Bioactive proteins and peptides in foods

Barbara Walther, Robert Sieber
Agroscope Liebefeld-Posieux Research Station ALP, Schwarzenburgstrasse 16, CH-3003 Bern

Summary

An increasing amount of data demonstrate a bioactive role of proteins and peptides beyond their nutritional impact. The focus of the investigations has mainly been on vitamin- and mineral-binding proteins, on antimicrobial, immunosuppressing/-modulatory proteins, on proteins with enzyme inhibitory activity as well as on hormones and growth factors from different food proteins; most research has been performed in milk proteins. Because of their molecular size intact absorption of proteins in the human gastrointestinal tract is limited. Therefore, most of the proteins with biological functions show physiological activity in the gastrointestinal tract by enhancing nutrient absorption, inhibiting enzymes and modulating the immune system to defend against pathogens.

Peptides are released during fermentation or digestion from food proteins by proteolytic enzymes, such peptides have mainly been found in milk. Some of these released peptides exert biological activities such as opiate-like, antihypertensive, mineral-binding, antioxidative, antimicrobial, immuno- and cytomodulating activity. Intact absorption of these smaller peptides is more likely than that of the larger proteins. Consequently, other organs than the gastrointestinal tract are possible targets for their biological functions. Bioactive proteins as well as bioactive peptides are part of a balanced diet. It is possible to accumulate bioactive peptides in food, for example by using specific microorganisms in fermented dairy products. Although bioactive peptides have been the subject of several studies *in vitro* and *in vivo*, their health potential is still under investigation. Up to now, the Commission of European Communities did not (yet) authorize any health claims for bioactive proteins and peptides from food.

Introduction

Proteins, along with carbohydrates and fats, are one of the three main macro-nutrients in food. Over the last century, protein research mainly investigated the importance of essential amino acids and their relevance for nutrition and health. As part of this work, nutritional science focused on other issues related to the biological value of proteins. Specifically, the biological functions of certain dietary proteins was investigated in greater detail. Bioactive proteins are referred to dietary proteins with special bioactivities that have the potential to influence health, mainly in a beneficial way. This statement, however, does not include the potentially damaging effects on human physiology such as

toxicity, allergenicity and mutagenicity, which are undoubtedly examples of “bioactivity” in its broadest sense [1]. Furthermore, it has been recognized that peptides with particular amino acid sequences which are inactive in the intact protein may exert biological functions after their release from the intact molecule. Such bioactive peptides have been defined as ‘peptides with hormone- or drug-like activity’ that eventually modulate physiological functions through binding interactions to specific receptors on target cells, leading to induction of physiological responses [2].

Bioactive proteins

Most of the dietary proteins demonstrating biological activity which have been investigated to date originated from milk (immunoglobulins, caseins, whey proteins). However, proteins from other animal sources as well as plant proteins have been reported to exert specific bioactivities. A broad spectrum of proteins shows physiological activity in the gastrointestinal tract. These activities range from enhancement of nutrient absorption, inhibition of enzymes, enzyme activity, growth stimulation to modulation of the immune system in defending against pathogens.

A number of proteins have been suggested to facilitate the uptake of essential nutrients. For example α- and β-caseins are thought to enhance calcium uptake by forming soluble casein phosphopeptides during digestion; lactoferrin appears to facilitate iron uptake, whereas other proteins such as vitamin B_{12}-binding protein (haptocorrin) and folate-binding protein improve the availability of vitamins. The bioactivities observed for a number of milk proteins such as immunoglobulins, vitamin- or mineral-binding proteins indicate that bioactive proteins are of importance for the development and protection of newborn mammals [3].

Vitamin-binding proteins

Folate and vitamin B_{12} from bovine milk are strongly bound to whey proteins. The involved proteins may favor the transition of these vitamins from the blood plasma into milk of the animal and facilitate their direct absorption in the gut. Additionally, the binding of vitamins prevents their degradation or uptake by the intestinal microflora and, as a result of the limited availability, inhibits the growth of pathogens. Bovine milk is not a rich source of folate but does contain an excess of folate-binding proteins. In combination with folate-rich foods, these proteins may improve the bioavailability of folate [4]. The protein β-lactoglobulin is able to bind small hydrophobic molecules like retinol and fatty acids. Its binding capacity is high at pH levels above 7.4 and decreases until a pH of 5.5 is reached. A reduction of pH to below 5.5 is accompanied by structural changes of β-lactoglobulin from an open to a closed conformation, whereby no further substances can be bound [3].

Vitamin-binding proteins for thiamin, riboflavin, biotin, cobalamin, retinol, and cholecalciferol have been found in egg yolk. Similarly, binding proteins for thiamin, riboflavin, biotin and cobalamin are present in egg white. Since vitamin binding proteins are usually not saturated with respect to their ligands, they

are able to scavenge nutrients and are, therefore, thought to have both nutritional and antimicrobial functions [5].

