Antihypertensive peptides: production, bioavailability and incorporation into foods

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Abstract

Bioactive food peptides are encrypted within the source protein but can exert physiological properties once released by enzymatic hydrolysis during gastrointestinal transit, fermentation or maturation during food processing, or proteolysis by food-grade enzymes derived from microorganisms or plants. Among the bioactive food peptides, those with antihypertensive activity are receiving special attention due to the high prevalence of hypertension in the Western countries and its role in cardiovascular diseases. This paper reviews the current literature on antihypertensive food peptides, focusing on the existing methodologies for their production, such as enzymatic hydrolysis, fermentation and genetic recombination in bacteria. This paper also evaluates the structure/activity relationship of angiotensin-converting enzyme (ACE) inhibitory peptides, as well as their bioavailability, physiological effects demonstrated by both *in vitro* and *in vivo* assays, and the existence of mechanisms of action other than ACE inhibition. Finally, current reported strategies for incorporation of antihypertensive peptides in foods and their effects on both availability and activity of these peptides are revised in this manuscript.

Keywords: Antihypertensive peptides, angiotensin-converting enzyme inhibitory activity, enzymatic hydrolysis, fermentation, genetic recombination, bioavailability.

1. Introduction

In recent years, increasing epidemiological evidence is linking the prevalence of diseases, such as cardiovascular disease, obesity, hypertension, diabetes, and even cancer to dietary factors. Manufacture of new foods termed functional foods is emerging in response to the increased perception about the relation of food and health. A functional food is generally any food which can provide a health benefit to one or more bodily functions beyond that of basic nutrition [1]. Recently, it has been recognized that apart from their basic nutritional role, many dietary proteins contain, encrypted within their primary structure, different peptide sequences that exert beneficial effects upon human health once released by digestive enzymes during gastrointestinal transit or by fermentation or ripening during food processing. Bioactive peptides range in size from 2 to 50 amino acid residues and exhibit different activities, such as antimicrobial, antioxidant, antithrombotic, antihypertensive, immunomodulatory, opioid, and antiproliferative activities, among others [2-4], affecting the major body systems – namely, the cardiovascular, digestive, endocrine, immune and nervous systems. The potential of these bioactive peptides to reduce the risk of chronic diseases and to promote human health has aroused increasing scientific and commercial interest over the past decade [5].

High blood pressure or hypertension, which is estimated to affect one third of the Western population [6], is a risk factor for cardiovascular diseases including coronary heart disease, peripheral artery disease and stroke. In view of its high prevalence and importance, changes in life-style, dietary approaches and pharmacological treatments are broadly applied to treat hypertension. It has been recognized that nutritional factors play a significant role in the prevention and/or treatment of hypertension, and therefore, efforts are being put into the production of foods with antihypertensive activity. Angiotensin-converting enzyme (ACE, EC 3.4.15.1) is one of the main regulators of blood pressure through its action on two body systems.

Firstly, ACE forms part of the rennin-angiotensin system (RAS), converting angiotensin I to a potent vasoconstrictor, angiotensin II, which also induces the release of aldosterone and therefore, increases the sodium concentration and blood pressure. ACE also takes part of the kinin-kallicrein system as it hydrolyzes bradykinin, which has a vasodilator action. By inhibiting this enzyme, bioactive peptides have been shown to lower blood pressure in animal and clinical studies. First ACE inhibitors were discovered in snake venom. Currently, different ACE inhibitors, such as Captopril and Enalapril, are being extensively used to treat essential hypertension. However, their undesirable effects, such as hypotension, cough, increased potassium levels, reduced renal function, angioedema, etc. [7], have promoted the search of ACE inhibitory peptides derived from food natural sources. To date, milk from different species is the main source of ACE inhibitory peptides [3, 8, 9]. Other animal protein sources of these peptides are muscle [10], ovalbumin [11], blood [12], and fish proteins [13, 14]. Plant protein sources include, among others, pea [15], garlic [16], rice [17], soybean [18, 19], wheat [20], and Amaranth proteins [21].

This article reviews current literature on the subject of ACE inhibitory and antihypertensive peptides, their structure-activity relationship, mechanism of action and bioavailability. Evaluation of their activity in humans as well as their possible incorporation into food products will be also covered.

2. Release and identification of antihypertensive peptides

Biologically active peptides can be released from their parent protein by enzymatic hydrolysis during gastrointestinal digestion, fermentation or maturation during food processing or proteolysis by food-grade enzymes derived from microorganisms, animals or plants [22]. If the peptidic sequence is known, it is also possible to synthesize the peptide by chemical or enzymatic synthesis or by recombinant DNA technology [23].

