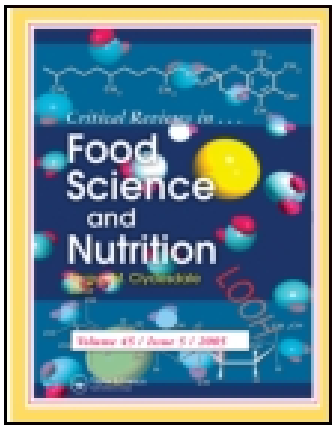


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# Isolation and Characterization of Peptides with Antihypertensive Activity in Foodstuffs

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REVIEW

# Isolation and Characterization of Peptides with Antihypertensive Activity in Foodstuffs

**PATRYCJA PUCHALSKA, M. LUISA MARINA ALEGRE, and M. CONCEPCIÓN GARCÍA LÓPEZ**

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*Hypertension is one of the main causes of cardiovascular diseases. Synthetic drugs inhibiting ACE activity present high effectiveness in the treatment of hypertension but cause undesirable side effects. Unlike these synthetic drugs, antihypertensive peptides do not show any adverse effect. These peptides are naturally present in some foods and since hypertension is closely related to modern diet habits, the interest for this kind of foods is increasing. Different methods for the purification, isolation, and characterization of antihypertensive peptides in foods have been developed. Nevertheless, there is no revision work summarizing and comparing these strategies. In this review, in vivo and in vitro pathways to obtain antihypertensive peptides have been summarized. The ACE mechanism and the methodologies developed to assay the ACE inhibitory activity have also been described. Moreover, a comprehensive overview on the isolation, purification, and identification techniques focusing on the discovery of new antihypertensive peptides with high activity has been included. Finally, it is worthy to highlight that the quantitation of antihypertensive peptides in foods is a new trend since genotype and processing conditions could affect their presence. Analytical methodologies using mass spectrometry constitute an interesting option for this purpose.*

**Keywords** ACE, chromatography, bioactivity, peptide, protein, enzyme

## *INTRODUCTION*

Traditionally, the dietetic value of a protein was evaluated from its nutritional quality, mainly by the presence of antinutrients and availability of essential amino acids. A new aspect to take into account is the possibility of generating bioactive peptides. Bioactive dietary components are defined as "*food components that can affect biological processes or substrates and hence have an impact on body function or condition and ultimately health*" (Schrezenmeir et al., 2000). Since any consumed dietary component in enough quantity could be described by this definition, two caveats should be added: the component should impart a measurable effect at a realistic physiological level and the measured "bioactivity" should show a potential beneficial health effect (Schrezenmeir et al., 2000; Moller et al., 2008). Bioactive peptides can be naturally presented in foods but the most usual scenario is that they are encrypted in parent proteins (Korhonen and Pihlanto, 2003; Wang and de Mejia, 2005; Iwaniak and Minkiewicz, 2008; Moller et al., 2008).

Several bioactive peptides from different origins such as milk (Zimecki and Kruzel, 2007; Hernández-Ledesma et al., 2008; Madureira et al., 2010) and soybean (Wang and de Mejia, 2005) have been released, isolated, characterized, and briefly reviewed. Moreover, peptides showing numerous bioactivities such as antihypertensive, antilipemic, anticarcinogenic, antioxidative, antimicrobial, antiamnestic, opiate, antithrombotic, osteoprotective, vasodilatative, immunomodulating were thoroughly described (Kitts and Weiler, 2003; Korhonen and Pihlanto, 2003; Wang and de Mejia, 2005; Iwaniak and Minkiewicz, 2008). According to the BIOPEP database, 37 different types of bioactivities have been gathered for more than 1950 peptides (Minkiewicz et al., 2008). Among them, peptides with antihypertensive activity are the most prevalent.