Mineral-binding and metal-binding proteins

Minerals in bovine milk are present either in free ionic form or in complexes with various components such as proteins, peptides, amino acids, or carbohydrates. Within a protein, the side chains of the amino acid residues can interact with ions. For example, milk proteins such as α- and β-casein strongly bind bivalent and trivalent cations like calcium, manganese, zinc, copper, and iron, whereas the affinity is highest for calcium (Ca^{2+}) and lowest for iron (Fe^{3+}). Similarly, whey and egg proteins are able to bind minerals: α-lactalbumin mainly binds calcium but also zinc, and lactoferrin as well as ovotransferrin iron. From animal studies but not yet from human studies some beneficial effects of these mineral-binding proteins on bone health could be reported.

Metal-binding proteins like metallothioneins, phytochelatins, metallochaperons, and several animal fibrous proteins are thought to play a role in metal homeostasis of zinc and copper, and to aid with the detoxification of heavy metals like cadmium and mercury. These proteins are broadly distributed among mammals, plants, yeasts, and microorganisms. Studies in mouse models have suggested that administration of metal-binding proteins has anti-inflammatory and neuroprotective effects and can reduce both the symptoms and incidence of multiple sclerosis and collagen-induced arthritis. Other reported effects of metallothioneins are protection against pathological damage caused by cadmium, arsenic, and anticancer agents, as well as *Helicobater pylori* induced gastritis. Accumulated trace elements like iron, znci, manganese, and selenium in plants achieve greater soluble concentrations and, therefore, a higher bioavailability than those provided in free form in supplements. The biochemical complexation between the metals and the metal-binding compounds maintains the metals in a soluble form that is readily available for the organism [7].

Antimicrobial proteins

A broad spectrum of antimicrobial proteins protects the gastrointestinal tract against pathogenic bacteria and viruses. They act indirectly by stimulating the growth of beneficial microorganisms in the gut or directly by exerting an antimicrobial activity or neutralizing the mechanisms of attachment or invasion of pathogens. Further, bioactive proteins can inhibit the growth of pathogens by withholding nutrients which are essential for the proliferation of bacteria.

Lactoferrin is an iron-binding glycoprotein that forms the antibiotic fragment lactoferricin during digestion. Another mechanism of the antimicrobial activity of lactoferrin is the growth inhibition of pathogens by iron scavenging. Furthermore, the bactericidal effect of lactoferrin has been related to direct interaction between the protein and the membrane of gram-negative bacteria. Although only a few clinical studies are found in the literature, it seems that bovine lactoferrin is not as effective as

human lactoferrin. This is probably due to the failure of the human receptor for lactoferrin to recognize bovine lactoferrin.

Lactoperoxidase is one of the most prominent enzymes in bovine milk and catalyzes the peroxidation of thiocyanate and some halides such as iodide and bromide to generate oxidizing agents like hypochlorite. This acts by oxidizing the cell membrane of microorganisms, which results in a loss of structure and leads to cell lysis and death [8].

Lysozyme (muramidase, E.C. No. 3.2.1.17) is an ubiquitous enzyme present in human serum, urine, tears, seminal fluid, as well as in milk and, in higher concentrations, in egg white. Lysozyme hydrolyzes β-(1-4)-glucosidic linkages between N-acetylmuramic acid and N-acetyl-D-glucosamine residues present in the mucopolysaccharide cell wall of a variety of microorganisms. This enzyme is effective against gram-negative bacteria by degrading the cell wall of these bacteria and is widely used as a food additive. It also improves the antimicrobial activity of lactoferrin and specific antibodies. However, its concentrations in bovine milk and the colostrum are very low [9].

Haptocorrin (vitamin B_{12}-binding protein) binds strongly to vitamin B_{12} stabilizing it and preventing its breakdown in the low-pH environment of the stomach. Furthermore, it demonstrates antimicrobial activity through vitamin B_{12} binding, thereby preventing its use by bacteria that need it as an essential nutrient.

Immunoglobulins (Igs) are present in elevated concentrations in the colostrum whereas only low concentrations are found in milk. The primary biological function is to protect the offspring against microbial pathogens and toxins and to prevent infections in the mammary gland. Due to their antigen-binding properties, immunoglobulins can directly bind and neutralize bacteria and viruses and make them non-infectious. In mammals, five major classes of Igs have been characterized: IgG, IgM, IgA, IgD and IgE. IgG and IgM can activate bacteriolytic reactions and augment recognition and phagocytosis of bacteria by leucocytes. IgA agglutinates antigens, neutralizes viruses and bacterial toxins, and prevents attachment of enteropathogenic bacteria to mucosal epithelial cells (9). Immune protection is mainly restricted to the gastrointestinal tract, but they may also protect against dental caries due to their activity against cariogenic mutans streptococci. IgG1 is relatively resistant to gastric acids and proteolytic enzymes such as trypsin. Various studies showed that 10 – 30% of orally administered bovine Igs can be recovered intact from the stool of human infants and adults (3). However, the uptake of bovine milk antibodies is of limited use in human immune defense since possible interactions with pathogens are restricted to the oral and gastrointestinal area [10].

