Review Article

Bioactive Peptides: A Review

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Abstract: Bioactive peptides have been defined as specific protein fragments that have a positive impact on body functions and conditions and may ultimately influence health [79]. According to Fitzgerald & Murray [46], bioactive peptides have been defined as peptides with hormone- or drug like activity that eventually modulate physiological function through binding interactions to specific receptors on target cells leading to induction of physiological responses. According to their functional properties, bioactive peptides may be classified as antimicrobial, antithrombotic, antihypertensive, opioid, immunomodulatory, mineral binding and antioxidative. These peptides play an important role human health. In this review, we describe above stated properties of bioactive peptides especially derived from milk.

Keywords: Milk derived bioactive peptides, Functional properties, Classification, Human health, Drug like activity, Binding interaction.

Introduction

Bioactive peptides are proteins synthesized in the cell in the form of large prepropeptides, which are then cleaved and modified to give active products. As signaling molecules, the bioactive peptides play important roles in physiological functions and pathogenesis. The emergence and fast development of bioinformatics, especially of the human genome project in 1980s and 1990s, have led to rapid accumulation of a huge amount of biological data organized in numerous biological databases. Besides, large protein and gene sequence repositories such as PIR [53], GeneBank [8] and Swiss-Prot [10]. There are also many databases with their focuses on special protein or gene sequences such as nuclear protein databases [32] and peptide database of immunology [11].

The major role of milk proteins is to supply amino acids and nitrogen to the young mammals and constitute an important part of dietary proteins for the adult. Intact milk proteins have also specific functions such as micelle formation. Furthermore, milk proteins have physiological importance, they facilitate uptake of several important nutrients such as trace elements and vitamins and contain a group of proteins which perform a protective function. This means that milk proteins are highly functional substances. During the last two decades it has become clear that milk proteins are a source of biologically active peptides. Milk proteins are a rich source of biologically active peptides such as antihypertensive, antithrombotic, opioid,
immune-stimulating, antimicrobial, mineral carrying and cholesterol lowering-peptides [149]. These peptides are inactive within the sequence of parent protein and can be released during gastrointestinal digestion or food processing. According to Kamau et al. [76], many bioactive peptide fragments can be obtained through hydrolysis of whole milk or precursor protein by digestive enzymes. This powerfully hypothesizes the existence of such peptides in the GIT after consumption of milk. The quantity and composition of milk, presence of additional food, instance, pH and enzymatic action utter the type and destiny of peptides released in digestive system. These factors are influenced by age, genetic makeup, nutritional patterns and health status of the consumer. A healthy diet is thus a prerequisite for attaining full benefits of these peptides.

Once bioactive peptides are liberated, they may act as regulatory compounds with hormone-like activity. This aspect has been studied since 1979 and numerous peptides, which exhibit various activities such as opiate, antithrombotic or anti-hypertension activity, immunomodulation or mineral utilization properties, has been found. Milk proteins are the most important source of bioactive peptides, though other animal as well as plant proteins also contain potential bioactive sequences. The first biologically active peptide found in milk was opioid peptides followed by immunomodulatory peptides.

According to Haque et al. [59], Milk derived bioactive peptides play vital roles in human health and nutrition. Many researchers are interested to solve the question about the importance of bioactive foods are as food constituents or as drugs and it needs careful examination. ACE inhibitory peptides, immunomodulating peptides, and caseinophosphophoproteptides are the most favourite bioactive peptides for application to foodstuffs formulated to provide specific health benefits. Casein derived peptides have already found interesting applications as dietary supplements and as pharmaceutical preparations such as tablets, toothpaste, and dental filling material. The efficacy and safe conditions of use of these peptides in animals and in humans remain yet to be proven.

**Opioid peptides**

According to Teschemacher et al. [161], the peptides exist in dairy products which play an active role in nervous system; they are known as opioid peptides. Opioid peptides are also having pharmacological similarity to opium (morphine). They are opioid receptor ligands with agonistic or antagonistic activities and are characterized by distinct N-terminal sequences and are called atypical opioid peptides different from that of the typical endogenous opioid peptides [161]. Opioid peptides are short sequences of amino acids that mimic the effect of opiates in the brain. Opioid peptides are also defined as peptides like enkephalins that have both affinity for opiate receptor and opiate-like effects which are inhibited by naloxone. The typical opioid peptides, all originate from three precursor proteins – proopiomelanocortin (endorphins), proenkephalin (enkephalin) and prodynorphin (dynorphins) [65]. Opioid peptides may be produced by the body itself, for example endorphins, or be absorbed from partially digested food (casomorphins, exorphins and rubiscolins). The effect of these peptides varies, but they all resemble opiates. The opioid food peptides have lengths of typically 4-8 amino acids. The body’s own opioids are generally much longer. Brain opioid peptide systems are known to play an important role in motivation, emotion, attachment behavior, the response to stress and pain, and the control of food intake. The human genome contains three homologous genes that are known to code for endogenous opioid peptides. Each gene codes for a large protein that can be processed to yield smaller peptides that have opiate-like activity.
All of these typical opioid peptides have the same N-terminal sequence i.e. Tyr-Gly-Gly-Phe. Opioid peptides exert their activity by binding to specific receptors of the target cell. Individual receptors are responsible for specific physiological effects, e.g., the m receptor for emotional behavior and suppression of intestinal motility, the s-receptor for emotional behavior and the k-receptor for sedation and food intake. The opioid peptides derived from a variety of precursor proteins are called “a typical” opioid peptides, since they carry various amino acid sequences at their N-terminal regions, only the N-terminal tyrosine is conserved. The N-terminal sequence of the atypical opioid peptides is Tyr-X-Phe or Tyr-X1-X2-Phe. The tyrosine residue at the N-terminal and the presence of another aromatic amino acid at third or fourth position is an important structural motif that fits into the binding site of the opioid receptors.

