementation of *C. arietinum* decreased the serum cholesterol level due to the isoflavone, biocharin, present in germinating *C. arietinum* (Madhavan *et al.*, 1971; Sharma, 1997).

F. *COMMIPHORA MUKUL* (SYNONYM: *C. WIGHTII*)

This plant is commonly known as ‘Indian Bdellium Tree’ and ‘Guggul’ in Ayurveda. Gum resin excreted from the plant is mostly used. Guggul is a spiny shrub or small tree with many branches, usually growing 2 or 3 m high and found in the arid, rocky tracts of Rajasthan, Gujarat and Karnataka in India. A healthy tree yields 250–500 g of resin in one season, and guggul plants typically begin yielding resin after 5 years (Raghunathan and

Mittra, 1982). The tree of *C. mukul* is mostly found in Sindh, Rajasthan, Hyderabad, Karnataka, Madhya Pradesh, Assam and Bangladesh regions of the Indian subcontinent. The medicinal activity has been attributed to the oleogum resin (guggul) of the stem bark, which has been in use for thousands of years. Ayurvedic literature is full of praise for guggul and its divine actions, right from healing bone fractures and inflammations to treating cardiovascular disease, obesity and lipid disorders. In Tibetan medicine, the plant is used for skin diseases, anemia, edema, salivation and heaviness of the stomach and has been proven helpful for regulating cholesterol levels. The plant's lipid-lowering properties have been noted among practitioners of Ayurvedic medicine, while modern scientific research is validating these observations. Guggul works to balance conditions of both low and high cholesterol whether brought on by diet, lack of exercise, chronic stress, or genetic predilection.

Gum guggul has been found to act as hypocholesterolemic and hypolipidemic agents in experimental animals like pigs, chicks, rabbits and rats (Arora *et al.*, 1972). Preliminary clinical studies carried out on 22 patients of hypercholesterolemia with associated obesity, ischemic heart disease, hypertension and diabetes showed a fall in total serum cholesterol and serum lipids in all cases treated with guggul (Dwarakanath and Satyavati, 1970). In another study, guggulipid, an oleoresin and mixture of several steroid lipids, in the dose of 1.2 g/day for 6 weeks reduced cholesterol by 15% and triglycerides by 20%. When guggulipid was administered in a dose of 1.5 g/day for 12 weeks, it brought down the levels of cholesterol by 16.9% and triglycerides by 27%. The effect of guggul powder (8 g/day) in 135 patients with ischemic heart disease for a duration of 12 weeks showed reductions in serum cholesterol (27%), serum triglycerides (36%), phospholipids (20%), and free fatty acids (37%) (Sharma, 1997). It is believed that the lipid-regulating effects of guggul resulted from its thyroid-regulating action, and its combined effects of inhibiting the biosynthesis of cholesterol's thyroid hormones. *Z*-guggalsterone, a ketosteroid and a component of guggul is such an agent. The plant is especially useful where T3 (Triiodothyronine) values of the thyroid are low. Guggul's thyroid stimulating property also explains the traditional use of the plant for thyroid-related problems. The hypolipidemic action of the gum resin of *C. mukul* is well accepted in Indian systems of medicine that can be explained on the following basis (Satyavati *et al.*, 1976):

- Significant increase in the rate of removal/extraction of cholesterol from the body.
- Mobilization of cholesterol from the tissues (as evident clinically by the resolution of xanthomas).
- Decrease in input/synthesis of cholesterol.

- Increase in the rate of degradation of cholesterol by activating the thyroid gland.
- Crude plant extracts contain ion-exchange resins, which combine with the bile acids and thereby trap cholesterol out of entero-hepatic circulation.

The oleoresin from *C. mukul* has been mentioned in the ancient Indian texts Atharvaveda, and in the early medical texts of Charaka, Sushruta, the Samhitas and Nighantus describing the old treatise, which are over a thousand years old. Textbooks of Ayurvedic Medicine distinguish between fresh and old varieties of guggul. The oleoresin contains 0.37% essential oil containing mainly myrecene, dimyrecene and polymyrecene. Alcohol extraction gives a soluble resin and an insoluble carbohydrate gum. The ethyl acetate-soluble portion of guggul was found to possess hypolipidemic and anti-inflammatory properties. The active ingredients responsible for the use of the plant in the maintenance of healthy cholesterol levels, are the guggulsterones, specifically guggulsterone *E* and guggulsterone *Z*, standardized to contain a minimum of 2.5% guggulsterones *E* and *Z*. The limits for the maximum level of guggulsterones (*E* and *Z* to 4.0–6.0%) in a soft extract have been specified in an official book (Anonymous, 1966). The extract of *C. mukul* was found to confer significant protection to albino rats against the development of experimental atherosclerosis; the extract not only prevented deteriorating changes in serum cholesterol, triglycerides, and plasma fibrinogen level but also favorably increased the plasma fibrinolytic activity (Nityanand *et al.*, 1973).

While performing clinical studies on 20 patients with hyperlipidemia who were administered with 4.5 g of purified gum guggul in two divided doses daily for 16 weeks, serum cholesterol and serum triglyceride levels were observed to decrease at the end of the 4th–8th week. HDL cholesterol showed a gradual increase while VLDL and LDL cholesterol showed a significant decrease at all time points (Satyavati *et al.*, 1969). Thus due to its use from ancient times, over the years, it has become well known for the lowering of blood cholesterol levels. It may also be used as part of a weight reduction program. Scientists have been studying the hypolipidemic activity of guggul for over 20 years. It began with animal trials in the late 1960s and because of its success, quickly progressed to human clinical studies. Guggul proved extremely effective in regulating cholesterol, triglycerides, and phospholipids in both types of research. Extracts of guggul have been shown to lower the serum cholesterol level in chicks and have shown similar effects in rabbits, rats and domestic pigs (Nityanand *et al.*, 1973; Satyavati *et al.*, 1976). Another study performed in India in the late 1970s researched the long-term effects of lipid-regulating substances on humans. The two substances compared were clofibrate—an effective and frequently used hypolipidemic drug, and an ether extract of guggul resin. The guggul extract (1.5 g per day)

was taken orally by 41 of the 51 subjects suffering from elevated cholesterol triglycerides while the remaining 10 took clofibrate (2.0 g per day). It was observed that cholesterol and triglyceride levels fell significantly, progressively, and equally in both groups. In a 1986 study, an ethyl acetate extract of guggul showed similar benefits to those highlighted in the study above. In this particular trial, the guggul extract compared favorably with other hypolipidemic drugs, significantly lowering serum cholesterol and triglyceride levels in 78.9% of the test subjects and it produced no noticeable side effects. Another study of guggul found that serum cholesterol and triglyceride levels were lowered by an average of 17.5 and 30.3%, respectively (Tripathi *et al.*, 1968; Satyavati *et al.*, 1976).

G. *CURCUMA LONGA*

Turmeric (*Curcuma longa*), synonymous with curcumin, is a native East Indian and Southeast Asian herb and one of the medicinal plants listed in an Assyrian herbal text dating from about 600 BC. *C. longa* is a perennial herb belonging to zingiberace family. It is 2–3 ft high with a short stem and tufted leaves (Panda, 1986). The rhizomes are short and thick. In India it is popularly known as haldi, halud, turmeric or halder. Turmeric was used by ancient practitioners in India as a stomachic, tonic and carminative. It is used as a household remedy for local application in inflammatory conditions and other painful affections. Its anti-platelet activity is equal to that of aspirin, but unlike aspirin does not increase prostacyclin synthesis. Studies on the effect of alcoholic and ethereal extracts of the rhizomes of *C. longa* and *C. amada* on the cholesterol level in experimental hypercholesterolemic rabbits revealed that the ethereal extract of *C. amada* lowered the blood cholesterol level (Pachauri and Mukherjee, 1970). Rhizome has been reported to contain the important coloring matter curcumin, which belongs to the dicinimolymethane group. Curcumin, isolated from the rhizomes of *C. longa*, caused a sharp but transient fall in blood pressure (Malhotra and Ahuja, 1974). It has also been reported that sodium curcuminate isolated from *C. longa* is an active choleretic which causes an increase in total excretion of bile salts, bilirubin and cholesterol. Ethanol extracts of *C. longa* (tuber) and *Nardostachyas jatamansi* (whole plant) feeding elevate the HDL cholesterol/TC ratio (Chaudhuri, 1998). The extract also caused a significant reduction in the ratio of TC/phospholipids. *C. longa* exhibited better cholesterol and triglyceride lowering activity (Ch = −85%, TG = −88%) as compared to *N. jatamansi* in Triton-induced hyperlipidemia. The cholesterol-lowering effects of curcumin span all levels of lipid-lowering mechanism, including lowering total and LDL cholesterol (by 11% normally), increasing HDL cholesterol (by 29% normally), and reducing

lipid peroxidation, thereby limiting the oxidation of LDL cholesterol rats (Pachauri and Mukherjee, 1970; Sharma, 1997; Kokate *et al.*, 1998).

H. *EMBLICA OFFICINALIS*

E. officinalis belonging to the Euphorbiaceae family is popularly known as amlaor, amlaki in India. It is cultivated in the Deccan region, Coastal districts and Kashmir region, Uttar Pradesh and the central part of India. Though all its parts including dried fruit seeds, leaves, root, bark and flower are used for medicinal purposes; its dried fruit seeds have been mainly in use for therapeutic purposes (Panda, 1986). *E. officinalis* has been reported to exert hypolipidemic activity. *E. officinalis* has been found to reduce serum TC, aortic cholesterol and hepatic cholesterol significantly, without any effect on the serum triglyceride levels in both normal and cholesterol induced hypercholesterolemic rabbits. The effect of *E. officinalis* on total serum cholesterol and its lipoprotein fractions was also studied in normal and hypercholesterolemic individuals aged 35–55 years. When the supplement was given for a period of 28 days in the raw form, both normal and hypercholesterolemic subjects showed a decrease in cholesterol levels. Two weeks after withdrawing the supplement, the total serum cholesterol levels of the hypercholesterolemic subjects rose significantly almost to the initial levels (Tewari *et al.*, 1968; Sharma, 1997).

I. *INULA RACEMOSA*

Inula racemosa belongs to the Asteraceae family and is commonly known as 'pushkarmool.' It is extensively cultivated in Jammu and Kashmir and abundantly found in Himalayan regions of India. Roots of this plant are used for medicinal purposes. The root powder of *I. racemosa* has been reported to be a hypolipidemic agent in clinical and experimental studies (Tripathi *et al.*, 1984). It has been investigated to have potential biochemical effects on isoprenaline-induced changes in rat's serum glutamic oxaloacetate transaminase, lactic dehydrogenase and creatinine phosphokinase. It has been found to possess a propranolol-like beneficial effect in prevention of coronary ischemia and has been reported for its potent hypolipidemic and cardioprotective activity (Sharma, 1997).

J. *TERMINALIA ARJUNA*

T. arjuna commonly known as 'arjun' in India belongs to the Combretacea family and is a large deciduous tree attaining a height of 66 ft. Its bark has been used as medicine in heart disease since 500 BC. The main constituents of

bark powder include glycosides (Arjunine, Arjunetin, Arjunoside I, Arjunoside II, Triterpene-*o*-glycoside). The alcoholic decoction of this bark powder significantly increases euglobin lysis time, prolongs prothrombin time and lowers the serum cholesterol levels in ischemic heart disease patients. The bark powder lowered the systolic blood pressure and body mass index, and increased the HDL-cholesterol. The administration of the bark powder of *T. arjuna* causes a significant decrease in circulating catecholamine levels, while in adrenal glands its concentration goes up. It might be acting by inhibiting the catecholamine release from adrenal glands into the circulation, thus protecting the heart from catecholamine toxicity (Sharma, 1997; Gauthamna *et al.*, 2001). The hypolipidemic action of *T. arjuna* coupled with the enhancement of PGE_2-like activity, negative inotropic, chrontropic, anti-arrhythmic, anti-hypertensive and HDL cholesterol-raising properties make it an imminently cardio-protective product for the overall management of CAD (Gauthamna *et al.*, 2001).

K. *TRIGONELLA FOENUMGRAECUM*

Fenugreek, otherwise known as *Trigonella foenumgraecum* has a rich 3000-year-old history of medicinal use in Egypt, Southern Europe, India, Asia, and Northern Africa. Fenugreek is mentioned in a variety of ancient writings, like the papyri found in Egyptian tombs and the records of the Roman emperor. The seeds of fenugreek (*T. foenumgraecum*), a condiment in India, are high in fiber content and are reported to have anti-diabetic and hypercholesterolemic properties in both animal models and humans. It is an aromatic herb, commonly known as 'Methi' in Hindi and fenugreek in English, belongs to family—leguminosae, and is cultivated in many parts of India. It has been used for many years as an insect repellent by agriculturists to protect gram from insect attacks. It was traditionally used to minimize the symptoms of menopause and arthritis, control diabetes, reduce cholesterol, control coughs and fevers, as an anti-inflammatory, and to improve heart and vascular health. Overall, fenugreek is an herb with a rich history in healthcare (Nadkarni and Nadkarni, 1976; Bruneton, 1995). The hypolipidemic effects of fenugreek seed on animals have been reported by many workers including effects on noninsulin-dependent diabetic patients; these studies have proven the potent thereapeutic potential of fenugreek in the treatment of hypercholesterolemia (Sharma, 1984, 1986a,b, 1996; Sharma *et al.*, 1990). The oral administration of *T. foenumgraecum* showed significant hypocholesterolemic and hypotriacylglycerolemic effects in cholesterol-induced hyperlipidemic rabbits, restoring the normal serum lipid levels and substantially lowering the tissue

lipids (Stark and Madar, 1993; Stark and Zechiaria, 1993; Sharma, 1997; Sowmya and Rajyalakshmi, 1998).