Immunosuppressing / immunomodulatory proteins

No clear answer exists as to whether and how milk influences the human immune system [11]. Neonatal ruminants are born with a poorly developed immune system and therefore need to build it up so that it suits their own requirements. During this development, the maternal milk plays a central role. Several studies indicate that bovine milk may influence the human immune system. Intact casein may

only modulate B-lymphocyte function, whereas κ-casein and its subfractions have the potential to affect T- and B-lymphocytes. Kappa-casein and caseinomacropeptides (CMP) could suppress production of the cytokine interferon-β, whereas α- and β-casein enhance production of this cytokine [11]. Lactoferrin can suppress the cytokine interleukin-6 in monocytic cell lines and inhibit the cell proliferation in bovine mammary epithelial cell lines. Furthermore, lactoferrin, and also bovine serum albumin, showed anticancerogenic activity inducing apoptosis in tumor and transformed cells *in vitro* [6].

However, bovine milk contains more than 25 protein components that may induce specific antibody production in humans. These antibodies are most frequently directed against β-lactoglobulin, followed by casein, α-lactalbumin, γ-globulin and serum albumin. Genetic predisposition and short or no breastfeeding may increase the risk of a cow's milk allergy in infancy. However, 85% will outgrow the milk allergy by the age of five [12].

Hormones and growth factors

A wide range of hormones have been identified in milk, including prolactin, somatostatin, insulin, and melatonin. Another group of hormones, known as growth factors, usually consist of proteins or steroid hormones. Many families of growth factors exist, but milk mainly contains insulin-like growth factor-1 (IGF-1) and epidermal growth factor (EGF). They can directly influence newborns' metabolism, and promote growth and differentiation of several organs and target tissues. Furthermore, they have a cytoprotective effect against toxic substances and reduce the risk of necrotizing enterocolitis. The amino acid composition of growth factors in human and bovine milk are rather similar, and recent studies support the idea that bovine growth factors may contribute to human body functions such as increased protein synthesis during and after physical exercise [3]. Bovine growth factors extracted from cheese whey reduced small bowel damage after oral ingestion in methotrexate-treated rats [13]. Similar to lactoferrin, IGF-1 and EGF stimulate the growth and proliferation of the mucosa that results in a larger surface area for nutrient absorption in the gut as well as a more developed barrier function. IGF-2, another growth factor present in bovine milk, exhibits anabolic activity, whereas transforming growth factor (TGF-α) is a protein that helps to maintain the normal epithelial function in the mucosa and TGF-β controls proliferation, cellular differentiation, and other functions in most cells [3].

Proteins with enzyme inhibitory activity in milk and other foods

Proteins from fish, wheat germ, and flour as well as soybean cotyledons exhibited a lipase-inhibitory effect in *in vitro* and in animal studies. Reduced lipase activity in the gut can result in reduced and/or delayed assimilation of fat. The consequences are lower postprandial triglyceride and LDL levels, and a better ratio of HDL to total cholesterol. These changes may be associated with improved insulin sensitivity and a lower risk for atherosclerosis, obesity, and other symptoms of the metabolic syndrome [6]. Feeding different sources of protein together with a diet containing cholesterol to rats showed a greater hypocholesterolemic effect for whey proteins in comparison to casein or soybean protein. The

cholesterol lowering effect of soybean protein seems to correlate with the bile-acid-binding capacity of these proteins whereas whey proteins affect the cholesterol absorption and the serum cholesterol level by influencing intestinal emulsification and the nature of the resulting micelles [14].

The cystatins are a family of cysteine protease inhibitors that typically comprise about 115 amino acids. They inhibit most cysteine endopeptidases, which are widely expressed in animals and plants. These peptidases are involved in a number of physiological processes, such as intracellular protein degradation, bone remodeling, control of antigen presentation; their activities are also increased in pathophysiological conditions, such as cancer metastasis and inflammation [15]. The presence of cysteine proteases inhibitors in food may influence the activity of digestive proteases.

Bioactive peptides

Bioactive peptides generally consist of between 3 and 20 amino acids and are encrypted within the primary structure of a dietary protein. Bioactive peptides are produced using dietary proteins by means of the following four mechanisms [16]: (1) during the fermentation of food using proteolytic starter cultures; (2) during the manufacture of protein hydrolysates; (3) as a result of the degradation of dietary proteins by digestive enzymes *in vivo*; or (4) as a result of the enzymatic action of digestive enzymes *in vitro*. For example lactic acid bacteria such as *L. helveticus* can release the tripeptides VPP[1] and IPP during the fermentation of milk. To demonstrate which peptides can result during digestion, β-casein was subjected to a two stage *in vitro* model of mammalian gastrointestinal digestion with pepsin, chymotrypsin / trypsin, pancreatin (named stage I digestion) and with brush-border peptidases on intestinal epithelial cells (stage II digestion with Caco-2 cells) [17]. The results of the *in vitro* study showed that the dipeptides NV, IV, QD, SK, VK, HK, PV, QS, VE, QS, QA, QE, PV and PI as well as the tripeptides PGE, INK, TED, IHP, FPP, YQE, PVL and GPF were released after the first stage of digestion. In the following stage II of the *in vitro* digestion the remaining casein-framework and the resulting peptides were degraded to only a few additional dipeptides and tripeptides (AQ, QS, PQ, VM, MP and HLP). The results of this study indicate that the resistance of peptides to gastrointestinal digestion is an important prerequisite in order to obtain physiological effects *in vivo* after oral administration of bioactive peptides.

