Review

Health Implications of Bioactive Peptides: A Review

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Abstract: Today, due to immobility, improper food habits, and changes in lifestyle, communities are faced with an increase in health problems such as blood pressure, cholesterol, diabetes, and thrombosis. Bioactive peptides are considered as being the main products of protein hydrolysis which exert high effects on the nervous, immune, and gastrointestinal systems. Unlike synthetic drugs, bioactive peptides have no side effects and this advantage has qualified them as an alternative to such drugs. Due to the above-mentioned properties, this paper focuses on the study of health-improving attributes of bioactive peptides such as anti-oxidative, anti-hypertensive, immunomodulatory, anti-microbial, anti-allergenic, opioid, anti-thrombotic, mineral-binding, anti-inflammatory, hypocholesterolemic, and anti-cancer effects. We also discuss the formation of bioactive peptides during fermentation, the main restrictions on the use of bioactive peptides and their applications in the field of functional foods. In general, food-derived biologically active peptides play an important role in human health and may be used in the development of novel foods with certain health claims.

Keywords: Enzymatic hydrolysis, bioactive peptides, health, functional foods

Introduction

Proteins are vital for health since they provide nitrogen, amino acids, and the energy required for normal body performance [1]. However, applications of proteins are limited due to certain properties, such as low solubility [2, 3]. Enzymatic hydrolysis of proteins is an extensively used approach to promote their chemical, functional, and nutritional properties [2–9]. Hydrolyzed proteins also have enhanced absorption and digestibility [10]. To date, casein, whey, and soy are the most studied protein sources to be hydrolyzed by enzymes [1].

Protein hydrolysis is the breakdown of proteins into smaller peptides and free amino acids. It is often preferred that hydrolysis be done with animal-based enzymes like trypsin (3.4.21.4), plant-based enzymes like papain (3.4.22.2), and some microbial enzymes rather than by acid or alkali hydrolysis, because the conditions of enzymatic hydrolysis are much milder and no amino acid is destroyed, while acid and alkali hydrolysis may destroy L-form amino acids and produce toxic substances, such as lysinoalanine [11].

Bioactive peptides are fractions of proteins 3–20 amino acids long with a potential positive influence on consumer health [12]. They exert their effects after release during food fermentation, degradation by digestive enzymes in the gastrointestinal tract or the enzymatic hydrolysis of proteins [2, 13–18]. Today, bioactive peptides are gaining more attention for their ability to produce functional foods [18]. Due to the numerous applications of bioactive peptides in human nutrition and health, the main focus of this review is to investigate the evidence for the health-promoting effects of bioactive peptides created during enzymatic hydrolysis.

Classifications of proteases

Proteases (enzymes used for protein hydrolysis) are classified into several groups: 1) Enzymes with a wide specificity like papain, ficin, and bromelain. 2) Enzymes with group specificity like pepsin, trypsin, and chymotrypsin – their activities are influenced by the type of target bond as well as by its surrounding structure. 3) Endopeptidases which cleave peptide bonds inside the protein chain. 4) Exopeptidases that break peptide bonds from the carboxyl or amino end of the protein chain. Based on the above-mentioned items, exopeptidases are capable to break down peptides into amino acids, while endopeptidases are not [11]. In case of enzymatic hydrolysis, exopeptidases are often preferred due to better control of hydroly-

sis conditions and the shorter time required to achieve the same degree of hydrolysis [19]; however, the use of endopeptidases was also reported particularly for the production of anti-oxidative peptides from fish protein [19]. It is well known that the final properties of bioactive peptides are affected by the enzyme specificities [18] as well as by hydrolysis conditions such as digestion temperature, time, and pH [19]. In another grouping, proteases are classified as proteolytic enzymes with microbial origins like alcalase, flavourzyme, and protamex; plant-based enzymes like papain; and animal-based enzymes such as pepsin and trypsin [19]. Despite different industrial applications, one of the main obstacles underlying the use of proteases are the high costs of enzymatic processes; therefore cheaper proteolytic enzymes are preferred. Animal-based proteases obtained from meat industry by-products like pancreases, proteases of microbial origin such as neutrase, subtilisin, orientase, protex 7L, and protamex 1.5 as well as enzymes produced by lactic acid bacteria (LAB) are some kinds of cost-effective proteases. Microbial proteases possess some advantages, since they are completely safe, have little nutritional requirements, and, in return, grow rapidly and can be purified by simple and inexpensive methods [20].

The applications of protein hydrolysates

Protein hydrolysates are applied as alternatives for intact proteins to provide special formulas in patient nutrition [21, 22] as well as for patients suffering from liver disease, phenylketonuria, food allergies, as diets for elderly individuals and in sports nutrition [11]. In addition they show multiple biological properties. They act as anti-oxidants [5, 11, 19, 23–30], anti-microbials [28, 31–34], immunomodulatories [18, 28, 34], anti-hypertensive [8, 14, 18, 28, 34, 35], anti-coagulant [36], and anti-carcinogenic agents [11, 18, 37], and act as regulatory agents via hormone-like activity [14, 17, 28, 30, 38]. Some other interesting characteristics are that they trigger anti-thrombotic, opioid and mineral-binding, that they have cholesterol-lowering effects [2, 3, 12, 13, 18, 28], and they act anti-fungal and anti-viral [2], anti-amnesic [18], anti-obesity [11, 12, 15], anti-inflammatory and anti-diabetic [19, 39], and can control stress [12, 15].

Health-promoting effects of bioactive peptides: Influence on the cardiovascular system

Angiotensin converting enzyme inhibitory peptides

ACE (Angiotensin Converting Enzyme) (EC 3.4.15.1) is a metallo peptidase binding to the membrane of endothelial or epithelial cells. It can also circulate in the blood and other body liquids. Blood pressure is controlled by a regulating system called the rennin–angiotensin system [8]. Rennin (3.4.23.15) acts on angiotensinogen (a protein synthesized in the liver) and releases a decapeptide named angiotensin-I [40] that is the precursor of angiotensin-II. Angiotensin-II is the main cause of increase in blood pressure because it has a vasoconstrictor effect and inhibits the vasodilator agent bradykinin [8, 40]. Furthermore, angiotensin-II raises the aldosterone level which causes water absorption and consequently increases blood pressure [40, 41]. The conversion of angiotensin-I to angiotensin-II is controlled by an ACE.

The ACE inhibitory activity of protein hydrolysates has been one of the most frequent fields of research so far, and different amino acid sequences have been discovered with ACE inhibitory activity in soy [14, 42, 43], broccoli [44], buckwheat and beef [43], amaranth and milk [45, 46], *Phaseolus lunatus* and *Phaseolus vulgaris* seeds [8], brown-striped red snapper [47], *Brassica carinata* [17], whey, α-lactalbumin and β-lactoglobulin [16, 46, 48, 49], casein [14, 40, 46], fish, meat, eggs [14], goat milk, sheep milk, cheese and yoghurt [50]. The presence of amino acid sequences Glu-Met-Pro-Phe-Pro-Lys and Tyr-Pro-Val-Glu-Pro-Phe-Thr-Glu is a common property of ACE inhibitory peptides [40]. In addition, peptides with the amino acid sequences Ile-Pro-Pro and Val-Pro-Pro showed ACE inhibitory activity [15].

The type of the enzyme is a key factor in ACE inhibitory peptides. As an example, the hydrolysis of whey protein by thermolysin (3.4.24.27) and proteinase-k (3.4.21.64) led to an increase in ACE inhibitory activity, while peptides made by actinase showed a low ACE inhibitory effect [16]. Lourenco da Costa et al. [16] reported that among the three enzymes alcalase (3.4.21.62), α-chymotrypsin (3.4.21.1), and proteomix, the most powerful ACE inhibitory activity was observed in hydrolysates produced by α-chymotrypsin.

The comparison of a DASH-like diet high in carbohydrates with diets high in proteins and diets high in unsaturated fatty acids indicated that high-protein and high-fat diets reduced the risk of cardiovascular diseases and the number of metabolic syndrome components [51]. In a

study, the decrease in systolic blood pressure of spontaneously hypertensive rats (SHR) fed by fermented milk containing tripeptides Val-Pro-Pro and Ile-Pro-Pro was examined. For this purpose, fermented milk containing Val-Pro-Pro and Ile-Pro-Pro was ingested at the level of 5 mL/kg body weight, and a decrease in systolic blood pressure was detected 6–8 hours after oral administration of sour milk, while the systolic blood pressure of the control group fed unfermented milk was not influenced. Furthermore, long-term (13 weeks) consumption of sour milk successfully reduced systolic blood pressure in SHR [18]. In a placebo-controlled study, 30 mild-hypertensive patients were treated with sour milk containing Val-Pro-Pro and Ile-Pro-Pro fractions. Patients were divided into two groups. The first group received sour milk at a concentration of 95 mL/day for 8 weeks and the second (control) group got the same amount of unfermented milk for the same period. The results confirmed a significant decrease in systolic and diastolic blood pressure of mild-hypertensive subjects who received sour milk, while the blood pressure of the control group was not influenced by unfermented milk [52]. In a randomized placebo-controlled trial, twice daily intake of 10 g of tryptic casein hydrolysates indicated an anti-hypertensive effect in normotensive and mildly hypertensive human volunteers after 4 weeks [18]. The investigation on the effect of enzymatic hydrolysis of salmon protein by protease S "Amano", Ewart et al. [53], showed a significant reduction in blood pressure of SHR that received salmon hydrolysates at a dose of 1500 mg/kg body weight. Protein hydrolysates with plant origin represented anti-hypertensive activity as well. For instance, anti-hypertensive activity of rice bran hydrolyzed by alcalase was studied by Li et al. [28]. According to their reports, single oral administration of rice bran hydrolysates at a concentration of 600 mg/kg body weight caused a significant decrease in systolic blood pressure in SHR. Moreover, a peptide with the amino acid sequence Thr-Gln-Val-Tyr isolated from rice bran hydrolysates exhibited a potent anti-hypertensive effect at a dose of 30 mg/kg body weight. Another study was conducted to evaluate the anti-hypertensive activity of tryptic hydrolyzed rice dreg protein in SHR and reduction in blood pressure was observed by 11 mmHg, 17 mmHg, 26 mmHg, and 17 mmHg at the dosage of 1 mg/kg, 10 mg/kg, and 50 mg/kg hydrolysates, and 1 mg/kg captopril (positive control), respectively, after 1 h [54].