2.1. *Gastrointestinal digestion*

It has been recognized that dietary proteins and peptides are susceptible to hydrolysis during the different stages of gastrointestinal digestion, namely ingestion, digestion and absorption [24]. Once ingested, these proteins and peptides are subjected to hydrolysis by different enzymes present in the gastrointestinal tract such as pepsin, trypsin, chymotrypsin and peptidases at the surface of epithelial cells to release peptides of various lengths. Some of these peptides may exert a direct function at the gastrointestinal tract. However, other peptides can be absorbed to reach target organs and tissues through systemic circulation [25].

In order to examine the effect of gastrointestinal proteases on the release of and breakdown of ACE inhibitory peptides from food proteins, simulated gastrointestinal digestion processes have been carried out on various protein sources, such as milk proteins (Table 1) [26-30], egg proteins [11], meat proteins [31, 32], fish proteins [33, 34], as well as vegetal proteins [21, 35-37]. As an example, Hernandez-Ledesma et al., [28] identified peptides with ACE inhibitory and antioxidant activity in hydrolyzates of several samples of human milk and infant formulas after digestion with pepsin and pancreatin simulating infant gastrointestinal conditions. Recently, Majumder and Wu [38] have studied the effect of simulated gastrointestinal digestion of cooked eggs on the release of ACE inhibitory peptides. These authors found that fried egg digests showed more potent ACE inhibitory activity than boiled egg digests, and postulated that the lower protein denaturation in boiled eggs may results in a lower protein digestibility.

2.2. *Fermentation and maturation process*

During fermentation process, lactic acid bacteria (LAB) hydrolyze milk proteins, mainly caseins, into peptides and amino acids which are used as nitrogen sources necessary for their growth [39]. Hence, bioactive peptides can be generated by starter and non-starter bacteria used

in the manufacture of fermented dairy products (Table 1) [40-43]. Many of these peptides have been reported to exert ACE inhibitory and antihypertensive properties. Proteolytic system of *Lactobacillus helveticus*, *Lactobacillus delbrueckii* ssp. *bulgaricus*, *Lactococcus lactis* ssp. *diacetylactis*, *Lactococcus lactis* ssp. *cremoris*, and *Streptococcus salivarius* ssp. *thermophilus* strains have been demonstrated to hydrolyze milk proteins releasing ACE inhibitory peptides [reviews 3, 43, 44]. Some of the peptides identified also have been shown to lower blood pressure in hypertensive rats [45-47], and humans [48, 49]. The best characterized ACE inhibitory peptides are VPP and IPP found in milk fermented with *Lactobacillus helveticus* and commercialized in Japan (Calpis, Calpis Co. Ltd., Tokyo, Japan) and Finland (Valio Evolus Double Effect, Valio Ltd., Finland). This fermented milk has shown beneficial effects on blood pressure in several rat models and human studies [47, 48, 50-55].

Ashar and Chand [42] identified an ACE-inhibitory peptide from milk fermented with *Lactobacillus delbrueckii* ssp. *bulgaricus*, and Pihlanto et al. [56] reported two peptides responsible for the ACE inhibitory activity of milk fermented with *Lactobacillus jensenii*. In combination with *Streptococcus salivarius* ssp. *thermophilus* and *Lactococcus lactis* biovar. *diacetylactis*, a hypotensive structure with a sequence of SKVYP was obtained from β -casein. Quirós and co-workers [57] identified two peptides in fermented milk with *Enterococcus faecalis* that corresponded to β -CN fragments LHLPLP and LVYFPFGPIPNSLPQNIPP, with potent ACE-inhibitory activity and proven antihypertensive effect when orally administered to spontaneously hypertensive rats after acute and long-term administration.

During the maturation of cheese, the major milk proteins are degraded into a large number of peptides due to the action of endogenous milk enzymes, added coagulants and microbial enzymes. A number of studies have shown that ACE inhibitory peptides can be produced during

cheese making, in particular during ripening process. These peptides have been characterized in different commercial cheeses, such as Edam, Gouda, Camembert, Havarti and Blue cheese [58], Italian and Spanish cheeses [29, 59, 60], and Asiago cheeses [30]. Tri-peptides VPP and IPP have also been identified and quantified in different cheese varieties by Butikofer and co-workers [61, 62]. A low-fat cheese containing ACE-inhibitory peptides derived from α_{s1} -casein and named as “Festivo” is commercialized in Eastern countries.

Fermented soy products, traditionally consumed in Eastern countries, have been also found to be an important source of ACE-inhibitory and antihypertensive peptides. A potent antihypertensive peptide has been identified and characterized in a Korean soy product denominated “chunggugjang” and obtained by soy fermentation with *Bacillus subtilis* CH-1023 [63]. Other ACE-inhibitory and antihypertensive peptides have been identified in soy paste [64], soy sauce [65, 66], natto and tempeh [67], and other fermented soy products [19, 68, 69].