The first studied food derived opioid peptides were the β-casomorphins [13]. Opioid properties have been demonstrated for β-casein f60-f70 as well as for fragments thereof. Morphiceptin, which is an amide derivative of β-casomorphin-4, is a highly specific opioid agonist for both m receptors in guinea pig ileum and morphine binding sites in rat brain. Other β-casomorphins has also been characterized as m ligands [19]. β-casomorphins were also found in analogous positions in sheep, water buffalo and human casein [145]. Other milk opioid agonist peptides are α-casein derived exorphins, corresponding to bovine αs1-casein f90-f96. These α-casein exorphins are selective receptor ligands and can be separated from pepsin hydrolysate of α-casein [93, 180]. All bovine k-casein fragments having opioid activities referred to αs-casoxins (k-casein f33-f39, f25-f34) as well as lactoferrin fragments termed lactoferroxin behave as opioid antagonists. The antagonistic activity of casoxin (k-casein f33-f38) was lower than that of naloxone, and it can be regarded as m and k selective receptors with low affinity [22, 176].

Opioid peptides from bovine caseins can be obtained by in vitro enzymatic hydrolysis [131]. Precursors of β-casomorphins have also identified in Parmesan cheese [1]. β-casomorphins have been detected in the duodenal chyme of mini pigs [101] and in the human small intestine (Svedberg et al. [158]) as a consequence of in vivo digestion.

Whey proteins contain opioid-like sequences in their primary structure, namely α-lactalbumin (both bovine and human) f50-f53 and β-lactoglobulin (bovine) f102-f105. These peptides were termed α-lactorphins and β-lactorphins [176]. Bovine blood serum albumin f399-f404 serorphins has also opioid activity [160]. α- and β-lactorphins can be released from parent protein using in vitro proteolysis and various proteolytic enzymes [4]. α-lactorphin exerts a weak but consistent opioid property in the smooth muscle and receptor binding, while β-lactorphin, in spite of the similar receptor binding affinity, exerts an apparently non-opioid stimulatory effect on the guinea pig ileum. α- and β-lactorphins were m receptor ligands [125].

Maruyama et al. [95, 96] have shown that tryptic peptides from bovine αs1-casein and β-casein are inhibitors of ACE. These peptides were termed casokinins and correspond to f23-f34, f23-f27 and f194-f199 of αs1-casein as well as f177-f183 of β-casein. Meisel and Schlimme [106] have shown that the synthetic peptides from β-casein-β-casomorphin-7 (f60-f66) as well as β-casokinin-10 (f193-f202) shows ACE-inhibitory activity, within the range of known food derived ACE-inhibitors. Synthetic β-casein peptide (f169-f175) indicated a strong antihypertensive activity in spontaneously hypertensive rats. The ACE-inhibitory activity of this peptide was quite low but increased after pancreatic digestion. αs1-casein peptide (f104-f109) had a strong ACE-inhibitory activity but no
significant antihypertensive effect [94]. Nakamura et al. [120] isolated two ACE-inhibitory peptides Val-Pro-Pro and Ile-Pro-Pro from Calpis sour milk. ACE inhibitory activity was also found in ripened cheese types and this activity increases during cheese maturation, but decreases when the proteolysis exceeds a certain level. Inhibitory activity in cheese may result from a complex mixture of small peptides [103].

Whey protein-derived opioid peptides (β-lactorphin and α-lactorphin) have been shown to moderately inhibit ACE activity. The N-terminal dipeptide (Tyr-Leu) of β-lactorphin was found to be the most potent inhibitor [115]. Tryptic β-lactoglobulin peptide, corresponding to β-lactoglobulin f142-f148, was found to be the most active ACE-inhibitory whey peptide so far reported [115].

The structure-activity relationship of ACE inhibitory peptides has not yet been established, because it has been observed that a large variety of peptides with different C-terminal amino acid sequence can serve as substrates. Structure activity correlations among different peptide inhibitors of ACE indicate that binding to ACE is strongly influenced by the C-terminal tripeptide sequence of the substrate. It has been suggested that peptides which contain hydrophobic amino acids at these positions are potent inhibitors.

The side chains of these amino acids are considered to interact with the subsites at the active site of ACE [123]. In studies on ACE inhibition by different structures, it was found that a C-terminal tryptophan, tyrosine, phenylalanine and proline residue was the most effective [21]. After the isolation of peptide inhibitors from snake venom it was realized that C-terminal proline can bind exceptionally well to ACE and can therefore provide good substrates or inhibitors depending on other features of the sequence [21]. Proline residues were also suggested as contributing to the potency of ACE inhibitory peptides from food proteins [120]. Furthermore, the positive charge – as in the guanidine group of the C-terminal Arg contributes to the ACE inhibitory potency of several peptides, indicating that the binding site may be different from the catalytic site in ACE. Using molecular modeling it has been shown that inhibitory peptides possess a characteristic pattern different from that of inactive molecules: positive potential is located in nearly the same region at the C-terminal [103].

Lactorphin peptides are m receptor ligands with a low affinity for opioid receptors [133]. α-lactorphin has been shown to exert a weak opioid activity to smooth muscles and β-lactorphin has been shown to have a smooth muscle contracting effect (Antila et al. [4]). Recently, β-lactorphin has shown to improve arterial function in SHR. Notably, β-lactorphin improved vascular relaxation in adult SHR in vitro, and additionally enhanced endothelium independent relaxation [153]. Opioid antagonists have been found in bovine and human k-casein (casoxins) and in human αs1-casein [22, 176]. Furthermore the opioid antagonist lactoferroxin has been found in human lactoferrin [176]. Opioid peptides derived from milk proteins appear to have physiological significance in the female organism (liberation of casomorphin in mammary gland) and in neonates [161]. Orally administered opioid peptides may modulate absorption processes in the gut and influence the gastrointestinal function in two ways: first, by affecting smooth muscles, which reduces the transit time, and second, by affecting the intestinal transport of electrolytes, which explains their anti-secretory properties. The enhancement of net water and electrolyte absorption by β-casomorphins in the small and large intestine is a major component of their anti-diarrhoeal action [20]. This effect could be mediated via subepithelial opioid receptors or specific luminal binding sites at their brush border membrane [12, 163]. β-casomorphins are claimed to be rapidly degraded once they
enter the bloodstream. However, β-casein-derived opioid peptides or their precursors have been detected in the duodenal chyme of mini pigs, in the plasma of newborn calves and in the human small intestine upon oral administration of casein or milk [47, 81, 104]. Opioid casein fragments have not been detected in the plasma of adult mammals [161]. Therefore, it is suggested that only the neonatal intestine is permeable to casomorphins, so babies may become calm and sleepy [155].