Several more recent animal and human clinical trials have confirmed the therapeutic application of fenugreek seeds. Antioxidant activity preventing lipid peroxidation has been reported for the seeds of fenugreek (Anuradha and Ravikumar, 1998). Combined with its long history of safe use, it is no surprise that fenugreek is gaining a reputation as a promising herbal supplement for optimal health. Fenugreek is endowed with a variety of natural therapeutic compounds. The seeds contain an excellent source of mucilaginous fiber mainly composed of galactomannans. Galactomannans are glyconutrients that help to slow the absorption of carbohydrates, help to create a feeling of fullness and also act to improve organ health at the cellular level. Fenugreek seeds contain 45–60% fiber. Fenugreek seeds are also rich in protein (20–30% of the seed) and free amino acids, such as arginine (which plays a key role in cardiovascular health), histidine, lysine and tryptophan (Sauvaire *et al.*, 1984, 1991, 1998). Fenugreek seeds also contain a unique amino acid called 4-hydroxyisoleucine (Fowden *et al.*, 1973; Hardman and Fazli, 1972). Recent scientific research has confirmed that this rare compound can actually mimic and stimulate the release of insulin thereby helping to control blood glucose levels (Ali *et al.*, 1995). This explains fenugreek's unique ability to help control diabetes. Fenugreek is also rich in flavonoids such as apigenin, luteolin, orientin, quercetin, vitexin, and isovitexin. These flavonoids act as powerful natural antioxidants and help to strengthen the immune system, improve cellular health and reduce the signs of aging. Even more impressive is the content of saponins in the seeds. Fenugreek contains natural saponins that can bind to cholesterol and fat in our diet and remove them from the body (Hardman and Fazli, 1972; Gupta *et al.*, 1986; Benichou *et al.*, 1999). Some of these saponins such as yamogenin, tigogenin, and neotigogenin can also act as natural phytoestrogens and help to support hormonal balance. In addition, fenugreek seeds contain vitamins A, B1, C, and nicotinic acid, therapeutic oils, pyridine-type alkaloids, such as trigonelline, choline, gentianine, and carpaine, and volatile oils such as *n*-alkenes and sesquiterpenes. Thus, fenugreek offers a diverse and effective natural therapeutic compound for curing so many ailments.

L. *APIUM GRAVEOLENS*

Apium graveolens known as celery belongs to the family Umbelliferae and is a biennial plant that is native to Europe. Celery generally grows about 1–2 ft tall, has tough-ribbed green stems and segmented dark green leaves containing toothed leaflets. It is high in mineral content, and contains pheromone steroids identified as boars and parsnips (Claus and Hoppen, 1979).

Besides this, it also contains isovalerinic aldehyde, propionic aldehyde and acetaldehyde (Leung, 1980). It has long been recommended in TCM orally for dizziness, headache and high blood pressure. In rats fed a high-fat diet for 8 weeks to induce hyperlipidemia, those given a celery juice supplement were reported to have significantly lower TC, LDL-cholesterol and triglycerides. An aqueous celery extract significantly lowers the systolic blood pressure (Leung, 1980).

M. *CICHORIUM INTYBUS*

Cichorium intybus, commonly known as 'Chicory' of the family Compositae or Astraceae, is a perennial plant indigenous to Europe, India and Egypt. Chicory flowers contain chichorin, which is 6,7-glucohydroxycoumarin. The root contains up to 8% inulin (a polysaccharide), a bitter principal containing one part protocatechuic aldehyde to three parts inulin (Balbaa *et al.*, 1973). Chicory's oligosaccharides are probiotic and are beneficial in maintaining healthy gastrointestinal flora. This plant is our best source of the cholesterol-lowering phytochemical, inulin. In France and Italy, the roots are not only consumed as a drink, but are also considered as a vegetable. Inulin is also reportedly lipolytic and may thereby decrease obesity and act as one of the contributors to cardiopathy. Improved lipid metabolism was demonstrated in rats fed an inulin containing chicory extract, possibly due to the changes in absorption or synthesis of cholesterol (Kim and Shin, 1996; Roberfroid and Delzenne, 1998; Roberfrodid, 1999).

N. *OENOTHERA BIENNIS*

Oenothera biennis, commonly known as Evening Primrose of the family Onagraceae, is a large delicate wild flower native to North America. It is not a true primrose; the blooms usually last for only one evening. Seeds of *O. biennis* contain 14% of a fixed oil known as OEP. This oil contains 50–70% *cis*-linoleic acid and 7–10% *cis*-gamma-linoleic acid (GLA) (Leung, 1980). Essential fatty acids are important as cellular and structural elements, and as precursors of prostaglandins, which help to regulate metabolic function. GLA may inhibit a number of cardiovascular pathologies, including cardiac arrhythmia, hypertensive responses, platelet aggregation and hyperlipidemia. Linoleic acid can reduce elevated serum cholesterol levels, but GLA has cholesterol-lowering activity about 170 times greater than the parent compound. GLA, the major active ingredient in the evening primrose oil (also in borage, currant and hempseed oil), inhibits platelet aggregation, reduces blood pressure and restores the motility of red blood cells (Marderosian and Lawerence, 1998).

O. *CRATAEGUS SPP.*

Crataegus spp. (*Crataegus oxyacantha*, *Crataegus laevigata*, and *Crataegus monogyna*), commonly known as Hawthorn of Rosaceae family, is a spiny bush or small tree that grows up to 7.5 m in height. The leaves, flowers, bark, and fruit contain the flavonoid pigments, hyperoside and vitex rhamnoside. The flavonoid constituents from hawthorn have been frequently reported (Fisel, 1965, 1966; Lewak, 1969). Hawthorn has been frequently studied in the prevention and treatment of atherosclerosis. A hawthorn preparation, when administered to animals, resulted in the lowering of blood viscosity and the fibrinogen level. Another report found that the tincture of hawthorn increased bile acid excretion and decreased the cholesterol synthesis in rats. The mechanisms involve an upregulation of hepatic LDL receptors, resulting in a greater influx of cholesterol into the liver. Hawthorn has also been found to enhance cholesterol degradation (He, 1990; Rajendran *et al.*, 1996).

P. *VITIS VINIFERA*

Vitis vinifera, which is commonly known as grapes, is a deciduous climbing plant, native to southern Europe and western Asia but is cultivated in warm temperature regions through the world (Ensminger *et al.*, 1994). Grapes are a rich source of polyphenolic compounds and flavonoids including quercetin, catechins, myricetin, kaempferol, and the isomers of resveratrol are the main constituents (Soleas *et al.*, 1995; Stein *et al.*, 1999). Flavonoids in red wine, especially quercetin, are said to be far more effective than vitamin E in preventing the oxidation of LDL cholesterol, which is a contributor to the development of atherosclerosis. Proanthocyanins in red wine are both radical scavengers and xanthine oxidase inhibitors. *In vivo* lipid peroxidation has been implicated in many coronary malfunctions, including atherosclerosis and other ailments like aging and cancer. Phenolic constituents also inhibit cyclooxygenase and LOX in platelets and macrophages, thereby reducing thrombotic tendencies.

Q. *PORTULACA OLERACEA*

Portulaca oleracea commonly known as purslane belongs to the family Portulacaceae and is an herbaceous cosmopolitan weed. *P. oleracea* is one of the major sources of omega-3 fatty acids and was found to produce beneficial effects on cholesterol and triglyceride levels in heart disease and in strengthening the immune system. The plant also possesses marked antioxidant activity (Reid, 1986; Chevallier, 1996; Hocking, 1997).

R. *CYNARA SCOLYMUS*

Cynara scolymus, also known as artichoke, belongs to the family Compositae and is a perennial herb widely cultivated in the Mediterranean regions and adjoining parts of central Europe. Artichoke is found to contain high amounts of flavone glycosides (Hammouda, 1993; Fleming, 1998; Gebhardt, 2001), volatile oils (Hammouda, 1993), bitter sesquiterpene principles (Gebhardt, 1998), phytosterol, tannins, and sugars (Hammouda, 1993). An *in vitro* study determined that artichokes inhibit cholesterol biosynthesis by indirectly modulating and inhibiting HMG-CoA reductase, the key enzyme in the biosynthetic pathway for cholesterol synthesis. The cynaroside and particularly the aglycone, luteolin, were mainly responsible for HMG-CoA reductase inhibition (Brown and Rice-Evans, 1998; Gebhardt, 1998; Anonymous, 1999; Gebhardt, 2001). The flavonoid of artichoke, luteolin, demonstrated anti-oxidant properties (Brown and Rice-Evans, 1998). A prospective study investigating 143 patients with TC greater than 280 mg/dl reported that patients given 1800 mg of dry antichoke extract per day versus placebo over a 6-week period experienced statistically significant changes in total and LDL cholesterol. TC was decreased by 18.5 versus 8.6% for the placebo, while TC was reduced 22.9 versus 6.3% for the placebo. Thus, dry artichoke extract was recommended to treat hypo-lipoproteinemia thereby reducing the risk of atherosclerosis and CHD (Brown and Rice-Evans, 1998).

S. *VACCINIUM MYRTILLUS*

Vaccinium myrtillus, commonly known as bilberry fruit, originates mainly from northern and central Europe (Bisset, 1994). This well known plant is rich in flavonoids, the polyphenolic compounds that promote anti-oxidant activity (Bisset, 1994). A study conducted on the antioxidative potential of *V. myrtillus* showed potent protective action on LDL particles during *in vitro* copper-mediated oxidation. The study concluded that this extract may be more potent than either ascorbic acid or butylated hydroxy-toluene in the protection of LDL particles from oxidative stress (Mitcheva, 1993; Bisset, 1994).

T. *GLYCINE MAX*

The soybean plant, *Glycine max*, belongs to the family Leguminosae, and is found to possess a number of health benefits including anti-carcinogenic effects and improvements in cardiovascular and intestinal problems. The mechanisms responsible for the effect of soy on serum lipoproteins are not well known. Caroll (1991) and Potter (1995) reviewed various hypotheses

which include the amino acid composition of soy protein, interruption of the intestinal absorption of bile acids and dietary cholesterol, direct effects on the hepatic metabolism of cholesterol, alteration of the concentration of the hormone involved in cholesterol metabolism, and the effects of components such as isoflavones, fiber and saponins in soybeans. The cholesterol-lowering effect may be due to another constituent associated with soy protein that is either lost or liberated during the hydrolysis of the protein (Potter, 1995). Greaves *et al.* (2000) have reported that a soy protein diet decreased both plasma cholesterol concentrations and intestinal cholesterol absorption. However, the addition of a semi-purified soy extract rich in isoflavones to casein-lactalbumin protein did not improve plasma lipids or reduce cholesterol absorption. Furthermore, the addition of a conjugated equine estrogen to casein-lactalbumin protein did not improve plasma lipids or affect intestinal cholesterol absorption. Thus, a bioactive component of soy protein other than or in addition to the isoflavones such as the saponins, phytic acid, protein components, or the amino acid composition of the soy protein or the protein–isoflavone interaction may be involved in the lipid-lowering effects (Greaves *et al.*, 2000).

U. *PLANTAGO PSYLLIUM*

Plantago psyllium, commonly known as plantain, belongs to the family Plantaginacea and is a perennial weed with worldwide distribution. About 250 different varieties are available which are mostly herbs and/or shrubs characterized by basal leaves and inconspicuous flowers (Greaves *et al.*, 2000). Plantain constituents include various acids like benzoic, caffeic, vanillic, and ursolic acids; alkaloids like boschniakine and amino acids. Flavonoids found in plantain include apigenin, baicalein, sctellarein and others (Greaves *et al.*, 2000; Der Marderosian and Beutler, 2002). Many reports on psyllium have concluded that it can be helpful in treating various hyperlipidemias (Der Marderosian and Beutler, 2002). In animal studies, plantain lowered the total plasma lipids, cholesterol and triglycerides in atherosclerotic rabbits (Der Marderosian and Beutler, 2002). In a study of 28 patients who took 3 doses (3.4 g/dose) per day compared with placebo for 8 weeks, the psyllium-treated patients showed decreases in total serum cholesterol levels compared with the placebo group after 4 weeks. Decreases were also seen in LDL cholesterol and LDL/HDL ratio. At the end of 8 weeks, values for TC, LDL cholesterol and the LDL/HDL ratio were 14, 20, and 15%, respectively, below baseline. This study suggested that a high-cholesterol level could be managed safely and easily by including psyllium preparations in the diet (Katcher and Koda, 1987). Psyllium seed was found to be more effective than *P. ovata* husk in reducing serum cholesterol in

normal patients. A report in 20 hypercholesterolemic pediatric patients on low-fat diets, however, found psyllium seed to be ineffective in lowering cholesterol or LDL levels (Der Marderosian and Beutler, 2002). A polyphenolic compound from the psyllium leaves was found to exhibit hypocholesterolemic activity, perhaps by the enhancement of cholesterol elimination as fecal bile acids (Der Marderosian and Beutler, 2002).

V. RED YEAST RICE

Red yeast dates back to 800 AD where it was described in the ancient Chinese Pharmacopoeia published during the Ming dynasty (Der Marderosian and Beutler, 2002). The yeast is grown on rice so that the crimson organism permeates the rice; then it is ground to a powder. It is a mild non-poisonous yeast thought to be useful for gastric problems such as indigestion. *Monascus purpureus* yeast is made by a fermentation process using cooked non-glutinous rice. It has been used traditionally in China as food and medicine (Der Marderosian and Beutler, 2002). The commercial product contains 0.4% naturally occurring HMG-CoA reductase inhibitors of which lovastatin and biologically active hydroxyl acids are the most abundant. One of the most significant aspects of red yeast rice is its 9 HMG-CoA reductase inhibitors, as well as the isoflavones, unsaturated fatty acids and trace elements such as selenium (Katcher and Koda, 1987). Over two dozen clinical studies demonstrate its effectiveness. For example, a TC decrease of 17% and a 22.4% decrease in LDLs were demonstrated in a clinical trial conducted at UCLA. Hypercholesterolemia is treated aggressively with statin drugs, very potent inhibitors of HMG-CoA reductase, and the rate-limiting enzyme in cholesterol biosynthesis at the mevalonate level. Lovostatin's action is its conversion to mevinolin in the body. Mevinolin is the active principle found in red yeast rice, which enzymatically inhibits mevalonate, thus lowering cholesterol. It also has anti-oxidant properties. Numerous clinical trials suggest that red yeast rice (*Monascus purpureus*) has comparable therapeutic effects without the side effects of the statin drugs (Der Marderosian and Beutler, 2002).

W. MILK THISTLE (*SILYBUM MARIANUM*)

Silybum marianum is commonly known as milk thistle or holy thistle. Silybum is indigenous to Kashmir, India but is also found in North America. It grows to a height of 5–10 ft and has large prickly leaves. The milk thistle seed extract contains a bioflavonoid complex known as Silymarin, which consists of silybin, silidianin, and silicristin (Leung, 1980; Katcher and Koda, 1987). Silybin is the most biologically active component with regard to its

anti-oxidant and hepatoprotective properties (Arnone *et al.*, 1979). It exerts anti-oxidant and membrane-stabilizing activities, attributes important for liver secretion and uptake of plasma lipoproteins. Inhibition of HMG-CoA reductase *in vitro* has been demonstrated with therapeutic application of milk thistle, implying its possible direct influence on liver cholesterol metabolism. Milk thistle can be compared to probucol, a hypocholesterolemic anti-oxidant drug. In contrast to probucol, milk thistle caused an increase in HDL lipoproteins and a decrease in liver cholesterol content, both additional benefits. Administration of silymarin at 420 mg/day for 3 months to 14 type II hyperlipidemic patients resulted in a slight decrease in TC and HDL-cholesterol levels (Benda *et al.*, 1980). The biliary cholesterol and phospholipid concentrations in rats were also slightly reduced. The silybin-induced reduction of biliary cholesterol both in rats and humans may be due in part to the decreased liver cholesterol synthesis (Lorenz *et al.*, 1982; Feher *et al.*, 1989; Schulz *et al.*, 1998).