Hypertension or high blood pressure is attributed by World Health Organization (WHO) as the fundamental source of

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cardiovascular mortality. Worldwide high blood pressure was estimated to be the cause of 7.6 million premature deaths (13.5% of the total premature deaths) (Lawes et al., 2008). Additionally, hypertension along with other cardiovascular risk factors (high cholesterol, high BMI (Body Mass Index), low fruit and vegetable intake, smoking, and alcohol intake) were established to be the cause of about 83–89% ischaemic heart disease cases and 70–76% of strokes in the world (Ezzati et al., 2003). Furthermore, hypertension can lead to cardiac arrhythmia, coronary heart disease, heart and renal failure, disability and death (Murray and FitzGerald, 2007) and, in accordance to the World Hypertension League (WHL), over 50% of the hypertension population is unaware of their condition (Chockalingam, 2008). Hypertension can be treated with distinct medications such as nitrates, diuretics, $\beta$-blockers, $\alpha$-adrenergic antagonist, vasodilators, dopamine agonists, calcium channel blockers (CCBs), and angiotensin converting enzyme (ACE) inhibitors (Perez and Musini, 2008). Among them, ACE inhibitors are mostly employed for showing greater effectiveness and lower side effects (Souza et al., 2009).

First ACE inhibitor was described by Ferreira et al. (1970). It was a bradykinin potentiator and was isolated from snake (*Bothrops jararaca*) venom (Ferreira et al., 1970). The first synthetic ACE inhibitor adopted for hypertension therapy was [2S]-1-[3-mercapto-2-methylpropionyl]-L-proline (captopril) (Cushman and Ondetti, 1991). Afterwards, several other synthetic ACE inhibitors were employed for treatment of hypertension (enalapril, lisinopril, alecepril or fosinopril) although they provoke adverse effects such as skin rashes, cough, angioedema, taste disturbances, hypotension, reduced renal function, increased potassium levels, and fetal abnormalities (Atkinson and Robertson, 1979; FitzGerald et al., 2004). Unlike these drugs, antihypertensive peptides do not yield any adverse effect but are usually less potent in comparison to synthetic substances (Lee et al., 2010). Indeed, seven dipeptides isolated from garlic showed decreasing systolic blood pressure (SBP) after oral administration of 200 mg/kg in spontaneously hypertensive rats (SHRs). However, none of these peptides lowered SBP as much as captopril that was used as a positive control test (Suetsuna, 1998). There are some exceptions to this fact such as a peptide isolated from tuna frame protein peptic hydrolysate and the milk peptides VPP and IPP that exert antihypertensive effects comparable with captopril (Pina and Roque, 2009; Lee et al. 2010).

Since hypertension is closely related to modern diet habits, interest in functional foods with antihypertensive activity is worth great consideration. Therefore, the aim of this work has been to review the methodologies used to isolate, purify, identify, and characterize food peptides with antihypertensive activity.

## *ACE AND BLOOD PRESSURE (BP)*

Several interacting biochemical pathways are associated with the control of blood pressure (BP) in living organisms, the *renin-angiotensin* system being the most important. Additionally, kinin-nitric oxide system, endothelin converting enzyme system, and neutral endopeptidase system are also recognized to have influence on BP.

*Renin-angiotensin system* is shown in Figure. 1. Angiotensinogen is the first link of the reaction chain in the renin-angiotensin system. It is the precursor of Angiotensin I (Ang I- DRVYIHPFHL). In fact, it converts to Ang I in the presence of renin (E.C. 3.4.23.15) in the bloodstream. On the other hand, Ang I hydrolyzes by the removal of the C-terminal dipeptide HL to Angiotensin II (Ang II- DRVYIHPF) through the action of angiotensin I converting enzyme (ACE; kinases II peptidyldipeptide hydrolase). Afterwards, Ang II is distributed in the blood until it is inactivated by aminopeptidase A (E.C. 3.4.11.7) or N (E.C. 3.4.11.2) enzymes and it is converted to Angiotensin III and IV (RVYIHPF and VYIHPF, respectively). Ang II peptide causes vasoconstriction by the activation of the AT1 receptor (AT1R) which leads to increase in the BP. Furthermore, Ang II negatively affects kidney retaining salts and water, causing an increase in extracellular fluid volume and, in a consequence, an increase in the BP (FitzGerald et al., 2004; Schmieder et al., 2007; Chen et al., 2009).