**Immunomodulatory peptides**

Immunomodulating peptides have been detected in human as well as in cow milk proteins (Migliore-Samour and Jolles, [110]). From human milk protein digests, two peptides, β-casein f54-f59 and α-lactalbumin f51-f53, enhance the phagocytic activity of macrophages both in mice and humans and enhance resistance against certain bacteria in mice [109, 128].

Among the immunomodulating peptides isolated from bovine caseins, the β-casein f191-f193, αs1-casein C-terminal hexapeptide (f194-f199) stimulated macrophages. β-casein f63-f68 stimulated in vitro phagocytosis, but this peptide as well as β-casein f191-f193 failed to exert protection of mice in vivo [43]. The physiological mode of action is not known, but they may stimulate the proliferation and maturation of immune system cells. Synthetic peptides derived from milk proteins have been shown to enhance proliferation of human peripheral blood lymphocytes. These peptides were Tyr-Gly and Tyr-Gly-Gly and they correspond to fragments of bovine k-casein and α-lactalbumin. β-casomorphin-7 and β-casokinin-10 showed suppression and stimulation of lymphocyte proliferation depending on the peptide concentration [77]. Laffieneur et al. [84] have shown that β-casein fermented by lactic acid bacteria have immunomodulatory activity which might be related to interaction with monocyte-macrophage and T helper cells, especially Th1 like cells. Sutas and co-workers [157] showed that caseins hydrolyzed with Lactobacillus GG and digestive enzymes generate compounds with suppressive effects on lymphocyte proliferation. Several known immunostimulating peptides were identified from these hydrolysates [139].

Another group of peptides which may be implicated in the stimulation of immune system are the ACE-inhibitors. Inhibition of ACE favors bradykinin formation and thus acts as immunomodulators. Bradykinin, known as a mediator of the acute inflammatory process, is able to stimulate macrophages to enhance lymphocyte migration and increase secretion of lymphokinines [126]. In this context it should be emphasized that peptides αs1-casein f194-f199, β-casein f60-f66 and f193-f202 have shown to have both immune stimulatory and ACE-inhibitory activities.

The structure-activity relationship and the mechanism by which milk-derived peptides exert their immunomodulatory effects are not yet defined. It has been suggested that arginine in the N- or C-terminal region of peptide is important structural entity recognized by specific membrane bound receptors [126]. A common structural feature among some immunomodulatory peptides is the presence of arginine in the C-terminal.

Immunomodulating peptides have also been found to stimulate the proliferation of human lymphocytes, the phagocytic activities of macrophages and antibody synthesis. The peptides may stimulate the proliferation and maturation of T cells and natural killer cells for defense of new born against a large number of bacteria, particularly enteric bacteria (Clare and Swaisgood, [25]). According to the findings of Migliore-Samour et al. [109], casein derived immune-peptides including fragments of αs1-casein and β-casein stimulate phagocytosis of
sheep red blood cells by murine peritoneal macrophages and exert a protective effect against *Klebsiella pneumoniae* infection in mice after intra venous administration.

According to Korhonen and Pihlanto [81], immunomodulatory milk peptides may alleviate allergic reactions in atopic humans and enhance mucosal immunity in the gastrointestinal tract. In this way immunomodulatory peptides may also be helpful in the regulation of the development of the immune system in newborn infants. Again, immunomodulatory peptides formed during milk fermentation have been shown to contribute to the antitumor effects [99].

Lactoferricin, one of the multifunctional peptide shows antimicrobial, antifungal, antitumor, and antiviral properties due to tryptophan/arginine rich proportion of the peptide, and an anti-inflammatory and immunomodulating properties because of its positively charged region of the molecule [169]. The peptides derived from lactoferricin hydrolysates can be useful for clinical applications because of their immunomodulatory effects or for chemoprevention of carcinogenesis. The use of lactoferricin derivatives in oral care and as food preservative has also been proposed by Expósito and Recio in their review [91].

**Mineral binding peptides**

Several phosphopeptides containing the cluster sequence -Ser(P)-Ser(P)-Ser(P)-Glu(E)-Glu(E)- have been identified from whole bovine casein. These sequences provide the peptides with the unique capacity to keep Ca, P and other mineral in a solution at intestinal pH. Several phosphopeptides have been identified from enzymatic digest of milk proteins, for example: αs1-casein f43-f58, f59-f79, f43-f79, αs2-casein f1-f24 and f46-f70 and β-casein f1-f28, f2-f28, f1-f25, f33-f48 [49, 75]. The highly anionic character of these peptides renders them resistant to further proteolytic attack, allows them to form soluble complexes with calcium and prevents the formation of insoluble calcium phosphate [6, 143]. The proportion of phosphopeptides interacting with colloidal calcium phosphate correlates with their relative content of phosphoserine residues [49]. Various phosphopeptide fractions revealed significant differences in their calcium-binding activities, which may be due to variant amino acid composition around the phosphorylated region [102].

The formation of caseinphosphopeptide has been observed during in vitro digestion of bovine caseins and specific caseinphosphopeptide residues have been identified in the intestinal content of minipigs after ingestion of a diet containing casein [100]. Caseinphosphopeptide can be formed also during cheese ripening due to plasmin and microbial protease activity during ripening [141, 152].

**Antithrombotic peptides**

Functional similarities between milk and blood coagulation as well as sequence homologies exist in the fibrinogen g-chain and k-casein [72]. Jolles et al. [73] showed that bovine k-casein f106-f116 inhibited platelet aggregation and combined with the receptor site, consequently preventing fibrinogen binding with blood platelets. This inhibition was dependent on peptide concentration. The two smaller tryptic peptides (k-casein f106-f112 and f113-f116) exerted a much more minimal effect on platelet aggregation and did not inhibit fibrinogen binding. These peptides are referred to as casoplatelins. The behavior of k-casein f106-f116 is similar to that of the C-terminal peptide of the human fibrinogen g-chain [43].

The mechanism involved in milk clotting, defined by interaction of k-casein with chymosin bear a remarkable similarity to the process involved in blood clotting, defined by interaction of fibrinogen with thrombin [74]. The k-casein fragment named casoplatelins, obtained from
tryptic hydrolysates, shows antithrombotic activity by inhibiting fibrinogen binding platelet [73, 74]. These peptides are released during gastrointestinal digestion and absorbed intact into the blood, which supports the concept that they exert an antithrombotic effect in vivo. The potential physiological effects of these antithrombotic peptides have not been established, but such peptides have been detected in the plasma of newborn children after breastfeeding or ingestion of cow milk-based infant formula [18].