2.3. Enzymatic hydrolysis

The most common way to produce bioactive peptides is through enzymatic hydrolysis of whole protein molecules (Table 1). A large number of studies have demonstrated the release of ACE inhibitory and/or antihypertensive peptides from food proteins, by hydrolysis with gastrointestinal enzymes, such as pepsin, trypsin, and chymotrypsin [24, 70-72]. Manso and Lopez-Fandino [73] described occurrence of ACE-inhibitory peptides in hydrolyzates of bovine, ovine and caprine κ -casein upon hydrolysis with various digestive enzymes. Pepsin was used by Contreras and co-workers [74] to hydrolyze total isoelectric casein and three peptide sequences derived from α_{s1} -casein and α_{s2} -casein were characterized as ACE inhibitors and antihypertensive peptides.

In addition to live microorganisms, proteolytic enzymes from bacterial and fungal sources have been used to generate bioactive peptides from various proteins. The use of commercially available microbial-derived food grade proteinases to hydrolyze food proteins is advantageous as these enzymes are low-cost and safe, and the product yields are very high [75]. Ueno et al. [76] purified and characterized an endopeptidase from *Lb. helveticus* CM4 and demonstrated that this peptidase can generate antihypertensive peptides using synthetic pro-peptides as substrates. Mizuno et al. [77] measured the ACE-inhibitory activity of casein hydrolyzates upon treatment with nine different commercially available proteolytic enzymes. Among these enzymes, a protease extracted from *Aspergillus oryzae* acted specifically on casein to release VPP and IPP, and the obtained casein hydrolyzate demonstrated a significant dose-dependent antihypertensive effect in a rat model with spontaneously hypertensive rats.

Recently, the interest of food technologists has turned to the use of different techniques, such as high-pressure and heat denaturing and power ultrasound to modify protein structure and increase enzymatic hydrolysis. As compared to the proteolysis at atmospheric pressure, qualitative and quantitative differences were detected in the hydrolysis pattern when proteolysis with trypsin was carried out under high pressure treatments [78-79]. Hernandez-Ledesma et al. [80] reported that heating of β -Lg during enzyme treatments with thermolysin enhances the formation of peptides with ACE-inhibitory activity, and one of the peptides released under these heat-denaturing conditions was LQKW that had previously been described as a potent ACE inhibitor [81]. Prolonged exposure to high-intensity ultrasound has been shown to inhibit the catalytic activity of a number of food enzymes [82]. However, in some cases, solutions containing enzymes have been found to have increased activity following short exposures to ultrasound [83]. Jia et al. [84] found that the use of ultrasonic treatment during proteolysis could

facilitate the enzymatic hydrolysis of peptide DWGP, whereas ultrasonic pre-treatment could promote the release of ACE inhibitory peptides from this peptide.

2.4. Genetic recombination in bacteria

Industrial preparation of ACE inhibitory and/or antihypertensive peptides by enzymatic hydrolysis and microbial fermentation showed to be a low efficient process because of low yield and high cost of separation and purification processes [85-88]. To solve these issues, during last years a new technique based on genetic engineering is being developed. One of the challenges of this technique is the susceptibility of short antihypertensive peptides to degradation by protease or peptidase. Moreover, the expression products may be harmful to the host, impacting the high-level expression of the gene. This shortcoming has been conquered by expression of antihypertensive peptides in the forms of a fusion protein or a tandem gene. Antihypertensive peptides with sequences HHL, HVLPVP, FFVAPFPEVFGK, and GHIATFQER have been expressed successfully in *Escherichia coli* [89-92], although special proteases are needed to release the target active protein, thus increasing the cost of separation and purification after enzymatic hydrolysis. Recently, Rao et al. [93] expressed an antihypertensive peptide multimer, a common precursor of 11 kinds of antihypertensive peptides, and the release was confirmed by simulated gastrointestinal digestion. Because of the fact that currently genetic modified microorganisms are difficult to be used in food products, further studies should be needed.

3. *In vitro* and *in vivo* assays

The search for ACE inhibitory activity is the most common strategy followed in the selection of antihypertensive peptides derived from food proteins. In order to study ACE inhibition, simple, rapid, sensitive and reliable analytical methods are desirable. *In vitro*

