Whey proteins and peptides

Emerging properties to promote health

Anne Pihlanto
MTT Agrifood Research Finland, Biotechnology and Food Research
Jokioinen, Finland

tel + 358 3 41883276 - fax +358 3 41883244
email anne.pihlanto@mtt.fi
website www.mtt.fi - www.mttt.fi/experts/anne_pihlanto

Key words

α-Lactalbumin
β-Lactoglobulin
Immunoglobulins
Lactoferrin
Lactoperoxidase
Growth factors
Glycomacropeptide

SUMMARY

The high nutritive value and diverse functional properties of milk proteins are well known. In recent years, intense scientific research has been focused on the identification of factors within bovine milk that may be relevant to improving human health. The best characterized whey-based bioactive proteins include α-lactalbumin, β-lactoglobulin, immunoglobulins, lactoferrin, lactoperoxidase and growth factors. These proteins exhibit a wide range of biological activities that may influence the digestive function, metabolic responses to absorbed nutrients, growth and development of organs and disease resistance. Some whey proteins may reduce the risks of chronic human diseases reflected by the metabolic syndrome. Whey proteins are a good source of various bioactive peptides which are encrypted within the proteins and can be released during gastric digestion or food processing by enzymes or microbes. Whey protein-derived peptides have been shown to exert a wide range of bioactivities affecting the cardiovascular, immune and nervous systems. The efficacy of a few peptides has been established in animal and human studies. A number of commercial whey-based protein products with potentital health benefits are on the market and this is envisaged to increase on a global scale.

INTRODUCTION

Because of their high nutritional quality and versatile functional properties, milk proteins have been studied in depth for well over 50 years. Normal bovine milk contains about 3.5% of protein, of which casein constitutes 80% and whey proteins 20%. The principal bovine casein fractions are α_{s1}-, α_{s2}-, β- and κ-casein, whereas human casein consists mainly of β-casein and a small fraction of κ-casein. Whey is separated from the curd during cheeses-making process. During cheese manufacture whole casein is removed from whole milk, what remains is whey which contains lactose, proteins and lipids. Advances in processing techniques have lead to industrial production of whey products with varying protein content. Due to good nutritional value and functional properties of these products, whey is sold as nutritional supplement, and is particularly popular in sport of body-building. The bovine whey protein fraction contains six major proteins, α-lactalbumin (α-La) and β-lactoglobulin (β-Lg), glycomacropeptide, proteose-peptone 3, immunoglobulins (Ig), and bovine serum albumin, which together make up 85% of whey protein. In addition, milk contain several proteins at very low or trace levels, the most abundant being lactoferrin and lactoperoxidase. These minor proteins are regarded as highly significant due to their bioactivities. The major whey proteins in human milk are IgA immunoglobulin, lactoferrin and α-la, whereas no indigenous β-Lg is present (**1-5**).

There is increasing interest in whey as a potentially rich source of bioactive compounds to reduce risk and/or to prevent disease development. Several methods, such as ultrafiltration, diafiltration and ion-exchange technology have been used to concentrate and separate the proteins from other components. The whey protein is then dried to obtain whey protein concentrate (WPC) or whey protein isolate (WPI), both of which are highly soluble, with protein levels ranging from 80-95%. Both WPC and WPI have a wide range of food applications and, because of their high protein content, can function as water-binding, gelling, emulsifying and foaming agents. Processing treatments used in the manufacture of WPC and WPI may sometimes cause some protein denaturation, which tends to affect their functionality.

Whey proteins have been reported to have utility in many different applications ranging from effects on bone, mus-

cle, blood, brain, immune, cancer, infection and wound healing.

Besides intact proteins, increasing interest is focused on peptides which may be released from proteins by enzymatic activity. Bioactive peptides may be encrypted in the amino acid sequence of larger protein and usually consist of 3-20 amino acids. Encrypted peptides can be released in three ways:

1. hydrolysis by digestive enzymes
2. fermentation of milk with proteolytic starter cultures
3. proteolysis by enzymes derived from microorganisms or plants.

In many studies, the above methods have been combined successfully and there are now more than two hundred biologically active peptides identified from milk proteins. Whey proteins are also an important source of bioactive peptides exerting various activities (**4,6-9**). Numerous bioactivities including antihypertensive, antilipemic, antimicrobial, antioxidative, opiate and immunomodulatory have been described for bovine whey protein-derived peptides. This article provides an overview the beneficial physiological effects of whey proteins and peptides and gives their current and potential applications on promotion of human health.

HEALTH EFFECTS AND APPLICATIONS

Table 1 shows the amino acid composition of main whey protein fractions as compared to caseins (**10**). Apart from being a balanced source of valuable amino acids which is comparable to egg proteins, whey proteins have multiple beneficial effects on human health (**5,7**). These emerging health properties and clinical implications have attained increasing industrial interest in recent years. The interest is based on scientific data indicating that whey proteins confer a number of physiological effects as shown in *Figure 1*.

Table 2 gives examples of whey proteins and peptides that have been employed commercially in recent years.

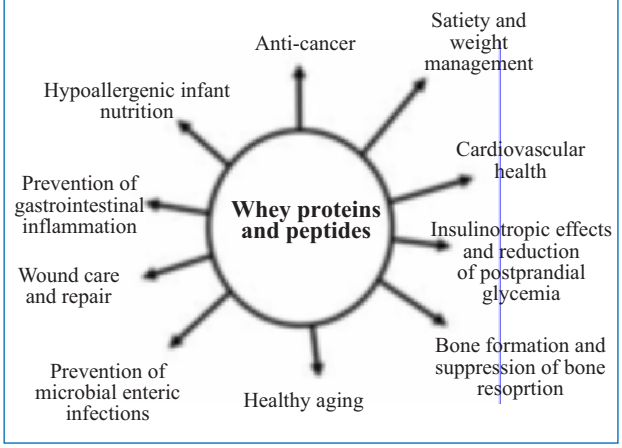
The potential of whey protein as a functional food component to contribute to the regulation of body weight by providing satiety signals that affect both short-term and long-term food intake regulation has received much attention during last years. More than 20 different regulatory peptide hormones are released in the gastro-intestinal (GI) system and many of them are recognized to be involved in the regulation of food intake and are sensitive to gut nutrient content and composition. The effect of whey proteins on food intake and body composition has

Table 1 *Amino acid composition of main whey protein fractions compared to α_{s1}-casein and β-casein*

Amino acid residue	***α-Lactalbumin*** (123 amino acid residues, MW 14,175)	***β-Lactoglobulin*** (162 amino acid residues, MW 18,277)	***BSA*** (582 amino acid residues, MW 66,267)	***αs1-Casein*** (199 amino acid residues, MW 23,614)	***β-Casein*** (209 amino acid residues, MW 23,983)
Aspartic acid	9	10	39	7	4
Asparagine	12	5	12	8	5
Asx[a]			3		
Threonine	7	8	334	5	9
Serine	7	16	25	16	16
Glutamic acid	8	9	59	24	18
Glutamine	5	2	19	15	21
Glx[b]			1		
Proline	2	8	28	17	35
Glycine	6	4	16	9	5
Alanine	3	12	46	9	5
Cysteine	8	5	35		
Valine	6	9	36	11	19
Methionine	1	4	4	5	6
Isoleucine	8	10	14	11	10
Leucine	13	22	61	17	22
Tyrosine	4	4	19	10	4
Phenylalanine	4	4	27	8	9
Lysine	12	15	59	14	11
Histidine	3	2	17	5	5
Tryptophane	4	2	2	2	1
Arginine	1	3	23	6	4

[a]), aspartic acid or asparagine undefined; [b]), glutamic acid or glutamine undefined; BSA, bovine serum albumin; Data taken from Eigen *et al* (**10**)

Figure 1 *Whey proteins and peptides exhibit various properties that can have positive effects on human health Information compiled from 1-7*



been investigated in several animal and human studies (**11**). Studies have demonstrated the effect of whey proteins on appetite control, muscle sparing and lipid metabolism in healthy adults. The local effects of whey proteins in the gastrointestinal tract, and in particular the interaction with the brain-gut axis may be important. Whey proteins (or their degradation fragments) have been shown to inhibit dipeptidyl peptidase IV (DPPIV) and thereby augment the insulinotropic effects of GLP-1 (**12**). This finding might be clinically very important. In a recent study Pal *et al* (**13**) found that supplementation of whey protein in overweight/obese individuals for 12 weeks decreased total cholesterol and LDL cholesterol levels compared with casein and control (glucose). Whey protein also decreased plasma TAG, insulin and homeostasis model assessment of insulin resistance scores compared with the control. These results indicated that whey protein supplementation can significantly improve metabolic risk factors associated with chronic diseases in overweight and obese individuals. Researchers studying the impact of dairy proteins on satiety, found that giving whey protein before a meal reduced food intake, post meal blood glucose and insulin, and the ratio of cumulative blood glucose to insulin AUCs in a dose-dependent manner (**14**).