**Antimicrobial peptides**

The antimicrobial activity of milk is mainly associated with minor whey proteins, namely lactoferrin. This protein has bacteriostatic and bactericidal properties attributed to its ability to chelate iron or to bind to bacterial surfaces. Tomita et al. [164] found out that pepsin digestion of bovine lactoferrin produces potent bactericidal peptide, and that the antimicrobial potency of hydrolysate was higher than that of undigested lactoferrin. Dionysius and Milne [33] have identified two peptides from the N-terminal of lactoferrin which displayed antimicrobial activity toward a number of pathogenic and food spoilage microorganisms.

According to the research of Lahov and Regelson [85], the bactericidal mechanism is independent of iron because the identified peptides are distinct from the iron-binding site of the molecule. It is possible that the active peptides have an affinity for the bactericidal cell surface and act by disrupting the essential membrane functions. No effect has been detected against bifidobacterium; therefore lactoferrin derived peptides may positively affect the intestinal flora. αs-casein f1-f23 obtained from chymosin hydrolysis has been shown to have antibacterial activity against *Staphylococcus aureus* and *Candida albicans* [85].

In reviews written by Lopez-Fandino et al. [92] and Murray & FitzGerald [117] describe that, among the different groups of bioactive peptides, angiotensin converting enzyme (ACE) inhibitory peptides are receiving special attention due to the prevalence and importance of hypertension in the Western population. ACE may play an important role in the prevention and treatment of hypertension. According to Jauhiainen & Korpela [68], the consumption of food products containing antihypertensive peptides produces a significant reduction in blood pressure [68]. Today, it is now widely recognized that the AMP concept could play a promising role in fighting the presently raging microbial resistance to conventional antibiotics [70, 178]. It was only a short period of time before selection pressures allowed these environmental resistance determinants to become incorporated into the pathogenic bacteria that were being treated with the new antibiotics [16]. In addition, medical and agricultural practices of the past 50 years have promoted resistance development and spread in both human and animal pathogens, compromising effective chemotherapy of infectious diseases [134]. Today, many important pathogens are resistant to multiple antimicrobial classes, covering most, sometimes all, clinically useable antimicrobials. Infections caused by these so-called multidrug resistant (MDR) organisms are costly to treat while the treatment is increasingly prone to failure [29, 31]. Bacterial resistance to antimicrobials comes in many flavors, relying typically on drug inactivation [170] or target site modification/mutation [86]. Reduced drug accumulation owing to limited uptake or enhanced efflux is also an important resistance mechanism for certain classes of antimicrobials [88]. Another mechanism is that of phenotypic resistance owing to specific growth modes (e.g., biofilm) during infection. It is prevalent amongst bacterial pathogens and likely plays a major role in in-vivo resistance and treatment failure despite indications of in-vitro drug susceptibility [154]. Overall, the clinical impact of resistance is immense, characterized by increased cost, length of hospital stay and mortality. Thus, it was time to
consider new classes of antibiotics such as the AMPs [57] whose mode of action promises both low susceptibility to MDR mechanisms and high activity against a vast range of microorganisms [41]. AMPs are produced by most species of life, from prokaryotes [5, 165] to plants [44, 52], insects [14, 56, 118], amphibians [20, 28, 114, 121, 177] and mammals including humans [50, 51], as part of the organism's host defense mechanism. As major effectors of the innate immune system, AMPs complement the highly specific but relatively slow adaptive immune system [51]. Unlike the acquired immune mechanisms, endogenous AMPs, which are constitutively expressed or induced, provide fast and effective means of defense. Most of these gene encoded peptides are mobilized shortly after microbial infection and act rapidly to neutralize a broad range of microbes [150].

Antioxidative peptide

The importance of oxidation in the body and in food stuffs has been widely recognized. Oxidative metabolism is essential for survival of cells. A side effect of this dependence is the production of free radicals and other reactive oxygen species that cause oxidative changes. When an excess of free radicals is formed, they can overwhelm protective enzymes like superoxide dismutase, catalase and peroxidase which cause destructive and lethal cellular effects (e.g. apoptosis) by oxidizing membrane lipids, cellular proteins, DNA, and enzymes thus shutting down cellular process. Recent studies have shown that anti-oxidative peptides can be released from caseins in hydrolysis by digestive enzymes and in fermentation of milk with proteolytic lactic acid bacteria strains [81]. Most of the identified peptides are derived from αs-casein and have been shown to possess free radical scavenging activities and to inhibit enzymatic and non enzymatic lipid peroxidation, most likely by being a preferred target over fatty acid free radicals [138]. The consumption of fermented goat milk improved antiatherogenicity in healthy subjects by prolonging the resistance of the lipoprotein fraction to oxidation, lowering the levels of peroxidized lipoproteins, oxidized LDL, 8-isoprostanes and the glutathione redox reaction, and enhancing total antioxidative activity [83]. Therefore, it is hypothesized that low antioxidant levels may increase coronary heart disease. More research is needed to elucidate the role of antioxidative peptides in the protective functions in human.

Antihypertensive peptides

Antihypertensive peptides have been found in processed dairy products (cheese, milk etc.) without any intentional functional role. Lactotripeptides isoleucine-proline-proline (Ile-Pro-Pro) and valine-proline-proline (Val-Pro-Pro) have been found from sour milk [120]. Also several cheeses from Swiss origin contain the same tripeptides. The concentration of Ile-Pro-Pro and Val-Pro-Pro seems to increase in the course of ripening process, reaching 100 mg/kg after 4-7 months. Whey fraction of yoghurt like product was found to contain a dipeptide Tyr-Pro, which produced a significant antihypertensive effect in spontaneously hypertensive rats (SHR) [173].

Although casein and whey have been shown to decrease blood pressure also as such, research has been focused on their degradation products, peptides [127]. Peptides may be deliberated from their parent protein by enzymatic hydrolysis during gastrointestinal digestion, fermentation of milk with proteolytic starter cultures or hydrolysis by enzymes obtained from microorganisms [130]. If the structure of the peptide is known, it is also possible to synthesize peptides by chemical synthesis, recombinant DNA technology or enzymatic synthesis.