X. *LINUM USITATISSIMUM*

Linum usitatissimum, commonly known as flaxseed, belongs to the family Linaceae. Flaxseed is the richest food source of lignans, one of the major groups of phytoestrogens (Thompson *et al.*, 1991) and is incorporated into human diets because of its reported health benefits. Lignans have been implicated as having anti-tumorigenic (Thompson *et al.*, 1996), estrogenic and/or anti-estrogenic, and antioxidant (Collins *et al.*, 1997; Prasad, 2000) properties. Prasad (2000) reported that rabbits receiving secoisolariciresinol diglucoside, the major lignan found in flaxseed, had reduced total and LDL-cholesterol concentrations. Lignans have also been shown to modulate activities of 7-hydroxylase and acyl CoA cholesterol transferase (Kitts *et al.*, 1999), two of the key enzymes involved in cholesterol metabolism. Sanghvi *et al.* (1984) concluded that the reduction in hypercholesterolemic atherosclerosis induced by flaxseed is due to a decrease in total serum cholesterol and LDL cholesterol and that the anti-atherogenic activity of flaxseed is independent of its linolenic acid content. However, the hypocholesterolemic effects of whole flaxseed can also be attributed, in part, to its linolenic acid and fiber components (Bierenbaum *et al.*, 1993; Cunnane *et al.*, 1995; Prasad, 1997; Jenkins *et al.*, 1999). The hypocholesterolemic effects of linolenic acid have been reported in both animals (Garg *et al.*, 1989) and humans (Chan *et al.*, 1991). Garg *et al.* (1989) demonstrated that feeding a linolenic acid-rich diet to rats lowered serum cholesterol levels more effectively than a diet rich in linoleic acid. Flaxseed reduced serum levels of both apo B and apo A-1 (Jenkins *et al.*, 1999). The soluble fiber mucilage present in flaxseed may also contribute to the observed

hypocholesterolemic properties (Edralin *et al.*, 2002; Brown *et al.*, 1999; Prasad, 1999; Lucas *et al.*, 2002).

Y. *ALLIUM SATIVUM*

1. Usage in medicine

Allium sativum, commonly known as garlic, is a perennial herb having bulbs with several cloves enclosed in a silky white or pink envelope of the skin. It is a popular spice added to several edible preparations all over the world since ancient times. It has also found its use as a folk remedy for a variety of ailments. The hypolipidemic effect of *A. sativum* is well documented. Garlic acquired a reputation in the folklore of many cultures over centuries as a formidable prophylactic and therapeutic medicinal agent. To date, many favorable experimental and clinical effects of garlic preparations, including garlic extract, have been reported. Some of the earliest references to this medicinal and culinary plant are found on Sumerian clay tablets dating from 2600 to 2100 BC (Banerjee and Maulik, 2002). Garlic was an important medicine to the ancient Egyptians listed in the medical text *Codex Ebers* (ca. 1550 BC), especially for the working class involved in heavy labor (Moyers, 1996; Lawson, 1998). There is evidence that during the earliest Olympics in Greece, garlic was fed to the athletes for increasing stamina (Lawson, 1998). In ancient Chinese medicine, garlic was prescribed to aid respiration and digestion, and most importantly, for treating diarrhea and worm infestations (Woodward, 1996). Three ancient medical traditions in India, i.e., Tibbi, Unani and Ayurveda, made extensive use of garlic as a central part of the healing efficacy of plants (Moyers, 1996). The leading Indian ancient medical text, *Charaka-Samhita* recommends garlic for the treatment of heart disease and arthritis. In another ancient Indian medical textbook, *Bower Manuscript* (~300 AD), garlic was used for fatigue, parasitic disease, digestive disorders, and leprosy (Rivlin, 1998). With the onset of the Renaissance period, increasing attention was paid in Europe to the medical use of garlic. A leading physician of the 16th century, Pietro Mattiali of Siena, prescribed garlic for digestive disorders, infestations with worms, and renal disorders, as well as to help mothers during difficult childbirth (Moyers, 1996). In England, garlic was used for toothache, constipation, dropsy, and plague (Rivlin, 1998). In the modern era, scientists have been trying to validate many of these properties of garlic, especially in terms of the identity of the active components, their mechanisms of action, and the exploration of the potential benefits as food supplements. The effects of *A. sativum* juice, as well as that of the essential oil extract of an equivalent amount of *A. sativum*, was studied on alimentary hyperlipidemia, blood coagulation time, and serum cholesterol

levels; both the juice and the essential oil of *A. sativum* were found to have significant protective action against fat-induced fibrinogen. Both garlic products caused a decrease in fibrinolytic activity as well as coagulation time. Both raw and boiled forms of *A. sativum* were reported to decrease total serum cholesterol (Sharma, 1997).

2. *Phytoconstituents in garlic*

Raw garlic homogenate has been the major preparation of garlic subjected to intensive scientific study because it is the commonest form for garlic consumption. Raw garlic homogenate is essentially the same as an aqueous extract of garlic, which has been used in various scientific studies. Allicin (allyl 2-propenethiosulfinate or diallyl thiosulfinate) is thought to be the principal bioactive compound present in aqueous garlic extract or raw garlic homogenate. When garlic is chopped or crushed, allinase enzyme, present in garlic, is activated and acts on alliin (present in intact garlic) to produce allicin. Other important sulfur-containing compounds present in garlic homogenate are allyl methyl thiosulfonate, 1-propenyl allyl thiosulfonate and γ-L-glutamyl-*S*-alkyl-L-cysteine. The adenosine concentration increases several-fold as the homogenate is incubated at room temperature. The enzyme allinase responsible for converting alliin (*S*-allyl cysteine sulfoxide) to allicin is inactivated by heat. Thus, the water extract of heat-treated garlic contains mainly alliin. Since garlic powder is simply a dehydrated, pulverized garlic clove, the composition, especially the allinase activity of garlic powder is identical to that of fresh garlic. However, the dehydration temperature should not exceed 60°C, above which allinase is inactivated (Lawson, 1998). Another widely studied garlic preparation is AGE. Sliced raw garlic stored in 15–20% ethanol for 20 months is referred to as AGE. This whole process is supposed to cause considerable loss of allicin and increased activity of certain newer compounds, like *S*-allylcysteine (SAC), *S*-allylmercaptocysteine, allixin and selenium which are stable, highly bioavailable and have significant antioxidant activity (Borek, 2001). Another recently identified antioxidant compound of AGE is *N*-α-(1-deoxy-D-fructos-1-yl)-L-arginine (Fru-Arg), which is not present in raw or heat-treated garlic (Ryu *et al.*, 2001). Medicinally used garlic oil is mostly prepared by steam-distillation process. Steam-distilled garlic oil consists of the diallyl (57%), allyl methyl (37%) and dimethyl (6%) mono- to hexasulfides. A typical commercial preparation of garlic oil contains diallyl disulfide (DADS, 26%), diallyl trisulfide (DATS, 19%), allyl methyl trisulfide (15%), allyl methyl disulfide (13%), diallyl tetrasulfide (8%), allyl methyl tetrasulfide (6%), dimethyl trisulfide (3%), pentasulfide (4%) and hexasulfide (1%). Oil-macerated garlic oil contains the vinyl-dithiins and ajoenes. Ether extracted garlic oil (essential oil) contains

nine times as much of the vinyl-dithiins (5.7 mg/g) and allyl sulfides (1.4 mg/g) and four times as much of the ajoenes (0.4 mg/g) (Lawson, 1998).

Garlic and its various preparations have been widely recognized as agents for prevention and treatment of atherosclerosis, hyperlipidemia, thrombosis, hypertension and diabetes. The effectiveness of garlic in the treatment of cardiovascular diseases appeared to be encouraging in experimental studies, which prompted several clinical trials. The efficacy of garlic for the treatment of various diseases has been increasingly subjected to rigorous scientific investigations. Garlic appears to be the cheapest way to prevent hyperlipedemia as well as cardiovascular disease (Banerjee and Maulik, 2002).

3. Therapeutic effect of garlic in hyperlipidemia and atherosclerosis

Garlic is best known for its lipid-lowering and anti-atherogenic effects. In cases where dietary therapy may not be sufficient to control lipid levels, natural compounds can lower cholesterol levels and, in general, are less expensive than drugs. The composition and method of preparation of garlic supplements may contribute to efficacy of garlic preparations in lowering cholesterol levels. There are conflicting studies on the effectiveness of garlic. Since the 1980s, four out of five studies have shown that garlic lowers cholesterol. Allicin, the major component of garlic imparting the characteristic garlic odor is mainly responsible for the hypolipidemic activity. The commercially prepared alliin or odorless garlic is converted to allicin in the body. Garlic reduces atherosclerosis by inhibiting platelet aggregation, increasing fibrinolysis, enhancing antioxidant activity, and reducing serum lipids in general to lower cholesterol levels and other significant risk factors for CAD (Banerjee and Maulik, 2002). Several animal and human studies have proven the efficacy of garlic as a herbal remedy to reduce a multitude of risk factors, which play a decisive role in the genesis and progression of arteriosclerosis including decreases in total and LDL-cholesterol, a decrease in HDL-cholesterol, a reduction of serum triglyceride and fibrinogen concentrations, the lowering of arterial blood pressure and the promotion of organ perfusion, and, finally, enhancement in fibrinolysis, inhibition of platelet aggregation, and diminution of plasma viscosity (Jain, 1975). Several groups of investigators (Jain, 1975, 1977; Bordia *et al.*, 1977; Chang and Johnson, 1980; Kamanna and Chandrasekhara, 1984; Mand *et al.*, 1985; Schwartz *et al.*, 1993) studied the effects of long-term (2–9 months) feeding of garlic and garlic preparations (2% garlic powder in diet) on experimental atherosclerosis induced by a high-cholesterol diet in rabbits. Most of these studies reported a statistically significant reduction in atheromatous lesions, particularly in the aorta, that averaged about 50%. The chronic effects of garlic on lipid metabolism in rats were also encouraging. The duration of

these studies was at least 4 weeks. Garlic (1–4% in diet) and garlic protein administration in hypercholesterolemic rats induced by a high-cholesterol diet, significantly reduced serum cholesterol, triglyceride and LDL cholesterol levels (Chang and Johnson, 1980; Rajasree *et al.*, 1999; Mathew and Daniel, 1996; Qureshi *et al.*, 1983; Kamanna and Chandrasekhara, 1982; Chi, 1982; Chi *et al.*, 1982) but had no effect on serum HDL. Total lipid content and cholesterol levels in liver were also decreased in rats after chronic garlic consumption. Since 1975, there have been more than 46 human studies on the lipid-lowering effects of garlic and garlic preparations. These studies were mostly randomized, double blind, and placebo-controlled using garlic powder rather than raw garlic for periods of 4–16 weeks, in hyperlipidemic patients. Most of these studies showed a significant decrease in serum cholesterol and serum triglyceride (Banerjee and Maulik, 2002). Only about one-third of these studies measured lipoproteins, where significant favorable changes in the LDL-cholesterol level (11–26% decrease) were consistently observed. A few studies using garlic powder (having low allicin yields) failed to show any lipid-lowering effects (Lutomski, 1984; Luley *et al.*, 1986). During the last decade, 18 clinical studies have been published regarding the hypolipidemic effect of garlic. Nine studies showed negative results, and garlic powder was used in seven of these studies (Simons *et al.*, 1995; Berthold and Sudhop, 1998; Isaacsohn *et al.*, 1998; McCrindle *et al.*, 1998; Byrne *et al.*, 1999; Rahman and Billington, 2000; Superko and Krauss, 2000; Gardner *et al.*, 2001; Ziaei *et al.*, 2001). The differences in the composition and quantity of sulfur components of different garlic preparations used in various studies could account for the inconsistent findings. It highlights the need for standardization of different garlic preparations. Other factors might also have affected the inconsistent results including subject recruitment, the duration of study, dietary control, lifestyle, and methods of lipid analyses (Warshafsky *et al.*, 1993; Silagy and Neil, 1994).

Four meta-analysis of randomized, placebo-controlled human studies on the hypocholesterolemic effects of garlic are available (Warshafsky *et al.*, 1993; Silagy and Neil, 1994; Neil *et al.*, 1996; Stevinson *et al.*, 2000). The analyses further detected that the extent of the cholesterol-lowering properties of garlic differed markedly from one study to another. Warshafsky and his colleagues deduced from five randomized clinical trials that hypercholesterolemic patients treated with garlic had a mean plasma cholesterol concentration that was 9% lower than that of patients treated with placebo (Warshafsky *et al.*, 1993). Silagy and Neil analyzed 16 trials, with data from 952 subjects in a meta-analysis (Silagy and Neil, 1994). Garlic, in powder and non-powder form, significantly lowered serum lipid levels over a 1–3 month period. Serum cholesterol fell by 8% with dried powder preparations and 15% with non-powder preparations. The serum triglyceride level also dropped

significantly, while HDL-cholesterol was essentially unchanged. Amongst the garlic powder preparations, these effects appeared to be similar across the daily dose-range of 600–900 mg. Another meta-analysis (Neil *et al.*, 1996) revealed that there was no significant difference in the mean concentrations of serum lipids, lipoproteins or apo A1 or B amongst the groups receiving garlic (900 mg/day of dried garlic powder standardized to 1.3% allicin) and placebo. In this meta-analysis, garlic was less effective in reducing TC than suggested by previous meta-analyses. However, in a more recent meta-analysis of 13 trials (Stevinson *et al.*, 2000), garlic reduced the TC level from baseline significantly more than placebo, while six diet-controlled trials with the highest scores for methodological quality revealed a nonsignificant difference between garlic and placebo groups. The available data suggest that garlic is superior to placebo in reducing TC levels. However, the size of the effect is modest, and the robustness of the effect is debatable. Therefore, the hypocholesterolemic effect of garlic remains to be firmly established (Banerjee and Maulik, 2002). The protective effect of garlic on atherosclerosis has been attributed to its capacity to reduce the lipid content of the arterial wall. Garlic causes direct anti-atherogenic (preventive) and anti-atherosclerotic (causing regression) effects at the level of the artery wall (Orekhov and Grunwald, 1997). Garlic depressed the hepatic activities of lipogenic and cholesterogenic enzymes such as malic enzyme, fatty acid synthase, glucose-6 phosphate dehydrogenase and HMG-CoA reductase (Yu-Yan and Liu, 2001). Garlic also increased the excretion of cholesterol, as manifested by enhanced excretion of acidic and neutral steroids after garlic feeding (Chi *et al.*, 1982). LDL isolated from human subjects given AGE (Munday *et al.*, 1999) and aqueous garlic extract (Lewin and Popov, 1994) was found to be significantly more resistant to oxidation. These data indicate that suppressed LDL oxidation may be one of the powerful mechanisms accounting for the benefits of garlic in atherosclerosis (LauBenjamin, 2001). The first investigated, active compound responsible for the anti-atherosclerotic effect was alicin, however, recent *in vitro* studies revealed that water-soluble organosulfur compounds, especially SAC, present in AGE and DADS, present in garlic oil are also potent inhibitors of cholesterol synthesis (Gebhardt and Beck, 1996).