Depending on their functionality bioactive peptides are divided into various groups such as opioids or casomorphins, angiotensin-converting enzyme (ACE = dipeptidyl carboxy peptidase; EC 3.4.15.1)-inhibitory peptides, phosphopeptides, antimicrobial peptides, immuno- and cytomodulating peptides, and peptides with antithrombotic activity [18]. Some sequences of bioactive peptides exhibit multifunctional effects.

[1] One-letter abbreviations of amino acids: A = alanine, C = cysteine, D = asparagic acid, E = glutamic acid, F = phenylalanine, G = glycine, H = histidine, I = isoleucine, K = lysine, L = leucine, M = methionine, N = asparagine, P = proline, Q = glutamine, R = arginine, S = serine, T = threonine, V = valine, W = tryptophan, Y = tyrosine.

Opioids

The late 1970s saw the first report detailing how a bioactive peptide released from food proteins was isolated. The protein in question was the bovine β-casomorphin-7 (YPFPGPI), an opioid peptide from a casein hydrolysate. Recently, the European Food Safety Agency (EFSA) published a comprehensive review on casomorphins, which are classified as opioids [19]. Unlike endomorphins found in human organisms, exorphins such as casomorphins from casein are also found in other milk proteins (β-lactoglobulin, α-lactalbumin, lactoferrin), in cereal proteins such as wheat (gluten, gliadin), barley (hordein, avenin, secalin, zein), rice (albumin), in vegetables such as soybeans (α-protein), spinach (rubisco protein), and in meat/poultry (albumin, hemoglobin, γ-globulin), egg (ovalbumin) (Table I). Homologous sequences have also been identified in both human and goat milk. In addition to the presence of casomorphins in hydrolyzed casein, casomorphins have been found in fermented milk products and in cheese, although the quantities found in cheese are significantly less than 1 mg/kg. Exorphins primarily affect the intestinal lumen and mucosa by regulating gastro-intestinal motility as well as gastric and pancreatic secretions. A discussion of the effect of exorphins on cerebral processes is not necessary in this context as these peptides must be supplied parenterally [19].

Antihypertensive (ACE-inhibitory) peptides

Angiotensin-converting enzyme (ACE)-inhibitory peptides represent an additional group of bioactive peptides. These are peptides which inhibit the activity of the angiotensin-converting enzyme *in vitro*. In humans, the peripheral blood pressure is regulated, amongst others, by the renin-angiotensin system of which ACE is a part of. Inhibiting this enzyme *in vivo* results in a reduction in blood pressure. ACE-inhibitory peptides with a chain length between 2 and more than 10 amino acids were first obtained from a range of milk proteins such as α_{s1}-, β-, κ-casein, β-lactoglobulin, α-lactalbumin, and serum albumin, although they have been found as well in other animal (non-milk) and plant proteins (Table II). Actually, the sequences of more than 150 ACE-inhibiting peptides obtained from cow's milk protein have been identified. The most studied peptides are the two tripeptides VPP and IPP, which are released by *L. helveticus* during the fermentation of milk. Similarly, various ACE-inhibitory peptides have been discovered in cheese [22]. Recently, seven ACE-inhibitory peptides from cooked eggs were subjected to an *in vitro* gastrointestinal digestion. The study included five tripeptides (VDF, LPF, MPF, IPF, and TTI) and two pentapeptides (YTAGV, ERYPI) [23]. In addition, ACE-inhibitory peptides originating from plant proteins were found in water-soluble extracts of broccoli, mushroom, garlic, buckwheat, and wine as well as in protein hydrolysates of soybean, mung beans, sunflower, rice, corn, wheat, buckwheat and spinach [20]. For example in the enzymatic hydrolysate of glycinin, the major storage protein of soybean, an ACE-inhibitory peptide with the sequence VLIVP was identified.

Mineral-binding peptides

Similarly to mineral-binding proteins, casein-derived phosphopeptides, that are reported as caseinophosphopeptides (CPP) show mineral-binding properties (Table I). This effect is related to the presence of the phosphorylated serine residues that can form salts with minerals such as calcium [16]. These peptides are involved in the remineralization of tooth enamel as well as in the increased absorption and bioavailability of calcium and other minerals such as zinc, copper, manganese and iron in the intestine.

Antioxidative peptides

Bioactive peptides which have an antioxidative effect have previously been obtained from various dietary proteins after enzymatic hydrolysis. The presence of such peptides, derived from hydrolyzed food proteins such as caseins, whey proteins, soybean (Table I), rice bran, quinoa seed protein, buckwheat protein, egg-yolk protein, porcine myofibrillar proteins and aquatic by-products proteins, has been investigated in a number of studies [24]. They are effective against enzymatic and nonenzymatic peroxidation of lipids and essential fatty acids, as free radical scavengers, in metal ions chelation and in adduct formation. The inhibition of oxidative processes is of particular importance for the survival of cells in an organism. However, undesired oxidative processes also occur in foods. The formation of free radicals results in a deterioration of food quality, for example rancid flavor, unacceptable taste, and shortening of shelf life.