The hydrolysis of isoelectric casein with pepsin generates antihypertensive peptides and the peptides responsible of this activity have been identified. The most potent ACE-inhibitory
peptides found in these hydrolysates corresponded to the sequences RYLGY, AYFYPEL, and YQKFPQY. These three sequences have in vitro radical scavenging activity and also exerted significant antihypertensive activity when administered orally at SHR at doses of 5 mg/kg of body weight. It must be highlighted that the antihypertensive effect found for peptides RYLGY and AYFYPEL is comparable to the activity of Val-Pro-Pro, an antihypertensive peptide already included in functional foods. These sequences together with their antihypertensive activity have been demonstrated here for the first time. Further studies to evaluate resistance of these sequences to gastrointestinal enzymes and chronic administration of the total hydrolysate in SHR are already in progress [26]. Hypertension is known to be one of the major risk factors for a number of cardiovascular diseases (CVD). The risk of developing CVD is directly related to blood pressure level. It is controlled by a number of interacting biochemical pathways. Blood pressure control is partly associated with renin-angiotensin system. Angiotensin I converting enzyme (ACE) is a multifunctional ectoenzyme that is located in different tissues and plays an important role in blood pressure regulation. Renin acts on angiotensinogen, inactive precursor, thus releasing decapetide angiotensin I. ACE further removes the C-terminal dipeptide HL from Angiotensin I resulting in the formation of Angiotensin II, a potent vasoconstrictor. ACE also removes the C-terminal dipeptide from bradykinin (potent vasodilator) resulting in the formation of inactive peptide fragment. Therefore ACE inhibition mainly results in hypotensive effect, but it may also influence different regulatory systems involved in immunodefence and nervous system activity [107]. ACE inhibitors derived from milk proteins represent different fragments of casein, named casokinins or whey proteins, named lactokinins [45]. Two potent ACE inhibitory peptides from β-casein, f84-f86, which corresponds to Val-Pro-Pro, and f74-f76, which corresponds to Ile-Pro-Pro and one from k-casein, f108-f110, which corresponds to Ile-Pro-Pro were purified from Japanese soft drink “Calpis” made from bovine skim milk fermented with Lactobacillus helveticus and Saccharomyces cerevisiae [120]. Single oral administration of sour milk containing these two tripeptides to spontaneously hypertensive rats (SHR) with dosage of 5 ml/kg of body weight significantly decreased the systolic blood pressure from 6 to 8 h after administration [120]. Antihypertensive effect of these chemically synthesized peptides was also observed from 2 to 8 h after administration and the effects were dose dependent. These two tripeptides have been also isolated from casein hydrolysate produced by extracellular proteinase enzyme of Lactobacillus helveticus CP790 [172]. Using the same proteinase, Maeno et al. [94] identified a β-casein-derived antihypertensive peptide from the casein hydrolysate. The antihypertensive effect of this peptide was dose dependent in SHR at a dosage level from 0.2-2 mg/kg of body weight. This peptide did not show strong ACE inhibitory activity as such, but a corresponding synthetic hexa-peptide deleted by Gln (Lys-Val-Leu-Pro-Val-Pro) exhibited strong ACE inhibitory activity as well as antihypertensive effect in SHR. This suggests possible activation of the peptides in the digestive tract. It has been demonstrated that a tetrapeptide isolated from β-lactoglobulin f142-f145, termed “β-lactosin B” had significant anti-hypertensive activity when administered orally to spontaneously hypertensive rats [116]. A study in normotensive and mildly hypertensive human volunteers reported that twice daily ingestion of 10 g of a tryptic digest of casein for 4 weeks had an antihypertensive effect [147]. In a placebo controlled study, the blood pressure of hypertensive patients decreased significantly after 4-8 weeks of daily ingestion of 95 ml of “Calpis” sour milk containing IPP and VPP tripeptides [62]. No major changes in blood pressure were observed in the placebo group. The ingested dose of these ACE inhibitory peptides was in the range of only 1.2-1.6 mg [62, 61]. These results were supported by a recent double-blind randomized controlled study in which the effect of “Calpis” was in borderline hypertensive men upon oral administration of 160 g of the product for 4 weeks [111]. Systolic blood pressure in the test group decreased
significantly after 2 and 4 weeks of ingestion of “Calpis”. No significant change in blood pressure was observed in the placebo group, which was administered unfermented acidified milk. Recently a study was conducted among patients with high-normal blood pressure and mild hypertension, who took different doses of casein hydrolysate produced with Aspergillus oryzae containing IPP and VPP [112]. Volunteers consuming a 1.8 mg daily dose of IPP and VPP exhibited a significant decrease in systolic blood pressure (SBP) after 6 weeks and in those receiving either 2.5 or 3.6 mg, this benefit was already recorded at 3 weeks. Moreover a significant difference in SBP between the placebo group and IPP and VPP group receiving 3.6 mg was also observed. Two different human studies with Evolus reported statically significant decrease in SBP of 10 and 6.7 mm Hg compared with controls (acidified milk and a mixed strain of Lactococcus-fermented milk) following 8 and 21 weeks ingestion of 150 ml, respectively, by mildly hypertensive volunteers. Two other commercial products, a casein hydrolysate (Casein DP, Kanebo, Ltd., Japan and C12 peptide, DMV, Netherlands), and a whey protein hydrolysate (Biozate, Davisco, US) were also claimed to lower blood pressure in humans [45]. ACE inhibitory peptides derived from dairy products are not as potent as the drugs used for hypertension treatment, but hold a promise as safe and natural therapeutic agent without any adverse side effect. The antihypertensive potential of milk protein-derived peptides is dependent on the ability of these peptides to reach their target site without being degraded and as a consequence inactivated by the action of intestinal or plasma peptidases. Resistance to peptidase degradation may be a prerequisite for an antihypertensive effect during the oral ingestion and the intravenous infusion of ACE inhibitory hydrolysates/peptides. For example, αs1-casein f23-f27, a potent ACE inhibitor in vitro, was shown to have no hypotensive effect in vivo [95]. The presence of Val-Pro-Pro and Ile-Pro-Pro in heat-treated solubilised aortal fractions of SHR fed on “Calpis” sour milk demonstrates the resistance of these peptides to intestinal and circulatory peptidases in addition to the absorption of these peptides from the intestine [98]. Proline-containing peptides are generally resistant to degradation by digestive enzymes [78, 2]. Furthermore, tripeptides containing C-terminal Pro-Pro are reported to be resistant to proline specific peptidases [113]. It is interesting that the tryptic peptide, β-lactoglobulin f142-f148, was resistant to further degradation by pepsin and chymotrypsin [115]. On the other hand, peptide degradation or fragmentation may result in more potent ACE inhibitory activities. For example, removal of C-terminal glutamine from β-casein f169-f175 increased the in vitro ACE inhibitory potency from 1000 to 5 mmol/l, however, both β-casein f169-f174 and f169-f175 had strong antihypertensive activities in SHRs [94]. These results emphasize the necessity of performing in vivo studies in all cases. It is very difficult to establish a direct relationship between in vitro and in vivo activity. This is mainly due to the bioavailability of the ACE inhibitory peptides after oral administration and the fact that peptides may influence blood pressure by mechanisms other than ACE inhibition. To exert an antihypertensive effect after oral ingestion, ACE inhibitory peptides have to reach the cardiovascular system in an active form. Therefore, they need to remain active during digestion by human proteases and be transported through the intestinal wall into the blood. The bioavailability of some ACE inhibitory peptides has been studied. It is also known that proline-containing peptides are generally resistant to degradation by digestive enzymes. Peptides can be absorbed intact through the intestine by paracellular and transcellular routes, but the potency of the bioactivity after absorption is inversely correlated to chain length [168]. Interestingly it has been seen that ACE inhibitory peptides are also related with bone health. Polymorphism in ACE causes lower ACE activity, and this has been shown to correlate with higher bone mineral density [129]. Ang II has been reported to affect bone by decreasing osteoblast differentiation and increasing osteoclastic bone resorption [58, 63]. The inhibition of ACE increases the formation of the vascular endothelial growth factor, which is important for bone formation [64].
On the other hand, bradykinin indirectly helps in the formation of bone as described by Van’t Hof et al. [167] in their review article. ACE inhibitors have been shown to multiply the activity of bradykinin. In conclusion, ACE inhibition can affect bone through Ang II inhibition or bradykinin activation. Immuno-defensing peptides. The systems involved in the human body’s defense against invaders are rather complex and diet is known to play an important role therein. Research concerning the role of functional peptides is quite recent but seems to be promising. The two main activities are the immunomodulatory (stimulation of immune system) and antimicrobial (inhibition of bacterial pathogens). Several casein and whey protein derived peptides display an immunomodulatory role in which case a totally separate cascade of host defense responses is initiated. Immunomodulating peptides have been found to stimulate the proliferation of human lymphocytes, the phagocytic activities of macrophages and antibody synthesis. The peptides may stimulate the proliferation and maturation of T cells and natural killer cells for defense of newborn against a large number of bacteria, particularly enteric bacteria [25]. Casein derived immunopeptides including fragments of αs1-casein and β-casein stimulate phagocytosis of sheep red blood cells by murine peritoneal macrophages and exert a protective effect against Klebsiella pneumoniae infection in mice after intravenous administration [109]. The C-terminal β-casein sequence 193-209 containing β-casokinin-10 induced significant a proliferative response in rat lymphocytes [27]. Depending on peptide concentration, β-casokinin-10 and β-casomorphin-7 showed a suppression as well as stimulation of lymphocyte proliferation. β-casomorphin-7 inhibits the proliferation of human colonic lamina propria lymphocytes where anti-proliferative effect was reversed by opiate receptor antagonist naloxone [40]. Also, it has been suggested that immunomodulatory milk peptides may alleviate allergic reactions in atopic humans and enhance mucosal immunity in the gastrointestinal tract [81]. In this way, immunomodulatory peptides may regulate the development of the immune system in newborn infants. Furthermore, immunopeptides formed during milk fermentation have been shown to contribute to the antitumor effects [99]. Recent studies have focused on immuno enhancing properties of casenophosphopeptides. Hata et al. [62] reported on the immuno stimulatory action of phosphopeptides 39-CN (f59-f79)5P, αs2-CN (f1-f32)4P and β-CN (f1-f25)4P, which enhanced immunoglobulin IgG production in mouse spleen cell cultures. Moreover, the level of serum and intestinal antigen specific IgA was higher in the mice fed the casenophosphopeptides than those fed the control diet. Another group of peptides which may be implicated in the stimulation of immune-system are ACE inhibitors. Inhibition of ACE favors bradykinin formation and thus acts as immunomodulators. Bradykinin, known as mediator of the acute inflammation process, is able to stimulate macrophages to enhance lymphocyte migration and increase secretion of lymphokinines [126]. The peptide fragments αs1-casein f194-f199 and β-casein f60-f66 and f193-f202 have shown to have both immuno stimulatory and ACE inhibitory activities.