197 inhibitory activity is generally measured by monitoring the conversion of an appropriate substrate
198 by ACE in the presence and absence of the potential inhibitors. There are several methods, but
199 those based on spectrophotometric and high-performance liquid chromatography (HPLC) assays
200 are most commonly utilized. The spectrophotometric method of Cushman and Cheung [94] is
201 based on the hydrolysis of Hippuryl-His-Leu (HHL) by ACE to hippuric acid and His-Leu, and
202 the extent of hippuric acid released is measured after its extraction with ethyl acetate. The
203 inhibitory potency is expressed as the IC_{50} value, or concentration needed to inhibit 50% of the
204 enzyme activity. Extraction of reaction product is tedious and may overestimate ACE activity if
205 unhydrolyzed HHL is also extracted. Another broadly used spectrophotometric method is based
206 in the hydrolysis of a furanocryloyl tripeptide (FAPGG) to FAP and the di-peptides GG [95].
207 Using HPLC methods, the peak of hippuric acid may be interfered with by the added ACE
208 inhibitors in the reaction mixture, so the mobile phase needs to be adjusted to different tested
209 compounds. Moreover, the HPLC method show lower detection sensitivity and longer analysis
210 time to obtain good results. Doig and Smiley [96] and Mehanna and Dowling [97] have improved
211 HPLC methods by applying ultraviolet detection, and Van Elswijk et al. [98] have developed an
212 alternative strategy for the screening of complex food samples applying an HPLC method with
213 biochemical detection. In that approach, separation and activity detection are combined within
214 one step. However, as spectroscopic detection is used to monitor the enzymatic conversion, these
215 methods are restricted to artificial substrates as well. Direct, extraction-free methods have been
216 published recently [99, 100]. Siemerink et al. [101] have optimized a new robust HPLC coupled
217 with electrospray ionization mass spectrometry (HPLC/ESI-MS)-based screening method for
218 ACE-inhibiting substances in crude samples. Similarly, an ultra-performance-liquid
219 chromatography (UPLC) coupled with MS (UPLC-MS) for determination of the ACE activity
220 has been recently developed by Geng et al. [102]. This new method is more sensitive, accurate

and reproducible. The small total reaction volume, the short analysis time, high selectivity and lower expense are the advantages of this method in comparison with the conventional methods.

The antihypertensive effects can only be reliably assessed by *in vivo* experiments using spontaneously hypertensive rats that constitute an accepted model for human essential hypertension [7]. A great number of studies have addressed the effects of both short-term and long-term administration of potential antihypertensive peptides using this animal model [88, 103-106]. Moreover, many *in vivo* studies include the evaluation of the effect of antihypertensive peptides on arterial blood pressure of normotensive Wistar-Kyoto rats. Recently, Nakahara et al. [66] have used the Dahl salt-sensitive rats as a model of salt-sensitive hypertension to evaluate the antihypertensive effect of a peptide-enriched soy-sauce like seasoning. The results of these tests have highlighted an important lack of correlation between the *in vitro* ACE inhibitory activity and the *in vivo* action. This fact has provided doubts on the use of the *in vitro* ACE inhibitory activity as the exclusive criteria for potential antihypertensive substances because other mechanisms of action than ACE inhibition might be responsible for the antihypertensive effect. Also, it should be needed to take into consideration the physiological transformations determining the bioavailability of the peptides.

The antihypertensive effect of some food proteins-derived peptides has been conducted in human studies to determine whether these peptides possess an antihypertensive effect on human subjects with high-normal blood pressure and mild hypertension [50, 107, 108]. The most substantiated antihypertensive activity in humans has been obtained for the commercial fermented milk products and hydrolyzates containing the ACE-inhibitory peptides IPP and VPP. The antihypertensive effect of the sour milk product Calpis, commercialized in Japan, was tested in a clinical study with mildly hypertensive patients [50]. Recently, a study has been conducted among patients with high-normal blood pressure and mild hypertension, evaluating the effect of

different doses of a casein hydrolyzate produced by *Aspergillus oryzae* containing IPP and VPP and commercialized as AmealPeptide[®] by Calpis [77]. Similarly, a milk product Evolus[®] fermented with *Lactobacillus helveticus* LBK-16H and produced by Valio Ltd. (Finland) has been tested in hypertensive humans [48]. This product, containing peptides IPP and VPP, showed to exert a long-term blood pressure-lowering effect after normal daily ingestion during a 21-weeks intervention period.

4. Structure-activity relationship

Although the structure-activity relationship of ACE-inhibitory peptides derived from foods has not yet been fully elucidated, several structural features influencing potency of these peptides have been identified [7, 109]. Recently, it has been reported that artificial neural networks (ANN) and quantitative structure-activity relationship (QSAR) modelling may be used to develop statistical computer models potentially capable of identifying ACE inhibitory peptides based on structure-activity data [110]. Several descriptor variables such as molecular mass and shape, hydrophobicity, charge and electronic properties have been recognized as critical in this QSAR modelling. The majority of ACE inhibitory peptides are relatively short sequences containing from 2 to 12 amino acids. This in agreement with the results of Natesh and coworkers [111], which demonstrated from crystallography studies, that the active site of ACE cannot accommodate large peptide molecules. However, some studies have identified ACE inhibitory peptides with up to 27 amino acids [58, 112, 113]. Of many ACE-inhibitory peptides identified from different food sources, structure-activity correlation indicated that C-terminal tri-peptide residues play a predominant role in competitive binding to the active site of ACE. It has been reported that this enzyme prefers substrates or inhibitors containing hydrophobic (aromatic or branched side chains) amino acid residues at each of the three C-terminal positions. The most