IV CONCLUSION

Atherosclerosis is a complex disease, characterized by an excessive inflammatory, fibro-fatty, proliferative response to damage of the artery wall involving several cell types, particularly smooth muscle cells, monocyte-derived macrophages, T-lymphocytes and platelets (Schwartz *et al.*, 1993).

Hyperlipidemia constitutes a major etiopathological factor for atherosclerosis. Most recent findings indicate a multi-faceted cause to the problem of cardiovascular disease, including excessive intake of saturated fats, carbohydrate metabolic dysfunction, nutritional deficiencies, hormonal imbalance, and a high-stress lifestyle. Different people seem to oxidize cholesterol differently. In addition, certain mechanisms in the body may have gone awry, such as impaired liver LDL receptor uptake in FH. Lowering, but not eliminating, the intake of saturated fats is advisable. Nature has provided specific compounds capable of augmenting dietary and lifestyle changes for improved cardiovascular health and may afford a way to lower cholesterol without resorting to synthetic drug preparations and their potential side effects.

Atherosclerosis results from multiple complex interactions among injurious stimuli and the healing or reparative response of the arterial wall occurring in a hyperlipidemic and dyslipoproteinemic environment (Schwartz *et al.*, 1993). Events in the atherogenic cascade involve both environmental and genetic factors (Epstein, 1992). Despite the decline in rates of mortality due to heart diseases during the past two decades, cardiovascular diseases remain the most frequent cause of death (Carlson *et al.*, 1979). Several large clinical trials have established that regulation of dyslipidemia through diet or diet plus pharmacotherapy reduces the incidence of CHD events (Carlson and Bottiger, 1985). The association of raised serum cholesterol with cardiovascular disease is well known. Some studies suggest that elevated serum triglyceride may also be a risk factor (Carlson *et al.*, 1979; Carlson and Bottiger, 1985) especially in individuals with diabetes, since there is often a marked hyperlipidemia in diabetes (Beteridge, 1989). Moreover, diabetic patients experience a two-to-three-fold increase in cardiovascular morbidity and mortality when compared with nondiabetics. The beneficial effect of lowering elevated serum cholesterol levels on the prevention of CHD has been well established (Lipid Research Clinic Program, 1984). Dietary intervention has been recommended for all subjects with a LDL level of more than 160 mg/dl (Huttunen *et al.*, 1991). In addition to the quantity of fat and the polyunsaturated/saturated fat ratio, other dietary factors also play a role in the management of hyperlipidemia. Several studies have shown that dietary fiber, particularly soluble fiber, has considerable influence on serum cholesterol levels (Dreher, 1987; Miettinen, 1987).

Herbs have been used as medical treatments since the beginning of civilization and some herbal derivatives (e.g., aspirin, reserpine, and digitalis) have become a mainstay of human pharmacotherapy. For cardiovascular diseases, herbal treatments have been used in patients with congestive heart failure, systolic hypertension, angina pectoris, atherosclerosis, cerebral insufficiency, venous insufficiency, and arrhythmia. Scientific validation of several plant species has proved the efficacy of the botanicals in reducing the

cholesterol levels. From the reports on their potential effectiveness against hypercholesterolemia, it is assumed that the botanicals have a major role to play in the management of hyperlipidemia, which need further exploration for necessary development of drugs and nutraceuticals from natural resources (Mukherjee, 2001, 2002). However, many herbal remedies used today have not undergone careful scientific assessment, and some have the potential to cause serious toxic effects and major drug-to-drug interactions. Continuing research is necessary to elucidate the pharmacological activities of the many herbal remedies now being used to treat hyperlipidemia, atherosclerosis and other cardiovascular diseases.

ACKNOWLEDGEMENTS

The author wishes to express his heartfelt thanks to Mr. Atul Wahiile, KFH Nazeer Ahamed, L. Manikandan and V. Kumar, Research scholars of the Dept of Pharmaceutical Technology, Jadavpur University for their assistance in the preparation of the manuscript. Thanks are due to All India Council for Technical Education (AICTE) New Delhi for the TAPTEC project grant and to the Department of Science and Technology (DST), Govt of India for financial assistance through a project grant.

REFERENCES

Abramovitz, D., Gavri, S., Harats, D., Levkovitz, H., Mirelman, D., Miron, T., Eilat-Adar, S., Rabinkov, A., Wilchek, M., Eldar, M., and Vered, Z. 1999. Allicin-induced decrease in formation of fatty streaks (atherosclerosis) in mice fed a cholesterol-rich diet. *Coron. Artery Dis.* **10**, 515–519.

Afanas'ev, I.B., Dorozhko, A.I., Brodskii, A.V., Kostyuk, V.A., and Potapovitch, A.I. 1989. Chelating and free radical scavenging mechanisms of inhibitory action of rutin and quercetin in lipid peroxidation. *Biochem. Pharmacol.* **38**, 1763–1769.

Agarwal, V. and Chauhan, B.M. 1988. *Plant Foods Hum. Nutr.* **38**, 189–197.

Ali, L., AzadKhan, A.K., Hassan, Z., Mosihuzzaman, M., Nahar, N., Nasreen, T., Nur-e-Alam, M., and Rokeya, B. 1995. Characterization of the hypoglycemic effects of *Trigonella foenum graecum* seed. *Planta Med.* **61**(4), 358–360.

Anderson, J.W. 1995. Cholesterol-lowering effects of soluble fiber in humans. *In* "Dietary Fiber in Health and Disease" (D. Kritchevsky and C. Bonfield, eds), pp. 126–145. Eagan Press, St. Paul, MN.

Anderson, J.W., Story, L., Sieling, B., Lin Chen, W., Petro, M.S., and Story, J. 1984. Hypocholesterolemic effects of oat-bran or beanintake for hypercholesterolemic men. *Am. J. Clin. Nutr.* **40**, 1146–1155.

Anderson, J.J., Ambrose, W.W., and Garner, S.C. 1995a. Orally dosed genistein from soy and prevention of cancellous bone loss in two ovariectomized rat models. *J. Nutr.* **125**, 799s.

Anderson, J.W., Johnstone, B.M., and Cook-Newel, M.E. 1995b. Meta analysis of the effects of soy protein intake on serum lipids. *N. Engl. J. Med.* **333**, 276–282.

Anonymous, 1966. "Indian Pharmacopoeia", 2nd edition. Ministry of Health and Family Welfare, Govt of India, New Delhi.

Anonymous, 1999. Renewed proof inhibition of cholesterol biosynthesis by dried extract of artichoke leaves. *Forsch. Komplementar Med.* **6**(3), 168–169.

Anonymous, 2000. "Quick Access-Professional Guide to Conditions, Herbs and Supplements", 1st edition. Integrative Medicine Communications, MA, USA.

Anthony, M.S., Clarkson, T.B., Bullock, B.C., and Wagner, J.D. 1997. Soy protein versus soy phytoestrogens in the prevention of diet-induced coronary artery atherosclerosis of male cynomolgus monkeys. *Arterioscler. Thromb. Vasc. Biol.* **17**, 2524–2531.

Anthony, M.S., Clarkson, T.B., Bullock, B.C., and Wagner, J.D. 1997. Soy protein versus soy phytoestrogens in the prevention of diet-induced coronary artery atherosclerosis of male cynomolgus monkeys. *Arterioscler. Thromb. Vasc. Biol.* **17**, 2524–2531.

Anuradha, C. and Ravikumar, P. 1998. Anti-lipid peroxidative activity of seeds of fenugreek (*Trigonella foenum graecum*). *Med. Sci. Res.* **26**, 317–321.

Arnone, A., Merlini, L., and Zanarotti, A. 1979. Constituents of *Silybum marianum*. Structure of isosylbin and stereochemistry of isosylbin. *J. Chem. Soc. (Chem. Commun.)*, 696–697.

Arora, R.B., Taneja, V., Sharma, R.C., and Gupta, S.K. 1972. Anti-inflammatroy studies on a crystalline steroid isolated from *Commiphora mukul*. *Indian J. Med. Res.* **60**, 920.

Augusti, K.T. and Mathew, P.T. 1974. Lipid lowering effect of allicin (di allyl disulphide oxide) on long term feeding in normal rats. *Experientia* **30**, 468–470.

Aviram, M. 1996. Interaction of oxidized low density lipoproteins with macrophages in atherosclerosis and anti atherogenecity of antioxidant. *Eur. J. Clin. Chem. Clin. Biochem.* **43**, 599–608.

Balbaa, S.I., Zaki, A.Y., Abdel-Wahab, S.M., El-Denshary, E.S., and Motazz-Bellah, M. 1973. Preliminary phytochemical and pharmacological investigations of the roots of different varieties of *Cichorium intybus*. *Planta Med.* **24**, 133–144.

Banerjee, S.K. and Maulik, S.K. 2002. Effect of garlic on cardiovascular disorders: a review. *Nutr. J.* **1**, 4 (http://www.nutritionj.com/content/1/1/4).

Barnes, S. 1998. Evolution of the health benefits of soy isoflavones. *Proc. Soc. Exp. Biol. Med.* **217**(3), 386–392.

Basarkar, P.W. and Nath, N. 1983. Hypocholesterolemic and hypolipidemic activity of quercetin, a vitamin P-like compound in rats. *Indian J. Med. Res.* **77**, 122–126.

Beena, A.K., Annie, A., and Leelamma, S. 1995. Haemtological and histological studies after curry leaf [*Murraya koenigii*] and mustered [*Brassica juncea*] feedings in rats. *Indian J. Med. Res.* **102**, 184–186.

Beena, A.K., Annie, A., and Leelamma, S. 1996. *Murraya koenigii* and *Brassica juncea* alteration on lipid profiles in 1,2-dimethyl hydrazine induced colon carcinogenesis. *Plant Food Human Nutr.* **14**, 365–369.

Benda, L., Dittrich, H., Ferenzi, P., Frank, H., and Wewalka, F. 1980. The influence of therapy with silymarin on the survival of patients with liver cirrhosis. *Wien. Klin. Wschr.* **92**(19), 678–683.

Benichou, A., Aserin, A., and Garti, N. 1999. Steroid-saponins from fenugreek seeds: extraction, purification and surface properties. *J. Dispers. Sci. Technol.* **20**, 581–605.

Berthold, H.K. and Sudhop, T. 1998. Garlic preparations for prevention of atherosclerosis. *Curr. Opin. Lipidol.* **9**, 565–569.

Beteridge, D.J. 1989. Diabetes, lipoprotein metabolism and atherosclerosis. *Br. Med. Bull.* **45**, 285–311.

Bierenbaum, M.L., Reichstein, R., and Watkins, T.R. 1993. Reducing atherogenic risk in hyperlipemic humans with FS supplementation: a preliminary report. *J. Am. Coll. Nutr.* **25**, 501–504.

Bisset, N.G. 1994. "Herbal drugs and Phytopharmaceuticals". Medpharm Scientific Publishers, Stuttgart, p 162.

Bone, K. and Bupleurum 1996. A natural steroid effect. *Can. J. Herbalism*, 22–41.

Booyens, J., Englebrecht, P., Le Roux, S., and Louwrens, C.C. 1984. Some effects of the essential fatty acid linoleic and alpha-linolenic acid and their metabolites gamma-linolenic acid, arachidonic acid, eicosapentaenoic acid, docosahexaenoic acid, and of prostaglandins A1 and E1 on the proliferation etc. *Prostaglandins Leukot. Med.* **15**, 15–33.

Bordia, A., Verma, S.K., Vyas, A.K., Khabya, B.L., Rathore, A.S., Bhu, N., and Bedi, H.K. 1977. Effect of essential oil of onion and garlic on experimental atherosclerosis in rabbits. *Atherosclerosis* **26**, 379–386.

Borek, C. 2001. Antioxidant health effect of aged garlic extract. *J. Nutr.* **131**, 1010S–1015S.

Borradaile, N.M., Carroll, K.K., and Kurowska, E.M. 1999. Regulation of Hep G2 cell apolipoprotein B metabolism by the citrus flavanones hesperetin and naringenin. *Lipids* **34**, 591–598.

Brown, J.E. and Rice-Evans, C.A. 1998. Luteolin rich artichoke extract protects low density lipoprotein from oxidation *in vitro*. *Free Radic. Res.* **29**, 247–255.

Brown, L., Rosner, B., Willett, W.W., and Sacks, F.M. 1999. Cholesterol-lowering effects of dietary fiber: a meta-analysis. *Am. J. Clin. Nutr.* **69**, 30–42.

Bruneton, J. 1995. "Pharmacognosy, Phytochemistry, Medicinal Plants". Lavoisier Publishing, Paris.

Bydlowski, S.P., D'Amico, E.A., and Chamone, D.A. 1991. An aqueous extract of guarana (*Paullinia cupana*) decreases platelet thromboxane synthesis. *Braz. J. Med. Biol. Res.* **24**, 421–424.

Byrne, D.J., Neil, H.A., Vallance, D.T., and Winder, A.F. 1999. A pilot study of garlic consumption shows no significant effect on markers of oxidation or sub-fraction composition of low-density lipoprotein including lipoprotein(a) after allowance for non-compliance and the placebo effect. *Clin. Chim. Acta* **285**, 21–33.

Capelli, R., Nicora, M., and Di Perri, T. 1988. Use of extract of *Ruscus aculeatus* in venous disease in the lower limbs. *Drugs Exp. Clin. Res.* **14**, 277–283.

Carlson, L.A. and Bottiger, L.E. 1985. Risk factor for ischemic heart diseases in man and women. *Acta Med. Scand.* **18**, 207–211.