The structure-activity relationship and mechanisms by which milk-derived peptides exert their immunomodulatory effects is not yet defined. It has been suggested that arginine in the N- or C-terminal region of peptide is important structural entity recognized by specific membrane bound receptors [126]. The immuno stimulatory activity casenophosphopeptides was attributable to phosphoseryl residues, [62] and the phosphorylation site appears to be an allergenic epitope in caseins [9]. The results obtained with human lymphocytes suggest that opioid peptides may affect the immuno-reactivity of lymphocytes via opiate receptor. Therefore, there is a remarkable relationship between the immune system and opioid peptides, because opioid µ receptors for endorphins are present on lymphocytes (Elitsur et al. [40]). It has been shown that glutamine containing peptides can substitute for the free amino acid glutamine, which is required for lymphocyte proliferation and utilized at a high rate by immuno competent cells, even in a resting state [17]. Therefore, such peptides exert nonspecific immuno stimulation as a result of their trophic properties. Immunopeptides have
potential applications as supplements in the maintenance of immune health. For example, they can potentially provide some protection against infections involving bacteria, viruses, and parasites. Alternatively, immunosuppressive peptides could be considered in some medical applications such as the prevention of graft or transplants rejection and in the regulation of inflammation process involved in various autoimmune disorders [54]. The antimicrobial properties of milk have been widely acknowledged for many years. The antimicrobial activity of milk is mainly attributed to immunoglobulins, and to non immune proteins, such as lactoferrin, lactoperoxidase, and lysozyme. It has been recognized for a long time that breast-feeding of infants provides protection from a range of enteric and respiratory infections. Antibacterial peptides are recognized as an important component of innate immunity, particularly at mucosal surfaces such as the lungs and small intestine that are constantly exposed to a range of potential pathogens. An amphiphilic and a positive charge are recognized as major structural motifs determining the interaction with bacterial membranes, which has been accepted as a common target in their mechanism of action. It has been demonstrated that some milk derived antibacterial peptides can reach intracellular targets. One of the most potent antimicrobial peptides described so far corresponds to a fragment of the whey protein lactoferrin, named lactoferricin [7]. The structure-activity of lactoferricin fragment has been studied during last decade. It has been suggested that while the antimicrobial, antifungal, antitumor and antiviral properties of lactoferricin can be related to tryptophan/arginine rich proportion of the peptide, the anti-inflammatory and immunomodulating properties are more related to a positively charged region of the molecule [169]. The peptides derived from LF hydrolysates can be useful for clinical applications because of their immunomodulatory effects or for chemoprevention of carcinogenesis. According to Exposito et al. [91], the use of LF-derivatives can also be used in oral care and as food preservative. Later on, other whey proteins such as α-lactalbumin and β-lactoglobulin have also been considered as potential precursors of bactericidal fragments. Similarly, antibacterial fragments have also been derived from αs1-, αs2-, and k-casein [85]. These peptides have been found to be active against broad range of pathogenic organisms, e.g., Escherichia, Helicobacter, Listeria, Salmonella and Staphylococcus, yeasts, and filamentous fungi. Depending on the target bacterial strains, inhibitory concentrations of peptides vary, e.g., antimicrobial peptides αs2-CN f183-f207 and f164-f179 exhibited inhibition against gram positive and negative bacteria with MICs (minimal inhibitory concentration) ranging from 8 to 95 µmol/l [85]. Bactericidal peptides may assist in protecting against a microbial challenge, especially in the neonatal intestinal tract, and thus support the non-immune defense of the gut. On the other hand, these peptides may find interesting applications in the field of food safety and as pharmaceuticals.

