BIOACTIVE PEPTIDES AND PROTEINS

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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

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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,

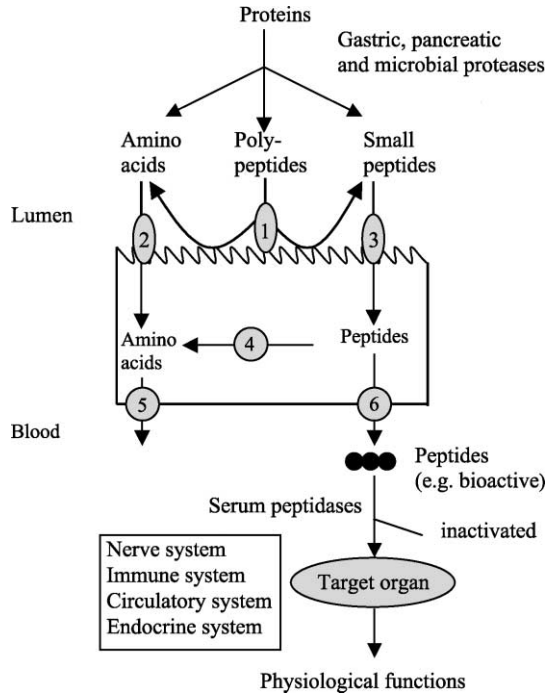

FIG. 1 Digestion and absorption of proteins in the small intestine. (1) Brush-border peptidases, (2) brush-border amino acid transport systems, (3) brush-border peptide transport systems, (4) cytoplasmic peptidases, (5) basolateral amino acid transport systems, (6) basolateral peptide transport systems.

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).

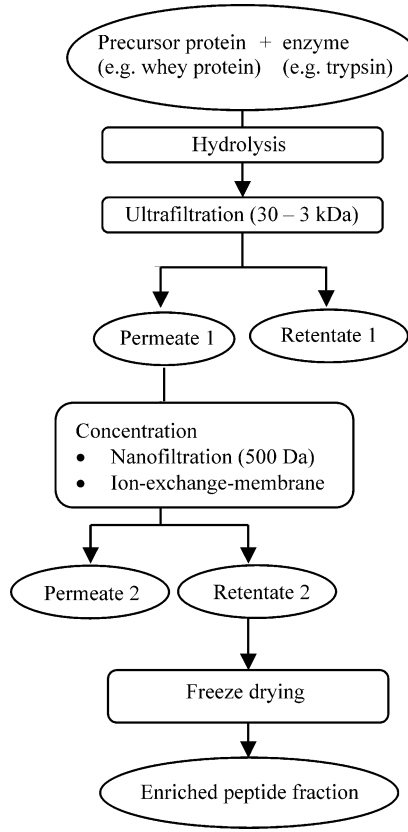

FIG. 2 Flow diagram for production and separation of bioactive peptides from food proteins obtained by enzymatic hydrolysis.

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.

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