Carlson, L.A., Bottiger, L.E., and Ahfeldt, P.E. 1979. Risk factor for myocardial infection. *Acta Med. Scand.* **206**, 351–360.

Carper, J. 1988. "The Food Pharmacy", pp. 202–223. Bantam Publishing, New York.

Caroll, K.K. 1991. Review of clinical studies on cholesterol lowering response to soy protein. *J. Am. Diet. Assoc.* **91**, 820–827.

Carroll, K.K., Giovannetti, P.M., Huff, M.W., Moase, O., Roberts, D.C., and Wolfe, B.M. 1978. Hypocholesterolemic effect of substituting soybean protein for animal protein in the diet of healthy young women. *Am. J. Clin. Nutr.* **31**(8), 1312–1321.

Chamorro, G., Salazar, M., Favila, L., and Bourges, H. 1996. Pharmacology and toxicology of Spirulina alga. *Rev. Investig. Clin.* **48**, 389–399.

Chan, J.K., Bruce, V.M., and McDonald, B.E. 1991. Dietary-linolenic acid is as effective as oleic acid and linoleic acid in lowering blood cholesterol in normolipidemic men. *Am. J. Clin. Nutr.* **53**, 1230–1240.

Chan, P.T., Fong, W.P., Cheung, Y.L., Huang, Y., Ho, W.K.K., and Chen, Z.Y. 1999. Jasmine green tea epicatechins and hypolipidemic in hamsters (*Mesocricetus auratus*) fed a high fat diet. *J. Nutr.* **129**, 1094–1101.

Chander, R., Singh, C., and Kapoor, N.K. 1988. Effect of chronic ethanol administration on serum lipoproteins lipid profile in rats. *Biochem. Life Sci. Adv.* **7**, 25–27.

Chang, M.L.W. and Johnson, M.A. 1980. Effect of garlic on carbohydrate metabolism and lipid synthesis in rats. *J. Nutr.* **110**, 931–936.

Chaudhuri, R.D. 1998. "Herbal Drug Industry", pp. 562. Eastern Publishers, New Delhi, India.

Chen, X. 1996. Cardiovascular protection by ginsenosides and their nitric oxide releasing action. *Clin. Exp. Pharmacol. Physiol.* **23**(8), 728–732.

Chen, J.D., Wu, Y.Z., Tao, Z.L., Chen, Z.M., and Liu, X.P. 1995. Drink and its lowering effect on blood lipid levels in humans and rats. *World Rev. Nutr. Diet* **77**, 147–154.

Chevallier, A. 1996. "Encyclopedia of Medicinal Plants", pp. 141–273. DK Publishing, New York.
Chi, M.S. 1982. Effect of garlic products on lipid metabolism in cholesterol-fed rats. *Proc. Soc. Exp. Biol. Med.* **171**, 174–178.
Chi, M.S., Koh, E.T., and Stewart, T.J. 1982. Effect of garlic on lipid metabolism in rats fed cholesterol or lard. *J. Nutr.* **112**, 241–248.
Chisaka, T., Matsuda, H., Kubomura, Y., Mochizuki, M., Yamahara, J., and Fujimura, H. 1988. The effect of crude drugs on experimental hypercholesterolemia; mode of action of (−) epigallocatechin gallatein tea leaves. *Chem. Pharm. Bull.* **36**, 227–233.
Chitra, V. and Leelamma, S. 2000. *Coraindrum sativum*—effect on lipid metabolism in 1,2-dimethyl hydrazine induced colon cancer. *J. Ethnopharmacol.* **71**(3), 457–463.
Choi, J.S., Yokozawa, T., and Oura, H. 1991. Antihyperlipidemic effect of flavonoids from *Prunus davidiana*. *J. Nat. Prod.* **54**, 218–224.
Choi, J.S., Yokozawa, T., and Oura, H. 1991. Improvement of hyperglycemia and hyperlipemia in streptozotocin-diabeticrats by a methanolic extract of *Prunus davidiana* stems and its main component, prunin. *Planta Med.* **57**, 208–211.
Chopra, R.N., Chopra, S.L., and Chopra, I.C. 1986. Glossary of Indian Medicinal Plants. CSIR, New Delhi.
Cicero, A.F.G. and Gaddi, A. 2001. Rice bran oil and gamma oricinol in the treatment of hyperlipoprotenimeas and other conditions. *Phytother. Res.* **15**(4), 277–287.
Claus, R. and Hoppen, H.O. 1979. The boar pheromone steroid identified in vegetables. *Experentia* **35**(12), 1674.
Cohen, B.I., Mosbach, E.H., Matoba, N., Suh, S.O., and McSherry, C.K. 1990. The effect of alfalfa-corn diets on cholesterol metabolism and gallstones in prairie dogs. *Lipids* **25**(3), 143–148.
Collins, B.M., Mclachlan, J.A., and Arnold, S. 1997. The estrogenic and antiestrogenic activities of phytochemicals with the human receptor expressed in yeast. *Steroids* **62**, 365–372.
Compassi, S., Werder, M., Weber, F.E., Boffelli, D., Hauser, H., and Schulthess, G. 1997. Comparison of cholesterol and sitosterol uptake in different brush border membrane models. *Biochemistry* **36**, 6643–6652.
Cook, N.C. and Samman, S. 1996. Flavonoids—chemistry, metabolism, cardioprotective effects, and dietary sources. *J. Nutr. Biochem.* **7**, 66–76.
Corvazier, E. and Maclouf, J. 1985. Interference of some flavonoids and non-steroidal anti-inflammatory drugs with oxidative metabolism of arachidonic acid by human platelets and neutrophils. *Biochem. Biophys. Acta* **835**, 315–321.
Cunnane, S.C., Hamadeh, M.J., Leide, A.C., Thompson, L.U., Wolever, T.M.S., and Jenkins, D.J.A. 1995. Nutritional attributes of traditional FS in healthy young adults. *Am. J. Clin. Nutr.* **61**, 62–68.
da Silva, E.L., Tsushida, T., and Terao, J. 1998. Inhibition of mammalian 15-lipoxygenase-dependent lipid peroxidationin low-density lipoprotein by quercetin and quercetin monoglucosides. *Arch. Biochem. Biophys.* **34**, 313–320.
de Whalley, C.V., Rankin, S.M., Hoult, J.R.S., Jessup, W., and Leake, D.S. 1990. Flavonoids inhibit the oxidative modification of low density lipoproteins by macrophages. *Biochem. Pharmacol.* **39**, 1743–1750.
Der Marderosian, A. and Beutler, J.A. 2002. "The Review of Natural Products". Facts & Comparisons, St Louis, USA.
DeSmet, P.A.G.M., Keller, K., Hänsel, R., and Chandler, R.F. 1997. "Adverse Effects of Herbal Drugs". Springer-Verlag, Berlin, Germany.
Dimo, T., Azay, J., Tan, P.V., Pellecuer, J., Cros, G., Bopelet, M., and Serrano, J.J. 2001. Effcets of the aqueous and methlene chloride extrats of Bidens pilosa leaf onilfructose-hypertensive rats. *J. Ethnopharmacol.* **76**, 215–221.
Divi, R.L. and Doerge, D.R. 1996. Inhibition of thyroid peroxidase by dietary flavonoids. *Chem. Res. Toxicol.* **9**, 16–23.

Dreher, M.L. 1987. "Handbook of Dietary Fibre—An Applied Approach", pp. 199. Dekker, NY.
Dreosti, I.E. 1996. Bioactive ingredients: antioxidants and polyphenols in tea. *Nutr. Rev.* **54**, S51–S58.
Duke, J. 1989. "Hand Book of Medicinal Herbs", pp. 417–418. CRC Press Inc, Boca Raton, FL.
Dwarakanath, C. and Satyavati, G.V. 1970. Research in some of the concept of Ayurvedha and application of modern chemistry and experimental pharmacology therefore. *Ayurveda Pradeepika* **1**, 69.
Edralin, A.L., Robert, D.W., Lisa, J.H., Dania, A.K., Shanil, J., Bruce, P.D., Barbara, J.S., and Bahram, H.A. 2002. Flaxseed improves lipid profile without altering biomarkers of bone metabolism in postmenopausal women. *Clin. Endocrinol. Metab.* **87**(4), 1527–1532.
Ensminger, A.H., Ensminger, M.E., Konlande, J.E., and Robson, J.R.K. 1994. "Foods and Nutrition Encyclopedia", 2nd edition. Vol 1. CRC press, Boca Raton, FL, pp. 1093–1099.
Epstein, F.H. 1992. Age and the cardiovascular system. *N. Engl. J. Med.* **327**, 173.
Farquhar, J.W., Smith, R.E., and Dempsey, M.E. 1956. The effect of beta sitosterol on the serum lipids of young men with arterosclerotic heart disease. *Circulation* **14**, 77–82.
Feher, J., Deak, G., Muezes, G., Lang, I., Niderland, V., Nekam, K., and Karteszi, M. 1989. Hepatoprotective activity of silymarin ligalon therapy in patients with chronic alcoholic liver diseases. *Orv. Hetil.* **130**(51), 2723–2727.
Filipek, J. 1992. Effect of *Alchemilla xanthochlora* water extract on lipid peroxidation and superoxide anion scanvenging activity. *Pharmazie* **48**(6), 335.
Fisel, J. 1965. New flavonoids from Crataegus 1. Isolation of an acetylated vitexin-4-rhamnoside from *Crataegus monogyna* L. *Arzneim-Forsch.* **15**(12), 1417–1421.
Fisel, J. 1966. New flavonoids from Crataegus 2. Isolation of a mixture from rutin and quercetin 3-rhamnoglycoside from *Crataegus monogyna* L. *Arzneim-Forsch.* **16**(1), 80–82.
Fleming, T. 1998. "PDR for Herbal Medicines", pp. 793–794. Medical economics company, Montvale, NJ.
Forsythe, W.A., Miller, E.R., Hill, G.M., Romsos, D.R., and Simpson, R.C. 1980. Effects of dietary protein and fat sources on plasma cholesterol parameters, LCAT activity and amino acid levels and on tissue lipid content of growing pigs. *J. Nutr.* **110**(12), 2467–2479.
Fowden, L., Pratt, H., and Smith, A. 1973. 4-Hydroxyisoleucine from seed of *Trigonella foenum-gracum*. *Phytochemistry* **12**, 1707–1711.
Fremont, L., Gozzelino, M.T., Franchi, M.P., and Linard, A. 1998. Dietary flavonoids reduce lipid peroxidation in rats fed polyunsaturated or monounsaturated fat diets. *J. Nutr.* **128**, 1495–1502.
Fuhrman, B., Lavy, A., and Aviram, M. 1995. Consumption of red wine with meals reduces the susceptibility of humanplasma and LDL to lipid peroxidation. *Am. J. Clin. Nutr.* **61**, 549–554.
Fuhrman, B., Elis, A., and Aviram, M. 1997. Hypocholesterolemic effect of lycopene and β-carotene is related to suppression of cholesterol synthesis and augmentation of LDL receptor activity in macrophages. *Biochem. Biophys. Res. Commun.* **233**, 658–662.
Fukuyo, M., Hara, Y., and Muramatsu, K. 1986. Effect of tea leaf catechin, (−)-epigallocatechin gallate, on plasma cholesterol level in rats. *Nippon Eiyo. Shokuryo. Gakkaishi* **39**, 495–500 (in Japanese).
Gardner, C.D., Chatterjee, L.M., and Carlson, J.J. 2001. The effect of a garlic preparation on plasma lipid levels in moderately hypercholesterolemic adults. *Atherosclerosis* **154**, 213–220.
Garg, M.L., Wierzbicki, A.A., Thomson, A.B.R., and Clandinin, M.T. 1989. Dietary saturated fat level alters the competition between linolenic and linoleic acid. *Lipids* **24**, 334–339.
Gauthamna, K., Maulik, M., Kumari, R., Manchanda, S.C., Dinda, A.K., and Maulik, S.K. 2001. Effect on chronic treatment with bark of *Terminalia arjuna*: a study of the isolated ischemic-reperfused rat heart. *J. Ethnopharmacol.* **75**, 197–201.
Gebhardt, R. 1998. Inhibition of cholesterol biosynthesis in primary cultured rat hepatocytes by artichoke (*Cynara scolymus* L.) extracts. *J. Pharmacol. Exp. Ther.* **286**(3), 1122–1128.

Gebhardt, R. 2001. Anticholestatic activity of flavonoids from artichoke (*Cynara scolymus* L.) and of their metabolites. *Med. Sci. Monit.* **7**(1), 316–320.

Gebhardt, R. and Beck, H. 1996. Differential inhibitory effects of garlic-derived organosulfur compounds on cholesterol biosynthesis in primary rat hepatocyte culture. *Lipids* **31**, 1269–1276.

Gehm, B.D., McAndrews, J.M., Chien, P.Y., and Jameson, J.L. 1997. Resveratrol, a polyphenolic compound found in grapes and wine, is an agonist for estrogen receptor. *Proc. Natl Acad. Sci.* **94**, 14138–14143.

Ghasi, S., Nwobodo, E., and Ofili, J.O. 2000. Hypocholesterolemic effects of crude extract of leaf of *Moringa oliefera* in high fat feed wistar rats. *J. Ethnopharmacol.* **69**(1), 21–25.

Ginsberg, H.N. 1990. Lipoprotein physiology and its relationship to atherogenesis. *Endocrinol. Metab. Clin. N. Am.* **19**, 211–228.

Glore, S.R., Van Treeck, D.V., Knehaus, A.W., and Guild, M. 1994. Soluble fiber and serum lipids: a literature review. *J. Am. Diet. Assoc.* **94**, 425–436.

Greaves, K.A., Willson, M.D., Rudel, L.L., Williams, J.K., and Wagner, J.D. 2000. Consumption of soy protein reduces cholesterol absorption compared to casein protein alone or supplemented with an isoflavone extract or conjugated equine estrogen in ovariectmized cynomolgus monkeys. *J. Nutr.* **130**, 820–826.

Gupta, R.K., Thain, D.C., and Thakur, R.S. 1986. Two furostanol saponins from *Trigonella foenum-graecum*. *Phytochemistry* **25**, 2205–2207.

Gylling, H. and Miettinen, T.A. 1994. Serum cholesterol and cholesterol and lipoprotein metabolism in hypercholesterolemic NIDDM patients before and during sitostanol ester-margarine treatment. *Diabetologia* **37**, 773–780.

Gylling, H. and Miettinen, T.A. 1996. Effects of inhibiting cholesterol absorption and synthesis on cholesterol and lipoprotein metabolism in hypercholesterolemic non-insulin-dependent diabetic men. *J. Lipid Res.* **37**, 1776–1785.