In a research study into the thermolysin (3.4.24.27) digestion of porcine skeletal muscle, proteins were shown to make two peptides with ACE inhibitory activity with the amino acid sequences Met-Asn-Pro-Pro-Lys and Ile-Thr-Thr-Asn-Pro [55]. Jamdar et al. [2] reported that the hydrolysis of peanut by alcalase positively affected the ACE inhibitory activity of hydrolysates. In cheese, an increase in ACE inhibitory effect was related to peptides with a higher degree of hydrolysis (DH), like Gouda cheese at 13 weeks or 2 years of age [12]. Peptide fractions with a molecular weight of less than 3 kD released during digestion, fermentation or enzymatic hydrolysis of koumiss (a traditional fermented product from mare milk) also demonstrated an ACE inhibitory effect [50]. Studying the structure of anti-hypertensive peptides, Erdmann et al. [14] reported that an interesting property of these peptides is their little effect on the blood pressure of normotensive persons. Furthermore, they do not have any side effects, as synthetic anti-hypertensive drugs do. The authors reported that the presence of Pro, Lys or Arg at the C-terminus of the peptide chain is a key factor for anti-hypertensive activity [14]; however, others believed that the structure-activity relationship of anti-hypertensive peptides has not been understood, because a large variety of bioactive peptides with different C-terminal amino acid sequences may exhibit this effect. For this reason, it was suggested that peptides containing hydrophobic amino acid residues at their C-terminus may act as anti-hypertensive substances due to their ability to interact with the subsites present at the active site of ACE [18]. Apart from ACE inhibitory activity, other mechanisms by which biopeptides exert anti-hypertensive effects may be taken into account. For instance, the release of vasodilatory substances such as prostaglandin I_2, NO, and CO by bioactive peptides is thought to provide an anti-hypertensive effect. It has been shown that bioactive peptides exert their anti-hypertensive properties via the inhibition of chymase activity as well [14]. Chymase forms vasoconstrictor angiotensin-II by hydrolysis of the Phe-His bond in angiotensin-I. Similarly, the formation of angiotensin-II will be facilitated through the hydrolysis of peptide bonds at the carboxyl end of hydrophobic aromatic amino acid residues such as Phe and Trp [56]. Prevention of the release of endothelin-1 by endothelial cells, stimulation of bradykinin activity, and enhancement of the production of nitric oxide by endothelium are other ways by which bioactive peptides exert their anti-hypertensive effect [57]. Table I indicates some food-derived anti-hypertensive peptides.

Behavior of ACE inhibitory peptides during absorption and uptake

In most cases it is not reasonable to expect a direct correlation between ACE inhibitory activity and the anti-hypertensive effect of bioactive peptides. Bioavailability is a key factor in this case. In other words, having anti-hypertensive effects, bioactive peptides must be resistant against degradation in the gastrointestinal tract. It is demonstrated that the presence of Pro and Pro-Pro residues at the C-terminus of peptide chain enables the peptide to stay intact during gastrointestinal digestion [18], confirming the isolation of

several anti-hypertensive peptides from casein and gelatin, as they possess a high amount of Pro [58]. The bioactivity of anti-hypertensive peptides is also reversely correlated to chain length [58]. The well-known tripeptide Val-Pro-Pro was detected in the abdominal aorta of SHR, indicating that this tripeptide is resistant against serum peptidases, while ACE inhibitory peptide purified from α-casein was not able to lower blood pressure due to susceptibility to digestive enzymes. Lactokinin, a β-lactoglobulin-derived ACE inhibitory peptide with the amino acid sequence Ala-Leu-Pro-Met-His-Ile-Arg was also reported to pass intact through the intestine but at a concentration too low to show

Table I. Food-derived bioactive peptides with anti-hypertensive activity.

Protein	Dietary source	Involved enzymes and processes	Bioactive peptide	Reference
Glycinin		Enzymatic hydrolysis	Val-Leu-Ile-Val-Pro	[113]
Casein		Protease from *Lactobacillus helveticus CP790*	Lys-Val-Leu-Pro-Val-Pro-Gln	[134]
Casein		Protease from *Aspergillus oryzae*	Ile-Pro-Pro and Val-Pro-Pro	[12, 18]
αs_1-casein		Pepsin	Arg-Tyr-Leu-Gly-Tyr and Ala-Tyr-Phe-Tyr-ProGlu-Leu	[46]
β-Casein		*Lactobacillus delbrueckii ssp. bulgaricus* protease	Ser-Lys-Val-Tyr-Pro-Phe-Pro-Gly-Pro-Ile	[12]
β-Casein		Combination of *Streptococcus salivarius ssp. thermophiles* and *Lactococcus lactis biovar. diacetylactis*	Ser-Lys-Val- Tyr-Pro	[12]
Whey		Trypsin	Tyr-Leu at the N-terminal	[18]
Whey		Alcalase, a-chymotrypsin or proteomix	amino acids at the N-terminal position, and branched-chain amino acid residues such as Val and Ile, residues of the hydrophobic amino acids Trp, Tyr, Phe or Pro at the C-terminus	[16]
Whey fraction of yoghurt like product		Fermentation	Tyr-Pro	[18]
Lactoferricin		Pepsin	Arg-Arg-Trp-Gln-Trp-Arg and Trp-Gln	[46]
β-Lactoglobulin		Trypsin	f (142–148), Ala-Leu-Pro- Met-His-Ile-Arg	[12, 18]
	Cheddar cheese	Fermentation	αs_1-Casein f (1–6), f (1–7), f (1–9), f (24–32), f (102–110), β-casein f (47–52), f (193–209)	[12]
	Cooked eggs	*In vitro* gastrointestinal digestion	Val-Asp-Phe, Leu-Pro-Phe, Met-Pro-Phe, Ile-Pro-Phe, and Thr-Thr-Ile and Tyr-Thr-Ala-Gly-Val, Glu-Arg-Tyr-Phe-Ile	[113]
	Red snapper	Mixed hydrolysis by alcalase or flavourzyme with pyloric caeca protease	Hydrolysate	[47]
	Amaranth	Pronase, papain, trypsin, chymotrypsin, and alcalase	Peptides with less than 5 amino acids and MW lower than 6 kD.	[45]
	Rice	Alcalase	Thr-Gln-Val-Tyr	[28]
	Fermented soybean	*Bacillus natto*	Low- and high-molecular-weight inhibitors	[135]
	Peanut	Alcalase	Hydrolysate	[2]
	Brassica carinata	trypsin, chymotrypsin, and carboxypeptidase A	1400–1800 D	[17]
	Milk	*Lactobacillus helveticus* and *Streptococcus cerevisiae*	Val-Pro-Pro, Ile-Pro-Pro	[12, 14, 18, 39, 46, 85, 134]

anti-hypertensive activity. *In vitro* incubation of human plasma with a casein-derived peptide with the sequence Leu-His-Leu-Pro-Leu-Pro prepared by different strains of *Enterococcus faecalis* indicated that the peptide remained intact and was degraded by half after 1 h and 2 h of incubation, respectively [59]. The resistance of anti-hypertensive peptides against digestive enzymes may be examined by treatment of these peptides with digestive enzymes such as pepsin, trypsin, α-chymotrypsin or pancreatin. As an instance, hydrolyzed wheat germ protein and its peptide with the sequence Ile-Val-Tyr were treated with pepsin, trypsin, and α-chymotrypsin, alone and in combination. The ACE inhibitory activity of wheat germ hydrolysates was increased by 27 % after combined digestion. Similar increase in ACE inhibitory activity was observed followed by *in vitro* gastrointestinal digestion of pea and whey proteins [58]. It seems possible to enhance bioavailability of ACE inhibitory peptides through the cross-linking of peptides to protein transduction domains, use of specific peptide carriers such as Pep-1 with the potential to deliver bioactive peptides into the mammalian cells and use of chemical enhancers and surfactant-like agents [58].

Anti-oxidative peptides

Free radicals and hydroperoxides produced during oxidation are among the reasons behind some diseases, such as cancer [47, 60], cystic fibrosis [61], neurological disorders, and the ageing process [47]. In addition, oxidation is one of the most important factors leading to deterioration and quality loss of foodstuff [47]. It is shown that different plant and animal protein hydrolysates have anti-oxidant effects [14, 19, 30, 62, 63]. The mechanism of action of anti-oxidative peptides is not completely clear; however, their effect is probably related to metal ion chelating, radical scavenging, and singlet oxygen quenching activities. Nevertheless, it seems that none of these features can be effective by itself, therefore the anti-oxidative activity of bioactive peptides is the result of the cooperative effects of these mechanisms [14]. In addition, bioactive peptides may induce genes responsible for the protection of cells against reactive oxygen species [14]. There are similar ambiguities about the exact mechanism by which bioactive peptides inhibit lipid peroxidation, but it is likely related to their metal chelating activity or the presence of some groups in the side chains of amino acids or hydrolysates, which preferentially bind to fatty acid free radicals [18]. It has been reported that anti-oxidant peptides also prevent Alzheimer's, myocardial disease and ethanol-induced liver injuries [60]. Anti-oxidative activity of biologically active peptides was studied in different animal models. Daily intake of egg white protein hydrolysates at a concentration of 0.5 g/kg body weight increased plasma radical-scavenging and lipid peroxidation inhibitory activities, and decreased the aorta malondialdehyde concentration in SHR [64]. A study on the effect of soy protein and its peptides revealed that intake of a diet containing 20 % soy protein as well as its hydrolysates had a significant inhibitory effect on thiobarbituric acid reactive substances and paraquat-induced oxidative stress in male Wistar rats [65]. Evaluation of anti-oxidative activities of freeze-dried douchi (a soybean-derived fermented food) extracts at concentrations of 2 and 4 % caused an increase in superoxide dismutase, catalase, and glutathione peroxidase activities and a significant decrease in thiobarbituric acid reactive substances in liver and kidney of 3-week-old male Wistar rats [66]. In a clinical trial, human consumption of fermented goat milk elevated plasma anti-oxidative activity and lipoprotein resistance against oxidation, while decreasing the glutathione redox ratio and the concentration of oxidized low density lipoprotein (LDL) [46]. Similar *in vivo* LDL oxidation and lipid peroxidation inhibitory effects were reported in rats fed with 9 % of either low-molecular weight viscous substance (LMWVS) or soybean water extract (SWE) of natto (some kind of fermented soybean) for 3 weeks [67]. Peptides derived from the enzymatic hydrolysis of milk, eggs, fish [8, 19, 68, 69], casein [15], buckwheat [8], *Phaseolus lunatus* and *Phaseolus vulgaris* seeds [8], zein, rice, and barley hordein and flaxseed protein [68], threadfin bream surimi by-products [30], sunflower protein isolate [11, 29] potato protein [60, 70], pumpkin seed extracts [71], brown-striped red snapper [47], gingerbread plum (*Neocarya macrophylla*) seeds [72], cuttlefish (*Sepia officinalis*) muscle protein [73], peanut protein [2], *Brassica carinata* [13, 17], whey protein [62, 74], and yellowfin sole *(Limanda aspera)* frame protein [25] have been proven to show anti-oxidant activity. Anti-oxidative peptides often have a molecular weight between 500 and 1800 D [19]. Peptides rich in Pro, Leu, Ala, Trp and Phe [30] as well as those containing Tyr, Met, Cys and His in the main sequence [30, 70] and Val or Leu at the N-terminus [19] show anti-oxidant properties. Again, peptides with the amino acid sequences Try-Phe-Try-Glu-Leu and Val-Lys-Glu-Ala-Pro-Lys demonstrate anti-oxidative characteristics [15]. In addition, acidic and basic amino acids have metal chelating activity because of their amino and carboxyl groups [30]. Sheep casein hydrolysates made by alcalase, pepsin, trypsin, and chymotrypsin indicated anti-oxidative activity [50]. This ability is attributed to the existence of high amounts of His and Leu residues. It is believed that hydrophobicity has a key role in multifunctional bioactive peptides such as anti-hypertensive and anti-oxidative peptides [50, 75]. According to Lahart et al. [15], as the hydrolysis process proceeded, the molecular weight of hydrolysates decreased and hydrolysates with lower molecular weight showed much more anti-oxidative activity. The results from the enzymatic hydrolysis of brown-striped red snapper showed an increase in anti-oxidant activities with increase in hydrolysis up until a degree of hydrolysis