Antimicrobial peptides

In addition to antimicrobial proteins such as lactoferrin, lysozyme, lactoperoxidase and immunoglobulins, antimicrobial peptides are also known to exist. The best investigated antimicrobial peptide is the fragment 17-41 of lactoferrin, more commonly known as lactoferricin (Table I). Antimicrobial peptides are effective against different bacteria and yeasts but only a few *in vivo* studies have been carried out to date [25].

A protection against pathogens has been attributed to α-lactalbumin and involves the release of peptides, which support the immune function in humans [9]. Different antimicrobiological functions have been attributed to the CMP that is formed during cheese manufacture or digestion from κ-casein (106-169). It binds enterotoxins (cholera and *E.coli*), modulates the immune system response, inhibits bacterial and viral adhesion, suppresses gastric secretions and promotes bifidobacterial growth [26].

Immuno- and cytomodulatory peptides

As previously mentioned, proteins can have an immunomodulatory effect and bioactive peptides from caseins and whey proteins are also known to have such an effect (Table I). These peptides can modulate the proliferation of human lymphocytes, down-regulate the production of certain cytokines, and stimulate the phagocytic activities of macrophages. As a result, they can regulate the development

of the immune system in newborn infants [27]. In addition, cytomodulatory peptides exist, such as the fragments 1-18 and 105-117 from β-casein, which have been shown to influence the viability as well as the proliferation, differentiation, and apoptosis of different cell types [16].

Other bioactive peptides

The CMP and human lactoferrin also contain peptide sequences which have an antithrombotic effect. These antithrombotic peptides can inhibit blood clotting and aggregation of platelets. A hypocholesterolemic peptide has been identified from the tryptic hydrolysate of β-lactoglobulin (IIAEK), from soybean glycinin (LPYPR), and from fish protein. Additionally, antiobesity peptides (or bioactive appetite suppressants) in β-conglycinin derived from soybean protein (VRIRLLQRFNKRS) and in the CMP as well as hypotriglyceridemic peptides from blood (globin) (VVP; VYP; VTL) are believed to exist [28]. There are also indications that the β-casein fragment 177-183 exhibits a cell growth-stimulating effect as it stimulates DNA synthesis in mouse fibroblast cells [16]. Other dietary proteins also promise additional surprising results. For example, marine organisms are a rich source of bioactive peptides with antihypertensive, antioxidant, anticoagulant and antimicrobial properties [29].

Examples of bioactive peptides with physiological effects in biological systems

The presence of the tripeptides VPP and IPP in milk which had been fermented using *L. helveticus a*nd *Saccharomyces cerevisiae* has been known since 1995. VPP is located in the sequence 84–86 and IPP in 74–76 both in the β-casein) as well as in f108–110 of κ-casein. These studies were the starting point for the development of hypotensive milk-drink products, such as *Ameal S*™ (Calpis Company, Japan) and *Evolus*® (Valio, Finland), and several patents have been filed in order to protect their commercial use. In our own in-depth studies, new insights into the prevalence and ripening-dependent formation of the two lactotripeptides VPP and IPP in various Swiss cheeses are being compiled. The results show that soft cheeses contain only traces of VPP and IPP whereas large differences in the content of the two tripeptides were obtained in samples of semi-hard, hard, and extra-hard cheeses. The total concentration of VPP and IPP varied from 1.6 mg/kg in a sample of Sbrinz cheese up to 424.5 mg/kg in a sample of Bernese Alpkäse. However, high levels of variation were found even within the samples of the same cheese variety: in Bernese Alpkäse the concentration varied between 10.7 and 424.5 while in Bernese Hobelkäse the concentration varied between 6.8 and 353.0 mg/kg [22].

The resistance of peptides to gastrointestinal digestion is an important prerequisite in order to obtain physiological effects *in vivo* after oral administration of bioactive peptides. Synthetic VPP and IPP were highly resistant to the treatment with different digestive enzymes in a two-stage *in vitro* model, and thus would reach the small intestine in intact form. Current knowledge indicates that absorption is only possible with dipeptides and tripeptides. In humans, IPP but not VPP was absorbed intact into the circulation after consumption of a lactotripeptide-enriched milk beverage, but bioavailability was low, and the elimination half-life from plasma rather short [22]. Only very little is known about the absorption

mechanism for peptides longer than three amino acids. It would appear possible that passive diffusion takes place through the intestinal mucosa, although the quantities absorbed are extremely small. Additionally, absorbed peptides are further degraded by peptidases in the blood. In the case of opioids, a passage through the blood-brain-barrier is necessary in order to allow activity in the central nervous system to take place. This type of passage is regarded as rather unlikely and if it does occur it is likely to be at very low levels [19].

Various animal studies concluded that consumption of fermented milk containing VPP and IPP results in a reduction in blood pressure. These observations were also confirmed in human studies including subjects with mildly elevated blood pressure. According to two meta-analyses, one including 12 randomized controlled trials published between 1996 and 2005 with a total of 623 participants, and the other including 15 placebo-controlled clinical studies, systolic blood pressure decreased by 4.8 and 5.13 mmHg, respectively, while diastolic blood pressure decreased by 2.2 and 2.42 mmHg respectively. However, three other placebo-controlled studies recently published found no evidence of this effect after administration of a dairy drink containing these lactotripeptides; as a result, the antihypertensive effect of fermented milk is still debatable [22].

