Antihypertensive peptides: production, bioavailability and incorporation into foods

Blanca Hernández-Ledesma[1,2], Isidra Recio[1,2,*]

[1] Instituto de Fermentaciones Industriales (CSIC). Juan de la Cierva, 3, 28006 Madrid, Spain

[2] Food Research Institute (CIAL, CSIC-UAM). Nicolás Cabrera, 9. Campus de la Universidad Autónoma de Madrid. 28049 Madrid, Spain

[*] Corresponding author: Dr. Isidra Recio

Phone: +34915622900

Fax: +34915644853

Email: recio@ifi.csic.es

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-kalicrein 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. *thermophylus* 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. *thermophylus* 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 LVYPFPGPIPNSLPQNIPP, 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*

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

316 contractions in rat-isolated aorta as compared with Captopril, but unlike this drug, peptides exert
317 a direct action on vascular smooth muscles [128]. Similarly, peptide lactokinin (ALPMHIR)
318 inhibits the release of ET-1, an endothelial factor that evokes contractions in smooth muscle cells
319 through mechanisms both dependent and independent of ACE-inhibition [129]. It is also likely
320 that opioid receptors are involved in the antihypertensive effect of some peptides, such as α-
321 lactorphin, β-lactorphin and human casein-derived fragments, as this was abolished by the opioid
322 receptor antagonist naloxone. As an example, it has been demonstrated that α-lactorphin, a tetra-
323 peptide (YGLF) formed by *in vitro* proteolysis of α-lactalbumin with pepsin and trypsin, lowers
324 blood pressure in spontaneously hypertensive rats and produces an endothelium-dependent
325 relaxation of their mesenteric arteries that is inhibited by an eNOS inhibitor [47]. Therefore, a
326 mechanism of action driven by the stimulation of peripheral opioid receptors and subsequent
327 nitric oxide (NO) release causing vasodilation has been proposed for this peptide. Although α-
328 lactorphin interacting with opioid receptors does no elicit effects typical of centrally active
329 opioids such as antinociception and sedation [130]. It has been suggested that these opioid
330 peptides might lower blood pressure through receptors expressed in the gastrointestinal tract,
331 which implies that no absorption is required [131].

332 Strong epidemiological evidence indicates that oxidative stress and associated oxidative
333 damage are mediators in cardiovascular diseases. In experimental and human hypertension
334 studies, it has been demonstrated an increased production of superoxide anion and hydrogen
335 peroxide, reduced NO synthesis, and decreased bioavailability of antioxidants [132]. Therefore,
336 food-derived peptides with antioxidant properties might also have effect on blood pressure
337 modulation. Many of these peptides have been identified and characterized from casein and whey
338 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 though 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 KVLPVPQ 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, alkanine/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 lysophopholipids [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

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

416 Processing provides an additional value to foods in improving their safety, shelf-life,
417 palatability, nutritive and functional value, but the conditions of processing and storage may be
418 detrimental to peptides. At this regard, changes in the molecular structure of an amino acid may
419 lead to changes in the bioactivity and in the absorption of the peptide of interest. The most
420 relevant degradation pathways of amino acids during processing were recently reviewed by
421 López-Fandiño & co-workers [3]. For example, thermal processing favors racemization, amino
422 acids decomposition (e.g. ornithine from arginine), glycation (non-enzymatic browning or
423 Maillard reaction), and cross-linking. Furthermore, amino acids can be also oxidized during food
424 processing [161], and even $_{D}$-amino acids can by synthesized out from $_{L}$-amino acids by LAB.
425 Dehydratation by spray-drying may produce some negatively effects on food protein
426 hydrolyzates, such as changes in peptide composition, reduction of amino acid content and non
427 enzymatic browning [162-164]. There are little data about the effects of other processes on
428 bioactive peptides. Recently, the stability of two α_{s1}-casein-derived antihypertensive peptides to
429 spray-drying, homogenization and pasteurization when they were incorporated into fermented
430 milk has been demonstrated [165]. Incorporation of active hydrolyzates to fermented milks
431 implies that these peptides have to survive in the presence of LAB because of their cell-
432 associated proteinases/peptidases systems that could further hydrolyze the bioactive sequences.
433 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 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.

Acknowledgments

This work has received financial supported from projects AGL-2007-65035, AGL2008-01713, Consolider Ingenio 2010 FUN-C-Food CSD2007-00063 and P2009/AGR-1469. The

457 authors want to acknowledge the European Commission and the Spanish National Research
458 Council for the Marie-Curie post-doctoral fellowship awarded to Blanca Hernández-Ledesma.

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.

References

[1] Diplock AT, Aggett PJ, Ashwell M, Bornet F, Fern EB, Roberfroid MB. Scientific concepts of functional foods in Europe: consensus document: Br J Nutr, 1999; 81:51.

[2] Meisel H, Fitzgerald RJ. Biofunctional peptides from milk proteins: mineral binding and cytomodulatory effects: Curr Pharm Des, 2003; 9:1289.

[3] Lopez-Fandino R, Otte J, van Camp J. Physiological, chemical and technological aspects of milk-protein-derived peptides with antihypertensive and ACE-inhibitory activity: Int Dairy J, 2006; 16:1277.

[4] López-Expósito I, Recio I. In Bosze Z (editor), Bioactive components of milk, Springer Science + Business Media Inc.; 2008:271.

[5] Hartmann R, Meisel H. Food-derived peptides with biological activity: from research to food applications: Curr Opin Biotechnol, 2007; 18:163.

[6] Kearney PM, Whelton M, Reynolds K, Muntner P, Whelton PK, He J. Global burden of hypertension: Lancet, 2005; 365:217.

[7] Fitzgerald RJ, Murray BA, Walsh DJ. Hypotensive peptides from milk proteins: J Nutr, 2004; 134:980S.

[8] Hernandez-Ledesma B, Recio I, Amigo L. β-lactoglobulin as source of bioactive peptides: Amino Acids, 2008; 35:257.

[9] Saito T. Antihypertensive peptides derived from bovine casein and whey proteins: Bioactive components of milk: Adv Exp Med, 2008; 606:295.