(DH) of 40% [27]. The effect of enzymatic hydrolysis of ginger bread plum seed protein isolates by pepsin (3.4.23.1) and trypsin (3.4.21.4) was investigated by Amza et al. [72]. An increase in nutritional parameters such as amino acid score, essential amino acid index, biological value, protein efficiency ratios, and anti-oxidant characteristics and a decrease in functional properties, such as the emulsifying index and foaming capacity, were observed as hydrolysis proceeded for 180 min. Bioactive peptides produced during the enzymatic hydrolysis of αs-casein by digestive enzymes have also been reported as having great potential for radical scavenging activity [18]. The hydrolysis of sunflower protein with pepsin, trypsin, chymotrypsin and a mixture of the three enzymes, and alcalase, flavourzyme, and a mixture of alcalase and flavourzyme, showed that peptides prepared with trypsin at 60 min hydrolysis had the highest anti-oxidant activity [11]. Comparing the anti-oxidative activities of 28 synthetic peptides with an anti-oxidative peptide obtained by the enzymatic hydrolysis of soybean, it was found that the elimination of His residue from the C-terminus decreased the anti-oxidative activities, while the elimination of Leu from the N-terminus had no effect. There was a direct correlation between the amounts of His and Pro, and the anti-oxidative activities of peptides. Among all peptides tested, Pro-His-His showed the highest anti-oxidative activity and synergistic effect on lipid-soluble anti-oxidants such as BHT and BHA [19]. DPPH radical scavenging and ferrous ion chelating activities also increased with increase in DH [2]. Two anti-oxidant peptides obtained from tilapia hydrolysates had Asp-Cys-Gly-Tyr (456.12 D) and Asn-Tyr-Asp-Glu-Tyr (702.26 D) amino acid sequences [23]. In another research study, an anti-oxidant peptide with the amino acid sequence of Ala-Cys-Ala-Lys-Asp-Lys-Val was obtained from a sunflower protein hydrolysate prepared by flavourzyme [29]. The existence of His and some hydrophobic amino acids is attributed to the anti-oxidant activity [14, 29]. Hydrophobic amino acids will facilitate the orientation to hydrophobic targets, such as fatty acids [14]. Trp residues have also been reported as showing radical scavenging activity in casein, whey, and cow and human milk protein hydrolysates [35].

Anti-thrombotic effect

Thrombosis happens when the level of fibrinogen increases or platelets accumulate and the body is unable to perform fibrinolysis [14]. Blood coagulation extremely resembles milk clotting, since milk coagulation takes place as a result of casein-rennet interaction, and the blood clotting occurs when fibrinogen is joint with thrombin [76]. Based on this similarity, bioactive peptides produced during hydrolysis or fermentation may exert anti-thrombotic activity. Until now, the most reported anti-thrombotic peptides have been isolated from cow or human κ-casein and lactoferrin [14, 19, 28]. It is understood that f [106–116], f [106–112] and f [113–116] of κ-casein – casoplatelins – have the potential to bind to f [400–411] of fibrinogen γ-chain and prevent the formation of fibrinogen-platelet interaction. Another mode of action of casoplatelins is attributed to their capability to inhibit the aggregation of ADP-activated platelets. Therefore the anti-thrombotic potential of casoplatelins is affected by the competition between human fibrinogen γ-chain and casoplatelins to bind to platelet receptors [14]. Presence of similar regions in bovine, ovine, and caprine milk as well as their tryptic hydrolysates confirmed their anti-thrombotic activity, while this characteristic was slightly higher in ovine κ-casein. In addition, f [106–111], f [106–112], f [112–116], f [113–116] and f [103–111] isolated from tryptic-hydrolyzed κ-casein inhibited platelets aggregation [76]. Similar peptides with anti-thrombotic activity were f [163–171] and f [165–171], both purified from bovine casein hydrolysates produced by trypsin and f [161–169] of tryptic hydrolyzed ovine casein [77]. Fractions f [106–112] and f [113–116] presented lower anti-thrombotic activity than that of f [106–116] [18]. It is also reported that some peptides have been separated from the N-terminus of casein that can inhibit platelet aggregation [78]. In a clinical trial, the anti-thrombotic activity of κ-casein was confirmed by a blood test on 5-day old newborns fed bovine milk [79]. In another study conducted by Shimizu et al. [80] on the anti-thrombotic activity of pork meat hydrolyzed with papain, oral administration of a fraction with the molecular weight of 2500 D at a dose of 210 mg/kg body weight caused a strong anti-thrombotic activity in mice. The similar effect was observed in a fraction with the molecular weight of 2517 D at a dose of 70 mg/kg body weight, with anti-thrombotic power equivalent to that of aspirin at a dosage of 50 mg/kg body weight. The authors claimed that daily intake of purified pork peptides can exert an anti-thrombotic effect in humans as well.

Hypocholesterolemic effects

Increases in blood cholesterol, especially LDL, may lead to cardiovascular diseases. It is claimed that milk [43], fish [28], soybeans [28, 43], whey protein and its subfractions α-lactalbumin and β-lactoglobulin [14, 46] as well as their hydrolysates (especially with hydrophobic residues) are rich in bioactive peptides with hypocholesterolemic properties. In this case, hydrolyzed proteins are more efficient than their intact form. The common characteristics of these peptides are the presence of low levels of Met-Gly and Lys-Arg residues [14]. It has been found that the amino acid composition of bioactive peptides is the main factor involved in hypocholesterolemic effects [46]. Ketogenic amino acids (amino acids that may produce acetyl CoA via ketogenesis) increase bad cholesterol (LDL) and very

low density lipoprotein (VLDL), hence they may increase the blood cholesterol level, while the amount of Cys has the opposite effect, e.g. an increase in Cys will decrease serum cholesterol [81].

It has been shown that soy protein is capable to reduce blood cholesterol. High molecular weight soy fraction 11-S globulin demonstrated a strong hypocholesterolemic effect and increased the rate at which steroids excreted into the feces compared to low molecular weight fractions [81]. Microbial or enzymatic hydrolysis of soy protein had a positive effect on hypocholesterolemic action of final hydrolysates [81]. Zhong et al. [43] studied soy protein hydrolysates with DH of 18% to examine the possibility of purification of a fraction with the highest hypocholesterolemic activity. The highest *in vitro* hypocholesterolemic effect was observed in a fraction eluted with 75% ethanol. More fractionation by Sephadex G15 showed the highest micellar cholesterol solubility inhibition rate of 81.3% by a fraction with a molecular weight of 300–800 D. In the final step, a fraction with the hypocholesterolemic activity of 94.3% was obtained during purification with RP-HPLC. The authors reported that hypocholesterolemic activity directly correlated with hydrophobicity, e.g. the cholesterol-lowering effect increased with increase in peptide hydrophobicity [43]. In addition to hydrophobicity, the hypocholesterolemic effect of bioactive peptides is associated with the amino acid composition and bile-acid-binding capacity [81]. Different animal studies have indicated that soybean-derived biologically active peptides activate LDL receptors or enhance the expression of genes in liver cells. In addition, regulation of synthesis and catabolism of LDL as a result of the presence of special amino acids is thought to be important in hypocholesterolemic activity [82]. It is said that fish proteins possess a hypocholesterolemic effect, while casein may lead to an increase in blood cholesterol level [14]. Investigating the effect of immobilized enzymes alcalase, trypsin, and chymotrypsin on the health-promoting properties of *Brassica carinata*, Pedroche et al. [17] reported that an *in vitro* reduction of 43% in micellar cholesterol was achieved by a hydrolysate with the DH of 36% and the molecular weight of less than 15 kD in comparison with protein isolate (with cholesterol-lowering effect of 10%), and the intact protein (with cholesterol-lowering effect of 7%). Based on these results, the hypocholesterolemic activity increased with increase in hydrolysates concentration from 0.1 to 4 mg/mL.