The remineralizing effect of cheese consumption on tooth health due to its high calcium and phosphorus content is well known. There is evidence, that casein itself has the same effect. In Western countries tooth loss is a growing problem among children and adolescents a consequence of their increased consumption of erosive foods such as citrus fruits and soft drinks. Caseinomacropeptide, a bioactive peptide, shows an inhibiting effect to tooth erosion. In an *in vitro* study hydroxyapatite was selected as a tooth model system. It was pretreated with CMP and then exposed to an acidic solution, a citrate buffer at three different pH level (2.3, 3.5 and 4.5). The conclusion of this study is a reducing potential of whole CMP and its fractions (a glycosylated and phosphorylated fraction and a non-glycosylated but phosphorylated fraction) against the erosive effect of acidic foods and drinks by 30 to 40% [30].

Safety aspects

If new, pharmacologically active substances are identified in human food, it is essential that both, potential benefits and risks of these substances are evaluated. For this reason, intensive toxicological studies have been carried out on VPP and IPP. The following toxicological methods were used: Short-term studies, single-dose and 4-week repeated-dose toxicity in rats, 8-week studies in dogs and rats, 13-week toxicity, fertility and reproductive performance in rats, micronucleus test in rats and mice, and evaluation of cytotoxicity, clastogenicity, as well as mutagenicity (*Salmonella-E.coli* microsome incorporation assay). Similarly, a commercial milk protein hydrolysate containing IPP was investigated in three *in vitro* genotoxicity tests and in a 90-day repeated-dose oral toxicity study in rats. Overall, these studies found no adverse effects as a result of the administered lactotripeptides [22]. According to a comprehensive review by the EFSA, casomorphin-7 does not pose any health risks. After it oral

consumption of this or related bioactive peptides did not demonstrate any cause-effect relationship with the aetiology or course of any suggested non-communicable diseases. Consequently, a formal EFSA risk assessment of food-derived peptides has not been recommended [19].

Application and regulation of bioactive protein and peptides in food

Intensive research on bioactive peptides being carried out around the world has already led to the introduction of a wide range of commercial products. Eleven of them are functional foods or food ingredients containing casein-derived bioactive peptides, such as the fermented milk Calpis or Evolus, identified by Phelan et al. [16], five claim to have hypotensive properties, four claim to aid mineral absorption, one claims to improve athletic performance, and one claims to reduce stress. Since 1991, the Ministry of Health and Welfare in Japan has awarded the status of *Food of Specific Health Use* (FOSHU) to foods with scientifically validated health claims. Since then, antihypertensive peptides such as VPP, IPP, VY and CPP have obtained FOSHU approval, according to Phelan et al. [16].

As a result of the regulation of health claims made on foods passed by the European Parliament which subsequently took effect, the EU can permit health claims (1) referring to reduction of disease risk (article 14(1)(a)), (2) referring to children's development and health (article 14(1)(b)), and (3) based on newly developed scientific evidence and/or including a request for the protection of proprietary data (article 13(5)). As the highest regulatory authority, the EFSA is responsible for verifying all applications submitted for permission for products to carry health claims. Regarding the applications already processed, the Commission of European Communities has not yet authorized any claims relating to the effect of bioactive peptides in foods. For example, the claim that Evolus reduces arterial stiffness in mildly hypertensive subjects, and consequently the risk of cardiovascular disease was rejected, as was the health claim related to dairy foods (milk and cheese) and dental health as well as the health claim related to the effects of a dairy product enriched with milk peptide and magnesium on the reduction of anxiety (Regulation (EC) No 1924/2006;
http://ec.europa.eu/food/food/labellingnutrition/claims/index_en.htm).

In addition to the formation of bioactive peptides in fermented foods previously mentioned, it is quite possible to enrich foods with bioactive peptides derived from the hydrolysates of various food proteins. An example in this direction would be the development of antioxidant-rich peptides from milk protein using microbial proteases which would then be used in cooked beef to prevent lipid peroxidation, or the addition of CPPs to soluble fractions of fruit beverages in order to improve iron transport in Caco-2 cells.

Conclusions

Bioactive proteins are a part of our daily food intake and their effects on the human body mainly take place in the lumen and mucosa of the digestive tract. Bioactive peptides, which are encrypted in native peptides, are primarily found in fermented foods, especially in fermented dairy products. The quantities

in which they are present are highly dependent on the specific effects of the lactic acid bacteria involved, which can result in substantial variations in traditional dairy products, as our studies on cheese have shown. In addition, bioactive peptides may be formed or degraded in the digestive tract by proteases and peptidases. The issue of whether bioactive peptides or proteins can have an effect outside the intestinal tract is questionable as their absorption is limited or impossible due to the size of their molecules.