[10] Muguruma M, Ahhmed, AM, Katayama K, Kawahara S, Maruyama M, Nakamura T. Identification of pro-drug type ACE inhibitory peptide sourced from porcine myosin B: Evaluation of its antihypertensive effects *in vivo*: Food Chem, 2009; 114:516.

[11] Miguel M, Aleixandre MA, Ramos M, Lopez-Fandino R. Effect of simulated gastrointestinal digestion on the antihypertensive properties of ACE-inhibitory peptides derived from ovalbumin: J Agric Food Chem, 2006; 54:726.

[12] Yu Y, Hu JN, Miyaguchi YJ, Bai XF, Du YG, Lin BC. Isolation and characterization of angiotensin I-converting enzyme inhibitory peptides derived from porcine hemoglobine: Peptides, 2006; 11:2950.

[13] Jung WK, Mendis E, Je JY, Park PJ, Son BW, Kim HC, Choi YK, Kim SK. Angiotensin I-converting enzyme inhibitory peptide from yellowfin sole (*Limanda aspera*) frame protein and its antihypertensive effect in spontaneously hypertensive rats: Food Chem, 2006; 94:26.

[14] Qian ZJ, Je JY, Kim SK. Antihypertensive effect of angiotensin I converting enzyme-inhibitory peptide from hydrolyzates of bigeye tuna dark muscle, *Thumus obesus*: J Agric Food Chem, 2007; 55:8398.

[15] Aluko RE. Determination of nutritional and bioactive properties of peptides in enzymatic pea, chickpea, and mung bean protein hydrolysates: J AOAC Int, 2008; 91:947.

[16] Suetsuna K. Isolation and characterization of angiotensin I-converting enzyme inhibitor dipeptides derived from *Allium sativum* L (garlic): J Nutr Biochem, 1998; 9:415.

[17] Li GH, Qu MR, Wan JZ, You JM. Antihypertensive effect of rice protein hydrolysate with in vitro angiotensin I-converting enzyme inhibitory activity in spontaneously hypertensive rats: Asia Pac J Clin Nutr, 2007; 16:275.

[18] Chiang WD, Tsou MJ, Tsai ZY, Tsai TC. Angiotensin I-converting enzyme inhibitor derived from soy protein hydrolysates and produced by using membrane reactor: Food Chem, 2006; 98:725.

512 [19] Rho SJ, Lee JS, Il Chung Y, Kim YW, Lee HG. Purification and identification of an
513 angiotensin I-converting enzyme inhibitory peptide from fermented soybean extract: Process
514 Biochem, 2009; 44:490.
515 [20] Motoi H, Kodama T. Isolation and characterization of angiotensin I-converting enzyme
516 inhibitory peptides from wheat gliadin hydrolysate: Nahrung-Food, 2003; 47:354.
517 [21] Tovar-Perez EG, Guerrero-Legarreta I, Farres-Gonzalez A, Soriano-Santos J. Angiotensin I-
518 converting enzyme-inhibitory peptide fractions from albumin 1 and globulin as obtained of
519 amaranth grain: Food Chem, 2009; 116:437.
520 [22] Phelan M, Aherne A, Fitzgerald RJ, O'Brien NM. Casein-derived bioactive peptides:
521 Biological effects, industrial uses, safety aspects and regulatory status: Int Dairy J, 2009; 19:643.
522 [23] Gill I, Lopez-Fandino R, Jorba X, Vulfson EN. Biologically active peptides and enzymatic
523 approaches to their production: Enzyme Microb Technol, 1996; 18:163.
524 [24] Vermeirssen V, Van Camp J, Verstraete A, Verstraete W. Bioavailability of angiotensin I
525 converting enzyme inhibitory peptides: Brit J Nutr, 2004; 92:357.
526 [25] Shimizu M. Food-derived peptides and intestinal functions: Biofactors, 2004; 21:43.
527 [26] Hernandez-Ledesma B, Amigo L, Ramos M, Recio I. Angiotensin converting enzyme
528 inhibitory activity in commercial fermented products. Formation of peptides under simulated
529 gastrointestinal digestion: J Agric Food Chem, 2004; 52:1504.
530 [27] Hernandez-Ledesma B, Amigo L, Ramos M, Recio I. Release of angiotensin converting
531 enzyme inhibitory peptides by simulated gastrointestinal digestion of infant formulas: Int Dairy J,
532 2004; 14:889.
533 [28] Hernandez-Ledesma B, Quiros A, Amigo L, Recio I. Identification of bioactive peptides
534 after digestion of human milk and infant formula with pepsin and pancreatin: Int Dairy J, 2007;
535 17:42.

[29] Gomez-Ruiz JA, Ramos M, Recio I. Angiotensin converting enzyme-inhibitory activity of peptides isolated from Manchego cheese. Stability under simulated gastrointestinal digestion: Int Dairy J, 2004; 14:1075.

[30] Lignitto L, Cavatorta V, Balzan S, Gabai G, Galaverna G, Novelli E, Sforza S, Segato S. Angiotensin-converting, enzyme inhibitory activity of water-soluble extracts of Asiago d'allevo cheese: Int Dairy J, 2010; 20:11.

[31] Jang A, Jo C, Lee M. Storage stability of the synthetic angiotensin converting enzyme (ACE) inhibitory peptides separated from beef sarcoplasmic protein extracts at different pH, temperature, and gastric digestion: Food Sci Biotechnol, 2007; 16:572.

[32] Escudero E, Sentandreu MA, Arihara K, Tolera F. Angiotensin I-Converting Enzyme Inhibitory Peptides Generated from in Vitro Gastrointestinal Digestion of Pork Meat: J Agric Food Chem, 2010; 58:2895.

[33] Cinq-Mars CD, Hu C, Kitts DD, Li-Chan ECY. Investigations into inhibitor type and mode, simulated gastrointestinal digestion, and cell transport of the angiotensin I-Converting enzyme-inhibitory peptides in pacific hake (Merluccius productus) fillet hydrolysate: J Agric Food Chem, 2008; 56:410.

[34] Samaranayaka AGP, Kitts DD, Li-Chan ECY. Antioxidative and Angiotensin-1-Converting Enzyme Inhibitory Potential of a Pacific Hake (Merluccius productus) Fish Protein Hydrolysate Subjected to Simulated Gastrointestinal Digestion and Caco-2 Cell Permeation: J Agric Food Chem, 2010; 58:1535.