It has been shown that whey protein and its derivatives such as β-lactoglobulin diminished serum cholesterol in rats [81]. In addition to β-lactoglobulin, its tryptic hydrolysates lowered serum cholesterol and increased fecal excretion of total steroids in rats, and this effect was much stronger than with casein tryptic hydrolysates. Further investigations exhibited that hydrolysates also inhibited micellar cholesterol absorption in Caco-2 cells. According to the authors, lactostatin, a novel β-lactoglobulin-derived hypocholesterolemic peptide with the amino acid sequence Ile-Ile-Ala-Glu-Lys, could surpass casein hydrolysates or even β-sitosterol in terms of hypocholesterolemic action [83]. β-Lactotensin, another example of hypocholesterolemic peptides obtained through enzymatic hydrolysis of β-lactoglobulin with chymotrypsin, caused a significant decrease in blood cholesterol level and LDL content in rats fed a cholesterol-enriched diet [46]. Additionally, natto water-soluble fractions, i.e. low-molecular weight viscous substance (LMWVS) and soybean water extract (SWE) showed the tendency to lower total cholesterol, free cholesterol, and LDL-cholesterol levels in rats fed cholesterol-enriched diets, indicating that natto fractions suppressed intestinal lipid absorption [67]. Egg white protein as well as its hydrolysates has also been proven to possess *in vivo* hypocholesterolemic effect. In contrast, the consumption of egg yolk may lead to an increase in serum cholesterol in humans and animals [81]. Some examples of anti-thrombotic and *hypocholesterolemic* peptides are listed in Table II.

Regulation of the immune system

Immunomodulatory and anti-microbial activity

Bioactive peptides released during hydrolysis may have immunomodulatory activity, as indicated by enhancement of phagocytosis and the maturation of immune cells, lymphocytes, antibodies, and T-killers [18]. These peptides will provide the newborn immunity against bacteria, infections, and risk factors [18]. Casein and whey protein hydrolysates are among the best-known peptides with immunomodulatory activity [84]. It has been reported that peptides obtained from hydrolysis of casein may have either immune-stimulating or -suppressive effects, depending on the concentration. As an example, β-casokinin-10 separated from the C-terminus of β-casein intensified lymphocyte proliferation in rats [84]. However, β-casokinin-7 had an opposite effect on human lymphocytes [84]. The structure-activity relationship and the exact mechanism of action of immunomodulatory peptides still remains unclear, but the common property of immunomodulating peptides is the existence of Tyr-Gly and Tyr-Gly-Gly sequences [18, 85] as well as Arg at the C- or N-terminus of the peptide chain [18]. It has been suggested that immunomodulatory peptides enhance the proliferation and maturation of immune cells such as human lymphocytes, stimulate antibody synthesis and phagocytic activity of macrophages, and intensify the proliferation and maturation of T-cells and natural killer cells [18].

Table II. Food-derived anti-thrombotic and hypocholesterolemic peptides

Protein	Dietary source	Involved enzymes and processes	Bioactive peptide	Health-promoting effect	Reference
κ-Casein		Trypsin	f (106–112) and f (113–116)	Anti-thrombosis	[18]
Bovine κ-casein		Enzymatic hydrolysis	Met-Ala-Ile-Pro-Pro-Lys-Lys-Asn-Gln-Asp-Lys, f (106–116)	Anti-thrombosis	[14]
β-Lactoglobulin		Trypsin	Ile-Ile-Ala-Glu-Lys	Anti-thrombosis	[113]
Lactoferrin		Enzymatic hydrolysis	Lys-Arg-Asp-Ser and Arg-Gly-Asp-Ser	Anti-thrombosis	[14]
Glycinin		Enzymatic hydrolysis	Leu-Pro-Tyr-Pro-Arg	Anti-thrombosis	[113]
β-Lactoglobulin		Trypsin, chymotrypsin	f (71–75)	Hypocholesterolemic	[46]
β-Lactoglobulin		Trypsin	Ile-Ala-Glu-Lys	Hypocholesterolemic	[43]
Glycinin		Enzymatic hydrolysis	Leu-Pro-Tyr-Pro-Arg, Ieu-Ala-Val-Pro-Gly-Glu-Val-Ala	Hypocholesterolemic	[14]
	Brassica carinata	Trypsin, chymotrypsin, and carboxypeptidase A	1400–1800 D	Hypocholesterolemic	[17]
	Pork meat	Papain	Fraction with the molecular weight of 2517 D	Anti-thrombosis	[80]
	Soy	Alcalase	Trp-Gly-Ala-Pro-Ser-Leu, Leu-Pro-Tyr-Pro	Hypocholesterolemic	[43]

Peptides with immunomodulatory activity are likely to have ACE inhibitory activity as well. For instance, f [194–199] of αs_1-casein as well as f [60–66] and f [193–203] obtained from β-casein have both immunomodulatory and anti-hypertensive activities [18]. Moreover, μ-receptors of endorphins are present in lymphocytes and therefore immunomodulatory peptides show some signs of opioid properties, too. Some immunomodulatory peptides are believed to show anti-tumour activity as well as decreasing allergic symptoms in atopic patients and increasing mucosal immunity in the gastrointestinal tract [18, 57, 84]. In a similar manner, immunomodulatory peptides can demonstrate mineral-binding effects. This status has been shown during digestion or hydrolysis of camel milk casein [50]. In a study, casein-derived peptides such as fragments of αs_1-casein and β-casein enhanced phagocytic activity of murine peritoneal macrophages against sheep red blood cells and exerted anti-microbial effects on *Klebsiella pneumonia* after intravenous administration in rats [18]. Glycomacropeptide obtained from κ-casein has also exhibited anti-microbial as well as immunomodulatory activities via phagocytosis enhancement. Moreover, the growth of beneficial bacteria such as *Bifidobacterium* or *Lactobacillus* was enhanced as a result of casein hydrolysates used in infant formulas [76].

It should be noted that both immune-stimulating and -suppressive effects are necessary, depending on the conditions. Immunostimulation is required for body protection against bacterial attacks and infections, while immunosuppression is necessary in some clinical cases such as prevention of graft rejection and control of inflammations [76]. Lactoferrin, β-lactoglobulin and α-lactalbumin [18, 86], wheat [85, 87], soybean, eggs, mushrooms [85], goat milk, whey protein, and serum albumin [50] are other examples of immunomodulatory peptides.

It has been well known that a large portion of microorganisms are going to become resistant against currently available antibiotics. This problem has proposed a new generation of natural, easy to use and effective anti-microbial agents [88]. Bioactive peptides produced by enzymatic hydrolysis, fermentation, or digestion possess these effects [31]. These are 5–10 kD peptides [85] with 12–50 amino acid residues [89], that are mostly isolated from milk proteins. By 2013, more than 2100 anti-microbial peptides had been separated from natural sources, and this is increasing daily [89].

It is thought that bioactive peptides mainly affect the cytoplasmic membrane of bacteria. The accumulation of peptides in the membrane causes leakage of bacterial cytoplasmic components and finally the death of the bacterium [31, 34]. Cell membrane permeability of microorganisms may increase due to excessive net positive charge of bioactive peptides released during hydrolysis [81]. Binding to enterotoxins and inhibiting cell adhesion to tissues are reported to be effective anti-microbial properties [81]. Furthermore, anti-microbial peptides deprive essential nutrients for bacterium regeneration and maintenance. One mode of action of anti-microbial (mainly lactoferrin-derived) peptides is the potential to attach to membrane lipopolysaccharides, the main components in the outer layer of Gram-negative

bacteria. The release of membrane lipopolysaccharides causes cell destruction [90]. In general, anti-microbial actions of bioactive peptides are the results of both their effect on the bacterial membrane and that they cause disorder in the operations of biopolymers [91]. The interaction of peptides and the biological membrane depends on the actions of bioactive peptides as well as on the composition of membrane lipids [11]. There are two other important factors affecting the anti-microbial activity of hydrolysates. The first is the existence of short-chain basic and hydrophobic amino acids [31, 85], the second is the surface hydrophobicity which is responsible for the interaction between anti-microbial peptides and the lipid core [31]. It is thought that at least 50 % of amino acids in anti-microbial peptides are hydrophobic [31]. Some authors believe that the existence of amphipathic structures allow peptides to bind to the membrane interface, interact with the bacterial membrane and cause its disruption [11, 89]. Trp, His [11], Leu, Ile, Val, and Phe [68] are often found in anti-microbial peptides.

Considering the net charge, anti-microbial peptides are divided into two groups: anionic and cationic peptides.

Cationic anti-microbial agents: this is the first reported and the largest group of natural antibiotics. Their mechanism of action is either through the interaction between their positively charged regions and the membranal negatively charged lipids or through membrane destruction by change in the net charge. Other mechanisms are penetration into the target cell, contribution to make channels for ions to pass through, and interference with membrane performance [88].

Anionic anti-microbial peptides: are often purified from mammalian tissues [88].