effective ACE inhibitory peptides identified contain Tyr, Phe, Trp, and/or Pro at the C-terminal. Gomez-Ruiz and coworkers [29] have suggested that amino acid Leu may contribute significantly to increase ACE inhibitory potential. Furthermore, other branched chain aliphatic amino acids such as Ile and Val are predominant in highly peptide inhibitors. In addition, structure-activity data suggest that the positive charge of Lys (ϵ -amino group) and Arg (guanidine group) as the C-terminal residue may contribute to the inhibitory potency [114-117]. Other characteristics have also been found to play important roles for ACE inhibition. It has been recognized that ACE inhibitory peptides possess a characteristic pattern (i.e. a similar positive potential located at the C-terminal end) different from that of inactive peptide molecules [112, 118]. For long chain peptides, it is expected that peptide conformation, i.e. the structure adopted in the specific environment of the binding site, will influence binding to ACE [117, 118]. It has also been demonstrated that ACE has a requirement for the L-configuration of the amino acid at position three from the C-terminal. Moreover, changes in cis-trans conformations of Pro at the C-terminal position of an ACE inhibitory peptide may cause significant changes in its interaction with the enzyme [119].

5. Mechanism of action

Blood pressure is determined by cardiac output and vascular peripheral resistance, and is regulated by a complex system involving the RAS, the sympathetic nervous system (SNS), and the kidney and fluid balance mechanism [118]. Most food-derived peptides usually display higher *in vivo* activities than the efficacy levels extrapolated from the *in vitro* ACE inhibitory activity. This may be an indication of the existence of an additional mode of action [120]. In fact, increasing evidence is being provided that different mechanisms, others than ACE inhibition, are

involved in the blood-pressure-modulating effect exerted by many of these peptides. *In vitro*, tri-peptides VPP and IPP have been shown to inhibit ACE at micromolar concentrations [40, 41]. *In vivo*, long-term treatment of spontaneously hypertensive rats with fermented milk containing these peptides has been found to decrease serum ACE activity [121-123]. However, according to Jauhiainen et al. [54], the mechanistic theory of ACE inhibition of IPP and VPP remains to be confirmed and other effects have to be taken into consideration. Some of these effects have been evaluated in animal models and clinical studies. Plasma rennin activity and levels have been found to be raised in spontaneously hypertensive rats receiving IPP and VPP for 14 weeks. This raising can be due to the lack of negative feedback by angiotensin II, which supports that ACE was inhibited [47]. Other authors have reported the protective effects exerted by these peptides on endothelial function of isolated mesenteric arteries of rats after 24 h incubation with them [124]. In humans with mild hypertension, administration of a casein hydrolyzate containing VPP and IPP increases maximum blood flow forearm during reactive hyperemia, thus demonstrating an improvement in the vascular endothelial dysfunction. Yamaguchi et al. [125] studied effect of a 5-day repeated administration of VPP and IPP on gene expression of spontaneously hypertensive rats abdominal aorta using DNA microarray microanalysis, reporting a significant increase for the endothelial nitric oxide synthase (eNOS) gene and the connexin 40 gene, which are involved in blood pressure regulation. Expression of these genes was restored in the aortic tissue after treatment with these tri-peptides [126, 127], suggesting that VPP and IPP might act *in vivo* as ACE inhibitors in the aorta and also have preventive potential in cardiovascular function.

Fuglsang et al. [46] reported that ingestion of two milks fermented with *Lactobacillus helveticus* provokes a decrease of the response to an intravenous injection of angiotensin I in unconscious normotensive rats, whereas response to bradikinin was increased, confirming the inactivation of ACE. Dried bonito-ACE inhibitory peptides slightly inhibit angiotensin I-induced

contractions in rat-isolated aorta as compared with Captopril, but unlike this drug, peptides exert a direct action on vascular smooth muscles [128]. Similarly, peptide lactokinin (ALPMHIR) inhibits the release of ET-1, an endothelial factor that evokes contractions in smooth muscle cells through mechanisms both dependent and independent of ACE-inhibition [129]. It is also likely that opioid receptors are involved in the antihypertensive effect of some peptides, such as α -lactorphin, β -lactorphin and human casein-derived fragments, as this was abolished by the opioid receptor antagonist naloxone. As an example, it has been demonstrated that α -lactorphin, a tetrapeptide (YGLF) formed by *in vitro* proteolysis of α -lactalbumin with pepsin and trypsin, lowers blood pressure in spontaneously hypertensive rats and produces an endothelium-dependent relaxation of their mesenteric arteries that is inhibited by an eNOS inhibitor [47]. Therefore, a mechanism of action driven by the stimulation of peripheral opioid receptors and subsequent nitric oxide (NO) release causing vasodilation has been proposed for this peptide. Although α -lactorphin interacting with opioid receptors does not elicit effects typical of centrally active opioids such as antinociception and sedation [130]. It has been suggested that these opioid peptides might lower blood pressure through receptors expressed in the gastrointestinal tract, which implies that no absorption is required [131].