Whey proteins and amino acid supplements have a strong position in the sports nutrition market based on the purported quality of proteins and amino acids they provide. Studies employing stable isotope methodology demonstrate the ability of whey proteins or amino acid mixtures of similar composition to promote whole body and muscle protein synthesis. Whey protein is a rich source of branched chain amino acids (BCAAs) and leucine (***Table 1***). BCAAs are important for athletes since unlike the other essential amino acids, they are metabolized directly into muscle tissue and are the first ones used during periods of exercise and resistance training. Leucine plays a distinct role in protein metabolism and has been identified as a key signal in the translation initiation pathway of muscle protein synthesis (**15**). In a recent study, whey proteins stimulated postprandial muscle protein accretion more effectively than casein and casein hydrolysate in older men. This effect was attributed to a combination of whey's faster digestion and absorption kinetics and higher leucine content (**16**).

Table 2 *Whey proteins and milk protein-derived peptides exploited in commercial applications*

Protein/peptide	***Application/health claim***	***Remarks***
Immunoglobulins	Prevention/treatment of oral or intestinal microbial infections	Commercial products against rotavirus and traveller's diarrhea, potential for Helicobacter and hospital infections
Glycomacropeptide	Weight management, dental caries	No conclusive results on efficacy
Lactoferrin	Prevention of enteropathogen and viral infections Stimulation of immune system Prevention of pathogen contamination of raw meat	Beneficial effect documented in several animal model studies Human studies inconclusive GRAS status and commercial use in USA
Lactoperoxidase system	Prevention of dental caries Preservation of raw milk	Oral hygiene products are on the market Approved by Codex Alimentarius Commission In use in many countries with insufficient milk cooling facilities Proven efficacy against mutans streptococci
Lactotripeptides (VPP, IPP)	Reduction of blood pressure	Beneficial effect documented in animal model and human studies Commercial products (fermented milk) are on the market
Whey peptides (β-Lg hydrolysate)	Reduction of blood pressure	Effects documented in animal and human studies Commercial products are on the market
Alpha-casozepine (α_{s1}-casein f91-100)	Reduces stress	Decreases anxiety in animal and clinical studies Commercial product (drink, confectionery) are on the market

Information compiled from Hartman and Meisel (**8**), Korhonen and Pihlanto (**9**)

WHEY PROTEINS

β-Lactoglobulin

β-Lg, a member of lipocalin family, is the most abundant whey protein in bovine milk accounting for about 50% of the proteins in whey but is not found in human milk. β-Lg displays a great variety of functional properties, such as excellent heat-set gelation properties and water-binding. The protein's behavior has been the focus of extensive experimental work questioning of the role of β-Lg as a transporter of hydrophobic molecules through the acidic environment of the gut (**17**).

The endogenous function, apart from being an important source of amino acids, is not known. It has been proposed to participate in the digestion of milk lipids in the neonate by enhancing the activity of pregastric lipase in the neonate through removal of the fatty acids that inhibit this enzyme. β-Lg binds to hydrophobic components, including retinol and long-chain fatty acids and it has been speculated that this protein may play a role in the absorption and subsequent metabolism of fatty acids. The biological activities of β-Lg have been associated with antiviral, prevention of pathogen adhesion, anticarcinogenic and hypocholesterolemic effects (**18**).
β-Lg has proven to be an excellent source of peptides with a wide range of bioactivities. Peptides, Ile-Pro-Ala (f78-80), Tyr-Leu (f102-103), Ala-Leu-Pro-Met-His-Ile-Arg (f142-148), termed lactokinins inhibit the angiotensin-I-converting enzyme (ACE) and represent potential ingredients for prevention and/or treatment of high blood pressure (**19**). Hernández-Ledesma et al (20,21) identified several antioxidant peptides in the β-Lg A hydrolysate by corolase PP. The peptides, Trp-Tyr-Ser-Leu-Ala-Met-Ala-Ala-Ser-Asp-Ile (f19-29), Tyr-Val-Glu-Glu (f42-46) and Met-His-Ile-Arg-Leu (f145-149) possessed higher radical scavenging activity than butylated hydroxyanisole. Peptide Trp-Tyr-Ser-Leu-Ala-Met-Ala (f19-25) and its derived peptides Trp-Tyr (f19-20), Trp-Tyr-Ser (f19-21), Trp-Tyr-Ser-Leu (f19-22) and Trp-Tyr-Ser-Leu-Ala (f19-23) have shown both ACE-inhibitory and radical radical-scavenging activity. β-Lactorpin (Tyr-Leu-Leu-Phe f102-105) is an ACE inhibitor; moreover it improves vascular relaxation in spontaneously hypertensive rats (SHR) and is an opioid receptor antagonist suggesting it can modulate absorption process in the intestinal tract (**22**). β-Lactotensin (His-Ile-Arg-Leu f146-149) is released by chymotrypsin hydrolysis and has ileum-contracting activity. β-lactotensin is a natural ligand for neurotensin NT2 receptor, has antistress properties, promotes the abolition of fear memory, reduces sensitivity to painful stimuli and consolidates memory (**23,24**). Hydrolysis of β-Lg by trypsin liberates peptides Ile-Ile-Ala-Glu-Lys (f71-75) and Ala-Leu-Pro-Met-His f(142-146), termed lactostatin, have hypocholesterolemic properties. Ile-Ile-Ala-Glu-Lys has shown to decrease micellar solubility of cholesterol which leads to lower absorption of intestinal cholesterol in animal studies. Lactostatin is also able to induce gene transcription of human 7α-hydroxylase, a cholesterol-metabolizing enzyme, resulting in a hypocholesterolemic effect (**25**).

α-Lactalbumin

In mature bovine milk, the concentration of α-lactalbumin (α-La) is 1-1.5g/L, comprising approximately 3.4% of the total protein or 20% of the whey proteins. On the other hand, α-La is the predominant whey protein in human milk, levels increasing from 21% to 34% between days 1 and 14 of lactation. α-La concentrations in mature human milk are 2.44±0.64 g/L, determined in a multinational study (**26**). α-La is fully synthesized in the mammary gland where it acts as coenzyme for biosynthesis of lactose. α-La catalyses the last step of the biosynthesis of lactose and controls the subsequent movement of water into the mammary secretory vesicles. It is therefore critical for lactational control and secretion of milk. α-La has a globular structure at aqueous solutions and exhibits a high affinity to metal ions, calcium in particular. Binding of calcium is of utmost importance for maintaining the structure of this protein. Calcium depletion at low pH causes structural changes to form the so-called molten globule state. This has important implications during purification processes and for the bioactivity of protein (**18**).

Recent research suggests that the health benefits of α-La for human consumption can be subdivided into three groups: those related to
i the intact whole protein
ii peptides derived from the partly hydrolysed protein
iii amino acids of the fully digested protein (**18**).

α-La is a good source of the essential amino acids tryptophan and cysteine which are precursors of serotonin and glutathione, respectively. Evening intake of α-La by human volunteers increased plasma trypthophan bioavailability and improved morning alertness and brain measures of attention (**27**). Scrutton *et al* (**28**) demonstrated that daily administration of 40 g α-La to healthy women increased plasma tryptophan levels and its ratio to neutral amino acids but no changes in emotional processing was observed. In rats, α-La ingestion enhanced serotonin release and induced anxiolytic and rewarding effects suggesting it has beneficial effect on mood. α-La can provide protective effect against induced gastric mucosal injury caused by intake of ethanol or non-steroid anti-inflammatory drugs (NSAID) in animal models (**29-31**). α-La is rich in sulphuric amino acid cysteine and it has been demonstrated that an intake of cysteine-rich whey protein diet tends to improve glycemic control and alleviate sucrose-induced oxidative stress and development of insulin resistance in rats fed a high sucrose diet (**32**).
Encouraging results in view of the potential use of α-La in dietary regimes targeting at reducing the risk of development of diabetes type two and obesity has been obtained. Pilvi *et al* (**33**) showed that mice on lactoferrin

diet had higher lost in weight and α-La and lactoferrin resulted lower body fat content compared to WPI diet. *Ad libitum* feeding after weight loss resulted in weight regain in all groups and only the α-La diet significantly reduced fat accumulation during weight regain. The authors concluded that a high-Ca diet with α-La significantly improved the outcome of weight loss and subsequent weight regain during the feeding of a high-fat diet in mice, in comparison with WPI. In a recent study, especially α-La enriched yoghurt has been shown to suppress hunger and the desire to eat more than the whey enriched yoghurt (**34**). However, according to the EFSA Journal (**35**), the proofs for increased satiety leading reduced energy intake are not convincing.