In a study carried out by Tan et al. [91], alcalase and tryptic-hydrolyzed palm kernel cake proteins showed anti-microbial effects against *Bacillus cereus*. Milk-derived peptides are among the most studied sources that have frequently shown anti-microbial, anti-fungal, and anti-viral properties. Sometimes these peptides are even able to destroy antibiotic-resistant microorganisms [11]. Casecidins and isracidins are two types of anti-microbial peptides separated from chymosin-hydrolyzed casein, with bactericidal effects on both Gram-negative and Gram-positive bacteria [33, 89]. Researchers demonstrated that bioactive peptides made by enzymatic hydrolysis of casein possessed anti-microbial activity against *Staphylococcus, Bacillus subtilis, Diplococcus pneumonia, Streptococcus pyogenes,* and *Escherichia coli* [40]. Similarly, peptic-generated lactoferrin hydrolysates are typical peptides with concurrent anti-microbial and positive effect on the natural intestinal microflora except for *Bifidobacterium* [18, 89]. In a study on the effects of peptic hydrolysis of buffalo αs_1- and αs_2-caseins, bioactive peptides with bactericidal effects on *Micrococcus leutenus, Escherichia coli,* and *Bacillus cereus* were isolated. Gastrointestinally digested goat milk had also the potential to make bioactive peptides with a potent bactericidal effect on *Listeria monocytogenes* [50]. Peptide fragments purified from ovine αs_2-casein, i.g. f [165–170], f [165–181], f [184–208], and f [203–208] were shown to have bactericidal effects against Gram-negative bacteria [92]. A 24-amino-acid synthetic peptide similar to shrimp anti-lipopolysaccharide factor was examined in cyclic and linear forms for anti-microbial activity. The cyclic compound enhanced the survival of murine infected by 10^6 colony-forming units (CFU) of *Pseudomonas aeruginosa* [93]. In an experimental model of septic shock caused by a multidrug-resistant (MDR) clinical isolate of *Escherichia coli,* the possible anti-microbial role of s-thanatin (a synthetic peptide with the amino acid sequence Ala-Met-Pro) was studied. After animal infection by 2×10^{10} CFU of *Escherichia coli*, s-thanatin was injected at doses of 10, 20 or 40 mg/kg body weight. The results revealed that s-thanatin successfully bound to LPS and reduced the number of inoculated bacteria [94]. To investigate the anti-microbial activity of a synthetic peptide with the amino acid sequence Lys-Lys-Ile-Arg-Val-Arg-Lue-Ser-Ala, mice were challenged by 1.5×10^9 CFU of *Escherichia coli* TG1, 1×10^7 CFU of *Pseudomonas aeruginosa* ATCC 27853, and 1.5×10^7 CFU of *Pseudomonas aeruginosa* VR-143/97, and treated with peptide by *intraperitoneal administration after 30 min.* The peptide was 100 % effective on *Escherichia coli,* 75 % against *Pseudomonas aeruginosa* ATCC 27853, and 100 % on *Pseudomonas aeruginosa* VR-143/97 at the concentrations 10, 25, and 25 mg/kg body weight, respectively [95]. Isracidin (a casein-derived bioactive peptide) showed protective effects against *Listeria monocytogenes*, *Streptococcus pyogens,* and *Staphylococcus aureus* in mice, and against chronic streptococcal infection in cows with mastitis [95]. Some examples of anti-microbial activities of bioactive peptides are: the effect of hydrolyzed lactoferrin against *Escherichia coli* and *Listeria monocytogenes* in milk; activities against putrefactive bacteria in mozzarella cheese, beef, and meat by lactoferricin B, isracidin and kappacin; as anti-microbial peptides released during ripening of Italian cheese; influence of lactoferrin-derived bioactive peptides on the microbial population of wine (*Saccharomyces cerevisiae*); the retarding effect of lactoferricin B on the spoilage of fruits and vegetables [90]; the influence of Italian goat milk hydrolysates on *Lactobacillus sakei*; multifunctional properties of sheep milk hydrolysates as anti-hypertensive, anti-oxidative, and anti-microbial agents against *Escherichia coli, Bacillus subtilis,* and *Staphylococcus aureus* [50]; and rapeseed protein hydrolysates [85], sunflower protein isolate, α-lactalbumin, β-lacto globulin, whey protein [11], lysozyme [33], and pacific oyster [85] actions against different types of bacteria. Some *immunomodulatory*, anti-microbial, and anti-inflammatory peptides are presented in Table III.

Table III. Immunomodulatory, anti-microbial and anti-inflammatory peptides derived from food proteins.

Protein	Dietary source	Involved enzymes and processes	Bioactive peptide	Health-promoting effect	Reference
κ-Casein and α-lactalbumin		Enzymatic hydrolysis	Tyr-Gly and Tyr-Gly-Gly	Immunomodulatory	[18]
Lactoferricin		Enzymatic hydrolysis, pepsin	Positively charged regions	Immunomodulatory	[18, 86]
α-Lactalbumin		Pepsin, trypsin, and chymotrypsin	f (1–5), f (17–31)-f (109–114), f (61–68) and f (75–80)	Immunomodulatory	[34, 136]
Whey		Trypsin and chymotrypsin	Hydrolysate	Immunomodulatory	[46]
Lactoferrin		Pepsin	Hydrolysate	Immunomodulatory	[46]
Casein		Trypsin	Hydrolysate, f (106–169)	Immunomodulatory	[86, 137]
Ovotransferrin		Enzymatic hydrolysis	Ile-Arg-Trp and Ile-Gln-Tyr	Anti-inflammatory	[39]
Yak κ-casein		Pepsin	Peptides rich in Lys, Pro, Phe and Leu	Anti-microbial	[31]
Casein		Chymosin	High molecular weight basic polypeptides	Anti-microbial	[136]
αs-Casein		Chymosin	f (1–23), f (183–207) and f (164–179)	Anti-microbial	[18, 31]
αs_1-Casein		Chymosin	f (21–29), f (30–37)	Anti-microbial	[31]
β-Casein		Enzymatic hydrolysis	f (1–18) and f (105–117)	Anti-microbial	[113]
Lactoferrin		Pepsin	Peptides with less than 100 amino acids, amphiphilic and positively charged, f (267–285), f (267–288), and f (277–288)	Anti-microbial	[18, 46, 136]
β-Lactoglobulin		Trypsin	f (15–20), f (25–40), f (78–83)	Anti-microbial	[31]
Lactoferricin		Pepsin	F (17–41) of lactoferrin, Trp/Arg-rich proportion	Anti-microbial	[18, 46, 136]
Lactoferrampin		Pepsin	f (268–284) of bovine lactoferrin, hydrophobic region with a Trp residue	Anti-microbial	[46, 136]
β-Casein		Protease from *Lactobacillusdelbrueckii* ssp. lactis	Hydrolysate	Anti-inflammatory	[46]
Casein		Corolase	Hydrolysate	Anti-inflammatory	[39]
	Lysozyme	Enzymatic hydrolysis	f (87–114) of chicken lysozyme and f (87–115) of human- milk lysozyme	Anti-microbial	[136]
	Pacific oysters (*Crassostrea gigas*)	Enzymatic hydrolysis	5–10 kD peptides	Anti-microbial	[85]
	Leatherjacket (*Meuschenia sp.*)	Bromelain	Molecular weight of 6 kD	Anti-microbial	[33]
	palm kernel	Alcalase, trypsin	Hydrolysate	Anti-microbial	[91]
	Sunflower	Pepsin, trypsin, chymotrypsin, alcalase and flavourzyme	Hydrolysate	Anti-microbial	[11]
	Mushroom	Enzymatic hydrolysis	Peptides with the molecular weight of 13.4 kD	Immunomodulatory	[85]
	Milk	Enzymatic hydrolysis	Val-Pro-Pro and Ile-Pro-Pro	Anti-inflammatory	[39]
	Soybean	Enzymatic hydrolysis	Val-Pro-Tyr	Anti-inflammatory	[39]
	Wheat	*Aspergillus oryzae* protease	Glu-Lue	Anti-inflammatory	[39]

Anti-allergenicity

One of the most frequent allergenicities is sensitivity to cow's milk protein in children. For more than 60 years extensively or partially hydrolyzed formulas, and even products containing amino acid mixtures have been successfully used for the treatment of children with cow's milk allergy [96]. An alternative formula based on casein hydrolyzed by pepsin, trypsin and a protease derived from *Lactobacillus casei* strain GG (ATCC 53103) was utilized to reduce the allergenic potential of cow's milk. The results indicated that β-casein and αs_1-casein hydrolysates prepared by pepsin and trypsin suppressed lymphocyte proliferation at 0.1 and 10 μg/mL, respectively, while κ-casein hydrolysates significantly induced lymphocyte proliferation at 10 μg/mL. Hydrolyzed αs_1-β- and κ-caseins produced by *Lactobacillus casei* strain GG (ATCC 53103) showed lymphocyte proliferation suppressive effects at 0.1, 10, and 100 μg/mL, respectively [97]. Others reported a reduction in allergenicity in individuals receiving α-lactalbumin, soy [98], extensively hydrolyzed casein [98–100], and whey with an average molecular weight of less than 1500 D [98–101]. Recently, the German Infant Nutritional Intervention Study reported that partially hydrolyzed whey formulas diminished eczema in children breast-fed until 4 months of age. However, the results of a skin prick test showed no logical relation between hydrolyzed cow's milk, the soy infant formula [99] or partially hydrolyzed whey formula and allergy reduction in children [101] in randomized controlled studies. In order to investigate the anti-allergenic effect of partially hydrolyzed whey formula, 620 infants with a family history of allergy were randomly selected, divided into three groups, i.e. infants nourished by conventional cow's milk, partially hydrolyzed whey formula or soy formula, and the reduction of allergic symptoms was investigated in a single-blind randomized controlled trial. The results of skin prick test for six common allergens, i.e. milk, egg, peanut, dust mite, rye grass, and cat dander after 6, 12, and 24 months demonstrated that there was no significant reduction in symptoms of allergy in infants treated with soy formula and partially hydrolyzed whey formula, compared to infants fed by conventional cow's milk [101]. The influence of soy, extensively hydrolyzed casein or partially hydrolyzed whey formulas in infants with food allergy was studied by Porch et al. [99]. For this purpose, a double-blind study with 113 formula-fed and 12 breast-fed infants was carried out and the symptoms of the incidence of allergy were evaluated during the first year of life. No significant differences were found between soy, extensively hydrolyzed casein or partially hydrolyzed whey formulas in the development of allergy symptoms. It has been reported that lymphocyte proliferation decreased in allergic patients fed by a cow's milk protein-free diet containing hydrolyzed whey-casein formula after 3 months of treatment [102]. Reduction in allergic signs after treatment with hydrolyzed formula is attributed to the lower molecular weight of protein hydrolysates in comparison to intact proteins. In this regard, the molecular weight of bioactive peptides is too low to stimulate the immune system [34].

Anti-inflammatory properties

Inflammation is a response of arteries to some occurrences, such as non-lethal injuries, which leads to endothelial permeability, leakage of protein-rich exudates, and infiltration of leukocytes into extravascular tissues. When inflammation occurs, cells and tissues attempt to eliminate the foreign material [39]. Although this mechanism is necessary for the body's resistance against infections, when the response gets out of control it may lead to a risk of increase in tissue damage due to the excessive production of enzymes and oxidants [12], and may cause problems such as chronic diseases, atherosclerosis, myocardial infarction, stroke, and even cancer [39, 46, 103]. Today, nonsteroidal drugs such as aspirin are extensively applied to prevent inflammation; however, undesirable side effects like gastrointestinal bleeding and ulcer have restricted the long-term use of these compounds [39]. Bioactive peptides have the potential to prevent the inflammation. Similar to some other health-promoting effects of bioactive peptides, anti-inflammatory activity is also influenced by peptide chain characteristics and amino acid composition. The presence of Arg at the N or C-terminus as well as positively charged portions in the peptide chain are important in this case [84].