[35] Vermeirssen V, Van Camp J, Decroos K, Van Wijimelbeke V, Verstraete W. The impact of fermentation and in vitro digestion on the formation of angiotensin-I-converting enzyme inhibitory activity from pea and whey protein: J Dairy Sci, 2003; 86:429.

559 [36] Akillioglu HG, Karakaya S. Effects of heat treatment and in vitro digestion on the
560 Angiotensin converting enzyme inhibitory activity of some legume species: Eur Food Res
561 Technol, 2009; 229:915.
562 [37] Jiménez-Escrig A, Alaiz M, Vioque J, Ruperez P. Health-promoting activities of ultra-
563 filtered okara protein hydrolysates released by in vitro gastrointestinal digestion: identification of
564 active peptide from soybean lipoxygenase: Eur Food Res Technol, 2010; 230:655.
565 [38] Majumder K, Wu J. Angiotensin I Converting Enzyme Inhibitory Peptides from Simulated
566 in Vitro Gastrointestinal Digestion of Cooked Eggs: J Agric Food Chem, 2009; 57:471.
567 [39] Juillard V, Guillot A, Le Bars D, Gripon JC. Specificity of milk peptide utilization by
568 *Lactococcus lactis*: Appl Environ Microbiol, 1998; 64:1230.
569 [40] Nakamura Y, Yamamoto N, Sakai K, Okubo A, Yamazaki S, Takano T. Purification and
570 characterization of angiotensin I-converting enzyme inhibitors from sour milk: J Dairy Sci, 1995;
571 78:777.
572 [41] Nakamura Y, Yamamoto N, Sakai K, Takano T. Antihypertensive effect of sour milk and
573 peptides isolated from it that are inhibitors to angiotensin I-converting enzyme: J Dairy Sci,
574 1995; 78:1253.
575 [42] Ashar MN, Chand R. Antihypertensive peptides purified from milks fermented with
576 *Lactobacillus delbrueckii* ssp *bulgaricus*: Milchwissenschaft, 2004; 59:14.
577 [43] Fitzgerald RJ, Murray BA. Bioactive peptide and lactic fermentations: Int J Dairy Technol,
578 2006; 59:118.
579 [44] Korhonen H, Pihlanto A. Bioactive peptides: Production and functionality: Int Dairy J, 2006;
580 16:945.

[45] Fuglsang A, Nilsson D, Nyborg NCB. Cardiovascular effects of fermented milk containing angiotensin-converting enzyme inhibitors evaluated in permanently catheterized, spontaneously hypertensive rats: Appl Environ Microbiol, 2002; 68:3566.

[46] Fuglsang A, Rattray FP, Nilsson D, Nyborg CN. Lactic acid bacteria: Inhibition of angiotensin converting enzyme in vitro and in vivo: Antonie van Leeuwenhoek, 2003; 83:27.

[47] Sipola M, Finckenberg P, Korpela R, Vapaatalo H, Nurminen ML. Effect of long-term intake of milk products on blood pressure in hypertensive rats: J Dairy Res, 2002; 69:103.

[48] Seppo L, Jauhiainen T, Poussa T, Korpela R. A fermented milk high in bioactive peptides has a blood pressure-lowering effect in hypertensive subjects: Am J Clin Nutr, 2003; 77:326.

[49] Tuomilehto J, Lindstrom J, Hyyrynen J, Korpela R, Karhunen ML, Mikkola L, Jauhiainen T, Seppo L, Nissine A. Effect of ingesting sour milk fermented using *Lactobacillus helveticus* bacteria producing tripeptides on blood pressure in subjects with mild hypertension: J Human Hypert, 2004; 18:795.

[50] Hata Y, Yamamoto M, Ohni M, Nakajima K, Nakamura Y, Takano T. Placebo-controlled study of the effect of sour milk on blood pressure in hypertensive subjects: Am J Clin Nutr, 1996; 64:767.

[51] Masuda O, Nakamura Y, Takano T. Antihypertensive peptides are present in aorta after oral administration of sour milk containing these peptides to spontaneously hypertensive rats: J Nutr, 1996; 126:3063.

[52] Mizushima S, Ohshige K, Watanabe J, Kimura M, Kadowaki T, Nakamura Y, Tochikubo O, Ueshima H. Randomized controlled trial of sour milk on blood pressure in borderline hypertensive men: Am J Hypert, 2004; 17:701.

603 [53] Aihara K, Kajimoto O, Hirata H, Takahashi R, Nakamura Y. Effect of powered milk
604 fermented with *Lactobacillus helveticus* on subjects with high-normal blood pressure or mild
605 hypertension: J Am Coll Nutr, 2005; 24:257.
606 [54] Jauhiainen T, Collin M, Narva M, Cheng ZJ, Poussa T, Vapaatalo H, Korpela R. Effect of
607 long-term intake of milk peptides and minerals on blood pressure and arterial function in
608 spontaneously hypertensive rats: Milchwissenschaft 2005; 60:358.
609 [55] Hirota T, Ohki K, Kawagishi R, Kajimoto Y, Mizuno S, Nakamura Y, Kitakaze M. Casein
610 hydrolysate containing the antihypertensive tripeptides Val-Pro-Pro and Ile-Pro-Pro improves
611 vascular endothelial function independent of blood pressure-lowering effects: Contribution of the
612 inhibitory action of angiotensin-converting enzyme: Hypertens Res, 2007; 30:489.
613 [56] Pihlanto AJ, Virtanen T, Korhonen H. Angiotensin I converting enzyme (ACE) inhibitory
614 activity and antihypertensive effect of fermented milk: Int Dairy J, 2010; 20:3.
615 [57] Quirós A, Ramos M, Muguerza B, Delgado MA, Miguel M, Aleixandre A, Recio, I.
616 Identification of novel antihypertensive peptides in milk fermented with *Enterococus faecalis*: Int
617 Dairy J, 2007; 17:33.
618 [58] Saito T, Nakamura T, Kitazawa H, Kawai Y, Itoh T. Isolation and structural analysis of
619 antihypertensive peptides that exist naturally in Gouda cheese: J Dairy Sci, 2000; 83:1434.
620 [59] Smacchi E, Gobbetti M. Peptides from several Italian cheeses inhibitory to proteolytic
621 enzymes of lactic acid bacteria, Pseudomonas fluorescens ATCC 948 and to the angiotensin I-
622 converting enzyme: Enz Microb Technol, 1998; 22:687.
623 [60] Gómez-Ruiz JA, Taborda G, Amigo L, Recio I, Ramos M. Identification of ACE-inhibitory
624 peptides in different Spanish cheeses by tandem mass spectrometry: Eur Food Res Technol,
625 2006; 223:595.