In recent years, new peptides demonstrating biological activity have steadily been discovered in different foods. Bioactive peptides from milk proteins have been studied most intensively so far. Nowadays, the application of proteolytic enzymes in combination with new technologies such as chromatographic and membrane separation techniques as well as the use of specific cultures allow the large scale production of bioactive peptides from various food proteins. This enables the enrichment of selected foods with bioactive peptides or the development of new functional foods. Although a large number of physiological effects of bioactive peptides have been described in *in vitro* assays, no clinical studies involving humans have been performed yet, with the exception of those on ACE-inhibitory peptides from milk proteins. For this reason, randomized controlled trials are needed in order to evaluate the health potential of bioactive peptides and proteins in the diet.

Reference List

1. Schrezenmeir J, Korhonen H, Williams C, et al. Foreword. Br J Nutr 2000; 84:S1.
2. FitzGerald RJ, Murray BA. Bioactive peptides and lactic fermentations. Int J Dairy Technol 2006; 59:118-125.
3. Pihlanto A, Korhonen H. Bioactive peptides and proteins. In: Steve LT, editor. Advances in Food and Nutrition Research. Academic Press, 2003: 175-276.
4. Parodi PW. Cow's milk folate binding protein: Its role in folate nutrition. Austr J Dairy Technol 1997; 52:109-118.
5. White HB. Vitamin-binding proteins in the nutrition of the avian embryo. J Exp Zool Suppl 1987; 1:53-63.
6. Möller NP, Scholz-Ahrens KE, Roos N, et al. Bioactive peptides and proteins from foods: indication for health effects. Eur J Nutr 2008; 47:171-182.
7. Kovacs-Nolan J, Mine Y. Heavy-Metal-Binding Proteins. In: Mine Y, Shahidi F, editors. Nutraceutical Proteins and Peptides in Health and Disease. Boca Raton: Taylor and Francis, 2006: 69-80.
8. Boots JW, Floris R. Lactoperoxidase: From catalytic mechanism to practical applications. Int Dairy J 2006; 16:1272-1276.
9. Lönnerdal B. Bioactive proteins: clinical applications for gastrointestinal health. Bull Int Dairy Fed 2002; 375:22-24.
10. Mehra R, Marnila P, Korhonen H. Milk immunoglobulins for health promotion. Int Dairy J 2006; 16:1262-1271.
11. Cross ML, Gill HS. Immunomodulatory properties of milk. Br J Nutr 2000; 84:S81-S89.
12. Kurowski K, Boxer RW. Food allergies: detection and management. Am Fam Physician 2008; 77:1678-1686.
13. Steijns JM. Milk ingredients as nutraceuticals. Int J Dairy Technol 2001; 54:81-88.

14. Nagaoka S. Cholesterol-Lowering Proteins and Peptides. In: Mine Y, Shahidi F, editors. Nutraceutical Proteins and Peptides in Health and Disease. Boca Raton: Taylor and Francis, 2006: 41-67.
15. Nakamura S. Cystatin: A novel Bioactive Protein. In: Mine Y, Shahidi F, editors. Nutraceutical Proteins and Peptides in Health and Disease. Boca Raton: Taylor and Francis, 2006: 243-266.
16. Phelan M, Aherne A, FitzGerald RJ, et al. Casein-derived bioactive peptides: Biological effects, industrial uses, safety aspects and regulatory status. Int Dairy J 2009; 19:643-654.
17. Ohsawa K, Satsu H, Ohki K, et al. Producibility and digestibility of antihypertensive beta-casein tripeptides, Val-Pro-Pro and Ile-Pro-Pro, in the gastrointestinal tract: Analyses using an in vitro model of mammalian gastrointestinal digestion. J Agric Food Chem 2008; 56:854-858.
18. Korhonen H. Milk-derived bioactive peptides: From science to applications. J Funct Foods 2009; 1:177-187.
19. DATEX Working Group. Review of the potential health impact of beta-casomorphins and related peptides. EFSA Sci Rep 2009; 231:1-107.
20. Guang C, Phillips RD. Plant food-derived angiotensin I converting enzyme inhibitory peptides. J Agric Food Chem 2009; 57:5113-5120.
21. Meisel H, Walsh DJ, Murray B, et al. ACE inhibiting peptides. In: Mine Y, Shahidi F, editors. Nutraceutical proteins and peptides in health and disease. CRC Press: New York, 2008: 269-315.
22. Sieber R, Bütikofer U, Egger C, et al. ACE-inhibitory activity and ACE-inhibiting peptides in different cheese varieties. Dairy Sci Technol 2010; 90, 47-73.
23. Majumder K, Wu J. Angiotensin I converting enzyme inhibitory peptides from simulated in vitro gastrointestinal digestion of cooked eggs. J Agric Food Chem 2009; 57:471-477.
24. Pihlanto A. Antioxidative peptides derived from milk proteins. Int Dairy J 2006; 16:1306-1314.
25. Lopez Exposito I, Recio I. Antibacterial activity of peptides and folding variants from milk proteins. Int Dairy J 2006; 16:1294-1305.
26. Thomä-Worringer C, Sorensen J, Lopez-Findino R. Health effects and technological features of caseinomacropeptide. Int Dairy J 2006; 16:1324-1333.
27. Korhonen H, Pihlanto A. Bioactive peptides: Production and functionality. Int Dairy J 2006; 16:945-960.
28. Erdmann K, Cheung BWY, Schröder H. The possible roles of food-derived bioactive peptides in reducing the risk of cardiovascular disease. J Nutr Biochem 2008; 19:643-654.
29. Kim S-K, Wijesekaraa I. Development and biological activities of marine-derived bioactive peptides: a review. J Funct Foods 2010; 2:1-10.
30. Setarehnejad A, Kanekanian A, Tatham A, Abedi AH. The protective effect of caseinomacropeptide against dental erosion using hydroxyapatite as a model system. Int Dairy J 2010; 20:652-656.