According to Jakala and Vapaatalo [67], as they describe in their review, the blood pressure lowering effect has been confirmed in different animal models of human hypertension. There is some evidence for their beneficial effect on vasculature as well. All these effects could have been related to ACE-inhibition. Clinical evidence for the antihypertensive effect of the lacto tripeptides is more controversial. Jakala and Vapaatalo [67] suggest that products containing lactotripeptides offer a valuable option as a non-pharmacological and nutritional treatment of elevated blood pressure.

**Commercial applications of bioactive peptides**

Bioactive peptides have attracted increasing interest as prominent candidates for various health-promoting functional foods. At present, milk proteins are the best known source of such ingredients but until recently the commercial production of milk derived bioactive peptides has been limited by a lack of suitable large-scale technologies. Membrane separation
techniques seem to provide the best technology available for the enrichment of peptides with a specific molecular weight range [79, 82, 135]. Nano filtration and ultra filtration techniques are now employed industrially to produce ingredients which contain specific bioactive peptides based on casein or whey protein hydrolysates. Such preparations are commercially available and are being introduced into different consumer products, such as dairy and fruit based drinks, confectionery, chewing gum, pastilles and capsules. Currently marketed products contain peptides with anti-cariogenic, antihypertensive, mineral binding, stress relieving and satiety inducing properties. Only a few among the great number of milk peptides identified as antihypertensive under in vitro conditions have so far proven clinically effective in animal and human studies as reviewed in recent articles [42, 68, 117, 144]. In most of the human studies moderate or significant reduction of blood pressure has been observed after consumption of specific milk protein hydrolysates or fermented dairy products. In these studies, reductions of 1.5-14.0 mm Hg for systolic blood pressure (SBP) and 0.5 to 6.8 mm Hg for diastolic blood pressure (DBP), compared with placebo have been recorded. The best studied commercial products contain the two ACE-inhibitory tripeptides VPP and IPP and are sold under the trademarks Calpis and Evolus, respectively. The Japanese product Calpis is fermented with a culture containing L. helveticus and S. cerevisiae [159] and the Finnish product Evolus is produced using a L. helveticus LBK-16 H strain in milk fermentation [148]. In addition to the reduction of blood pressure via the inhibition of the renin-angiotensin system, recent clinical studies suggest that these products improve the vascular function of hypertensive subjects also by reducing arterial stiffness [66, 69]. The potential health effects of CCP’s have been studied in many animal model and human studies (Meisel and FitzGerald, [103]). Due to conflicting results obtained in these studies, the CPP’s role in enhancement of calcium bioavailability in the body remains to be established. Instead, the anti cariogenic effect of CPP’s has been well documented and various dental care products containing CPP’s have been launched on the market in some countries [137].

Yamauchi [175] has reported that two peptides derived from serum albumin and β-lactoglobulin induced contraction of guinea pig ileum longitudinal muscle when the test was completed without electric stimulation in the absence of agonist. The peptides were referred as to peptides acting on smooth muscle and they contained serum albumin f208-f216 (albutensin A) and β-lactoglobulin f146-f149 (β-lactotensin). β-lactotensin was released during hydrolysis with chymotrypsin and it had a non-opioid contracting effect on guinea pig smooth muscle [132].

Some of the peptides derived from milk proteins have more than one functional significance, peptides from the sequence 60-70 of β-casein show immuno-stimulatory, opioid and ACE-inhibitory activities. Such sequence is defined as strategic zone [110]. This sequence is protected from proteolysis because of its high hydrophobicity and the presence of proline residues. In addition to the strategic zone, some other multifunctional peptides can be liberated from milk proteins, for example αs1-casein f194-f199 and β-casein f177-f183 have immunomodulatory and ACE-inhibitory activity. Also from whey proteins multifunctional peptides can be liberated, e.g. β-lactoglobulin f102-f105 (β-lactorphin) shows both ACE-inhibitory and opioid activity.

Possible physiological importance
Bioactive peptides are widely distributed among milk proteins. This fact suggests the physiological importance of these peptides. Although the potency of these milk derived peptides is smaller than those of endogenous peptides or peptide-based drugs, they may well have physiological effects, because milk proteins are usually ingested in fairly large amounts.
To exert physiological effects in vivo, bioactive peptides must be released during intestinal digestion and then reach their target sites at the luminal side of the intestinal tract or after reabsorption, in the peripheral organs (Fig. 1).