626 [61] Butikofer U, Meyer J, Sieber R, Wechsler D. Quantification of the angiotensin-converting
627 enzyme-inhibiting tripeptides Val-Pro-Pro and Ile-Pro-Pro in hard, semi-hard and soft cheeses:
628 Int Dairy J, 2007; 17:968.
629 [62] Butikofer U, Meyer J, Sieber R, Walther B, Wechsler D. Ocurrence of the angiotensin-
630 converting enzyme-inhibiting tripeptides Val-Pro-Pro and Ile-Pro-Pro in different cheese varieties
631 of Swiss origin: J Dairy Sci, 2008; 91:29.
632 [63] Korhonen H, Pihlanto A. Food-derived bioactive peptides- opportunities for designing future
633 foods: Curr Pharm Des, 2003; 9:1297.
634 [64] Shin ZI, Yu R, Park SA, Chung DK, Ahn CW, Nam HS, Kim KS, Lee HJ. His-His-Leu, an
635 angiotensin I converting enzyme inhibitory peptide derived from Korean soybean paste, exerts
636 antihypertensive activity in vivo: J. Agric. Food Chem, 2001; 49:3004.
637 [65] Okamoto A, Hanagata H, Matsumoto E, Kawamura Y, Koizumi Y, Yanadiga F. Angiotensin
638 I converting enzyme inhibitory activities of various fermented foods: Biosci Biotech Biochem,
639 1995; 59:1147.
640 [66] Nakahara T, Sano A, Yamaguchi H, Sugimoto KRI, Chikata H, Kinoshita E, Uchida R.
641 Antihypertensive Effect of Peptide-Enriched Soy Sauce-Like Seasoning and Identification of Its
642 Angiotensin I-Converting Enzyme Inhibitory Substances: J Agric Food Chem, 2010; 58:821.
643 [67] Gibbs BF, Zoygman A, Masse R, Mulligan C. Production and characterization of bioactive
644 peptides from soy hydrolysate and soy-fermented food: Food Res Int, 2004; 37:123.
645 [68] Li FJ, Yin LJ, Cheng YQ, Yamaki K, Fan JF, Li LT. Comparison of Angiotensin I-
646 converting enzyme inhibitor activities of pre-Fermented Douchi (a Chinese traditional fermented
647 soybean food) started with various cultures: Int J Food Eng, 2009; 5:10.

[69] Ibe S, Yoshida K, Kumada K, Tsurushiin S, Furusho T, Otobe K. Antihypertensive effects of natto, a traditional Japanese fermented food, in spontaneously hypertensive rats: Food Sci Technol Res, 2009; 15:199.

[70] Pihlanto-Leppala A, Koskinen P, Piilola K, Tupasela T, Korhonen H. Angiotensin I-converting enzyme inhibitory properties of whey protein digests: Concentration and characterization of active peptides: J Dairy Res, 2000; 67:53.

[71] Roufik S, Gauthier SF, Turgeon SL. In vitro digestibility of bioactive peptides derived from β-lactoglobulin: Int Dairy J, 2006; 16:294.

[72] deCosta EL, Rocha Montijo JA, Netto FM. Effect of heat and enzymatic treatment on the antihypertensive activity of whey protein hydrolysates: Int Dairy J, 2007; 17:632.

[73] Manso MA, Lopez-Fandino R. Angiotensin I converting-enzyme-inhibitory activity of bovine, ovine, and caprine k-casein macropeptides and their tryptic hydrolysates: J Food Prot, 2004; 66:1686.

[74] Contreras MM, Carrón R, Montero MJ, Ramos M, Recio I. Novel casein-derived peptides with antihypertensive activity. Int Dairy J, 2009; 19:566.

[75] Mao XY, Ni JR, Sun WL, Hao PP, Fan L. Value-added utilization of yak milk casein for the production of angiotensin-I-converting enzyme inhibitory peptides: Food Chem, 2007; 103:1282.

[76] Ueno K, Mizuno S, Yamamoto N. Purification and characterization of an endopeptidase that has an important role in the carboxyl terminal processing of antihypertensive peptides in Lactobacillus helveticus CM4: Lett Appl Microbiol, 2004; 39:313.

[77] Mizuno S, Nishimura S, Matsuura K, Gotou T, Yamamoto N. Release of short and proline-rich antihypertensive peptides from casein hydrolysate with an *Aspergillus oryzae* protease: J Dairy Sci, 2004; 87:3183.