Strong epidemiological evidence indicates that oxidative stress and associated oxidative damage are mediators in cardiovascular diseases. In experimental and human hypertension studies, it has been demonstrated an increased production of superoxide anion and hydrogen peroxide, reduced NO synthesis, and decreased bioavailability of antioxidants [132]. Therefore, food-derived peptides with antioxidant properties might also have effect on blood pressure modulation. Many of these peptides have been identified and characterized from casein and whey proteins hydrolyzed with different enzymes [133, 134].

Taking together the results of all these studies, more thorough mechanistic research should be probably needed to detect the changes in the factors affecting blood pressure and vascular tone to show the exact mechanisms also *in vivo* of antihypertensive peptides.

6. Bioavailability and clinical studies

The physiological effects of bioactive peptides depend on the ability to reach in an active form their target organs. This implies resistance to gastrointestinal enzymes and brush border membrane peptidases and absorption through the intestinal epithelium. The resistance of peptides to these processes is usually performed by sequential hydrolysis with pepsin and pancreatic extracts mimicking the gastrointestinal conditions and with *in vitro* studies with epithelial intestinal cells. Although peptides were thought to be rapidly metabolized to constituent amino acids, these studies have demonstrated that several peptides are resistant to these physiological processes and can reach the circulation. This is the case of the short tri-peptides IPP and VPP [135, 136], but also has been demonstrated for longer proline-rich peptides, such as, LHLPLP. This latter peptide resists simulated gastrointestinal digestion but it is hydrolysed to a shorter active form, HLPLP, by cellular peptidases prior to transport across the intestinal epithelium [137, 138]. Figure 1 shows the formation of the shorter peptide during incubation of the peptide LHLPLP in the apical chamber of the Caco-2 cell culture. The penta-peptide appeared in approximately 3 min and its concentration increased with the incubation time up to 60 min. This shorter form has also been detected in human plasma after oral administration which demonstrates intestinal absorption of the pentapeptide in humans [139]. In some cases, the active form is released during gastrointestinal processes. For instance, the active form of peptide KVLPPVQ is generated by hydrolysis of the glutamine residue at the C-terminal end during pancreatic digestion [140]. This is also the case of the egg-derived antihypertensive peptides

YAEERYPIL and RADHPFL that were hydrolysed to other active forms after simulated gastrointestinal digestion [11]. The pharmacokinetic behaviour of the tri-peptides IPP and VPP has also been studied and an absolute bioavailability of 0.1% respect the administered dose has been calculated in pigs [141]. In humans, these two tri-peptides were detected in plasma after oral administration at picomolar concentrations and their absorption was enhanced when ingested in the form of an enriched yogurt beverage. In addition, a further increase (1.2-fold) in the plasmatic concentration of IPP was found when the enriched yogurt was administered after a meal [142].

Several clinical studies have evaluated the antihypertensive effect of the tri-peptides IPP and VPP after long-term administration in humans. Most of them are included in two meta-analyses recently published [143, 144]. The meta-analysis by Xu et al. [143] includes 12 trials with a total of 623 participants and found significant decreases in systolic and diastolic blood pressure (4.8 mmHg and 2.2 mmHg, respectively). Similar results arose from the meta-analysis published by Pripp et al. [144] with a total of 15 clinical trials included. Although two long-term studies have not found statistical differences with these tri-peptides [145, 146], most recent reviews on the subject identify several factors such as component of the final product, dose, method for blood pressure measurement that can influence the results in different trials [147, 148].

7. Incorporation into food products

For an industrial application of protein hydrolyzates containing antihypertensive peptides, main considerations would be the organoleptic characteristics of these ingredients and the evaluation of the resistance of the active peptides to processing conditions. The practical use of protein hydrolyzates in food systems is hindered due to the presence of low molecular weight peptides composed mainly of hydrophobic amino acids that results in a bitter taste [149]. In fact, this problem has limited the use of some of the developed hydrolyzates with proved