Anti-inflammatory activity is linked to ACE inhibitory and anti-oxidative activities [39, 46]. It seems that inflammation is deeply affected by bradykinin [84]. The effect of bradykinin on inflammation is attributed to the enhancement of lymphokine production as the result of bradykinin-induced migration of lymphocytes [84]. This is why ACE inhibitory peptides are thought to have anti-inflammatory activity [84]. For instance, whey hydrolysates are effective as both anti-hypertensive and anti-inflammatory peptides. Similarly, anti-hypertensive tripeptides Val-Pro-Pro and Ile-Pro-Pro obtained by fermentation of casein [39], f [194–199] of αs_1-casein, as well as f [60–66] and f [193–202] separated from β-casein [84] are believed to have anti-inflammatory activity. Anti-inflammatory peptides defensins and LL-37 show their activities through binding and neutralizing cell debris such as lipopolysaccharide and lipotechoic acid, which subsequently lead to downregulation of pro-inflammatory cytokines [82]. Trip-

eptides Ile-Arg-Trp and Ile-Gln-Trp produced during hydrolysis of ovotransferrin and a tripeptide Val-Pro-Tyr derived from soybean and Chungkookjang (a Korean product obtained from fermented soybean) [39], lactoferrin and its hydrolysate lactofericin [39, 46], as well as hydrolysates made by the enzymatic hydrolysis of human and cow's milk, egg, fish, meat [32, 39], and gluten [32] showed anti-inflammatory properties.

Anti-cancer peptides

At present, cancer is the second leading cause of death worldwide. It has been reported that 35% of cancer deaths are related to diet; therefore it seems possible to reduce the risk of cancer and increase the sensitivity of tumor cells against anti-cancer therapies by food components [104]. In this regard, food-derived bioactive peptides are considered as preventive components inhibiting cancer initiation and development [105]. Among them, biopeptides derived from cereals and legumes are of great interest [106].

Diets rich in soybean and its products show preventive effects on different types of cancers, especially cancers of the colon, breast, and prostate. Lunacin is a soybean-derived anti-cancer peptide with 9 aspartic acid residues, an Arg-Gly-Asp cell adhesion motif, and a structurally conserved helix region at the carboxyl end [107]. In recent decades the presence of lunacin has been also reported in barley, rye, triticale, *Solanum* and *Amaranthus* seeds; however, there was no evidence to confirm the presence of lunacin or its precursor protein in wheat [108].

Lunacin anti-cancer activity was confirmed for the first time by Galvez and de Lumen [109] in 1999, when *Escherichia coli* was transfected by lunacin-encoding cDNA, and subsequently mitotic arrest was observed [106]. Once the lunacin gene is transferred and expressed inside the mammalian cells, it inhibits mitosis and subsequently leads to cell death. It has been shown that lunacin is able to prevent the transformation of murine fibroblast cells to cancerous foci [106]. Moreover, lunacin may cause unusual elongation of spindle fibers, chromosomal fragmentation, and cell lysis in murine hepatoma and human breast cancer cells [106].

Galvez et al. [107] reported that dermal application of 250 μg/week of lunacin resulted in a 70-% reduction of skin tumor occurrence and a delay in the appearance of tumors in murine after 2 weeks. Soybean contains variable amounts of lunacin, from 5.48 mg/g in defatted soy flour to 16.52 mg/g in soy concentrate. Based on the FDA recommendations, daily intake of 25 g soy protein will provide about 250 μg lunacin; however, the effectiveness of this amount is still in doubt. It seems that lunacin is inactivated in the absence of carcinogenic agents, hence does not affect cell morphology, but inhibits cell transformation in the presence of carcinogenic agents; however, which genes or proteins are affected by lunacin in the presence or absence of carcinogenic agents is still unclear [107].

The mechanism of action of lunacin is attributed to its inhibitory effect on histone H3 and H4 acetylation in both transformed and non-transformed cells through binding to deacetylated histones, and competing with acetyltransferase as an acetyltransferase inhibitor [106]. Lunacin internalizes into the cell by its Arg-Gly-Asp cell adhesion motif, colocalizes with hypoacetylated chromatin, binds to deacetylated histone H4, prevents histone H3 and H4 acetylation in the presence of histone deacetylase inhibitor, and eventually causes apoptosis. Due to lunacin preference for hypoacetylated chromatin, it is suggested that a chromatin modification mechanism plays an important role in lunacin anti-carcinogenic activity and cell cycle control [107].

The lactoferrin-derived peptide lactoferricin has great anti-cancer potential against oral, colon, breast, and ovarian cancers through apoptosis induction, angiogenesis inhibition, and carcinogen metabolizing enzymes modulation. One of the factors involved in the effectiveness of lactoferricin is attributed to its surface charge. Due to its positively charged structure, lactoferricin can connect to negatively charged cancer cells through the outer membrane. Anti-tumor activity of lactoferricin has been confirmed in different animal models: for instance, a significant inhibition of liver and lung metastases of L5178Y-ML25 cells was observed by subcutaneous administration of 0.5 mg/mouse bovine milk lactoferricin 1 day after tumor inoculation [105]. Similarly, investigation on bovine and murine lactoferricin's anti-tumor activity showed cytotoxicity, cell membrane disruption, and cell lysis in tumor cells 1 day after bovine lactoferricin treatment (500 μg/50 μL), while there was no evidence showing murine lactoferricin anti-tumor activity at the same concentration [110]. An anti-cancer pentapeptide with the amino acid sequence Glu-Gln-Arg-Pro-Arg and a molecular weight of 685.378 D was isolated from alcalase-treated heat-stabilized defatted rice bran [111]. Based on the reports, doses of 600–700 mg/mL of this purified peptide showed inhibitory effects of 84, 80, and 84% on colon, breast, and liver cancers, respectively.

The presence of anti-cancer peptides has also been proven in other sources. Egg yolk [20], beef sarcoplasmic protein [112], α-lactalbumin [104], hydrolyzed casein [104], bovine serum albumin [113], fermented milk [34], yoghurt [40], whey protein hydrolysates [114], Bowman-Birk inhibitor [106], and lectins [106] are shown to have anti-cancer properties.

Influence on the nervous system

Opioid-like peptides

Opioid peptides are defined as 4–8 amino acid long peptides with a strong effect on the breathing system [40], nervous system (acting in the same way as sedative drugs and narcotics like morphine), and the gastrointestinal tract [40]. They can be made by the body as well as through food digestion and protein hydrolysis [18]. Opioid peptides were obtained from cow's milk β-casein hydrolysates (β-casomorphins) for the first time at the end of the 19th century [40]. In a similar way α-casomorphin, casoxin, exorphin, α-lactorphin, β-lactorphin, and lactoferroxin were derived from peptic-hydrolyzed bovine and human αs_1-casein, κ-casein, αs_1-casein, α-lactalbumin, β-lactoglobulin, and Lactoferrin, respectively [84, 115]. Opioid peptides are derived from three precursors named endorphins (proopiomelancortin), enkephalin (proenkephalin) or dynorphins (prodynorphin) [18, 40, 115]. Their activity takes place either by agonistic or antagonistic effects. They exert their effect by binding to specific receptors in target cells responsible for emotional behavior (m and s receptors), food intake (k receptor), intestinal motility (m receptor), appetite control, changes in body temperature, body response to pain and fear as well as water and mineral absorption. Showing the tendency to bind to opioid receptors, these peptides have to possess the amino acid sequence Tyr-Gly-Gly-Phe at the N-terminus and Phe or Tyr residues at the third or fourth position [18, 40]. Opioid peptides are also involved in satiety because they slow down the intestinal discharge [14]. They are believed to be responsible for breathing disorders, such as apnea, which occur during sleep and are thought to have an ACE inhibitory activity [40].

Opioid peptide efficacy on the gastrointestinal performance may appear in two ways: first by the reduction of transition time via adjustment of the smooth muscles motility, and second by the regulation of electrolyte transportation through the gastrointestinal tract [18]. According to the reports, opioid peptides (particularly casomorphins) cause an increase in water and electrolyte absorption in large and small intestines, hence they are thought to have anti-diarrheal ability [18]. Opioid peptides may be digested in the gastrointestinal tract after consumption; however, there is some evidence for their existence in piglet's duodenum, calf plasma, and neonates' small intestine following milk consumption. Based on the reports, α-lactorphin showed a weak but consistent opioid effect; however, β-lactorphin did not indicate opioid-stimulatory activity in pig ileum [116]. The presence of β-casein-derived opioid peptide β-casomorphin-11 was observed in duodenal chyme of minipigs fed 100 g acid-precipitated casein. Similarly, high amounts of β-casomorphin-7 and small amounts of β-casomorphin-4 or -6 were found in the small intestine of adult humans who received row cow's milk [18]. According to Darewicz et al. [79], the presence of Pro residues in β-casomorphin protects it against degradation by digestive enzymes and helps it linking to opioid receptors. Calmness and sleepiness in neonates after breast-feeding and also sudden infant death syndrome (SIDS) are attributed to these peptides [79, 84]. Lactoferrin [18], gluten hydrolysates, milk proteins, barley, rice, soybeans, spinach, meat/poultry, eggs [113], and Italian buffalo cheese whey [50] have been reported as showing opioid properties.