671 [78] Chicon R, Belloque J, Recio I, Lopez-Fandino R. Influence of high hydrostatic pressure on
672 the proteolysis of beta-lactoglobulin A by trypsin: J Dairy Res, 2006; 73:121.
673 [79] Chicon R, Lopez-Fandino R, Quiros A, Belloque J. Changes in chymotrypsin hydrolysis of
674 beta-lactoglobulin A induced by high hydrostatic pressure: J Agric Food Chem, 2006; 54:2333.
675 [80] Hernandez-Ledesma B, Ramos M, Recio I, Amigo L. Effect of β-lactoglobulin hydrolysis
676 with thermolysin under denaturing temperatures on the release of bioactive peptides: J Chrom A,
677 2006; 1116:31.
678 [81] Hernandez-Ledesma B, Recio I, Ramos M, Amigo L. Preparation of ovine and caprine β-
679 lactoglobulin hydrolysates with ACE-inhibitory activity. Identification of active peptides from
680 caprine β-lactoglobulin hydrolysed with thermolysin: Int Dairy J, 2002; 12:805.
681 [82] Kadkhodaee R, Povey MJW. Ultrasonic inactivation of Bacillus alpha-amylase. I. Effect of
682 gas content and emitting face of probe: Ultrason Sonochem, 2008; 15:133.
683 [83] Lee SH, Nguyen HM, Koo YM, Ha SH. Ultrasound-enhanced lipase activity in the synthesis
684 of sugar ester using ionic liquids: Proc Biochem, 2008; 43:1009.
685 [84] Jia JQ, Ma HL, Zhao WR, Wang ZB, Tian WM, Luo L, He RH. The use of ultrasound for
686 enzymatic preparation of ACE-inhibitory peptides from wheat germ protein: Food Chem, 2010;
687 119:336.
688 [85] Megias C, Yust MDM, Pedroche J, Lquari H, Giron-Calle J, Alaiz M, Millan F, Vioque J.
689 Purification of an ACE inhibitory peptide after hydrolysis of sunflower (*Helianthus annuus* L.)
690 protein isolates: J Agric Food Chem, 2004; 52:1928.
691 [86] Megias C, Pedroche J, Yust MDM, Alaiz M, Giron-Calle J, Millan F, Vioque J. Affinity
692 purification of angiotensin converting enzyme inhibitory peptides using immobilized ACE: J
693 Agric Food Chem, 2006; 254:7120.

694 [87] Chen GW, Tsai JS, Pan, BS. Purification of angiotensin I-converting enzyme inhibitory
695 peptides and antihypertensive effect of milk produced by protease-facilitated lactic fermentation:
696 Int Dairy J, 2007; 17:641.
697 [88] Wang JP, Hu JE, Cui JZ, Bai XF, Du YG, Miyaguchi Y, Lin BC. Purification and
698 identification of an ACE inhibitory peptide from oyster proteins hydrolysate and the anti
699 hypertensive effect of hydrolysate in spontaneously hypertensive rats: Food Chem, 2008;
700 111:302.
701 [89] Jeong DW, Shin DS, Ahn CW, Song IS, Lee HJ. Expression of antihypertensive peptide,
702 His-His-Leu, as tandem repeats in *Escherichia coli*: J Microbiol Biotechnol, 2007; 17:952.
703 [90] Liu D, Sun HY, Zhang LJ, Li SM. High-level expression of milk-derived antihypertensive
704 peptide in *Escherichia coli* and its bioactivity: J Agric Food Chem, 2007; 55:5109.
705 [91] Lv GS, Huo GC, Fu XY. Expression of milk derived antihypertensive peptide in *Escherichia*
706 *coli*: J Dairy Sci, 2003; 86:1927.
707 [92] Park CJ, Lee JH, Hong SS, Lee HS, Kim SC. High-level expression of the angiotensin-
708 converting-enzyme inhibiting peptide, YG-1, as tandem multimers in *Escherichia coli*: Appl
709 Microbiol Biotechnol, 1998; 50:71.
710 [93] Rao S, Su Y, Li J, Xu Z, Yang Y. Design and expresión of recombinant antihypertensive
711 peptide multimer gene in *Escherichia coli* BL21: J Microb Biotechnol, 2009; 19:1620.
712 [94] Cushman DW, Cheung HS. Spectrophotometric assay and properties of the angiotensin
713 converting enzyme of rabbit lung: Biochem Pharmacol, 1971; 20:1637.
714 [95] Vermeirssen V, Van Camp J, Verstraete W. Optimisation and validation of an angiotensin-
715 converting enzyme inhibition assay for the screening of bioactive peptides: J Biochem Biophys
716 Methods, 2002; 51:75.

717 [96] Doig MT, Smiley JW. Direct injection assay of angiotensin-converting enzyme by high-
718 performance liquid-chromatography using a shielded hydrophobic phase column: J Chrom-
719 Biomed Appl, 1993; 613:145.
720 [97] Mehanna AS, Dowling M. Liquid chromatographic determination of hippuric acid for the
721 evaluation of ethacrynic acid as angiotensin converting enzyme inhibitor: J Pharm Biomed Anal,
722 1999; 19:967.
723 [98] van Elswijk DA, Diefenbach O, van der Berg S, Irth H, Tjaden UR, Van Der Greef J. Rapad
724 detection and identification of angiotensin-converting enzyme inhibitors by on-line liquid
725 chromatography-biochemical detection, couple to electrospray mass spectrometry: J Chromat A,
726 2003; 1020:45.
727 [99] Li GH, Liu H, Shi Y-H, Le GW. Direct spectrophotoetric measurement of angiotensin I-
728 converting enzyme inhibitory activity for screening bioactive peptides: J Pharm Biom Anal,
729 2005; 37:219.
730 [100] Shalaby SM, Zakora M, Otte J. Performance of two commonly used angiotensin-
731 converting enzyme inhibition assays using synthetic peptide substrates: J Dairy Res, 2006;
732 73:178.
733 [101] Siemerink M, Schebb NH, Liesener A, Perchuc A-M, Schoni R, Wilmer M, Hayen H,
734 Karst U, Vogel M. Development of a fast liquid chromatography/mass spectrometry screening
735 method for angiotensin converting enzyme (ACE) inhibitors in complex natural mixtures like
736 snake venom: Rapid Commun Mass Spectrom, 2010; 24:687.
737 [102] Geng F, He Y, Yang L, Wang Z. A rapid assay for angiotensin-converting enzyme activity
738 using ultra-performance liquid chromatography-mass spectrometry: Biomed Chromatogr, 2010;
739 24:312.