In terms of cancer, α-La inhibits the growth of colon adenocarcinoma cell lines in culture (**36**). A folding variant of human α-La selectively enters tumour cells and induces an apoptosis-like effect. This kinetically trapped protein-lipid complex was named HAMLET/BAMLET for human/bovine α-La made lethal to tumour cells. The specific therapeutic effect of HAMLET in vivo has been demonstrated on several examples such as human skin papillomas, human glioblastoma tumour in mice and mammary cells of mice (**37,38**).

α-La is an immunostimulator. It stimulates the production of IL-1β by ovine bronchoalvelolar lavage macrophages in culture (**39**). Immunopeptides Tyr-Gly (f18-19; f50-51) and Tyr-Gly-Gly (f18-20) from the N-terminus of α-La stimulate the proliferation of human blood lymphocytes in culture (**40**). An immunostimulating peptide, Gly-Leu-Phe (f51-53) from bovine α-La binds to specific sites on human neutrophils and monocytes, stimulates superoxide anion production by neutrophils and human monocyte-macrophage adherence and phagocytosis of human senescent red blood cells. Moreover it protects mice against *Klebsiella pneumonia* infection (**41**).

α-La derived-peptides have shown ACE-inhibitory potencies, highest activity has been found with peptide Trp-Leu-Ala-His-Lys α-La (f104-108) produced by trypsin hydrolysis (**42**). α-lactorphin (Tyr-Gly-Leu-Phe, f50-53), released by pepsin treatment is an opioid-like peptide. The peptide inhibits the contraction of stimulated guinea pig ileum and lowers blood pressure in conscious adult SHR. Both phenomena were naloxone-sensitive suggesting that opioid receptors may be involved in these effects. Moreover, α-lactorphin improved the impaired vascular function in mesenteric arterial rings of adult SHR. The beneficial effect was directed towards endothelial function (**43**).

Glycomacropeptide

Glycomacropeptide (GMP) is a C-terminal glycopeptide (f106-169) released from the κ-casein molecule by the action of chymosin. GMP is hydrophilic and pure GMP can be recovered in large quantities from cheese. GMP contains a significant (50-60% of total GMP) carbohydrate fraction which is composed of galactose, N-acetyl-galactosamine and N-neuraminic acid. The non-glycosylated form of GMP is often termed caseinomacropeptide or CMP (**44**). GMP is high in branched amino acids and lacks the aromatic amino acids pheynylalanine, tryptophan and tyrosine. A lack of phenylalanine makes it one of the few natural proteins that can be safely ingested by individual with phenylketonuria. The potential biological activities of GMP have received much attention in recent years.

Oral GMP stimulates cholesystokinin (CCK) release, a candidate satiety hormone, which may make this protein useful component of a weight loss diet because CCK slows gastric emptying, which may in turn promote satiety (**45**). GMP has also been detected in the plasma of volunteers after milk or yoghurt ingestion, suggesting that GMP can be formed in the gut and then can be absorbed intact into intestinal cells (**46**). GMP has been associated with reduced daily food intake (**47**) decreased weight gain and effect on fat accumulation when combined with WPI (**48**). Based on these results, commercial GMP or CMP containing products have been launched on the market in recent years for the purpose of appetite control and weight management. However, the clinical efficacy of such products remains to be established. A short-term intervention trial failed to show any effect on satiety when 20 grams of CMP was administered daily as a solution to human subjects (**49**). Recent study by Koegh *et al* (**50**) suggests that GMP at doses employed did not influence satiety levels or energy intake at a subsequent meal.

GMP has been reported to bind cholera and Escherichia coli enterotoxins, to inhibit bacterial and viral epithelial adhesion, to promote bifidobacterial growth and to modulate the immune system response (**51,52**). Recent studies support the hypothesis that GMP may limit intestinal inflammation acting, at least in part, on lymphocytes (**53**). GMP was found to fully normalize the colonic expression of interleukin (IL) 1β, IL17, IL23, IL6, transforming growth factor β, IL10, and Foxp3 in the dextran sulfate sodium (DSS)-induced model of rat colitis. In addition, the production of interferon-γ by mesenteric lymph node cells ex vivo was also normalized by GMP treatment. These results suggest that GMP exerts intestinal anti-inflammatory activity in this model, which may be primarily related to actions on Th1 and Th17 lymphocytes and perhaps macrophages (**54**). All these results indicate that GMP has a therapeutic value in preclinical models of intestinal inflammation, however, more studies have to be done to prove the efficacy in humans.

Immunoglobulins

The immunoglobulin (Ig) proteins form a diverse family whose members, when in milk, protect the gut mucosa against pathogenic micro-organisms. In bovine milk, the predominant species of Ig proteins are members of the IgG subfamily, in particular IgG1. Colostrums contain Ig up to 70-80% of the total protein content, whereas in

milk they account for only 1-2% of total protein. In colostrums Ig's role is to confer passive immunity to the neonate while its own immune system is developing (**55**). IgG proteins have multiple functions, including complement activation, bacterial opsonization (rendering bacterial cells susceptible to immune response) and agglutination. They inactivate bacteria by binding to specific sites on the bacterial surface, inhibiting bacterial adhesion to epithelial cells. Ig can inhibit or reduce the production of toxins and other harmful components by inhibiting bacterial metabolism and by blocking enzymes and receptors. Specific Ig can protect against viral infections by binding viruses and preventing the virus replication by blocking the receptor mediated internalization of viruses in the host cells (**56**).

Most of the commercial Ig products are prepared from colostrum by removing the fat, followed by microfiltration or pasteurization under conditions that remain the biological activity (**4**). These supplements are promoted to boost natural immunity against microbial infections and general well-being without any specific microbial target. Some products have been tested clinically for certain physiological function or prevention or treatment of microbial functions. Few products boost specific health or function claims, such as boosting immunity against microbial infections or speeding recovery from physical endurance exercises (**57,58**). The scientific clinical evidence related to colostral supplements remains, however, disputed and seems to be associated e.g. with faster recovery from long endurance physical training among athletes (**59**).

Low abundance proteins

Low abundance proteins include those proteins representing 1% or less of the protein content of whey. The amounts of lactoferrin (approx. 0.2 mg/L) and lactoperoxidase (approx.0.03 mg/L) in milk are close to the limit in terms of recovery of whey proteins for sale as nutraceutical or food supplements. Growth factors are present only in trace amounts, but since they retain their activity during extraction they can have commercial interest. Especially, bovine colostrum is rich source of low abundance proteins, containing high amounts of immunoglobulins, growth factors, cytokines and nucleosices (**60**).