antihypertensive effect. In addition to the bitterness, the pH of the hydrolysis reaction needs to be regulated because the substrate susceptibility and the enzyme activity are strongly influenced by the pH. In order to achieve the desired hydrolysis degree to obtain biologically active peptides, the addition of some alkali or acid is required to neutralize the hydrolysis products. This leads to undesirable high ash build up in the hydrolyzates and the development of salty off-flavors. Different strategies have been applied for debittering protein hydrolyzates. These include absorption of bitter peptides on activated carbon, chromatographic removal using different matrices and selective extraction with alcohols [149]. The most extended approaches include hydrolysis of bitter peptides with enzymes such as aminopeptidase, alkaline/neutral protease and carboxypeptidase, condensation reactions of bitter peptides using protease and use of *Lactobacillus* as debittering starter adjunct [150]. However, the application of all these methods in biologically active hydrolyzates is limited because the enzymatic activity used for debittering can hydrolyze the previously generated bioactive peptides. Although it has not been found a significant correlation between bitterness and the ACE-inhibitory activity of di- and tri-peptides [151], it is recognized the importance of hydrophobic amino acid residues for both bitterness and ACE-inhibitory peptides. Therefore, those methods based in the removal of bitter peptides (either absorption, extraction or hydrolysis) have to be carefully applied to hydrolyzates containing, for instance, ACE-inhibitory peptides. For bioactive hydrolyzates, the application of masking methods by using monosodium glutamate or glutamylglutamic acid [152], the addition of cyclodextrins [153], encapsulation [154], or the addition of phospholipids and lysophospholipids [155] could be preferably used. For instance, for the encapsulation of casein hydrolyzates, different materials such as soy proteins isolates alone or in mixtures with gelatin [154, 156], maltodextrins [157], or lipospheres [158] have been successfully applied. The presence of proteins and reducing carbohydrates in a food ingredient will lead to the formation of Maillard

compounds that can have a positive effect on flavor but this reaction has to be controlled to avoid the generation of undesirable compounds [159, 160]. Therefore, the improvement of the flavor of biologically active protein hydrolyzates including possible protein-flavor interactions and the effect of these processes on biological activity are of interest in relation to the development of novel protein foods.

Processing provides an additional value to foods in improving their safety, shelf-life, palatability, nutritive and functional value, but the conditions of processing and storage may be detrimental to peptides. At this regard, changes in the molecular structure of an amino acid may lead to changes in the bioactivity and in the absorption of the peptide of interest. The most relevant degradation pathways of amino acids during processing were recently reviewed by López-Fandiño & co-workers [3]. For example, thermal processing favors racemization, amino acids decomposition (e.g. ornithine from arginine), glycation (non-enzymatic browning or Maillard reaction), and cross-linking. Furthermore, amino acids can be also oxidized during food processing [161], and even D-amino acids can be synthesized out from L-amino acids by LAB. Dehydration by spray-drying may produce some negative effects on food protein hydrolyzates, such as changes in peptide composition, reduction of amino acid content and non enzymatic browning [162-164]. There are little data about the effects of other processes on bioactive peptides. Recently, the stability of two α_{s1} -casein-derived antihypertensive peptides to spray-drying, homogenization and pasteurization when they were incorporated into fermented milk has been demonstrated [165]. Incorporation of active hydrolyzates to fermented milks implies that these peptides have to survive in the presence of LAB because of their cell-associated proteinases/peptidases systems that could further hydrolyze the bioactive sequences. For instance, it has been reported that an 12 amino acid long antihypertensive peptide can be

digested by exposure to yogurt starter culture strains and therefore, the incorporation of this bioactive peptide should preferably be done at the end of the yogurt-making process [166].

8. Future prospects

Among the different groups of bioactive peptides defined, antihypertensive peptides have received special attention, their activity has been tested *in vitro*, animal models and humans, and they have been incorporated into different food products. Controversial results on clinical studies and the different health claim legislations will contribute to increase research in this area. In this sense, different studies have been performed to demonstrate stability of the peptide, absorption and identification of the active form in the organism. It has been postulated that physiologically relevant concentrations and elimination kinetics will be mandatory for food derived bioactive components as it is now for pharmaceutical compounds. At the same time, the recent advances on specific analytical techniques able to follow small amounts of the peptides or derivatives from them in complex matrices and biological fluids will allow performing these kinetic studies in model animals and humans. Similarly, advances in new disciplines such as nutrigenomic and nutrigenetic will open new ways to follow bioactivity in the organism by identifying novel and more complex biomarkers of exposure and/or of activity. All these advances will be done simultaneously with the knowledge food technologists since the final formulation of the food product is crucial to ensure activity and bioavailability of bioactive peptides.

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Figure captions

Figure 1: Changes of the synthetic peptide LHLPLP when added to apical chamber of a Caco-2 cell culture at different periods of time. Extracted ion chromatograms obtained by HPLC-MS analysis of the apical chamber after a) 5 min, b) 10 min, c) 30 min, and d) 60 min of incubation. The extracted ion chromatogram was obtained by extraction of ions with m/z 689.4, 711.4 and 727.4, which correspond to molecular ion of peptide LHLPLP and its sodium and potassium adducts, and ions with m/z 598.3, 614.3 and 620.3, corresponding to molecular ion of peptide HLPLP and its sodium and potassium adducts. Reproduced from [137] with permission.