740 [103] Nakano D, Ogura K, Miyakoshi M, Ishii F, Kawanishi H, Kurumazuka D, Kwak CJ,
741 Ikemura K, Takaoka M, Moriguchi S, Jino T, Kusumoto A, Asami S, Shibata H, Kiso Y,
742 Matsumura Y. Antihypertensive effect of angiotensin I-converting enzyme inhibitory peptides
743 from a sesame protein hydrolysate in spontaneously hypertensive rats: Biosci Biotechnol
744 Biochem, 2006; 70:1118.
745 [104] Hernandez-Ledesma B, Miguel M, Amigo L, Aleixandre MA, Recio I. Effect of simulated
746 gastrointestinal digestión on the antihypertensive properties of synthetic beta-lactoglobulin
747 peptide sequences: J Dairy Res, 2007; 74:336.
748 [105] Miguel M, Manso M, Aleixandre A, Alonso MJ, Salaices M, Lopez-Fandino R. Vascular
749 effects, angiotensin I-converting enzyme (ACE)-inhibitory activity, and antihypertensive
750 properties of peptides derived from egg white: J Agric Food Chem, 2007; 55:10615.
751 [106] Takai-Doi S, Hashimoto K, Yamamura M, Kamei C. Antihypertensive activities of royal
752 jelly protein hydrolysate and its fractions in spontaneously hypertensive rats: Acta Med Okayama,
753 2009; 63:57.
754 [107] Kawasaki T, Seki E, Osajima K, Yoshida M, Asada K, Matsui T, Osajima Y.
755 Antihypertensive effect of Valyl-Tyrosine, a short chain peptides derived from sardine mucle
756 hydrolyzate, on mild hypertensive subjects: J Human Hypert, 2000; 14:519.
757 [108] Matsui T, Tamaya K, Seki E, Osajima K, Matsumoto K, Kawasaki T. Absorption of Val-
758 Tyr with *in vitro* Angiotensin I-converting enzyme inhibitory activity into the circulating blood
759 system of mild hypertensive subjects: Biol Pharm Bull, 2002; 25:1228.
760 [109] Meisel H. Biochemical properties of regulatory peptides derived from milk proteins:
761 Biopolymers, 1997; 43:119.

762 [110] Pripp AH, Isaksson T, Stepaniak L, Sorhaug T. Quantitative structure-activity relationship
763 modelling of ACE-inhibitory peptides derived from milk proteins: Eur Food Res Tech, 2004;
764 219:579.
765 [111] Natesh R, Schwager SLU, Sturrock ED, Acharya KR. Crystal structure of the human
766 angiotensin-converting enzyme-lisinopril complex: Nature 2003; 421:551.
767 [112] Yamamoto N, Akino A, Takano T. Purification and characterization of an antihypertensive
768 peptide from yoghurt-like produce fermented by *Lactobacillus helveticus* CP790: J Dairy Sci,
769 1994; 77:917.
770 [113] Robert MC, Razamane A, Mutter M, Juillerat MA. Peptides derived from sodium caseinate
771 Hydrolysates produced *by Lactobacillus helveticus* NCC 2765: J Agric Food Chem, 2004;
772 52:6923.
773 [114] Ondetti MA, Rubin B, Cushman DW. Design of specific inhibitors of angiotensin-
774 converting enzyme : new class of orally active antihypertensive agents: Science, 1977; 196:441.
775 [115] Cheung H-S, Wang F-L, Ondetti MA, Sabo EF, Cushman DW. Binding of peptide
776 substrates and inhibitors of angiotensin-converting enzyme: importance of the COOH-terminal
777 dipeptide sequence: J Biol Chem, 1980; 255:401.
778 [116] Ariyoshi Y. Angiotensin-converting enzyme inhibitors derived from food proteins: Trends
779 Food Sci Technol, 1993; 4:139.
780 [117] Meisel H. Casokinins as inhibitors of angiotensin-converting enzyme. In Sawatzki G,
781 Renner B (editors), New perspectives in Infant Nutrition, Thieme; 2003;153.
782 [118] Fitzgerald RJ, Meisel H. Milk protein derived inhibitors of angiotensin-I-converting
783 enzyme: Br J Nutr, 2000; 84:S33.

[119] Gomez-Ruiz JA, Recio I, Belloque J. ACE-inhibitory activity and structural properties of peptide Asp-Lys-Ile-His-Pro [beta-CN f(47-51)]. Study of the peptide forms synthesized by different methods: J Agric Food Chem, 2004; 52:6315.

[120] Vermeirssen V, van der Bent A, Van Camp J, van Amerongen A, Verstraete W. A quantitative in silico analysis calculates the angiotensin I converting enzyme (ACE) inhibitory activity in pea and whey protein digests: Biochimie, 2004; 86:231.

[121] Nakamura Y, Masuda O, Takano T. Decrease of tissue angiotensin I-converting enzyme activity upon feeding sour milk in spontaneously hypertensive rats: Biosci Biotechnol Biochem, 1996; 60:488.

[122] Jakala P, Pere E, Lehtinen R, Turpeinen A, Korpela R, Vapaatalo H. Cardiovascular activity of milk casein-derived tripeptides and plant sterols in spontaneously hypertensive rats: J Physiol Pharmacol, 2009; 60:11.

[123] Jakala P, Hakala A, Turpeinen A, Korpela R, Vapaatalo H. Casein-derived bioactive tripeptides Ile-Pro-Pro and Val-Pro-Pro attenuate the development of hypertension and improve endothelial function in salt-loades Goto-Kakizaki rats: J Functional Foods, 2009; 1:366.

[124] Jakala P, Jauhiainen T, Korpela R, Vapaatalo H. Milk protein-derived bioactive tripeptides Ile-Pro-Pro and Val-Pro-Pro protect endothelial function *in vitro* in hypertensive rats: J Functional Foods, 2009; 1:266.

[125] Yamaguchi N, Kawaguchi K, Yamamoto N. Study of the mechanism of antihypertensive peptide VPP and IPP in spontaneously hypertensive rats by DNA microarray analysis: Eur J Pharmacol, 2009; 620:71.

[126] De Gennaro CV, Rossoni G, Rigamonti A, Bonomo S, Manfredi B, Berti F, Muller E. Enalapril and quinapril improve endotelial vasodilator function and aortic eNOS gene expresión in L-NAME-treated rats: Eur J Pharmacol, 2002; 450:61.