Regulation of the gastrointestinal system

Mineral-binding capacity

There are some bioactive peptides that have been proven to show calcification effects on bones and preventive properties on rickets. It has been found that proteolytic enzymes from LAB can produce peptides with the same ability during the ripening of cheese. Furthermore, a cytomodulatory effect was observed in peptides produced during the enzymatic hydrolysis of proteins like casein. The hydrolysis of casein produces some bioactive peptides, namely caseinophosphopeptides (CPPs), which are said to have a potent mineral-binding ability. CPPs possess the peptide sequence Ser-Ser-Ser-Glu-Glu which is responsible for absorption of calcium, phosphorus, and other minerals in the gastrointestinal tract [40]. They bind to some minerals such as magnesium and iron, barium, chrome, nickel, cobalt, and selenium [40], and this capacity is influenced by the composition of amino acid residues around the phosphorylated region [18, 84]. An interesting ability of bioactive peptides is the binding to iron, which reduces the formation of ferric hydroxide (a low absorbable molecule) and partly solves problems such as iron deficiency [117]. Zinc and calcium are also better absorbed in the presence of bioactive peptides, because they bind to hydrolyzed portions of proteins. Mineral-binding peptides such as CPPs are believed to be resistant against gastrointestinal digestion due to their negatively charged portions [18, 84]. Several anionic amino acids enable CPP to change insoluble calcium to its soluble form, hence inhibit its precipitation in the ileum. In contrast, CPP doesn't participate in the formation of insoluble calcium phosphate [84]. Phosphorylated portions of CPP (particularly phosphoserine residues) form an acidic and polar area around the peptide chain and reinforce

the uptake of calcium, iron, zinc, and other minerals. It has been shown that CPP may be released during bovine milk digestion and cheese ripening by plasmin and microbial proteases [18]. In an *in vivo* study, the presence of CPP in digests of the distal jejunum was confirmed in minipigs fed with diets containing 15% casein [118]. Again, the occurrence of CPP was detected in the human stomach and small intestine after ingestion of milk, αs_1- αs_2- or β-caseins. In a human feeding trial, CPP demonstrated the potential to enhance calcium and zinc absorption in rice-based infant foods and also improved calcium absorption in human intestinal HT-29 tumor cells [40]. It has been suggested that the mineral-binding capacity of CPP is influenced by the concentration, the type of calcium salt, and the amount of zinc. CPP presented zinc-binding ability at a concentration of 14 μmol/L, while the opposite effect was observed in higher amounts, e.g. 36 μmol CCP/L or 72 μmol CPP/L. In case of calcium salt, among three salts tested ($Ca_3(PO_4)_2$, $CaCl_2$, and calcium glycerophosphate calcium gluconate), the highest zinc absorption was observed in the presence of CPP and calcium glycerophosphate calcium gluconate [119]. CPP is also beneficial in case of tooth decay through enhancing enamel calcification as well as depriving calcium from plaque-forming bacteria [84]. In a randomized controlled trial, casein hydrolysates and calcium phosphates were examined as anti-cariogenic agents in 63 volunteers, and the results showed a significant reduction in coronal caries after 12 weeks [79]. The influence of collagen hydrolysates on bone metabolism and the prevention of osteoporosis was investigated by Guillerminet et al. [120]. For this purpose, 12-week-old ovariectomized mice were fed a diet containing 0, 10 or 25 g/kg hydrolyzed collagen for 12 weeks. The results indicated that collagen hydrolysates at the level of 25 g/kg had an ameliorative effect on bone mineral density (BMD) and stability in ovariectomized mice [120]. In a research study by Barac et al. [121], partial hydrolysis of soy concentrate by pepsin and trypsin was found to have no significant effect on anti-trypsin activity, but a significant reduction in phytic acid content was observed. Hydrolyzing gingerbread plum seed protein by pepsin in the first step and trypsin in the second step, Amza et al. [72] reported that the nutritional properties of hydrolyzed gingerbread plum seed protein such as amino acid score, essential amino acid index, biological value, and protein efficiency ratios were positively affected as a function of hydrolysis time (180 min). These results suggested that hydrolyzed gingerbread plum seed protein can provide all essential amino acids excluding Thr and Lys, and may be considered as a nutrient supplement. Examples of opioid and mineral-binding peptides are presented in Table IV.

Formation of bioactive peptides during fermentation

Fermentation (mostly by LAB) is one of the oldest food preservation methods and is thought to improve the shelf life and nutritional value of fermented foods [122]. Most

Table IV. Some food-derived peptides with opioid and mineral-binding activities.

Protein	Dietary source	Involved enzymes and processes	Bioactive peptide	Health-promoting effect	Reference
β-Casein, α-casein, lactoferrin, α-lactalbumin, and β-lactoglobulin		Pepsin	Peptides with 4–8 amino acids, Tyr-Gly-Gly-Phe at the N- terminus	Opioid-like	[18]
αs_1- casein		Enzymatic hydrolysis	Tyr-Leu-Gly-Tyr-Leu-Glu-Gln-Leu-Leu-Arg, f (91–97)	Opioid-like	[46]
α-Casein		Pepsin	Hydrolysate	Opioid-like	[138]
Wheat gluten		Pepsin	Hydrolysate	Opioid-like	[138]
Bovine casein		Enzymatic hydrolysis	Ser-Ser-Ser-Glu-Glu,	Mineral-binding[1]	[40, 85]
αs_1-Casein		Enzymatic hydrolysis	f (43–58), f (59–79), f (43–79)	Mineral-binding[2]	[18, 119]
αs_2-Casein		Enzymatic hydrolysis	f (1–24) and f (46–70)	Mineral-binding[2]	[18]
β-Casein		Enzymatic hydrolysis	f (1–28), f (2–28), f (1–25), f (33–48)	Mineral-binding[2]	[18, 119]
	Shrimp processing by-product	Flavourzyme	Molecular mass of 699 D, Leu-Pr-Thr-Gly-Pro-Lys-Ser.	Mineral-binding[3]	[139]

[1] Calcium, phosphorus, magnesium, iron, barium, chrome, nickel, cobalt, and selenium,
[2] Calcium,
[3] Iron

dairy starter cultures are strongly proteolytic, which causes the release of bioactive peptides with different chain lengths [119]. Several health-promoting properties such as anti-oxidative, immunomodulatory, ACE inhibitory, and anti-microbial activities have been reported to occur in fermented foods, and in most cases production of bioactive peptides is considered as the main reason behind the formation of these properties [122].

Production of biofunctional peptides by LAB is often performed by a cell-wall-associated peptidase, and different intracellular peptidases such as endopeptidases, aminopeptidases, tripeptidases, and dipeptidases. Among LAB, *Lactobacillus* and *Bifidobacterium* have been the best-known genera applied to produce bioactive peptides. Since peptidase activities are influenced by the microorganisms' growth conditions, the production of bioactive peptides can be controlled to some extent [85].

The type of bacteria involved in the fermentation is the most important factor affecting the nature of the final peptides. For example, production of anti-tumor peptides or antibodies against O157:H7 *Escherichia coli* may occur as a result of milk fermentation by *Lactobacillus helveticus*. Similarly, bioactive peptides such as casomorphines, lactorphines, casokinines or immunomodulatory peptides may be produced through *Lactobacillus lacti*s fermentation [34].

Milk and its derivatives are the most documented protein sources used for the production of bioactive peptides by fermentation. *Lactobacillus helveticus* and *Saccharomyces cerevisiae* strains indicated a great potential to make well-known anti-hypertensive peptides Val-Pro-Pro and Ile-Pro-Pro [12]. Bioactive peptides with the amino acid sequences Leu-His-Leu-Pro-Leu-Pro and His-Leu-Pro-Leu-Pro released during *Enterococcus faecalis* fermentation of β-casein exhibited potent anti-hypertensive activities in rats. Additionally, significant reductions in systolic and diastolic blood pressure were reported in milk fermented by *Lactococcus lactis* 188 NRRLB-50 571 and NRRLB-50 572. Bioactive peptides derived from milk fermented by *Lactobacillus helveticus* R389 demonstrated positive effects on the mucosal immune system and a preventive effect on the fibrosarcoma system in infected and non-infected mice [46]. A placebo-controlled study on the anti-hypertensive effect of Dahi (fermented milk) indicated a significant reduction in systolic blood pressure in subjects receiving 100 mL of Dahi for 2 and 4 weeks [57].

To investigate the possible role of fermentation in the preparation of ACE inhibitory peptides, casein and cheese whey were fermented by starter cultures used in the production of yoghurt, ropy milk, and sour milk, and no ACE inhibitory peptide was detected. However, further enzymatic hydrolysis by pepsin and trypsin caused the release of ACE inhibitory peptides particularly from αs_1-casein and β-casein. A potent ACE inhibitory dipeptide with the amino acid sequence Tyr-Pro was isolated from a yoghurt-like fermented product made by *Lactobacillus helveticus*. It was reported that this dipeptide was present in all major fractions of casein, and its concentration was increased during fermentation [57]. Fermented milk produced by *Lactobacillus helveticus* CPN4, R211, R389, and LP01; *Enterococcus faecalis* CECT5827, 5727, 5826, and 5728; *Lactobacillus rhamnosus* CECT287, LP09, and LP17; *Lactobacillus acidophilus* LP18, LP20, LP24, and LP28, as well as *Lactococcus lactis* ssp. *cremoris* LP25 showed ACE inhibitory activity. In addition, whey protein fermented by a combination of *Lactobacillus casei* TCM0409 and *Streptococcus thermophilus* TCM1543 exhibited anti-hypertensive activity in human [123].

Fermentation of milk by industrial starter cultures produced anti-oxidative peptides from whey fraction. It was believed that the anti-oxidative activity was directly correlated to DH [12]. Sourdough fermentation of different cereal (wheat, spelt, rye, and kamut) flours by a mixture of LAB also caused improvement in anti-oxidative activity of fermented sourdoughs compared to chemically acidified doughs [39]. In a study, three anti-microbial peptides caseicins, A, B, and C, were released during fermentation of milk by *Lactobacillus acidophilus*. These peptides were active against different Gram-negative bacteria such as *Cronobacter sakazakii, Cronobacter muytjensii, Salmonella enterica* ssp. 1 serovar Typhimurium, *Escherichia coli, Klebsiella pneumoniae,* and *Pseudomonas fluorescens,* and one Gram-positive pathogen, *Staphylococcus aureus* [34]. Anti-tumor activity was also observed in fermented milk, which was associated with immunomodulatory peptides formed during fermentation [119]. Mineral-binding CPPs were identified during ripening of Comté, Cheddar, and Herrgard cheese, and the occurrence of these compounds was affected as a function of the ripening stage. In this way, prolonged ripening caused a significant increase in formation of mineral-binding CPPs. Similarly, gradual development of peptides with ACE inhibitory, mineral-binding, anti-microbial, anti-hypertensive, and immunostimulatory activities was observed in Gouda and Emmental cheese during ripening; however, active opioid peptides were not detected in mature Cheddar cheese [12].

In addition to microbial fermentation by live microorganisms, it is possible to use proteolytic enzymes isolated from LAB. For instance, casein fermentation by cell-wall-related proteinase from *Lactobacillus helveticus* demonstrated anti-hypertensive activity in SHR. In another study, nine commercial proteases were examined to form ACE inhibitory peptides from casein, and maximal *in vitro* ACE inhibitory and potent *in vivo* anti-hypertensive activities were observed in bioactive peptides released by a protease isolated from *Aspergillus oryzae* [57].