Lactoferrin

Lactoferrin is a monomeric, globular, Fe(III)-binding glycoprotein comprising 680 amino acids, giving a molecular mass of 80 kDa. Lactoferrin is a member of the transferring family, but unlike the eponymous protein, to date there is no strong evidence that lactoferrin is involved in iron transport or metabolism under normal circumstances. Indeed, the protein is only lightly loaded with iron (III) in milk, allowing it to perform a major bacteriostatic role by sequestering iron (III). Lactoferrin is present in exocrine secretins such as milk, tears, nasal exudates, saliva, bronchial and cervico-vaginal mucus, seminal plasma and gastrointestinal fluid. It is synthesized in various exocrine glands such as submucosal glands of doducting airways, epithelial cells in mammary glands and in neutrophils. Lactoferrin is promptly delivered by circulating neutrophils to sites of microbial invasion. Lactoferrin is a multifunctional protein, with antimicrobial, antioxidative, antiinflammatory, anticancer and immune regulatory properties (**61,62**).
Commercial applications utilizing bovine lactoferrin and its partially digested peptides are appearing as nutraceuticals in infant formulas, health supplements, oral care products and animal feeds. In addition, its reputed antioxidant properties are being exploited in cosmetic products. For a review on the remarkable properties of lactoferrin and their commercial applications, see Marnila and Korhonen (**62**).
Antimicrobial properties The first recognized antibacterial property of lactoferrin was its ability to bind iron from medium, hence preventing bacterial growth. Lactoferrin has bacteriostatic and bactericidal activity against Gram-negative and Gram-positive bacteria. Fungicidal activity particularly against *Candida* species has also been described. Moreover, lactoferrin is capable of inhibiting replication of viruses. Proteolysis of lactoferrin by pepsin produces N-terminal arginine-rich fragments, called lactoferricin(s), which have antimicrobial activity as well as activity as antiviral and antiparasitic agents ([61,62]).
Cancer prevention and immunological effects Lactoferrin has marked effects on immune cells in culture, being an immunostimulator and immunoregulator. Both bovine and human lactoferrin inhibit the proliferative response and cytokine production of Th1, but not Th2 cell lines (63). Oral ingestion of bovine lactoferrin stimulates the immune system of the gut, and enhances and reconstitutes the peripheral immune system. There are over 30 studies demonstrating that orally ingested bovine lactoferrin reduces tumor growth. Orally administered lactoferrin reduced the development of neoplastic cells in the colon, oesophagus, and lungs of rats treated with carcinogens (**64**). Black tea polyphenols and bovine lactoferrin had a synergistic effect in the prevention of chemically induced buccal pouch carcinogenesis in a hamster model (**65**). In healthy human volunteers, a treatment with oral bovine lactoferrin of 50 g/day increased significantly the proportion of neutrophilic precursors in the peripheral blood and reduced the spontaneous production of IL-6 and TNF-α by cultured peripheral blood mononuclear cells and enhanced chemotactic reactions by promoting the recruitment of leukocytes to the inflammatory site (**63**). Oral ingestion of bovine lactoferrin that has been saturated with iron, designated Lf+, appears to display enhanced anti-tumor activity in combination with chemotherapy. This combination was capable completely eradicating tumors that are otherwise completely insensitive to chemotherapy (for a review see **61**).

Other activities Lactoferrin protects against lethal endotoxin shock in germ-free piglets as it blocks the binding of LPS to monocytes (**66**), and has myelosuppressive effects in mice when injected intravenously (**67**). Lactoferrin is a protease inhibitor which inhibits tryptase, and cysteine proteases. Aerosolized lactoferrin inhibited airway hypersensitivity in sheep asthma model (**68**). Cornish (**69**) demonstrated that both bovine and human lactoferrin are anabolic factors for the bone in a mouse model. Local injections of bovine lactoferrin into adult mice resulted in an increased bone growth. In human studies reviewed by Wakabayashi *et al* (**61**) lactoferrin has been shown to increase eradication rate of *H. pylori* gastritis when administered in connection of triple therapy. Also, lactoferrin ingestion has decreased the incidence of bacteremia and severity of infection in neutropenic patients. In further human studies, lactoferrin has been shown to alleviate symptoms of hepatitis C virus infection, and to reduce small intestine permeability in drug induced intestinal injury. Also, lactoferrin ingestion and topical application have proven beneficial in the cure of *tinea pedis*. Clinical studies in infants have demonstrated that oral administration of bovine lactoferrin preparations increases the number of bifidobacteria in fecal flora and the serum ferritin level while the ratios of *Enterobacteriaceae, Streptococcus* and *Clostridium* tended to decrease.

OTHER WHEY PROTEINS

Lactoperoxidase

Lactoperoxidase (LPO) is a glycoprotein which occurs naturally in colostrum, milk and many other human and animal secretions (**70**). LPO acts as a protective factor against infectious microbes; it catalyzes an antimicrobial system consisting of the thiocyanate anion (SCN-) and hydrogen peroxide to generate short-lived oxidation products, primarily hypothiocyanate (OSCN-), which kill or inhibit the growth of a wide range of microorganisms, including bacteria, viruses, fungi, molds and protozoa (**71**).

Growth factors

It is now well documented that colostrum and milk contain several factors which promote or inhibit the growth of different cell types. The best characterized growth factors are beta cellulin (BTC), epidermal growth factor (EGF), fibroblast growth factors (FGF1 and FGF2), insulin-like growth factors (IGF-I and IGF-II), transforming growth factors (TGF-β1 and TGF-β2) and platelet-derived growth factor (PDGF). The concentration of growth factors is highest in colostrum during the first hours after calving and decrease substantially thereafter (**72,73**). The growth factors have been associated with many physiological functions affecting e.g. skin, intestinal tract and bone health.

Animal model studies have shown that EGF, IGF-I, and both TGF forms can provoke various local effects on the gut mucosa and can be absorbed intact or partially from intestine into blood circulation (**72**). Several health-related applications have been proposed for growth factors extracted from bovine colostral or cheese whey. An acid casein extract rich in TGF-β2 has been tested successfully in children suffering from Crohn's disease.

WHEY BIOACTIVE PEPTIDES

There is increasing evidence from animal and human studies that milk-derived bioactive peptides may deliver many physiological effects *in vivo*, for example on the gastrointestinal, cardiovascular, endocrine, immune and central nervous systems. For this reason, milk-derived bioactive peptides are considered as prominent candidates for various functional foods targeted at promoting specific body functions. As stated above, whey proteins are good source of peptides. However, most studies with whey protein-derived peptides have shown the activity only *in vitro* and only few studies have been done *in vivo*. The structural properties of peptides play an important role in the bioactivity and bioavailability.

However, in most cases this is not yet well characterized and only some general features have been found. The examples of structure activity relationship and peptide sequences of whey-derived bioactive peptides with different bioactivities are presented in *Table 3* (data compiled from references **8, 19,20,74,75**).

Production of bioactive peptides

1. Enzymatic hydrolysis

Enzymatic hydrolysis is the most common way to release bioactive peptides from whey proteins. Hydrolysis of milk proteins allows the selection of the protein substrate and enzyme specificity to optimize the yield of bioactive peptides. Pancreatic enzymes, especially trypsin and other endoproteinases, such as chymotrypsin, pepsin and thermolysin as well as proteinase extracts derived from bacteria, moulds, plants and animal have been used (**4**). Mullally *et al* (**76**) used gastric and pancreatic proteases to hydrolyze whey proteins in order to obtain ACE-inhibitory peptides. Hydrolysis of α-La and β-Lg with pepsin and trypsin separately or in combination resulted similar ACE-inhibitory activities. The order of enzyme additions affected types of peptides present in final hydrolysates but not on ACE-inhibition.

The amino acid sequence of milk proteins has been reported, and can be used to evaluate the presence of bioactive peptides by computer-aided models. *In silico* screening was used to rank individual milk proteins according to their potential to generate ACE-inhibitory peptides and to evaluate the effect of initial proteolysis on the formation of ACE-inhibitory peptides during gas-

Table 3 Structural elements and activities of bioactive peptides

Activity	*Structural elements*	*Remarks*	*Encrypted protein*	*Identified peptides/names*
Opioid	Tyr at N-terminus, coupled with another aromatic residue, e.g., Phe or Tyr in positions 3or 4	Fits into the binding site of opioid receptors	α-La β-Lg Serum alb	α-lactorphin (Tyr-Gly-Leu-Phe) β-lactorphin (Tyr-Leu-Leu-Phe) Serorphin (Tyr-Gly-Phe-Asn-Ala)
ACE inhibitory	Hydrophobic amino acids or positive charge of Lys or Arg as C terminus	Influences the binding to ACE	α-La β-Lg	Lactokinins (Tyr-Gly-Leu, Leu-Ala-His-Lys-Ala-Leu, Trp-Leu-Ala-His-Lys) Lactokinins (Ala-Leu-Pro-Met-His-Ile-Arg, Leu-Ala-Met-Ala, His-Ile-Arg)
Antithrombotic	Ile^{108}, Lys^{112} and Asp^{115} residues of casoplatelins Sugar content	Important antithrombotic activity	κ-casein	Glycomacropeptide
Antioxidant	High amounts of His and hydrophobic amino acids	Peptides with a Pro-His-His sequence show greatest antioxidant activity	β-Lg	Met-His-Ile-Arg-Leu, Tyr-Val-Glu-Glu-Leu
Hypocholesterolemic	Low ratios of methionine-glycine and lysine-arginine in the dietary proteins. High amount of hydrophobic amino acids	Hydrophobic amino acids bind bile acids and enhance fecal steroid excretion	β-Lg	Lactostatins (Ile-Ile-Ala-Glu-Lys, Gly-Leu-Asp-Ile-Gln-Lys, Ala-Leu-Pro-Met-His)
Antiobesity	Peptide length Multiple Arg residues	Influences on CCK releasing activity Necessary condition for CCK release through binding to intestinal cells		
Antimicrobial	Positively charged residues and hydrophobic region that contains tryptophan	Trp involved in membrane interactions	Lactoferrin β-Lg α-La	Lactoferricin Ala-Gly-Thr-Trp-Tyr, Ile-Pro-Ala-Val-Phe-Lys Gly-Tyr-Gly-Gly-Val-Ser-Leu-Pro-Trp-Val-Cys-Thr-Thr-Phe Ala-Leu-Cys-Ser-Glu-Lys