808 [127] Rummery NM, Grayson TH, Hill CE. Angiotensin-converting enzyme inhibition restores
809 endothelial but not medial connexion expression in hypertensive rats: J Hypertens, 2005; 23:317.
810 [128] Kuono K, Hirano S, Kuboki H, Kasai M, Hatae K. Effects of dried bonito (*Katsuobushi*)
811 and captopril, and angiotensin I-converting enzyme inhibitor, on rat isolated aorta: a possible
812 mechanism of antihypertensive action: Biosci Biotechnol Biochem, 2005; 69:911.
813 [129] Maes W, Van Camp J, Vermeirssen V, Hemeryck M, Ketelslegers JM, Schrezenmeir J,
814 Van Oostveldt P, Huyghebaert A. Influence of the lactokinin Ala-Leu-Pro-Met-His-Ile-Arg
815 (ALPMHIR) on the release of endothelin-1 by endothelial cells: Regul Pept, 2004; 118:105.
816 [130] Ijäs H, Collin M, Finckenberg P, Pihlanto-Leppala A, Korhonen H, Korpela R, Vapaatalo
817 H, Nurminen ML. Antihypertensive opioid-like milk peptide alpha-lactorphin: lack of effect on
818 behavioural tests in mice: Int Dairy J, 2004; 14:201.
819 [131] Yamada Y, Matoba N, Usui H, Onishi K, Yoshikawa M. Design of a highly potent anti-
820 hypertensive peptides base don ovokinin(2-7): Biosci Biotechnol Biochem, 2002; 66:1213.
821 [132] Touyz RM. Reactive oxygen species, vascular oxidative stress and redox signalling in
822 hypertension: what is the clinical significance? Hypertension, 2004; 44:248.
823 [133] Rival SG, Boeriu CG, Wichers HJ. Caseins and casein hydrolyzates. 2. Antioxidant
824 properties and relevance to lipoxygenase inhibition: J Agric Food Chem, 2001; 49:295.
825 [134] Hernandez-Ledesma B, Davalos B, Bartolome B, Amigo L. Preparation of enzymatic
826 hydrolysates from α-lactalbumin and β-lactoglobulin. Identification of active peptides by HPLC-
827 MS/MS: J Agric Food Chem, 2005; 53:588.
828 [135] Ohsawa K, Satsu H, Ohki K, Enjoh M, Takano T, Shimizu M. Producibility and
829 digestibility of antihypertensive b-casein tripeptides, Val-Pro-Pro and Ile-Pro-Pro, in the

gastrointestinal tract: Analyses using an in vitro model of mammalian gastrointestinal digestion. J Agric Food Chem, 2008; 56:854.

[136] Foltz M, Cerstiaens A, van Meensel A, Mols R, van der Pijl PC, Duchateau GSMJE, Augustijns P. The angiotensin converting enzyme inhibitory tripeptides Ile-Pro-Pro and Val-Pro-Pro show increading permeabilities with increasing physiological relevance of absorption models: Peptides, 2008; 12:1312.

[137] Quirós A, Dávalos A, Lasunción MA, Ramos M, Recio I. Bioavalilability of the antihypertensive peptide LHLPLP: Transepithelial flux of HLPLP: Int Dairy J, 2008; 18:279.

[138] Quirós A, Contreras MM, Ramos M, Amigo L, Recio I. Stability to gastrointestinal enzymes and structure-activity relationship of b-casein peptides with antihypertensive properties: Peptides, 2009; 30:1848.

[139] van Platerink C, Janssen H-GM, Horsten R, Haverkamp J. Quantification of ACE inhibiting peptides in human plasma using high performance liquid chromatography-mass spectrometry: J Chromatogr B, 2006; 830:151.

[140] Maeno M, Yamamoto N, Takano T. Identification of an antihypertensive peptide from casein hydrolysate produced by a proteinase from *Lactobacillus helveticus* CP790: J Dairy Sci, 1996; 79:1316.

[141] van der Pijl PC, Kies AK, Ten Have GAM, Duchateau GSMJE, Deutz NEP. Pharmacokinetics of proline-rich tripeptides in the pig: Peptides, 2008; 29:2196.

[142] Foltz M, Meynen EM, Bianco V, van Platerink C, Koning TMMG, Kloek J. Angiotensin converting enzyme inhibitory peptides from a lactotripeptide-enriched milk beverage are absorbed intact into the circulation: J Nutr, 2007; 137:953.

[143] Xu J-Y, Qin L-Q, Wang P-Y, Li W, Chang C. Effect of milk tripeptides on blood pressure: A meta-analysis of randomized controlled trials: Nutrition, 2008; 28:933.

854 [144] Pripp AH. Effect of peptides derived from food proteins on blood pressure: a meta-analysis
855 of randomized controlled trials: Food Nutr Res, 2008; 52:doi:10.3402/fnr.v52i0.1641
856 [145] Engberink MF, Schouten EG, Kok FJ, van Mierlo LAJ, Brouwer IA, Geleijnse JM.
857 Lactotripeptides show no effect on human blood pressure. Results from a double-blind
858 randomized controlled trial: Hypertension, 2008; 51:399
859 [146] van Mierlo LAJ, Koning MMG, van der Zander K, Draijer R. Lactotripeptides do not lower
860 blood pressure in untreated whites: Results from 2 controlled multicenter crossover studies: Am J
861 Clin Nutr, 2009; 89:617.
862 [147] Boelsma E, Kloek J. Lactotripeptides and antihypertensive effects: a critical review: Br J
863 Nutr, 2009; 101:776.
864 [148] Jakala P, Vapaatalo H. Antihypertensive peptides from milk proteins: Pharmaceuticals,
865 2010; 3:251.
866 [149] Saha BC, Hayashi K. Debittering of protein hydrolysates: Biotechnol Adv, 2001; 19:355.
867 [150] FitzGerald RJ, O´Cuinn GO. Enzymatic debittering of food protein hydrolysates:
868 Biotechnol Adv, 2006; 24:234
869 [151] Wu J, Aluko RE. Quantitative structure-activity relationship study of bitter di-and tri-
870 peptides including relationship with angiotensin I-converting enzyme inhibitory activity: J Pept
871 Sci, 2007; 13:63
872 [152] Matsuura Y, Yugeta R, Noda K. Bitter-masking effect of glutamylglutamic acid isomers:
873 Seikatsu Kagaku, 1980; 12:199
874 [153] Tamura M, Mori N, Miyoshi T, Koyama S, Kohri H, Okai H. Studies on flavoured peptides.
875 7. Practical debittering using model peptides and related-compounds: Agric Biol Chem, 1990;
876 54:41.