Many studies have shown in vitro formation of bioactive peptides and in some studies in vivo formation have also been found. In addition to liberation during in vitro or in vivo digestion, bioactive peptides may also be liberated during the manufacture of milk products (Fig. 2). For example, hydrolyzed milk proteins used for hypoallergenic infant formulae, for clinical application and as food ingredients, consist exclusively of peptides. Proteolysis during milk fermentation and cheese ripening leads to the formation of various peptides. Indeed, casomorphins, ACE-inhibitory peptides and phosphopeptides have been found from fermented milk products.

There are different possible intestinal and peripheral target sites of the active substance (Fig. 3). As opioid receptor ligands, these peptides can be expected to behave like other opioids i.e. to act as agonist or antagonist, to bind to receptors and elicit effects at all cells or tissues where opioids are known to do this, mediated via signal transduction pathways as known for opioids already. Indirect evidence suggest the presence of β-casomorphins in the intestinal of humans after milk ingestion [158], whereas milk derived opioid peptides do not seem to permeate into the cardiovascular compartment, in more than negligible amounts, in adult mammals. Enzymatic degradation of peptides in the intestinal wall and in the blood appears to prevent. It seems likely that casomorphins participate in the control of gastrointestinal function in adults [163]. Casomorphins have been found to prolong gastrointestinal transit time and exert anti-diarrhoeal action [30].

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**Fig. 1 Physiological functionality of milk derived peptides**

| Antihypertensive | CARDIOVASCULAR SYSTEM |
| Antioxidative | |
| Antithrombotic | |
| Hypocholesterolemic | |
| Opioid | NERVOUS SYSTEM |
| • agonist activity | |
| • antagonist activity | |
| Mineral-binding | GASTROINTESTINAL SYSTEM |
| Anti-appetizing | |
| Antimicrobial | |
| Immunomodulatory | IMMUNE SYSTEM |
| Cytomodulatory | |
The physiological significance of opioid peptides may be different in pregnant, puerperal women and in neonates. \( \beta \)-casomorphin immuno reactive materials, obviously representing \( \beta \)-casein cleavage products larger than \( \beta \)-casomorphins, i.e., potential \( \beta \)-casomorphin precursors, have been found in plasma and in the cardiovascular compartment in women during pregnancy and lactation and new-born animals [161]. These findings indicate the pharmacological activity of \( \beta \)-casomorphins not for physiological significance.

Fig. 2 Illustrations of several functionalities of milk proteins useful for delivery tasks [89]

Inhibition of ACE, which is located in different tissues (e.g. plasma, lung, kidney, heart, skeletal muscle, pancreas brain) may influence different regulatory systems [123]. When ACE-inhibitory peptides (Val-Pro-Pro and Ile-Pro-Pro) were given to spontaneously hypertensive rats, the blood pressure was reduced dose dependently. The peptide mixture or the fermented milk containing the peptides did not change the blood pressure [120]. Masuda et al. [98] detected two ACE-inhibitory tripeptides, present in fermented milk product, in aorta after oral administration of fermented milk in spontaneously hypertensive rats. Also the ACE activity in fractions from aorta was lower in rats given fermented milk than in control group. The results indicate that these tripeptides are absorbed directly without being decomposed by digestive enzymes, reach the abdominal aorta, inhibit the ACE and show antihypertensive activity.

Casein phosphor peptides have been shown to have anticariogenic properties, based on their ability to localize amorphous phosphate in dental plaque [136]. Caseinphosphopeptide residues have been isolated in the intestinal contents of pigs and rats fed casein, indicating the in vivo formation. Most minerals are dissociated from the food, as a result of the low pH in the stomach, subsequently transferring to the duodenum. These ions may gradually become insoluble as pH increases. When phosphopeptides are present, metal ions may be rebound in soluble complexes instead of precipitated with other compounds and thereby rendering them more absorbable [143]. There is, however considerable controversy as to the physiological significance of the enhancement of intestinal calcium paracellular absorption by caseinphosphopeptide. Disagreement centers on the conclusions drawn from the various experimental methods used to evaluate calcium bioavailability in vivo, which may involve different endpoint measurements. The disagreements are partly due to different compositions
of phosphopeptide preparations, which have been in the studies and might lead to different calcium-binding activities as shown by Meisel et al. [102].

Peptides with biological activity could be produced in several ways. The most common methods are: processing of foods using heat, alkali or acid conditions that hydrolyze proteins, enzymatic hydrolysis of food proteins and microbial activity of fermented foods. Although
bioactive peptides do exist in a number of processed and fermented foods, their true physiological functions in humans are unknown. In healthy individual, eating a varied diet, the presence of bioactive peptides may help keep the nervous, immune and digestive systems in a well-maintained state. The future potential value of bioactive peptides in the diet may be their ability to affect certain pathological conditions, although this has yet to be proven.

Casein derived peptides have already found interesting applications as dietary supplements (phosphopeptides) and as pharmaceutical preparations (phosphopeptides, β-casomorphins) [15, 136]. The efficacy and safe conditions of use of these peptides in animals and in humans remain to be proven. At present, ACE-inhibitory peptides and phospho-peptides are an important area in which bioactive peptides may be found to be useful ingredients for dietary applications.

Milk derived peptides can bind a variety of molecules and ions according to their specific sites. Bovine derived caseins like α and β have binding affinities to bind with calcium, as well as calcium phosphate nano-particles through their serine-phosphate residues [48]. Basic function of Lactoferrin is to bind and carry ferric ion, but it can also bind other metal ions like copper, chromium, manganese and aluminum in vivo.

Guilloteau et al. [55] determined that an inhibition of gastric secretions seemed more important when phosphopeptides (PP) was given in association with caseinomacropeptide (CMP) in the diet rather than alone. CMP and phosphopeptides (PP) may have short and long term action respectively over the 24 h day. To our knowledge, it is the first time that phosphopeptides coming from milk casein digestion are demonstrated to inhibit gastric secretion. Co-binding of one bicarbonate anion is essential for metal ion binding [48]. Function of α-la is regulation of lactose synthesis. It also has natural affinity to bind with calcium [48], besides of calcium delivery, it could potentially be utilized for this purpose in food systems. Milk proteins bind with hydrophobic molecules by using several mechanisms; basically use hydrophobic interactions, Vander Waals forces and hydrogen bonding (Fig. 2).

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