Information compiled from Hartmann and Meisel (**8**), Meisel *et al* (**19**), Hernandes-Ledesma *et al* (**20**), Erdman et al (**74**) and Pellegrini *et al* (**75**)

trointestinal digestion. A database containing the amino acid sequence of milk proteins was combined with the database containing the sequence and IC_{50}-values of 498 ACE-inhibitory peptides reported in literature. Fifty different ACE-inhibitory peptides were identified in β-Lg, giving the highest ranking among the milk proteins (**77**).

Dzuiba and Iwaniak (**78**) developed similar method, a database containing protein and bioactive peptide sequences. In another study, initial proteolytic cleavage at the C-end of amino acids isoleucine and proline increased ACE-inhibitory peptides after gastrointestinal proteolysis of milk proteins. Cleavage after most other amino acid residues had little or no effect (**79**). Theoretical predictions and simulations are considered as an emerging tool in peptide science. Although these models give some insight into the number and type of active peptide sequences in milk and other food proteins, they do not take into account the release of active peptides from the source proteins and therefore the results have to interpret with caution (**80**).

The production of bioactive peptides using enzymatic digestion can be studied using response surface methodology. Van de Ven *et al* (**81**) showed that the ACE-inhibitory activity of whey protein hydrolysates could be controlled by systematic regulation of process conditions. Roufik *et al* (**82**) demonstrated that the *in vitro* digestibility of bioactive peptides derived from bovine β-Lg is not only influenced by pH, time of incubation and enzyme/substrate ratio, but also by the length and the nature of the peptides.

Also, other peptides present in the medium can influence the hydrolysis by gastric enzymes. A higher degree of proteolysis was obtained for pepsin and chymotrypsin when the β-lactorphin (β-Lg f102-105) was combined with β-Lg f92-105, than when peptides were hydrolysed separately. It was suggested that in a mixture, peptides may form complexes that interact more efficiently with

the hydrolyzing enzymes compared with peptides alone.

2. Fermentation

Many industrially-used dairy starter cultures are highly proteolytic. The peptides and amino acids liberated during fermentation contribute to the typical flavor, aroma and texture of dairy products. The proteolytic system of lactic acid bacteria contribute to the liberation of bioactive peptides during fermentation. The cell-wall bound proteinases, which have a very broad specificity, initiate the proteolysis and release of oligopeptides. Oligopeptides that cannot be transported into the cells can be further degraded after lysis of bacterial cell, which allows release of intracellular peptidases, such as di-, tri-, amino- or endopeptidases. It is important to choose the right strains or combination of strains with optimal proteolytic activity and lysis at the right time, as the number of bioactive peptides relies on a balance between formation and degradation into inactive peptides and amino acids (**4**). Over the last years several articles and book chapters have reviewed the release of bioactive peptides during fermentation. Many studies have demonstrated that *Lactobacillus helveticus* strains are capable of releasing antihypertensive peptides, the best known of which are ACE-inhibitory tripeptides Val-Pro-Pro (VPP) and Ile-Pro-Pro (IPP). Pihlanto-Leppälä *et al* (**83**) studied the potential formation of ACE-inhibitory peptides from cheese whey during fermentation with various commercial lactic acid starters used in the manufacture of yoghurt, ropy milk and sour milk. No ACE-inhibitory activity was observed after fermentation, but further digestion with pepsin and trypsin was needed to produce ACE-inhibitory properties. Belem *et al* (**84**) fermented whey with Kluyvermyces *marxinaus* var *marxianus* and identified peptide corresponding to β-lactorphin sequence in the hydrolysate. In a recent study, Pan and Guo (**85**) fermented whey with *Lb. helveticus* LB10 and identified a novel ACE-inhibitory peptide from β-Lg (f148-153, Arg-Leu-Ser-Phe-Asn-Pro). Paul and Somkuti (**86**) demonstrated that antimicrobial and hypotensive polypeptides released from lactoferrin by pepsin remain mostly intact at pH 4.5 when added into yoghurt at the end of the fermentation process.

3. Enrichment and separation of bioactive peptides

Various technologies have been applied for the enrichment and separation of bioactive peptides from the hydrolysates. Membrane filtration shows most potential to be used in pilot and industrial scale preparation. Use of precipitation is limited due to low specificity and chromatography due to high cost.

Stepwise filtration *Stepwise* filtration has been found to be useful to separate small peptides. Pihlanto-Leppälä (**87**) enriched successfully opioid and ACE-inhibitory peptides from α-La and β-Lg using membranes with molecular weight cut-off of 1 and 30 kDa. The peptide separation from a tryptic hydrolysate of whey protein using nano/ultra-filtration is influenced by the characteristics of the membrane and the ionic strength and pH of the solution (**88**). Moreover peptide-peptide interactions can influence the transmission of bioactive peptides (**89**). Electro-membrane filtration (EMF) was applied to selective separate cationic peptides from a tryptic hydrolysate of β-Lg (**90**).

Chromatography Chromatographic separatory methods have been applied to purify lactoferricin from pepsin treated lactoferrin. Recio and Visser (**91**) described a method where the protein of interest was concentrated in a chromatographic medium and hydrolysed *in situ* with an appropriate enzyme. Alternative method is absorptive membrane chromatography, which combines the use of chromatography with membrane filtration. The strong acidic membrane has been applied for the purification of lactoferricin (**92**).

Efficacy of peptides

The potential physiological role of bioactive peptides derived from milk proteins and the *in situ* formation of these peptides in the gastrointestinal tract is still largely unknown. It is known that di- and tri-peptides are absorbed easily in the intestine. Little, however, is known about the absorption of bioactive peptides with larger molecular weight in the intestine. In order to exert a beneficial effect, for example anti-hypertensive peptides have to be absorbed from the intestine and reach the target cell in the blood vessels in substantial concentrations. Many studies *in vitro* have been performed to determine the stability of different ACE inhibitory peptides to survive gastrointestinal passage and to determine if ACE inhibitory peptides can be transported through intestinal cells. Walsh *et al* (**93**) have studied the stability of β-Lg (f142-148). The peptide was degraded by gastric enzymes and human serum. Moreover, β-Lg (f142-148) could not be detected in the sera of 2 human volunteers following its ingestion or in sera from these volunteers subsequently spiked with β-Lg (f142-148). Vermeirssen *et al* (**94**) demonstrated that this peptide could be transported intact through a Caco-2 Bbe cell monolayer. However, the concentrations transported were reported to be too low to exert an ACE-inhibitory effect *in vitro*. While valuable information can be obtained from *in vitro* model systems with respect to the proteolytic/peptidolytic stability and susceptibility to intracellular passage, however, it is only through *in vivo* studies that the hypotensive affects of a given peptide or peptide preparation can be reliably assessed. Based on data from clinical and preclinical in vivo studies, the absorption and bioavailability of milk-derived tripeptides is poor due to extensive hydrolysis in the gastrointestinal tract (95). The peptides absorbed in the circulation are also eliminated very rapidly. Furthermore, it should be mentioned that in vivo kinetic studies investigating the absorption, distribution, metabolism and excretion of the biopeptides are, in general, very sparse.