The production of bioactive peptides was also reported from protein sources other than dairy products. Fermentation of marine blue mussels made an anti-oxidative heptapeptide with the amino acid sequence His-Phe-Gly-Pro-Asp-Phe-His and a molecular weight of 962 kD with superoxide scavenging activity of 98%, hydroxyl radical scavenging effect of 96%, carbon-centered radicals scavenging activity of 91%, and DPPH radical scavenging activity of 72% at a concentration of 200 μg/mL [122]. Investigating the possible formation of anti-oxidative peptides during fermentation of *Ganoderma lucidum,* Sun et al. [124] reported that fermented *Ganoderma lucidum* had a potent dose-dependent anti-oxidative effect in both soybean oil and lard systems, and even surpassed BHT in terms of anti-oxidative activity in the soybean oil system. Moreover, the production of *malondialdehyde* (MDA) was inhibited in mouse liver by 52–63% at doses of 0.035–0.7 mg/mL [124]. Fermented soybean products such as douchi, natto, and tempeh also contain anti-oxidative peptides [19]. The Korean fermented soybean product Chungkookjang had potent anti-inflammatory activity in breast cancer cells through the activation of transforming growth factor beta signaling, and downregulation of cytokine/chemokine expression [39]. Seacure, a fish product made through controlled fermentation of pacific hake by yeast, enhanced cell growth at a dosage of 1 mg/mL and decreased cell injury at a concentration of 25 mg/mL in cultured rat epithelia and human colon cells. Seacure also reduced human small intestinal damage caused by anti-inflammatory drugs such as indomethacin [19].

Applications of bioactive peptides in functional foods

Nowadays, biologically active peptides have attracted increasing attention as proper candidates for the preparation of functional foods and nutraceuticals [18]. The main purpose of functional food production is to either enhance the bioavailability of final hydrolysates in comparison with source proteins, or to create novel health-improving products through the incorporation of isolated or enriched fractions of bioactive peptides [119]. Today, milk-derived proteins and peptides are the best-studied ingredients to develop health-promoting functional foods [18]. Casein-derived products, hypotensive products, functional foods to enhance mineral absorption, products to help athletic performance, items for stress reduction, and products containing anti-cariogenic peptides are among the suggested functional foods [113]. Calpis and Evolus are Japanese and Finnish fermented milks containing two anti-hypertensive tripeptides Val-Pro-Pro and Ile-Pro-Pro. Calpis is sour milk produced by *Lactobacillus helveticus* and S*treptococcus cerevisiae,* and Evolus is a calcium-enriched fermented milk drink that is mainly produced by *Lactobacillus helveticus* LBK-16 H strain [57]. Animal model studies indicated that single oral administration of Calpis containing 2.5–3.5 mg/kg/day of Val-Pro-Pro and Ile-Pro-Pro decreased blood pressure in SHR after 12 weeks [125]. Similarly, in a randomized placebo-controlled study, reduction of 6.7 ± 3 mmHg in systolic and 3.6 ± 1.9 mmHg in diastolic blood pressure was reported in mild hypertensive human subjects receiving 150 mL/day Evolus for 21 weeks [126].

Casein DP Peptio Drink, Kotsu Kotsu calcium, and Tekkotsu Inryou are some brands of soft drinks produced in Japan that show anti-hypertensive, mineral absorption, and mineral absorption activities, respectively [119]. Other examples of currently available health-promoting functional foods are listed in Table V.

In addition to direct fermentation, it is possible to enrich different food systems using bioactive substances derived from other food proteins to achieve the desired features. For example, anti-oxidative peptides produced during fermentation of milk can be successfully applied in cooked beef to prevent oxidation, or the addition of CPP into the

Table V. Commercial brands of functional foods and their health claims. EFSA: European Food Safety Authority.

Product name	Manufacturer	Type of the product	EFSA health claim
Capolac	Arla Foods, Denmark	Ingredient	Mineral absorption
Cardi-04	Chr. Hansen A/S, Denmark		Anti-hypertensive
PeptoPro DSM Food Specialists	Netherlands	Ingredient	Athletic performance
CE90CPP DMV	Netherlands	Ingredient	Mineral absorption
BioZate	Davisco, USA	Hydrolyzed whey protein	Anti-hypertensive
Vivinal Alpha	Borculo Domo Ingredients (BDI), the Netherlands	Hydrolyzed whey protein	Opioid
C12	DMV International, the Netherlands	Hydrolyzed casein	Anti-hypertensive
BioPURE-GMP	Davisco, USA	Whey protein isolate	Anti-cariogenic

soluble fraction of fruit juices may enhance iron absorption in Caco-2 cells [113]. Another example is the enrichment of yoghurt by Val-Pro-Pro and Ile-Pro-Pro anti-hypertensive tripeptides, which has been successfully conducted in USA and enhanced the daily intake of these peptides by 118.1 g/person [119].

In order to produce functional foods many scientific, technological, and regulatory aspects must be considered. In the first step, the development of new technologies such as chromatographic and membrane separation methods is required for isolation and enrichment of bioactive peptides from protein hydrolysates. It is well known that due to their lower molecular weight, bioactive peptides are more reactive than the intact proteins and may interact with other food components such as lipids and carbohydrates. On the other hand, the process conditions, e.g. heating, not only have a negative influence on peptide activity and bioavailability, but also may cause the production of toxic compounds; therefore the second step is the creation of novel food systems to guarantee the maintenance of bioactive peptide activity during processing and storage, and the development of modern analytical methods to confirm the safety of the final products. Eventually, studying the mechanism of action of bioactive peptides is of great importance to investigate the influence of proteins and peptides on gene expression and to increase their nutritional value and health benefits [57].

Restrictions in use of protein hydrolysates

Despite the numerous advantages of bioactive peptides, their usage in foods is limited for several reasons such as some organoleptic defects like color, taste, and flavor. In addition, their activity may be influenced by other food components, such as carbohydrates and lipids, which can affect their stability and bioactivity during processing and storage [19]. They may even act as pro-oxidants under specific conditions [19]. Although some hydrolyzed formulas can reduce the risk of product allergenicity, it cannot ensure that another formulation with different DH represents hypoallergenic properties as well. In fact, the primary stages of hydrolysis increase allergenicity, while in higher DH allergenicity is diminished. In addition, the preparation of these formulations is not economic. In case of consumer acceptability, the biggest challenge is bitter taste, which is often related to hydrophobic peptides produced during hydrolysis [98, 127, 128]. In most cases, bitterness will increase with an increase in DH [127, 129]. For instance, soy hydrolysates with a molecular weight of 2–4 kD have a potent bitter taste [127]. Fish, meat, and gelatin hydrolysates have a lower bitterness in comparison with other sources [130]. Most peptides with a molecular weight of less than 1 kD and free amino acids have no bitter taste. The intensity of bitterness is also influenced by the proteases used. However, some authors believe that bitterness is primarily affected by the amino acid sequence and the peptide chain length. Lemieux and Simard [128] reported that there is a correlation between bitterness and the presence of amino acids Phe and Leu. Pedrosa et al. [98] believed that bitterness is associated with peptide size, hydrophobicity, and the type of the enzyme used. For instance, exopeptidases produce less bitter taste because of their degrading effect on hydrophobic peptides present at the end of the amino acid chain. Two ways to decline bitterness which are applied in gluten hydrolysates are treatment with microbial transglutaminase [129] and plastein reaction [131]. Yeast powder and flavorease (endopeptidase and exopeptidase) may also be useful for reducing the bitter taste [132]. Villanueva et al. [133] examined the influence of an exo-enzyme (flavourzyme) followed by an endo-enzyme (alcalase) on the bitterness of hydrolyzed sunflower protein. They found that hydrolysates with a degree of hydrolysis of 50.7% were white and less bitter. In a study on the acceptability of soy and rice formulas, and casein and whey hydrolysates and their mixture, a correlation was observed between the weight of peptides and their scores for smell, taste, and palatability, which was attributed to the production of hydrophobic (bitter) peptides during hydrolysis. The highest scores were assigned to soy and rice formulas followed by whey hydrolysates, while casein hydrolysates and the mixture of whey and casein hydrolysates earned the lowest sensory scores [98]. The use of glutamic acid, polyphosphates, starch, proteins and peptides, acidic amino acids, plastein reaction, cyclodextrin, activated carbon and precipitation in isoelectric point are also ways to diminish the bitterness of hydrolysates [127].

There are some challenges for the production of bioactive peptides due to the lack of an efficient technology for large-scale production [17, 85]. Nanofiltration, ultrafiltration, and chromatographic procedures are appropriate methods to enrich products with bioactive peptides based on casein and whey. However, fouling is a big problem in this case, especially in the ultrafiltration process [85]. Another problem in extensively hydrolyzed formulas is the increase in osmolarity which may cause diarrhea and even the destruction of enterocytes [127]. On the other hand, the health-promoting properties of bioactive peptides are dependent on their stability against digestion in the gastrointestinal tract, but some bioactive peptides have been proven to be sensitive to digestive enzymes, which make them ineffective [85]. New technologies, such as nano- or micro-encapsulation can be successfully applied so as to improve peptide stability in foods, as well as in the gastrointestinal tract [75].

Conclusions

Today, lifestyle-related diseases as well as cardiovascular system defects are rising due to the accumulation of free radicals. In keeping with the idea that food is the best medicine, many attempts have been made to produce medicines based on foodstuffs. Bioactive peptides are applicable substances with abundant helpful effects on cardiovascular, immune, nervous, and gastrointestinal systems, etc. Bioactive peptides have anti-hypertensive, hypocholesterolemic, opioid, anti-microbial, anti-diabetic, anti-cancer, mineral-binding and many other characteristics, therefore foods enriched with protein hydrolysates and their peptides can be considered as functional foods, meaning these compounds promote consumer health and body maintenance. Despite the above-mentioned interesting properties, due to some limitations such as their sensitivity to digestive enzymes, bitter taste, and the absence of a comprehensive method to produce bioactive peptides, more efforts are needed to completely eliminate the bitter taste, create an innovative and economic procedure for bioactive peptide production, improve their bioavailability and to hold more clinical trials to prove hydrolyzed proteins are completely safe.

Conflict of interest

The authors declare that there is no conflict of interest.

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