Table I. Sequences of bioactive peptides (without ACE-inhibiting peptides) derived from food proteins [19;24]

Protein	Primary structure or peptide fragment
Opioids	
α_{s1}-casein	α_{s1}-casein exorphin (90-96)
β-casein	casomorphin 4, 5, 7, 8 (60-63, -64, -66, -67)
κ-casein	casoxin A, B, C (25-34: YIPIQYVLSR, 35-42: YPSYGLNN; 58-61: TPTY)
β-lactoglobulin	β-lactorphin (102-105)
α-lactalbumin	α-lactorphin (50-53)
serum albumin	serorphin (399-404: YGFNA),
bovine hemoglobin	hemorphin-4, -5, -6 (34-37, -38, -40: YPLSTQEF),
gluten	exorphin A4, A5, B4, B5 (GYYP, -T and YGGW, -L),
spinach	rubiscolin-5, -6 (YPLDL, -F)
soybean	soymorphin-5 (YPFVV)
Mineral-binding peptides	
α_{s1}-casein	CPP (59-79, 64-84)
α_{s2}-casein	CPP (1-21, 46-70)
β-casein	CPP (1-25)
κ-casein	CPP (147-153)
Antioxidative peptides	
α_{s1}-casein	YFYPEL (144-149)
β-casein	VKEAMAPK (89-105), AVPYPQR (177-183), KVLPVPEK (169-, 170-176)
β-lactoglobulin	WYSLAMAASDI (19-29), MHIRL (145-149), YVEEL (42-46)
fermented milk	ARHPHPHLSFM (κ-casein 96-106)
soybean	peptides with LLPHH sequence
Antimicrobial peptides	
α_{s1}-casein	isracidin (1-23), (99-109)
α_{s2}-casein	casocidin-I (150-188), (164-179, 164-, 175-, 181-, 182-207)
β-casein	(184-210)
κ-casein	(18-24, 30-32, 139-146), CMP (106-169)
β-lactoglobulin	(15-20, 25-40, 78-83, 92-100)
lactoferrin	lactoferricin (17-41)
Immuno- and cyto-modulatory peptides	
α_{s1}-casein	(1-23, 23-34, 90-95, 90-96, 194-199)
β-casein	(60-66, 60-70, 63-68, 177-183, 191-193, 193-202, 193-209)
κ-casein	(17-21, 38-39)
α-lactalbumin	YG
lactoferrin	lactoferricin

The figures in brackets indicate the amino acid sequence of the related protein.

Table II. Sequences of ACE-inhibitory peptides mainly derived from food proteins (compiled according to [20] and [21])

Protein	Primary structure or peptide fragment
Bovine milk	
α_{s1}-casein	23-34, 23-, 24-, 25-27, 27-30, 28-, 32-34, 104-109, 142-147, 143-148, 157-164, 194-, 197-, 198-199
β-casein	48-, 49-, 50-, 52-, 53-, 54-, 55-, 56-, 57-, 58-, 59-61, 59-64, 60-66, 74-76, 84-86, 108-113, 169-174, 169-175, 177-179, 177-, 179-181, 177-, 181-183, 193-198, 193-202
κ-casein	25-34 (YIPIQYVLSR), 35-41 (YPSYGLNY), 58-59 (YP), 108-110 (IPP)
β-lactoglobulin	9-14, 15-20, 102-103, 102-, 104-105, 142-, 146-, 147-148, 146-, 148-149
α-lactalbumin	50-51, 50-, 52-53, 105-110
serum albumin	208-216 (ALKAWSVAR)
Animal (nonmilk)	
chicken muscle	LKP, FKGRYYP, IVGRPHQG,LAP, LKA, FQKPKR
sardine	LKVGKQY, KVLAGM, HQAAGW, VKAGF, LKL
tuna muscle	IF, VWIG, LTF, IFG
porcine myosin	79-81, 80-82, 79-, 81-83 (MNPPK)
porcine hemoglobin	GKKVLQ, FQKVVA(K), FQKVVAG
ovalbumin	(F)FGRCVSP, ERKIKVYL, LW, FCF, NIFYCP
Plant	
broccoli	YPK
buckwheat	GPP
garlic	FY, NY, NF,SY, GY, SF
mushroom	VIEKYP, GEP
pea albumin	LGP, YW, VY, DG, LY, MF, GP, GS, GK
pea vicilin	LKP, IY,VK, AF, GYK, IR, QK, FG, SG, GK
α-zein	LRP, LSP, LQP, LAQ, IRA, VSP, LAA, VAA, VAY, FY, LNP, LLP, LQQ, IRAQQ
soybean protein	DLP, HHL, DG