Hain, R., Biescler, B., Kindl, H., Schroder, G., and Stodker, R. 1990. Expression of a stilbene synthese gene in *Nicotiana tabacum* results in synthesis of the phytolaxin resveratrol. *Plant Mol. Biol.* **15**, 325–335.

Hallikainen, M.A. and Uusitupa, M.I.J. 1999. Effect of 2 low-fat stanol ester-containing margarines on serum cholesterol concentrations as part of a low-fat diet in hypercholesterolemic subjects. *Am. J. Clin. Nutr.* **69**, 403–410.

Hammouda, F. 1993. Flavonoids of *Cynara scolymus* cultivated in Egypt. *Plant Foods Hum. Nutr.* **44**, 163–169.

Hardman, R. and Fazli, F.R. 1972. Labeled steroidal sapogenins and hydrocarbons from *Trigonella foenum graecum* by acetate mevalonate and cholesterol feeds to seeds. *Planta Med.* **21**, 188–195.

Havel, R.J. and Kane, J.P. 1995. Introduction: structure and metabolism of plasma lipoproteins. *In* "The Metabolic and Molecular Bases of Inherited Disease", pp. 1841–1851. McGraw-Hill, New York.

Hayek, T., Fuhrman, B., and Vaya, J. 1997. Reduced progression of atherosclerosis in apolipoprotein E-deficientmice following consumption of red wine, or its polyphenols uercetin or catechin, is associated with reduced susceptibility of LDL to oxidation and aggregation. *Arterioscler. Thromb. Vasc. Biol.* **17**, 452–456.

He, D. 1990. Effect of the prevention and treatment of atherosclerosis of a mixture of hawthorn and motherwort. *Chung His Chieh Ho Tsa Chih* **10**, 361.

Heinecke, J.W. 1997. Mechanisms of oxidative damage of low density lipoprotein in human atherosclerosis. *Curr. Opin. Lipidol.* **8**, 268–274.

Heinemann, T., Leiss, O., and von Bergmann, K. 1986. Effect of low-dose sitostanol on serum cholesterol in patients with hypercholesterolemia. *Atherosclerosis* **61**, 219–223.

Heinemann, T., Kullak-Ublick, G.A., Pietruck, B., and von Bergmann, K. 1991. Mechanisms of action of plant sterols on inhibition of cholesterol absorption: comparison of sitosterol and sitostanol. *Eur. J. Clin. Pharmacol.* **40**, S59–S63.

Heinemann, T., Axtmann, G., and von Bergmann, K. 1993. Comparison of intestinal absorption of cholesterol with different plant sterols in man. *Eur. J. Clin. Investig.* **23**, 827–831.

Hemingway, S.R. and Phillipson, J.D. 1974. Proceedings: alkaloids from S. American species of *Uncaria* (*Rubiaceae*). *J. Pharm. Pharmacol.* (Suppl), 113.

Henry, N. and Ginsberg, M.D. 2002. New perspectives on atherogenesis: role of abnormal triglyceride-rich lipoprotein metabolism. *Circulation* **106**, 2137–2140.

Herber, D. 2001. Herbs and atherosclerosis. *Curr. Atheroscler. Rep.* **3**, 93–96.

Hocking, G. 1997. "A Dictionary of Natural Products", pp. 625. Plexus publishing, Inc., Medford, NJ.

Horrobin, D.F. and Manku, M.S. 1983. How do polyunsaturated fatty acids lower plasma cholesterol levels. *Lipids* **18**, 558–562.

Huang, Z., Fasco, M.J., and Kaminsky, L.S. 1997. Inhibition of estrone sulfatase in human liver microsomes by quercetin and other flavonoids. *J. Steroid Biochem. Mol. Biol.* **63**, 9–15.

Husain, S.R., Cillard, J., and Cillard, P. 1987. Hydroxyl radical scavenging activity of flavonoids. *Phytochemistry* **26**, 2489–2491.

Huttunen, J.K., Manninen, V., and Manttary, M. 1991. The Helsinki heart study, central findings and clinical implications. *Ann. Med.* **53**, 155–159.

Hwang, S.B., Chang, M.N., Garcia, M.L., Han, Q.Q., Huang, L., King, V.F., Kaczorowski, G.J., and Winquist, R.J. 1987. L-652,469—a dual receptor antagonist of platelet activating factor and dihydropyridines from *Tussilago farfara* L. *Eur. J. Pharmacol.* **141**, 269–281.

Ikeda, I., Imasato, Y., Sasaki, E., Nakayama, M., Nagao, H., Takeo, T., Yamabe, F., and Sugano, M. 1992. Tea catechins decrease micellar solubility and intestinal absorption of cholesterol in rats. *Biochim. Biophys. Acta* **1127**, 141–146.

Isaacsohn, J.L., Moser, M., Stein, E.A., Dudley, K., Davey, J.A., Liskov, E., and Black, H.R. 1998. Garlic powder and plasma lipids and lipoproteins: a multicenter, randomized, placebo-controlled trial. *Arch. Intern. Med.* **158**, 1189–1194.

Ishikawa, T., Suzukawa, M., and Ito, T. 1997. Effect of tea flavonoid supplementation on the susceptibility of low-density lipoprotein to oxidative modification. *Am. J. Clin. Nutr.* **66**, 261–266.

Iwakami, S., Wu, J.B., Ebizuka, Y., and Sankawa, U. 1992. Platelet activating factor (PAF) antagonists contained in medicinal plants: lignans and sesquiterpenes. *Chem. Pharm. Bull. (Tokyo)* **40**(5), 1196–1198.

Jacob, A., Pandey, M., Kapoor, S., and Saroja, R. 1988. Effect of the Indian gooseberry (amla) on serum cholesterol levels in men aged 35–55 years. *Eur. J. Clin. Nutr.* **42**, 939–944.

Jahromi, M.A.F., Ray, A.B., and Chansouria, J.P.N. 1993. Antihyperlipidemic effect of flavonoids from *Pterocarpus marsupium*. *J. Nat. Prod.* **56**, 989–994.

Jain, R.C. 1975. Onion and garlic an experimental cholesterol atherosclerosis in rabbits. *Artery* **1**, 115–125.

Jain, R.C. 1977. Effect of garlic on serum lipids, coagulability and fibrinolytic activity of blood. *Am. J. Clin. Nutr.* **30**, 1380–1381.

Jenkins, D.J., Kendall, C.W., Vidgen, E., Agarwal, S., Rao, A.V., Rosenberg, R.S., Diamandis, E.P., Novokmet, R., Mehling, C.C., Perera, T., Griffin, L.C., and Cunnane, S.C. 1999. Health aspects of partially defatted flaxseed, including effects on serum lipids, oxidative measures, and *ex vivo* androgen and progestin activity: a controlled crossover trial. *Am. J. Clin. Nutr.* **69**, 395–402.

Johnson, J.E. 2002. The role of carotenoids in human health. *Nutr. Clin. Care* **5**, 56–65.

Jones, K. 1994. The herb report: uña de gato, life-giving vine of Peru. *Am. Herb Assoc.* **10**(3), 4.

Jones, P.J., Howell, T., MacDougall, D.E., Feng, J.Y., and Parsons, W. 1998. Short-term administration of tall oil phytosterols improves plasma lipid profiles in subjects with different cholesterol levels. *Metabolism* **47**, 751–756.

Kabir, Y., Yamaguchi, M., and Kimura, S. 1987. Effect of shiitake and maitake mushrooms on blood pressure and plasma lipids of spontaneously hypertensive rats. *J. Nutr. Sci. Vitaminol.* **33**(5), 341–346.

Kamanna, V.S. and Chandrasekhara, N. 1982. Effect of garlic on serum lipoproteins cholesterol levels in albino rats rendered hypercholesteremic by feeding cholesterol. *Lipids* **17**, 483–488.

Kamanna, V.S. and Chandrasekhara, N. 1984. Hypocholesteromic activity of different fractions of garlic. *Indian J. Med. Res.* **79**, 580–583.

Katcher, Y. and Koda, K. 1987. "Applied Therapeutics", 3rd Ed, pp. 651–652. Applied therapeutic Inc., Spokene, WA.

Kerry, N. and Abbey, M. 1998. The isoflavone genistein inhibits copper and peroxyl radical mediated low density lipoprotein oxidation *in vitro*. *Atherosclerosis* **140**, 341–347.

Khanna, A.K., Chander, R., Sing, C., Srivastav, A.K., and Kapoor, N.K. 1992. Hypolipidemic activity of *Achyranthus aspera* in normal and triton induced hyperlipidemic rats. *Indian J. Exp. Biol.* **30**, 128–130.

Khanna, A.K., Rizvi, F., and Chander, R. 2002. Lipid lowering activity of *Phylanthus niruri* in hyperlipemic rats. *J. Ethnopharmacol.* **82**, 19–22.

Kiho, T., Yamane, A., Hui, J., Usi, S., and Ukai, S. 1996. Polysaccharides in fungi XXXVI. Hypoglycaemic activity of a polysaccharide (CS-F30) from the cultural mycelium of *Cordyceps sinensis* and its effect on glucose metabolism in mouse liver. *Biol. Pharm. Bull.* **19**(2), 294–296.

Kim, M.H. and Shin, H.K. 1996. The water-soluble extract of chicory reduces glucose uptake from the perfused jejunum in rat. *J. Nutr.*, 2236–2242.

Kim, O.W., Yokozawa, T., Hattori, M., Kadota, S., and Namba, T. 2000. Inhibitory effects of an aqueous extract of *Apocynum veretum* leaves and its constituents on Cu 2 + induced oxidative modification on low density lipoprotein. *Phytother. Res.* **14**(7), 501–504.

Kitts, D.D., Yuan, Y.V., Wijewickreme, A.N., and Thompson, L.U. 1999. Antioxidant activity of the flaxseed lignan secoisolariciresinol diglycoside and its mammalian lignan metabolites enterodiol and enterolactone. *Mol. Cell. Biochem.* **202**, 91–100.

Kiuchi, F., Iwakami, S., Shibuya, M., Hanaoka, F., and Sankawa, U. 1992. Inhibition of prostaglandin and leukotriene biosynthesis by gingerols and diarylheptanoids. *Chem. Pharm. Bull. (Tokyo)* **40**(2), 387–391.

Kokate, C.K., Purohit, A.P., and Gokhale, S.B. 1998. "Pharmacognosy", pp. 540. Nirali Prakashan, Pune, India.

Kono, S., Shinchi, K., Yakabayashi, K., Honjo, S., Todoroki, I., Sakurai, Y., Imanishi, K., Nishikawa, H., Ogawa, S., and Katsurada, M. 1996. Relation of green tea consumption to serum lipids and lipoproteins in Japanese men. *J. Epidemiol.* **6**, 128–133.

Kritchevsky, D. 1991. The effect of dietary garlic on the development of cardiovascular disease. *Trends Food Sci. Technol.* **2**, 141–144.

Kubo, K. and Nanba, H. 1996. The effect of maitake on liver and serum lipids. *Altern. Ther. Health Med.* **2**(5), 62–66.

Kubo, K. and Nanba, H. 1997. Anti-hyperliposis effect of maitake fruit body (*Grifola frondosa*). *Biol. Pharm. Bull.* **20**(7), 781–785.

Kuhnau, J. 1976. The flavonoids: a class of semi-essential food components and their role in human nutrition. *World Rev. Nutr. Diet.* **24**, 117–191.

Landolfi, R., Mower, R.L., and Steiner, M. 1984. Modification of platelet function and arachidonic acid metabolism by bioflavonoids—structure–activity relations. *Biochem. Pharmacol.* **33**, 1525–1530.

Lata, S., Saxena, K.K., Bhasin, V., Saxena, R.S., Kumar, A., and Srivastava, V.K. 1991. Beneficial effects of *Allium sativu*, *Allium cepa* and *Commiphora mukul* on experimental hyperlipidemia and atherosclerosis—a comparative evaluation. *J. Postgrad. Med.* **37**(3), 132–135.

LauBenjamin, H.S. 2001. Suppression of LDL oxidation by garlic. *J. Nutr.* **131**(3S), 958S–988S.

Laughton, M.J., Evans, P.J., Moroney, M.A., Hoult, J.R.S., and Halliwell, B. 1991. Inhibition of mammalian 5-lipoxygenaseand cyclo-oxygenase by flavonoids and phenolic dietary

additives—relationship to antioxidant activity and to iron ion-reducing ability. *Biochem. Pharmacol.* **42**, 1673–1681.

Lawson, L.D. 1998. Garlic: a review of its medicinal effects and indicated active compounds. "Phytomedicines of Europe. Chemistry and Biological Activity", Series 691 (L.D. Lawson and R. Bauer, eds), pp. 176–209. American Chemical Society, Washington, DC.

Lees, A.M., Mok, H.Y.I., Lees, R.S., McCluskey, M.A., and Grundy, S.M. 1977. Plant sterols as cholesterol-lowering agents: clinical trials in patients with hypercholesterolemia and studies of sterol balance. *Atherosclerosis* **28**, 325–338.

Leung, A.Y. 1980. "Encyclopedia of Common Natural Ingredients". John Wiley, New York.

Lewak, S. 1969. Flavonoids of hawthorn. *Postepy Biochem.* **15**(3), 425–434.

Lewin, G. and Popov, I. 1994. Antioxidant effects of aqueous garlic extract. 2nd communication: inhibition of the Cu(2 +)-initiated oxidation of low density lipoproteins. *Arzneim-For sch.* **44**, 604–607.

Lichtenstein, A.H., Ausman, L.M., Carrasco, W., Gualtieri, L.J., Jenner, J.L., Ordovas, J.M., Nicolosi, R.J., Goldin, B.R., and Schaefer, E.J. 1994. Rice bran oil consumption and plasma lipid levels in moderately hypercholesterolemic humans. *Arterioscler. Thromb.* **14**, 549–556.

Ling, W.H. and Jones, P.J. 1995. Dietary phytosterols: a review of metabolism, benefits and side effects. *Life Sci.* **57**, 195–206.

Lininger, S. 1998. "The Natural Pharmacy". Prima Health Publishing, Rocklin, CA.

Lipid Research Clinic Program. 1984. The lipid research clinics coronary primary prevention trial results. *J. Am. Med. Assoc.* **251**, 351–364.

Lorenz, D., Mennicke, W.H., and Behrendt, W. 1982. Untersuchungen zur Elimination von Silymarin bei cholecystektomierten patienten. *Planta Med.* **45**, 216–233.

Low, T. and Rodd, T. 1994. "Magic and Medicine of Plants Surry Hills", NSW Reader's Digest, p. 282.