Numerous studies in spontaneously hypertensive rats (SHR) as well as in hypertensive human volunteers have been performed to determine the antihypertensive effect of milk-derived peptides. These studies demonstrated that several ACE-inhibitory peptides significantly reduce blood pressure, either after intravenous or oral administration. The best characterized ACE-inhibitory peptides are Val-Pro-Pro and Ile-Pro-Pro, found in fermented milk. About twenty human studies have been published linking the consumption of products containing these two tripeptides with a significant reduction in both systolic blood pressure (SBP) and diastolic blood pressure (DBP). Maximum blood pressure reductions approximate 13 mmHg (SBP) and 8 mmHg (DBP) after active treatment compared to placebo, and are likely to reach after 8-12 weeks of treatment. Effective dosages of tripeptides range from 3.07 to 52.5 mg/day. *In vivo* comparative studies with captopril, a clinically used ACE inhibitor, have shown that ACE-inhibitory peptides with an antihypertensive effect exhibit higher in vivo activity than could be expected from their *in vitro* activity (**8,74,96-99**). Although data from *in vitro* studies suggest that tripeptides act as ACE inhibitors at high concentration, it is unlikely that tripeptides at dosages used in the clinical studies produces such high plasma levels due to very poor bioavailability of the peptides. As the dairy products supplemented with biopeptides contain substantial amounts of potassium, calcium and magnesium, increased intakes of these electrolytes may explain, at least in part, the antihypertensive effects of the tripeptides reported in clinical and preclinical studies.
Animal studies have shown that whey hydrolysates and peptides have antihypertensive (**43,100**) and hypocholesterolemic activity (**25**). Pins and Keenan (**101**) evaluated the effects of a whey hydrolysate in 30 subjects with mild hypertension. After 6 weeks of treatment the whey hydrolysate (20g/day) group significantly reduced SBP by 8 mmHg and DBP by 6 mm Hg compared to control. An additional benefit of lower LDL cholesterol and C-reactive protein (a marker of inflammation) was also noted in the whey hydrolysate group. The most likely mechanism for this blood pressure-lowering effect is inhibition of ACE activity because milk has been shown to be a rich source of ACE inhibitory peptides. A recent study by Ballard *et al* (**102**) suggested that individuals with normal endothelial function, the acute ingestion of a peptide derived from whey improves both conduit and resistance vascular response. The results indicate that the peptide could be of value in populations with vascular dysfunction or as a method to attenuate vascular dysfunction associated with postprandial period.

CONCLUSIONS AND FUTURE PROSPECTS

Our appreciation of milk has grown substantially from a time when it was seen purely as an excellent source of protein and calcium. Dairy-derived bioactive ingredients have attracted growing attention in the food and pharmaceutical industries over the last decade. Technologies have been developed for recovery of bioactive components from milk, whey and colostrum and studies have been carried out to establish the activities of these components in different food matrices. However, there are many investigations of the effects of milk proteins and peptides in vitro and on cells in culture, but whether the results are relevant for oral feeding outcomes remains to be established in many cases.

The data for claiming effect on human health is still insufficient in many cases. The active components may be degraded during digestion, may not be absorbed or not attain the appropriate concentrations in blood or target tissues that are required for acting significantly. Processing procedures, especially heating, may influence on the bioactivity and may also lead to formation of undesired toxic, allergic or carcinogenic substances. Moreover, the bioactivity of the molecules may be reduced through alteration during processing or interaction with other food components. Protein hydrolysates can have bitter taste which prevents use as food additive.
There is a challenge for food technologist to develop functional foods and nutraceutical without the undesired side effects of added hydrolysates or peptides and to stabilize the peptides during processing and shelf life. Moving from science to commercial applications seems now appropriate as the fundamental biological properties and mechanism of action of milk proteins, bioactive peptides and growth factors are reasonably well established. It appears that the wide biological diversity of these components make them an excellent source for a number of applications in health-promoting foods. However, controlled trials in humans are mandatory when claiming a health effect for a food component. So far, the sour milk products containing the tripeptides, are examples for food products available on market whose effects on blood pressure has proven in human trials. Also effects of whey proteins and peptides have been demonstrated in human trials to prevent microbial infections, stimulation of immune system and reduction of blood pressure (***Table 2***).

In view of the current global trend of increasing prevalence of obesity and type two diabetes, more experimental research should be focused on whey proteins and peptides which can regulate appetite, reduce inflammation signals and manage blood glucose balance. More research is also warranted about the impairment of cognitive functions, memory-related diseases and mood control. In this context, antioxidative and opioid properties of whey proteins and peptides are worth further investigations.
It can be envisaged that several special whey protein formulations and peptide-based products will be developed and marketed as part of a regular healthy diet.

REFERENCES

1 **Schrezenmeir J, Korhonen H, Williams M et al (2000)**
Foreword
Br J Nutr **84** S1

2 **Goldman AS (1993)**
The immune system of human milk: antimicrobial, antiinflammatory and immunomodulating properties
Pediatr Infect Dis J **12** 664-671

3 **Fox P (2009)**
Milk: an overview. In: *Milk Proteins from Expression to Food* Thompson A, Boland M, Singh H eds, Academic Press, San Diego, CA, p 1-54

4 **Korhonen H, Pihlanto A (2007)**
Technological options for the production of health-promoting proteins and peptides derived from milk and colostrum
Curr Pharm Des **13** 829-843

5 **Korhonen HJ (2009)**
Bioactive components in bovine milk. In: *Bioactive Components in Milk and Dairy Products* Park YW ed, Wiley-Blackwell, Iowa, p 15-42

6 **Smithers GW (2008)**
Whey and whey proteins – from 'gutter-to-gold'
Int Dairy J **18** 695-704

7 **Krissansen GW (2007)**
Emerging health properties of whey proteins and their clinical implications
J Am Coll Nutr **26** 713S-723S

8 **Hartmann R, Meisel H (2007)**
Food-derived peptides with biological activity: from research to food applications
Curr Opin Biotechnol **18** 1-7

9 **Korhonen H, Pihlanto A (2006)**
Bioactive peptides: Production and functionality
Int Dairy J **16** 945-960

10 **Eigel VN, Buytler JE, Ernstrom CA *et al* (1984)**
Nomenclature of proteins of cow's milk: fifth revision
J Dairy Sci **67** 1599-1631

11 **Luhovyy BL, Akhavan T, Anderson H (2007)**
Whey proteins in the regulation of food intake and satiety
J Am Coll Nutr **26** 704S-712S

12 **Gunnarsson PT, Winzell MS, Deacon CF *et al* (2006)**
Glucose-induced incretin hormone release and inactivation are differently modulated by oral fat and protein in mice
Endocrinology **147** 3173-3180

13 **Pal S, Ellis V, Dhaliwal S (2010)**
Effects of whey protein isolate on body composition, lipids, insulin and glucose in overweight and obese individuals
Br J Nutr **104** 716-723

14 **Akhavan T, Luhovyy BL, Brown PH, Cho CE, Anderson GH (2010)**
Effect of premeal consumption of whey protein and its hydrolysate on food intake and postmeal glycemia and insulin responses in young adults
Am J Clin Nutr **91** 966-975

15 **Ha E, Zemel MB (2003)**
Functional properties of whey, whey components, and essential amino acids: mechanisms underlying health benefits for active people (review)
J Nutr Biochem **14** 251-258

16 **Pennings B, Boirie Y, Senden JM, Gijsen AP *et al* (2011)**
Whey protein stimulates postprandial muscle protein accretion more effectively than do casein and casein hydrolysate in older men
Am J Clin Nutr **93**(5) 997-1005

17 **Edwards PB, Creamer LK, Jameson GB (2009)**
Structure and stability of whey proteins In: *Milk Proteins from Expression to Food* Thompson A, Boland M, Singh H eds, Academic Press San Diego, CA p 163-203

18 **Chatterton DEW, Smithers G, Roupas P, Brodkorb A (2006)**
Bioactivity of β-lactoglobulin and α-lactalbumin β-Technological implications for processing
Int Dairy J **16** 1229-1240

19 **Meisel H, Walsh D, Murray B, FitzGerald RJ (2009)**
ACE inhibitory peptides In: *Nutraceutical Proteins and Peptides in Health and Disease* Mine Y, Shadi F eds, Taylor Francis Group, Boca Raton FL, p 269-315

20 **Hernández-Ledesma B, Recio I, Amigo L (2008)**
β-Lactoglobulin as source of bioactive peptides
Amino Acids **35** 257-265

21 **Hernández-Ledesma B, Dávalos A *et al* (2005)**
Preparation of antioxidant enzymatic hydrolysates from α-lactalbumin and β-lactoglobulin Identification of active Peptides by HPLC-MS/MS
J Agric Food Chem **53** 588-593

22 **Sipola M, Finckenberg P, Vapaatalo H *et al* (2002)**
[alpha]-lactorphin and [beta]-lactorphin improve arterial function in spontaneously hypertensive rats
Life Sci **71** 1245-1253