877 [154] Favaro-Trindade CS, Santana AS, Monterrey-Quintero ES, Trindade MA, Netto FM. The
878 use of spray drying technology to reduce bitter taste of casein hydrolysate: Food Hydrocolloids,
879 2010; 24:336.
880 [155] Sugira Y. Control of bitter taste by acid phopholipds in health food: Japanese Patent App,
881 08173093, 1996.
882 [156] Molina Ortiz SE, Mauri A, Monterrey-Quintero ES, Trindade MA, Santana AS, Favaro-
883 Trindade CS. Production and properties of casein hydrolysate microencapsulated by spray drying
884 with soybean protein isolate: LWT Food Sci Technol, 2009; 42:919.
885 [157] Rocha GA, Trindade MA, Netto FM, Favaro-Trindade CS. Microcapsules of a casein
886 hydrolysate: Production, characterization, and application in protein bars: Food Sci Tech Int,
887 2009; 15:407.
888 [158] Barbosa CMS, Morais HA, Delvivo FM, Mansur HS, De Oliveira MC, Silvestre MPC.
889 Papain hydrolysates of casein: molecular weight profile and encapsulation in liposphere: J Sci
890 Food Agric, 2004; 84:1891.
891 [159] Ogasawara M, Katsumata T, Egi M. Taste properties of Maillard-reaction products
892 prepared from 1000 to 5000 Da peptide: Food Chem, 2006; 99:600.
893 [160] Van Boeckel MAJS, Weerens CNJM, Holstra A, Scheidtweiler CE, Alink GM.
894 Antimutagenic effects of casein and its digestion products: Food Chem Toxicol, 1993; 31:731
895 [161] Elias RJ, Kellerby SS, Decker EA. Antioxidant activity of proteins and peptides: Crit Rev
896 Food Sci Nutr, 2008; 48:430.
897 [162] Abdul-Hamid A, Bakar J, Bee GH. Nutritional quality of spray dried protein hydrolysate
898 from Black Tilapia (*Oreochromis mossambicus*): Food Chem, 2002; 78:69

[163] Bueno-Solano C, López-Cervantes J, Campas-Baypoli ON, Lauterio-García R, Adan-Bante NP, Sánchez-Machado DI. Chemical and biological characteristics of protein hydrolysates from fermented shrimp by-products: Food Chem, 2009; 112:671.

[164] He HL, Wu H, Chen XL, Shi M, Zhang XY, Sun CY, Zhang Y-Z, Zhou B-C. Pilot and plant scaled production of ACE inhibitory hydrolysates from *Acetes chinensis* and its in vivo antihypertensive effect: Bioresource Technol, 2008; 99:5956.

[165] Contreras MM, Sevilla MA, Monroy-Ruiz J, Amigo L, Gómez-Sala B, Ramos M, Recio I. Characterization of an antihypertensive hydrolysate and resistance of active peptides to drying and during shelf-life: Int Dairy J, 2010 (sent for publication)

[166] Paul M, Somkuti GA. Degradation of milk-based bioactive peptides by yogurt fermentation bacteria: Lett Appl Microbiol, 2009; 49:345.

[167] Karaki H, Doi K, Sugano S, Uchiwa H, Sugai R, Murakami U, Takemoto S. Antihypertensive effect of tryptic hydrolysate of milk casein in spontaneously hypertensive rats. Comp Biochem Physiol C-Pharmacol Toxicol Endocrinol, 1990; 96:367.

[168] Recio I, Quirós A, Hernández-Ledesma B, Gómez-Ruiz JA, Miguel M, Amigo L, López-Expósito I, Ramos M, Aleixandre MA. European Patent 200501373, 2005.

[169] Abubakar A, Saito T, Kitazawa H, Kawai Y, Itoh T. Structural analysis of new antihypertensive peptides derived from cheese whey protein by proteinase K digestion. J Dairy Sci, 1998; 81:3131.

[170] Nurminen M-L, Sipola M, Kaarto H, Pihlanto-Leppälä A, Piilola K, Korpela R, Tossavainen O, Korhonen H, Vapaatalo H. Alpha-lactorphin lowers blood pressure measured by radiotelemetry in normotensive and spontaneously hypertensive rats. Life Sci, 2000; 66:1535.

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 *Lb. helveticus* CPN4	[140]
β-CN f(58-76)	LVYPFPGPIPNSLPQ NIPP	5.2	-15.0	Fermentation with *En. faecalis*	[11, 57]
β-CN f(60-68)	YPFPGPIPN	14.8	-7.0	Gouda cheese	[58]
β-CN f(74-76)	IPP	5.0	-28.3 (-10.1)[d]	Fermentation with *Lb. helveticus* y *Sc. cerevisiae*	[40, 41]
β-CN f(84-86)	VPP	9.0	-32.1 (-10.1)[d]	Fermentation with *Lb. helveticus* y *Sc. cerevisiae*	[40, 41]
β-CN f(133-138)	LHLPLP	5.5	-21.9	Fermentation with *En. faecalis*	[11, 57]
β-CN f(133-139)	LHLPLPL	425	-7.7	Fermentation with *En. faecalis*	[11, 57]
β-CN f(197-206)	VLGPVRGPFP	137	-16.2	Fermentation with *En. faecalis*	[11, 57]
β-CN f(201-209)	VRGPFPIIV	599	-16.1	Fermentation with *En. faecalis*	[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 *Lb. helveticus* 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 *Lb. helveticus* CP790	[140]
α_{s2}-CN f(190-197)	MKPWIQPK	300	-3.0	Hydrolysis with a proteinase from *Lb. helveticus* CP790	[140]
α_{s2}-CN f(198-202)	TKVIP	400	-9.0	Hydrolysis with a proteinase from *Lb. helveticus* 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)	VYPFPG	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 *Lb. helveticus* CP790	[140]
β-CN f(169-174)	KVLPVP	5	-32.2	Hydrolysis with a proteinase from *Lb. helveticus* CP790	[140]
β-CN f(169-175)	KVLPVPQ	1000	-31.5	Hydrolysis with a proteinase from *Lb. helveticus* CP790	[140]

Table 1. (Continuation). 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	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)[e]	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]

[a]: Peptide concentration needed to inhibit 50% ACE activity
[b]: Systolic blood pressure
[c]: Antihypertensive effects in humans
[d]: Ovine protein
[e]: Caprine protein