Lucas, E.A., Wild, R.D., Hammond, L.J., Khalil, D.A., Juma, S., Daggy, B.P., Stoecker, B.J., and Arjmandi, B.H. 2002. Flaxseed improves lipid profile without altering biomarkers of bone metabolism in postmenopausal women. *J. Clin. Endocrinol. Metab.* **87**, 1527–1532.

Luley, C., Lehmann-Leo, W., Moller, B., Martin, T., and Schwartzkopff, W. 1986. Lack of efficacy of dried garlic in patients with hyperlipoproteinemia. *Arzneim-Forsch. Drug Res.* **36**, 766–768.

Lupton, J.R., Robinson, M.C., and Morin, J.L. 1994. Cholesterol-lowering effect of barley bran flour and oil. *J. Am. Diet. Assoc.* **94**(1), 65–70.

Lutomski, J. 1984. Klinische Untersuchungen Zur therapeutischen wirksamkeit von llya Rogiff knoblanchpillen mit Rutin. *Z. Phytother.* **5**, 938–942.

Ma, Y.S. and Chen, S.Y. 1989. Observation on the anti aging, antihypertensive, and antilipemic effect of *Apocynum venetum* leaf extract. *Chin J. Mod. Dev. Trad. Med.* **9**, 335–337.

Madhavan, M., Chandra, K., and Reddy, D.J. 1971. Lipodiac activity of Bengal gram. *Indian J. Med. Sci.* **25**, 771.

Malhotra, S.C. and Ahuja, M.M.S. 1974. Proc. Fifth Asia Oceania Congr. Endocrinol. (G.K. Rastogi, ed.), Vol. 2, p. 504.

Malinow, M.R., McLaughlin, P., Papworth, L., Stafford, C., Kohler, G.O., Livingston, A.L., and Cheeke, P.R. 1977. Effect of alfalfa saponins on intestinal cholesterol absorption in rats. *Am. J. Clin. Nutr.* **30**(12), 2061–2067.

Malinow, M.R., McLaughlin, P., Naito, H.K., Lewis, L.A., and McNulty, W.P. 1978. Effect of alfalfa meal on shrinkage (regression) of atherosclerotic plaques during cholesterol feeding in monkeys. *Atherosclerosis* **30**(1), 27–43.

Malinow, M.R., Connor, W.E., McLaughlin, P., Stafford, C., Lin, D.S., Livingston, A.L., Kohler, G.O., and McNulty, W.P. 1981. Cholesterol and bile acid balance in *Macaca fascicularis*. Effects of alfalfa saponins. *J. Clin. Investig.* **67**(1), 156–162.

Manach, C., Regerat, F., and Texier, O. 1996. Bioavailability, metabolism and physiological impact of 4-oxo-flavonoids. *Nutr. Res.* **16**, 517–544.

Mand, J.K., Gupta, P.P., Soni, G.L., and Singh, R. 1985. Effect of garlic on experimental atherosclerosis in rabbits. *Indian Heart J.* **37**, 183–188.

Marderosian, A.D. and Lawerence, L.M. 1998. "Natural Product Medicine", pp. 293–295. George F. Stickley Co., Philadelphia, USA.

Marsch, D., Knowles, P.F., and Rattle, H.W. 1976. "Magnetic Resonance of Bimolecules", pp. 237. Wiley, New York.

Mary, J.M., Richard, A.V., and Pamela, C.C. 2000. "Lippincott's Illustrated Reviews: Pharmacology", 2nd edition. Lippincott Williams & Wilkins, Philadelphia.

Mathew, B.C. and Daniel, R.S. 1996. Hypolipidemic effect of garlic protein substituted for caseinin diet of rats compared to those of garlic oil. *Indian J. Exp. Biol.* **34**, 337–340.

Mathur, K.S., Sharma, R.D., and Ram, N. 1968a. Chromatographic aminoacid pattern of various protein fractions of Bengal gram. *Indian J. Med. Res.* **56**, 563.

Mathur, K.S., Sharma, N.S., and Sharma, R.D. 1968b. A comparative study of hypocholesterlemic activity of liid extrct of Bengal gram with safflower oil, mustard oil and ground-nut oil in albino rats. *Indian J. Med. Res.* **56**, 125.

Mathur, K.S., Khan, M.A., and Sharma, R.D. 1968c. Hypochlesterolemic effect of Bengal gram—a long term study in man. *Br. Med. J.* **1**, 30.

Mathur, R., Sharma, A., Dixit, V.P., and Varma, M. 1996. Hypolipidaemic effect of fruit juice of *Emblica officinalis* in cholesterol-fed rabbits. *J. Ethnopharmacol.* **50**, 61–68.

Matsuda, H., Chisaka, T., Kubomura, Y., Yamahara, J., Sawada, T., Fujimura, H., and Kimura, H. 1986. Effects of crude drugs on experimental hypercholesterolemia. I. Tea and its active principles. *J. Ethnopharmacol.* **17**, 213–224.

Matsumoto, M., Fukuyo, M., and Hara, Y. 1998. Effect of green tea catechins on plasma cholesterol level in cholesterol-fed rats. *J. Nutr. Sci. Vitaminol.* **44**, 337–342.

Mattson, F.H., Grundy, S.M., and Crouse, J.R. 1982. Optimizing the effect of plant sterols on cholesterol absorption in man. *Am. J. Nutr.* **35**, 697–700.

McCrindle, B.W., Helden, E., and Conner, W.T. 1998. Garlic extract therapy in children with hypercholesterolemia. *Arch. Pediatr. Adolesc. Med.* **152**, 1089–1094.

Miek, C., Jong, M.H., Hofker, L., and Havekes, M. 1999. Role of ApoCs in lipoprotein metabolism: functional differences between ApoC1, ApoC2, and ApoC3. *Arterioscler. Thromb. Vasc. Biol.* **19**, 472–484.

Miettinen, T.A. 1987. Dietary fibres and lipids. *Am. J. Clin. Nutr.* **45**, 1237–1242.

Miettinen, T.A., Puska, P., Gylling, H., Vanhanen, H., and Vartiainen, E. 1995. Reduction of serum cholesterol with sitostanol-ester margarine in a mildly hypercholesterolemic population. *N. Engl. J. Med.* **333**, 1308–1312.

Mitcheva, M. 1993. Biochemical and morphological studies on the effects of anthocyans and vitamin E on carbontetrachloride induced liver injury. *Cell Microbiol.* **39**, 443.

Mokady, S. 1992. Nutritional, toxicological and therapeutic aspects. *In* "Dunaliella: Physiology, Biochemistry and Biotechnology" (M. Avron and A. Ben-Amotz, eds), pp. 217. CRC Press, Boca Raton, FL.

Morel, I., Lescoat, G., and Cogrel, P. 1993. Antioxidant and iron-chelating activities of the flavonoids catechin, quercetin and diosmetin on iron-loaded rat hepatocyle cultures. *Biochem. Pharmacol.* **45**, 13–19.

Moyers, S. 1996. "Garlic in Health, History and World Cuisine", pp. 1–36. Suncoast Press, St. Petersburg, FL.

Mukherjee, P.K. 2001. Evaluation of Indian traditional medicine. *Drug Inf. J.* **35**(2), 623–631.

Mukherjee, P.K. 2002. "Quality Control of Herbal Drugs", 1st Ed, pp. 543–545. Business Horizons, New Delhi, India.

Mukherjee, P.K., Bhakta, T., Saha, B.P., Pal, S., Pal, M., and Das, A.K. 1995. Studies on anticholesterolmic activity of a fraction of *Azadirachta indica* leaf extract in rats. *Ancient Sci. Life* **14**, 150–153.

Munday, J.S., Jamesm, K.A., Fray, L.M., Kirkwood, S.W., and Thompson, K.G. 1999. Daily supplementation with aged garlic extract, but not raw garlic, protects low density lipoprotein against *in vitro* oxidation. *Atherosclerosis* **143**, 399–404.

Muramatsu, K., Fukuyo, M., and Hara, Y. 1986. Effect of green tea catechins on plasma cholesterol level in cholesterol-fed rats. *J. Nutr. Sci. Vitaminol.* **32**, 613–622.

Nadkarni, K.M. and Nadkarni, A.K. 1976. "Indian Materia Medica", pp. 414. Popular Prakashan, Bombay.

Nagy, E., Papay, V., Litkei, G., and Dinya, Z. 1985. Investigation of the chemical constituents, particularly the flavonoid components, of propolis and *populi gemma* by the GC/MS method. *In* "Flavonoids and Bioflavonoids" (L. Farkas, M. Gabor, and F. Kallay, eds), pp. 223–232. Elsevier, New York.

Navab, M., Berliner, J.A., Watson, A.D., Hama, S.Y., Territo, M.C., Lusis, A.J., Shih, D.M., Van Lenten, B.J., Frank, J.S., Demer, L.L., and Edwards, P.A. 1996. The Yin and Yang of oxidation in the development of the fatty streak. *Arterioscler. Thromb. Vasc. Biol.* **16**, 831–842.

Neil, H.A., Silagy, C.A., Lancaster, T., Hodgeman, J., Vos, K., Moore, J.W., Jones, L., Cahill, J., and Fowler, G.H. 1996. Garlic powder in the treatment of moderate hyperlipidaemia: a controlled trial and meta-analysis. *J.R. Coll. Phys. Lond.* **30**, 329–334.

Nityanand, S., Kapoor, N.K., and Dev, S. 1973. Cholesterol lowering activity of the various fractions of *Commiphora mukul* (Guggul). *Indian J. Pharmacol.* **5**, 259.

Orekhov, A.N. and Grunwald, J. 1997. Effects of garlic on atherosclerosis. *Nutrition* **13**, 656–663.

Pachauri, S.P. and Mukherjee, S.K. 1970. Effect of *Curcuma longa* (Haridra) and *Curcuma amcada* (Amaragandhi) on the cholesterol level in experimental hypercholesterolemia of rabbits. *J. Res. Indian. Med.* **5**, 27.

Pal, S.K., Mukherjee, P.K., Saha, K., Pal, M., and Saha, B.P. 1996. Studies on some psycho-pharmacological actions of *Moringa oleifera* Lam (*Moringaceae*) leaf extract. *Phytotherapy Research* **10**, 402–405.

Panda, H. 1986. "Herbs Cultivation and Medicinal Uses", pp. 269. Publication Division, National Institute of Industrial Research, Delhi, India.

Peluso, M.R. and Schneeman, B.O. 1994. A food-grade silicon dioxide is hypocholesterolemic in the diet of cholesterol-fed rats. *J. Nutr.* **124**, 853–860.

Petrovich, D.P., Waring, A.J., and Farbes, J.L. 1984. Liver ischemia increases the molecular order of microsomal membrane by inceasing the cholesterol to phospholipids ratio. *J. Biol. Chem.* **259**, 13217–13220.

Potter, S.M. 1995. Overview of possible mechanism for hypocholesterolemic effect of soy protein. *J. Nutr.* **125**, 606s–611s.

Potter, S.M., Bakhit, R.M., Essex-Sorue, D., Wein-Gartner, K.E., Champman, K.M., Nelson, R.A., Prabhudesai, M., Savage, W.B., Nelson, A.I., Winter, L.W., and Erdmann, J.W. 1993. Depression of plasma cholesterol in men by consumption of backed products containing soy protein. *Am. J. Clin. Nutr.* **58**, 501–506.

Prasad, K. 1997. Hydroxyl radical-scavenging property of secoisolariciresinol diglucoside (SDG) isolated from flax-seed. *Mol. Cell. Biochem.* **168**, 117–123.

Prasad, K. 1999. Reduction of serum cholesterol and hypercholesterolemic atherosclerosis in rabbits by secoisolariciresinol diglucoside isolated from flaxseed. *Circulation* **99**, 1355–1362.

Prasad, K. 2000. Antioxidant activity of secoisolariciresinol diglucoside-derived metabolites, secoisolariciresinol, enterodiol, and enterolactone. *Int. J. Angiol.* **9**, 220–225.

Price, K.R., Fenwick, G.R., Oleszek, W., Jurzysta, M., Oszyñski, M., and Colquhoun, I.J. 1992. Zanhic acid tridesmoside and other dominant saponins from alfalfa (*Medicago sativa* L.) aerial parts. *J. Agric. Food Chem.* **40**, 191–196.

Puolakka, J. and Makarainen, L. 1985. Biochemical and clinical effects of treating the premenstrual syndrome with prostaglandin synthesis precursors. *J. Reprod. Med.* **30**, 149–153.

Qian, C.J. 1996. Niruriside a new HIV REV/RRE binding inhibitor from *Phylanthus niruri*. *J. Nat. Prod.* **59**(2), 196–199.

Qureshi, A.A., Din, Z.Z., Abuirameileh, N., Burger, W.C., Ahmed, Y., and Elson, C.E. 1983. Suppression of avian hepatic lipid metabolism by solvent extracts of garlic: impact on serum lipids. *J. Nutr.* **113**, 1746–1755.

Raghunathan, K. and Mittra, R. 1982. "Pharmacognosy of Indigenous Drugs". Central Council for Research in Ayurveda & Siddha, New Delhi.

Rahman, K. and Billington, D. 2000. Dietary supplementation with aged garlic extract inhibits ADP-induced platelet aggregation in humans. *J. Nutr.* **130**, 2662–2665.

Rajasree, C.R., Rajmohan, T., and Agusti, K.T. 1999. Biochemical effects of garlic on lipid metabolism in alcohol fed rats. *Indian J. Exp. Biol.* **37**, 243–247.

Rajendran, S., Deepalakshmi, P.D., Parasakthy, K., Devaraj, H., and Devaraj, S.N. 1996. Effect of tincture of Crataegus on the LDL-receptor activity of hepatic plasma membrane of rats fed an atherogenic diet. *Atherosclerosis* **123**(1–2), 235–241.

Ramachandran, C., Peter, K.V., and Gopalkrishnan, P.K. 1980. Drumstick [*Moringa oleifera*]—a multipurpose Indian vegetable. *Economic Botany* **34**(3), 276–282.

Rang, H.P., Dale, M.M., and Ritter, J.M. 1999. "Pharmacology", 9th edition. Churchill Livingstone, UK.

Reid, D. 1986. "Chinese Herbal Medicine", pp. 56. Shambala Publishing Inc., Boston, MA.

Renaud, S.C., Beswick, A.D., Fehili, A.N., Sharp, D.S., and Elwood, P.C. 1992. Alcohol and platelate aggregation: the caerphilly prospective heart diseases study. *Am. J. Clin. Nutr.* **55**, 1012–1017.

Rivlin, R.S. 1998. Patient with hyperlipidemia who received garlic supplements. Lipid management. *Report from the Lipid Education Council* **3**, 6–7.