23 **Yamauchi R, Ohinata K, Yoshikawa M (2003)**
Beta-lactotensin and neurotensin rapidly reduce serum cholesterol via NT2 receptor
Peptides **24** 1955-1961

24 **Yamauchi R, Wada E, Yamada D, Yoshikawa M *et al* (2006)**
Effect of [beta]-lactotensin on acute stress and fear memory
Peptides **27** 3176-3182

25 **Nagaoka S (2006)**
Cholesterol lowering proteins and peptides In: *Nutraceutical Proteins and Peptides in Health and Disease* Mine Y, Shahidi T eds, Taylor Francis Group, Boca Raton FL, p 41-68

26 **Jackson JG, Janszen DB, Lonnerdal B *et al* (2004)**
A multinational study of α-lactalbumin concentrations in human milk
J Nutr Biochem **15** 517-521

27 **Markus CR, Jonkman LM, Lammers JHCM *et al* (2005)**
Evening intake of α-lactalbumin increases plasma tryptophan availability and improves morning alertness and brain measures of attention
Am J Clin Nutr **81** 1026-1033

28 **Scrutton H, Carbonnier A, Cowen PJ, Harmer C (2007)**
Effects of α-lactalbumin on emotional processing in healthy women
J Psychopharmacol **21** 519-524

29 **Ushida Y, Shimokawa Y, Matsumoto H *et al* (2003)**
Effects of bovine α-lactalbumin on gastric defense mechanisms in naive rats
Biosci Biotechnol Biochem **67** 577-583

30 **Rosaneli CF, Bighetti AE, Antonio MA *et al* (2004)**
Protective effect of bovine milk whey protein concentrate on the ulcerative lesions caused by subcutaneous administration of indomethacin
J Med Food **7** 309-314

31 **Mezzaroba LFH, Carvalho JE, Ponezi AN *et al* (2006)**
Antiulcerative properties of bovine [alpha]-lactalbumin
Int Dairy J **16** 1005-1012

32 Blouet C (2007)
Meal cysteine improves postprandial glucose control in rats fed a high-sucrose meal
J Nutr Biochem **18** 519-524

33 Pilvi TK, Harala S, Korpela R, Mervaala EM (2009)
Effects of high-calcium diets with different whey proteins on weight loss and weight regain in high-fat-fed C57BL/6J mice
Br J Nutr **102** 337-341

34 Hursel R, van der Zee L, Westerterp-Plantenga MS (2010)
Effects of a breakfast yoghurt, with additional total whey protein or caseinomacropeptide-depleted alpha-lactalbumin-enriched whey protein, on diet-induced thermogenesis and appetite suppression
Br J Nutr **103** 775-780

35 EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA) (2010)
Scientific Opinion on the substantiation of health claims related to whey protein and increase in satiety leading to a reduction in energy intake (ID 425), contribution to the maintenance or achievement of a normal body weight (ID 1683), growth or maintenance of muscle mass (ID 418, 419, 423, 426, 427, 429, 4307), increase in lean body mass during energy restriction and resistance training (ID 421), reduction of body fat mass during energy restriction and resistance training (ID 420, 421), increase in muscle strength (ID 422, 429), increase in endurance capacity during the subsequent exercise bout after strenuous exercise (ID 428), skeletal muscle tissue repair (ID 428) and faster recovery from muscle fatigue after exercise (ID 423, 428, 431), pursuant to Article 13(1) of Regulation (EC) No 1924/20061
EFSA J **8** 1818

36 Sternhagen LG, Allen JC (2001)
Growth rates of a human colon adenocarcinoma cell line are regulated by the milk protein alphalactalbumin
Adv Exp Med Biol **501** 115-120

37 Gustafsson L, Leijonhufvud I, Aronsson A *et al* (2004)
Treatment of Skin Papillomas with Topical α-Lactalbumin-Oleic
Acid N Engl J Med **350** 2663-2672

38 Gustafsson L, Hallgren O, Mossberg A *et al* (2005)
HAMLET kills tumor cells by apoptosis: structure, cellular mechanisms, and therapy
J Nutr **135** 1299-1303

39 Wong CW, Seow HF, Husband AJ, Regester GO *et al* (1997)
Effects of purified bovine whey factors on cellular immune functions in ruminants
Vet Immunol Immunopathol **56** 85-96

40 Kayser H, Meisel H (1996)
Stimulation of human peripheral blood lymphocytes by bioactive peptides derived from bovine milk proteins
FEBS Lett **383** 18-20

41 Jaziri M, Migliore-Samour D, Casabianca-Pignède MR *et al* (1992)
Specific binding sites on human phagocytic blood cells for Gly-Leu-Phe and Val-Glu-Pro-Ile-Pro-Tyr, immunostimulating peptides from human milk proteins
Biochim Biophys Acta **1160** 251-261

42 **Pihlanto-Leppälä A, Koskinen P, Piilola K *et al* (2000)**
Angiotensin I-converting enzyme inhibitory properties of whey protein digests: concentration and characterization of active peptides
J Dairy Res **67** 53-64

43 Nurminen M-L ,Sipola M, Kaarto H *et al* (2000)
Alpha-lactorphin lowers blood pressure measured by radiotelemetry in normotensive and spontaneously hypertensive
rats Life Sci **66** 1535-1543

44 Thomä-Worringer C, Sörensen J, López-Fandino R (2006)
Health effects and technological features of caseinomacropeptide
Int Dairy J **16** 1324-1333

45 Yvon M, Beucher S, Guilloteau P, Le Huerou-Luron I *et al* (1994)
Effects of caseinomacropeptide (CMP) on digestion regulation
Reprod Nutr Dev **34** 527-537

46 Chabance B, Marteau P, Rambaud JC *et al* (1998)
Casein peptide release and passage to the blood in humans during digestion of milk or yogurt
Biochimie **80** 155-165

47 Burton-Freeman BM (2008)
Glycomacropeptide (GMP) is not critical to whey-induced satiety, but may have a unique role in energy intake regulation through cholecystokinin (CCK)
Physiol Behav **93** 379-387

48 Royle PJ, McIntosh GH, Clifton PM (2008)
Whey protein isolate and glycomacropeptide decrease weight gain and alter body composition in male Wistar rats
Br J Nutr **100** 88-93

49 Gustafson DR, McMahon DJ, Morrey J, Nan R (2001)
Appetite is not influenced by a unique milk peptide: caseinomacropeptide (CMP)
Appetite **36** 157-163

50 Keogh JB, Woonton BW, Taylor CM *et al* (2010)
Effect of glycomacropeptide fractions on cholecystokinin and food intake
Br J Nutr **104** 286-290

51 Brody EP (2000)
Biological activities of bovine glycomacropeptide
Br J Nutr **84** S39-S46

52 Manso MA, López-Fandino R (2004)
K-Casein macropeptides from cheese whey: Physicochemical, biological, nutritional, and technological features for possible uses
Food Rev Int **20** 329-355

53 Requena P, González R, López-Posadas R *et al* (2010)
The intestinal antiinflammatory agent glycomacropeptide has immunomodulatory actions on rat splenocytes
Biochem Pharmacol **79** 1797-1804

54 López-Posadas R, Requena P, González R *et al* (2010)
Bovine glycomacropeptide has intestinal antiinflammatory effects in rats with dextran sulfate-induced colitis
J Nutr **140** 2014-2019

55 Gapper LW, Copestake DE J, Otter DE, Indyk HE (2007)
Analysis of bovine immunoglobulin G in milk, colostrum and dietary supplements: a review
Anal Bioanal Chem **389** 93-109

56 Korhonen HJT, Marnila P (2009)
Bovine milk immunoglobulins against microbial human diseases In: *Dairy Ingredients Food and Nutraceutical Uses* Corredig M ed, Woodhead Publishing Limited Oxford, p 269-289

57 Scammel AW (2001)
Production and uses of colostrums
Aust J Dairy Technol **56** 74-82

58 Tripathi V, Vashishtha B (2006)
Bioactive compounds of colostrum and its application
Food Rev Int **22** 225-244

59 Crittenden R, Buckley J, Cameron-Smith D *et al* (2009)
Functional dairy protein supplements for elite athletes
Aust J Dairy Technol **64** 133-137