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Table 1. ACE inhibitory and antihypertensive activity in spontaneously hypertensive rats of peptide derived from caseins and whey proteins by fermentation and enzymatic hydrolysis

Peptide	Sequence	IC ₅₀ (μM) ^a	Decrease of SBP (mmHg) ^b	Origin	Reference
α _{s1} -CN f(1-9)	RPKHPIKHQ	13.4	-9.3	Gouda cheese	[58]
α _{s1} -CN f(146-147)	YP	720	-32.1	Fermentation with <i>Lb. helveticus</i> CPN4	[140]
β-CN f(58-76)	LVYPPFGPIPNSLPQ NIPP	5.2	-15.0	Fermentation with <i>En. faecalis</i>	[11, 57]
β-CN f(60-68)	YPPFGPIP	14.8	-7.0	Gouda cheese	[58]
β-CN f(74-76)	IPP	5.0	-28.3 (-10.1) ^d	Fermentation with <i>Lb. helveticus</i> y <i>Sc. cerevisiae</i>	[40, 41]
β-CN f(84-86)	VPP	9.0	-32.1 (-10.1) ^d	Fermentation with <i>Lb. helveticus</i> y <i>Sc. cerevisiae</i>	[40, 41]
β-CN f(133-138)	LHLPLP	5.5	-21.9	Fermentation with <i>En. faecalis</i>	[11, 57]
β-CN f(133-139)	LHLPLPL	425	-7.7	Fermentation with <i>En. faecalis</i>	[11, 57]
β-CN f(197-206)	VLGPVRGPFP	137	-16.2	Fermentation with <i>En. faecalis</i>	[11, 57]
β-CN f(201-209)	VRGPFPIIV	599	-16.1	Fermentation with <i>En. faecalis</i>	[11, 57]
α _{s1} -CN f(23-34)	FFVAPFPGVFGK	77	-34.0	Hydrolysis with trypsin	[167]
α _{s1} -CN f(104-109)	YKVPQL	22	-13.0	Hydrolysis with a proteinase from <i>Lb. helveticus</i> CP790	[140]
α _{s1} -CN f(194-199)	TTMPLW	16	-13.6	Hydrolysis with trypsin	[167]
α _{s2} -CN f(189-192)	AMPKPW	580	-5.0	Hydrolysis with a proteinase from <i>Lb. helveticus</i> CP790	[140]
α _{s2} -CN f(190-197)	MKPWIQPK	300	-3.0	Hydrolysis with a proteinase from <i>Lb. helveticus</i> CP790	[140]
α _{s2} -CN f(198-202)	TKVIP	400	-9.0	Hydrolysis with a proteinase from <i>Lb. helveticus</i> CP790	[140]
α _{s2} -CN f(203-208) ^d	PYVRYL	1.9	23.4	Hydrolysis with pepsin	[168]
β-CN f(59-61)	VYP	288	-21.0	Hydrolysis with proteinase K	[169]
β-CN f(59-64)	VYPPFG	221	-22.0	Hydrolysis with proteinase K	[169]
β-CN f(80-90)	TPVVVPPFLQP	749	-8.0	Hydrolysis with proteinase K	[169]
β-CN f(140-143)	LQSW	500	-2.0	Hydrolysis with a proteinase from <i>Lb. helveticus</i> CP790	[140]
β-CN f(169-174)	KVLPVP	5	-32.2	Hydrolysis with a proteinase from <i>Lb. helveticus</i> CP790	[140]
β-CN f(169-175)	KVLPVPQ	1000	-31.5	Hydrolysis with a proteinase from <i>Lb. helveticus</i> CP790	[140]

925

926 **Table 1. (Continuation).** ACE inhibitory and antihypertensive activity in spontaneously hypertensive rats of peptide derived from caseins and whey proteins
 927 by fermentation and enzymatic hydrolysis
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 929

Peptide	Sequence	IC ₅₀ (μM) ^a	Decrease of SBP (mmHg) ^b	Origin	Referente
β-CN f(177-183)	AVPYPQR	15	-10.0	Hydrolysis with trypsin	[167]
α-La f(50-53)	YGLF	733	-23.0	Hydrolysis with gastric and pancreatic enzymes	[170]
β-Lg f(58-61) ^c	LQKW	34.7	-18.1	Hydrolysis with thermolysin	[103]
β-Lg f(78-80)	IPA	141	-31.0	Hydrolysis with proteinase K	[169]
β-Lg f(103-105) ^e	LLF	79.8	-29.0	Hydrolysis with thermolysin	[104]
BSA f(221-222)	FP	315	-27.0	Hydrolysis with proteinase K	[169]

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 931
 932 ^a: Peptide concentration needed to inhibit 50% ACE activity

933 ^b: Systolic blood pressure

934 ^c: Antihypertensive effects in humans

935 ^d: Ovine protein

936 ^e: Caprine protein
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