Robbers, J.E., Speedie, M.K., and Tyler, V.E. 1996. "Pharmacognosy and Pharmacobiotechnology", pp. 139. Williams and Wilkins, Baltimore, USA.

Roberfrodid, M. 1999. Concepts in functional foods: the case of inulin and oligofructose. *J. Nutr.* **129**, 1398–1401.

Roberfroid, M.B. and Delzenne, N.M. 1998. Dietary fructans. *Ann. Rev. Nutr.* **18**, 117–143.

Robinson, T. 1991. "The Organic Constituents of Higher Plants", 6th edition. Cordus press, North Amherst, MA.

Rukmini, C. and Raghuram, T.C. 1991. Nutritional and biochemical aspects of the hypolipidemic action of rice bran oil: a review. *J. Am. Coll. Nutr.* **10**, 593–601.

Russell, R. 1999. Atherosclerosis—an inflammatory disease. *N. Engl. J. Med.* **340**, 115–126.

Ryu, K., Ide, N., Matsuura, H., and Itakura, Y. 2001. N alpha-(1-deoxy-D-fructos-1-yl)-L-arginine, an antioxidant compound identified in aged garlic extract. *J. Nutr.* **131**, 972S–976S.

Saija, A., Scalese, M., Lanza, M., Marzullo, D., Bonina, F., and Castelli, F. 1995. Flavonoids as antioxidant agents: importance of their interaction with biomembranes. *Free Radic. Biol. Med.* **19**, 481–486.

Sanghvi, A., Divven, W.F., Seltman, H., Warty, V., Rizk, M., Kritchevsky, D., and Setchell, K.D.R. 1984. Inhibition of rat liver cholesterol 7-hydroxylase acyl CoA: cholesterol acyltransferase activities by enterodiol and enterolactone. *In* "Proceedings of the Symposium on Drugs Affecting Lipid Metabolism" (D. Krietchevsky, ed.), pp. 450. Plenum Press, New York, (Abstract).

Satyavati, G.V., Dwarkanath, C., and Tripathi, S.N. 1969. Experimental studies on the hypocholesterolemc effect of *Commiphora mukul* Engl. (Guggul). *Indian J. Med. Res.* **57**, 1950.

Satyavati, G.V., Raina, M.K., and Sharma, M. 1976. "Medicinal Plants of India", Vol 1 & 2. Indian Council of Medical Research, New Delhi, India.

Sauvaire, Y., Girardon, P., Baccou, J., and Risterucci, A. 1984. Changes in growth, proteins and free amino acids of developing seed and pod of fenugreek. *Phytochemistry* **23**, 479–486.

Sauvaire, Y., Ribes, G., Baccou, J., and Loubatieres-Mariani, M. 1991. Implication of steroid saponins and sapogenins in the hypercholesterolemic effect of fenugreek. *Lipids* **26**, 191–197.

Sauvaire, Y., Petit, P., and Broca, C. 1998. 4-Hydroxyisoleucine: a novel amino acid potentiator of insulin secretion. *Diabetes* **47**, 206–210.

Schulz, V., Rudolf, H., and Tyler, V.E. 1998. "Rational Phytotherapy", pp. 219. Springer Verlag, Berlin, Germany.

Schwartz, C.J., Valente, A.J., and Sprague, E.A. 1993. A modern view of atherogenesis. *Am. J. Cardiol.* **71**, 9b–14b.

Seetharamaiah, G.S. and Chandrasekharan, S. 1989. Studies on hypocholesterolemic activity of rice bran oil. *Atherosclerosis* **78**, 219–223.

Seo, E.K., Silva, G.L., and Chai, H.B. 1997. Cytotoxic prenylated flavanones from *Monotes engleri*. *Phytochemistry* **45**, 509–515.

Sharma, R.D. 1979. Effect of various isoflavones on lipid levels in triton treated rats. *Atherosclerosis* **33**, 371–375.

Sharma, R. 1984. Hypocholesterolemic activity of fenugreek (*T. foenum graecum*): an experimental study in rats. *Nutr. Rep. Int.* **33**, 660–677.

Sharma, R.D. 1986. An evaluation of hypocholesterolemic factor of fenugreek seeds (*T. foenum graecum*) in rats. *Nutr. Rep. Int.* **33**, 669–677.

Sharma, R.D. 1986. Effect of fenugreek seeds and leaves on blood glucose and serum insulin responses in human subjects. *Nutr. Res.* **6**, 1353–1364.

Sharma, R.D. 1996. Hypolipidaemic effect of fenugreek seeds: a chronic study in non-insulin dependent diabetic patients. *Phytother. Res.* **10**, 332–334.

Sharma, S.B. and Dwivedi, S. 1997. Medicinal plants with hypolipidaemic activities. *Indian Drugs* **34**(5), 242–251.

Sharma, R.D., Raghuram, T.C., and Rao, N.S. 1990. Effect of fenugreek seeds on blood glucose and serum lipids in type I diabetes. *Eur. J. Clin. Nutr.* **44**, 301–306.

Silagy, C. and Neil, A. 1994. Garlic as a lipid lowering agent—a meta-analysis. *J. R. Coll. Phys. Lond.* **28**, 39–45.

Simons, L.A., Balasubramanian, S., VonKonigsmark, M., Parfitt, A., Simons, J., and Peters, W. 1995. On the effects of garlic on plasma lipids and lipoproteins in mild hypercholesterolemia. *Atherosclerosis* **113**, 219–225.

Smith, T.J. and Yang, C.S. 1994. Effects of food phytochemicals or xenobiotic metabolism. *In* "Food Phytochemicals for Cancer Prevention I. Fruits and Vegetables" (M.T. Huang, T. Osawa, C.T. Ho, and R.T. Rosen, eds), pp. 17–48. American Chemical Society, Washington, DC.

Soleas, G.J., Goldberg, D.M., Diamandis, E.P., Karumanchiri, A., Yan, J., and Ng, E. 1995. A derivatized gas chromatographic–mass spectrometric method for the analysis of both isomers of resveratrol in juice and wine. *Am. J. Enol. Vitic.* **46**, 346–352.

Soleas, G.J., Ciamandis, E.P., and Goldberg, D.M. 1997. Resveratrol: a molecule whose time has come. *Clin. Biochem.* **30**, 91–113.

Sowmya, P. and Rajyalakshmi, P. 1998. Hypocholesterolemic effect of germinated fenugreek seeds in human subjects. *Plant Foods Hum. Nutr.* **53**, 359–365.

Srivastava, K.C. 1988. Extract of a spice—*Trachyspermum ammi* shows anti-aggregatory effects and alters arachidonic acid metabolism in human platelets. *Prostaglandins Leukot. Essent. Fatty Acids* **33**, 1–6.

Stark, A. and Madar, Z. 1993. The effect of an ethanol extract derived from fenugreek (*Trigonella foenum graecum*) on bile acid absorption and cholesterol levels in rats. *Br. J. Nutr.* **69**, 277–287.

Stark, A. and Zechiaria, M. 1993. The effect of an ethanol extract derived from fenugreek (*Trigonall foenum graecum*) on bile acid absorption and cholesterol levels in rats. *Br. J. Nutr.* **69**, 277–287.

Stein, J.H., Keevil, J.G., Wiebe, D.A., Aeschlimann, S., and Folts, J.D. 1999. Purple grape juice improves endothelial function and reduces the susceptibility of LDL cholesterol to oxidation in patients with coronary artery disease. *Circulation* **100**, 1050–1055.

Stevinson, C., Pittler, M.H., and Ernst, E. 2000. Garlic for treating hypercholesterolemia. A meta analysis of randomized clinical trials. *Ann. Intern. Med.* **133**, 420–429.

Story, J.A., LePage, S.L., Petro, M.S., West, L.G., Cassidy, M.M., Lightfoot, F.G., and Vahouny, G.V. 1984. Interactions of alfalfa plant and sprout saponins with cholesterol *in vitro* and in cholesterol-fed rats. *Am. J. Clin. Nutr.* **39**(6), 917–929.

Subbiah, M.T.R. 1971. Significance of dietary plants sterols in man and experimental animals. *Mayo Clin. Proc.* **46**, 549–559.

Sun, W. 1992. Determination of arctiin and arctigenin in *Frustus arctii* by reverse phase HPLC. *Acta Pharm. Sin.* **27**(7), 549.

Superko, H.R. and Krauss, R.M. 2000. Garlic powder, effect on plasma lipids, postprandial lipemia, low-density lipoprotein particle size, high-density lipoprotein subclass distribution and lipoprotein(a). *J. Am. Coll. Cardiol.* **35**, 321–326.

Suzuki, S. and Oshima, S. 1970. Influence of blending of edible fats and oils on human serum cholesterol level. *Jpn. J. Nutr.* **28**, 3–9.

Takano, S., Matsuhima, M., Eruturk, E., and Bayan, G.T. 1981. Early induction of rat colonic epithelial ornithine and *S*-adenosyl-L-methionine decarboxilase activities by bile salts. *Cancer Res.* **41**, 624–628.

Tewari, A., Sen, S.P., and Guru, L.V. 1968. The effect of amlaki rasayana on biological system. *J. Res. Indian Med.* **2**, 189.

Thakur, C.P. 1985. *Emblica officinalis* reduces serum, aortic and hepatic cholesterol in rabbits. *Experientia* **41**, 423–424.

Thakur, C.P. and Mandal, K. 1984. Effect of *Emblica officinalis* on cholesterol-induced atherosclerosis in rabbits. *Indian J. Med. Res.* **79**, 142–146.

Thakur, C.P., Thakur, B., Singh, S., Sinha, P.K., and Sinha, S.K. 1988. The Ayurvedic medicines Haritaki, Amala and Bahira reduce cholesterol-induced atherosclerosis in rabbits. *Int. J. Cardiol.* **21**(2), 167–175.

Thompson, L.U., Robb, P., Serraino, M., and Cheung, F. 1991. Mammalian lignan production from various foods. *Nutr. Cancer* **16**, 43–52.

Thompson, L.U., Seidl, M., Rickard, S.E., Orcheson, L.J., and Fong, H.H.S. 1996. Antitumorigenic effect of mammalian lignan precursor from flaxseed. *Nutr. Cancer* **26**, 159–165.

Tijburg, L.B., Wiseman, S.A., Meijer, G.W., and Weststrate, J.A. 1997. Effects of green tea, black tea and dietary lipophilic antioxidants on LDL oxidizability and atherosclerosis in hypercholesterolaemic rabbits. *Atherosclerosis* **135**, 37–47.

Tilvis, R.S. and Miettinen, T.A. 1986. Serum plant sterols and their relation to cholesterol absorption. *Am. J. Clin. Nutr.* **43**, 92–97.

Todd, P.A., Benfield, P., and Goa, K.L. 1990. Guar gum. A review of its pharmacological properties, and use as a dietary adjunct in hypercholesterolaemia. *Drugs* **39**(6), 917–928.

Torel, J., Cillard, J., and Cillard, P. 1986. Antioxidant activity of flavonoids and reactivity with peroxy radicals. *Phytochemistry* **25**, 383–385.

Tripathi, K.D. 1995. "Essentials of Medical Pharmacology", 3rd edition. Jaypee Brothers Medical Publishers Ltd., New Delhi, India.

Tripathi, S.N., Shastri, V.V.S., and Satyavati, G.V. 1968. Experimental and clinical studies on the effect of Guggulu in hyperlipaemia and thrombosis. *J. Res. Indian Med.* **2**, 10.

Tripathi, S.N., Tiwari, C.M., Upadhyay, B.N., and Gupta, V.K. 1984. Beneficial effect of *Inula racemosa* in angina pectoris. *Indian J. Physiol. Pharmacol.* **28**, 73–76.

Tunon, H., Olavsdotter, C., and Bohlin, L. 1995. Evaluation of anti-inflammatory activity of some Swedish medicinal plants. Inhibition of prostaglandin biosynthesis and PAF-induced exocytosis. *J. Ethnopharmacol.* **48**, 61–76.

Valsa, A.K., Ushakumari, B., and Vijayalakshmi, N. 1995. Effect of green tea catechins on plasmacholesterol level in cholesterol-fed rats. *J. Nutr. Sci. Vitaminol.* **32**, 613–622.

Valsa, A.K., Asha, S.K., and Vijayalakshmi, N.R. 1998. Effect of catechin on intestinal lipidmetabolism. *Indian J. Physiol. Pharmacol.* **42**, 286–290.

Wang, H.X. and Ng, T.B. 1999. Natural product with hypoglycemic, hypotensive, hypocholesterolemic, and antithrombotic activities. *Life Sci.* **65**, 2663–2677.

Warshafsky, S., Kamer, R.S., and Sivak, S.L. 1993. Effect of garlic on total serum cholesterol, a meta-analysis. *Ann. Intern. Med.* **119**, 599–605.

Wilcox, L.J., Borradaile, N., Kurowska, E., Telford, D.E., and Huff, M.W. 1998. Naringenin, a citrus flavonoid, markedly decreases apoB secretion in HepG2 cells and inhibits acyl CoA: cholesterol acyltransferase. *Circulation* **98**, 531–537.

Woodward, P.W. 1996. "Garlic and Friends: The History, Growth and Use of Edible Alliums pp. 2–22. Hyland House, Melbourne, Australia.

Yokozawa, T. and Dong, E. 1997. Influence of green tea and its three makor components upon low-density lipoprotein oxidation. *Exp. Toxicol. Pathol.* **49**, 329–335.

Yotsumoto, H., Yanagita, T., Yamamoto, K., Ogawa, Y., Cha, J.Y., and Mori, Y. 1997. Inhibitory effects of oren-gedoku-toand its components on cholesteryl ester synthesis in cultured human hepatocyte HepG2 cells: evidence from the cultured HepG2 cells and *in vitro* assay of ACAT. *Planta Med.* **63**, 141–145.

Yu-Yan, Y. and Liu, L. 2001. Cholesterol lowering effect of garlic extracts and organosulfur compounds: human and animal studies. *J. Nutr.* **131**, 989S–993S.

Zambon, D., Sabaté, J., Muñoz, S., Campero, B., Casals, E., Merlos, M., Laguna, J.C., and Ros, E. 2000. Substituting walnuts for monounsaturated fat improves the serum lipid profile of hypercholesterolemic men and women. *Ann. Intern. Med.* **132**, 538–546.

Ziaei, S., Hantoshzadeh, S., Rezasoltani, P., and Lamyian, M. 2001. The effect of garlic tablet on plasma lipids and platelet aggregation in nulliparous pregnants at high risk of preeclampsia. *Eur. J. Obstet. Gynecol. Reprod. Biol.* **99**, 201–206.

INDEX