60 Kelly GS (2003)
Bovine colostrums: a review of clinical uses
Altern Med Rev **8** 378-394, 2003

61 Wakabayashi H, Yamauchi K, Takase M (2006)
Lactoferrin research, technology and application
Int Dairy J **16** 1241-1251

62 Marnila P, Korhonen HJT (2009)
Lactoferrin for human health In: *Dairy Ingredients Food and Nutraceutical Uses* Corredig M ed, Woodhead Publishing Limited Oxford, p 290-307

63 Zimecki M, Kruzel ML (2007)
Milk-derived proteins and peptides of potential therapeutic and nutritive value
J Exp Ther Oncol **6** 89-106

64 Ushida Y, Sekine K, Kuhara T *et al* (1999)
Possible chemopreventive effects of bovine lactoferrin on esophagus and lung carcinogenesis in the rat Jpn
J Cancer Res **90** 262-267

65 Mohan KV, Letchoumy PV, Hara Y, Nagini S (2008)
Combination chemoprevention of hamster buccal pouch carcninogensis by bovine lactoferrin and black tea polyphenols
Cancer Invest **26** 193-201

66 Lee WJ, Farmer JL, Hilty M, Kim YB (1998)
The protective effects of lactoferrin feeding against endotoxin lethal shock in germfree piglets
Infect Immun **66** 1421-1426

67 Broxmeyer HE, Williams DE, Hangoc G *et al* (1987)
The opposing actions in vivo on murine myelopoiesis of purified preparations of lactoferrin and the colony stimulating factors
Blood Cells **13** 31-48

68 Elrod KC, Moore WC, Abraham WM, Tanaka RD (1997)
Lactoferrin, a potent tryptase inhibitor, abolishes late-phase airway responses in allergic sheep
Am J Respir Crit Care Med **156** 375-381

69 Cornish J (2004)
Lactoferrin promotes bone growth
BioMetals **17** 331-335

70 Kussendrager KD, van Hooijdonk ACM (2000)
Lactoperoxidase: physico-chemical properties, occurrence, mechanism of action and applications
Br J Nutr **84** 19-25

71 Seifu E, Buys EM, Donkin EF (2005)
Significance of the lactoperoxidase system in the dairy industry and its potential applications: a review
Trends Food Sci Technol **16** 137-154

72 Gauthier SF, Pouliot Y, Maubois J-L (2006)
Growth factors from bovine milk and colostrum : composition, extraction and biological activities
Lait **86** 99-12

73 Montoni A, Gauthier SF, Richard C *et al* (2009)
Bovine colostrum as substrate for the preparation of growth factor-enriched protein extracts: Identifying the optimal collection period during lactation
Dairy Sci Technol **89** 511-518

74 Erdmann K, Cheung BW, Schroder H (2008)
The possible roles of food-derived bioactive peptides in reducing the risk of cardiovascular disease
J Nutr Biochem **19** 643-654

75 Pellegrini A (2003)
Antimicrobial peptides from food proteins
Curr Pharm Des **9** 1225-1238

76 Mullally MM, Meisel H, FitzGerald RJ (1997)
Angiotensin-I-converting enzyme inhibitory activities of gastric and pancreatic proteinase digests of whey proteins
Int Dairy J **7** 299-303

77 Vermeirssen V, van der Bent A, Van Camp J *et al* (2004)
A quantitative in silico analysis calculates the angiotensin I converting enzyme (ACE) inhibitory activity in pea and whey protein digests
Biochimie **86** 231-239

78 Dzuiba J, Iwaniak A (2006)
Database of protein and bioactive peptide sequences In: N*utraceutical Proteins and Peptides in Health and Disease* Mine Y, Shahidi T eds, Taylor Francis Group, Boca Raton FL, p 543-563

79 Pripp AH (2005)
Initial proteolysis of milk proteins and its effect on formation of ACE-inhibitory peptides during gastrointestinal proteolysis: a bioinformatic, in silico, approach
Eur Food Res Technol **221** 712-716

80 80. Minkiewicz P, Dziuba J, Iwaniak A, Dziuba M, Darewicz M (2008)
BIOPEP database and other programs for processing bioactive peptide sequences
J AOAC Int **91** 965-980

81 Ven van der C, Gruppen H, de Bont DBA, Voragen AGJ (2002)
Optimisation of the angiotensin converting enzyme inhibition by whey protein hydrolysates using response surface methodology
Int Dairy J **12** 813-820

82 Roufik S, Gauthier SF, Turgeon SL (2006)
In vitro digestibility of bioactive peptides derived from bovine [beta]-lactoglobulin
Int Dairy J **16** 294-302

83 Pihlanto-Leppala A, Rokka T, Korhonen H (1998)
Angiotensin I Converting Enzyme Inhibitory Peptides Derived from Bovine Milk Proteins
Int Dairy J **8** 325-331

84 Belem MAF, Gibbs BF, Lee BH (1999)
Proposing sequences for peptides derived from whey fermentation with potential bioactive sites
J Dairy Sci **82** 486-493

85 Pan D, Guo Y (2010)
Optimization of sour milk fermentation for the production of ACE-inhibitory peptides and purification of a novel peptide from whey protein hydrolysate
Int Dairy J **20** 472-479

86 Paul M, Somkuti GA (2009)
Degradation of milk-based bioactive peptides by yogurt fermentation bacteria
Lett Appl Microbiol **49** 345-350

87 Pihlanto-Leppälä A (2001)
Bioactive peptides derived from bovine whey proteins: opioid and ACE-inhibitory peptides Trends
Food Sci Technol **11** 347-356

88 Pouliot Y, Gauthier SF, L´Heureux J (2000)
Effect of peptide distribution on the fractionation of whey protein hydrolysate using nanofiltraton membranes
Lait **80** 113-120

89 Lapointe JF, Gauthier SF, Pouliot Y, Bouchard C (2003)
Effect of hydrodynamic conditions on fractionation of beta-lactoglobulin tryptic peptides using nanofiltration membranes
J Membr Sci **212** 55-67

90 Lapointe JF, Gauthier SF, Pouliot Y, Bouchard C (2006)
Selective separation of cationic peptides from a tryptic hydrolysate of beta-lactoglobulin by electrofiltration
Biotechnol Bioeng **94** 223-233

91 Recio I, Visser S (1999)
Two ion-exchange chromatographic methods for the isolation of antibacterial peptides from lactoferrin In situ enzymatic hydrolysis on an ion-exchange membrane
J Chromatogr A **831** 191-201

92 Plate K, Beutel S, Buchholz H et al (2006)
Isolation of bovine lactoferrin, lactoperoxidase and enzymatically prepared lactoferricin from proteolytic digestion of bovine lactoferrin using adsorptive membrane chromatography
J Chromatogr A **1117** 81-86

93 Walsh DJ, Bernard H, Murray BA et al (2004)
In vitro generation and stability of the lactokinin β-lactoglobulin fragment (142-148)
J Dairy Sci **87** 3845-3857

94 Vermeirssen V, Deplancke B, Tappenden K A, Camp J Van, Gaskins HR, Verstraete W (2002)
Intestinal transport of the lactokinin Ala-Leu-Pro-Met-His-Ile-Arg through a Caco-2 Bbe monolayer
J Peptide Sci **8** 95-100

95 Foltz M, van der Pijl PC, Duchateau GSMJE (2011)
Current in vitro testing of bioactive peptides is not valuable
J Nutr **140** 117-118

96 Jauhiainen T, Korpela R (2007)
Milk peptides and blood pressure
J Nutr **137** 825S-829S

97 Murray BA, FitzGerald RJ (2007)
Angiotensin converting enzyme inhibitory peptides derived from food proteins: Biochemistry, bioactivity and production
Curr Pharm Des **13** 773-791

98 Saito T (2008)
Antihypertensive peptides derived from bovine casein and whey proteins
Adv Exp Med Biol **606** 295-317

99 Boelsma E, Kloek J (2009)
Lactotripeptides and antihypertensive effects: A critical review
Br J Nutr **101** 776-786

100 Scholthauer R-C, Schollum LM, Reid JR *et al* (2006)
Bioactive whey protein hydrolysate *US Patent No* US 7,148,034 B2

101 Pins JJ, Keenan JM (2006)
Effects of whey peptides on cardiovascular disease risk factors
J Clin Hypertens **8** 775-782

102 Ballard K, Bruno R, Seip R *et al* (2009)
Acute ingestion of a novel whey-derived peptide improves vascular endothelial responses in healthy individuals: a randomized, placebo controlled trial
Nutr J **8** 34