Moreover, ACE also removes a dipeptide from C-terminus of bradykinin (RPPGFSPFR) resulting in the inactivation of this vasodilator. Therefore, ACE inhibitors decrease the BP not only by lowering the level of Ang II but also by increasing the level of bradykinin. Since the inhibition of ACE causes an

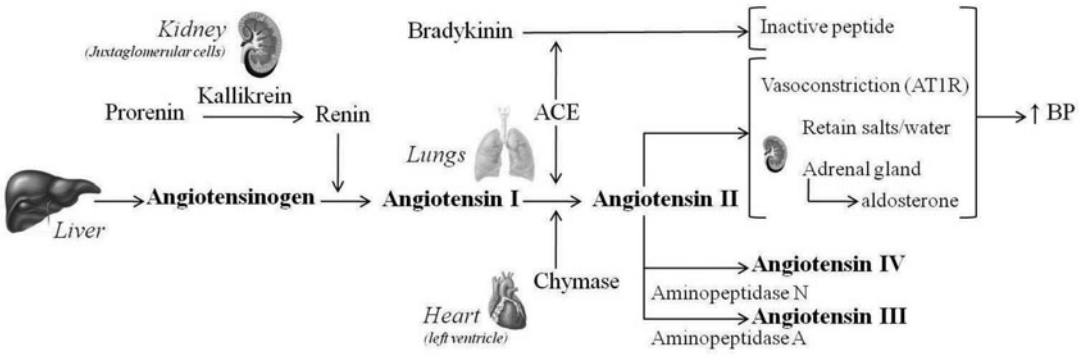


**Figure 1** Scheme of renin-angiotensin system.

effective decrease in BP, most antihypertensive drugs employ this mechanism for the treatment of hypertension.

## *RELEASE OF ANTIHYPERTENSIVE PEPTIDES FROM FOODSTUFFS*

Antihypertensive peptides used to be encrypted in a parent protein from which they need to be released in order to exert its ability to inhibit ACE. Two main proteolytic pathways can be distinguished, in vivo and in vitro. The first one involves the in vivo digestion of the parent protein by the action of gastrointestinal enzymes while the second one involves food or protein processing before its ingestion. Moreover, some bioactive peptides cannot be liberated by gastrointestinal enzymes and so have to be synthetically produced, added to foods, and supplied as functional foods (Meisel, 1997).

The first antihypertensive peptide isolated and identified from food was described in 1982 by Maruyama and Suzuki (1982). Casein from bovine milk was subjected to hydrolysis by trypsin and purified by several chromatographic steps. A peptide with 12 amino acids and sequence FFVAPFPEVFGK was identified. The knowledge about preparation, purification, and identification of antihypertensive peptides from food steadily increased since this first discovery, especially in the case of milk-derived peptides (Yamamoto and Takano, 1999; Lopez-Fandiño et al., 2006; Ricci et al., 2010). In this case, advances have even enabled the development of commercial milk products enriched with antihypertensive peptides (Table 1) (Sirtori et al., 2009; Ricci et al., 2010).

In addition to milk and dairy products, several other foodstuffs have been examined as potential sources of peptides with ACE inhibition activity. Marine foods (shrimps, sea cucumber, blue mussel), fishes (alaska pollock, bonito, salmon, pacific hake), meat (pork, bullfrog, chicken), vegetable foods (soybean, wheat products, rice, garlic, aramanth grain), mushrooms or processed products (miso paste, douche, wakame, royal jelly, soy sauce or paste) are some examples. The most common way to induce the in vitro release of antihypertensive peptides is enzymatic digestion (hydrolysis) and fermentation with bacterial organisms.

With regard to enzymatic digestion, the composition of hydrolysate depends on several parameters such as the enzyme to substrate ratio, hydrolysis time, pH, temperature of hydrolysis, etc. but it mostly depends on the type of proteolytic enzyme. Most commonly used enzymes are pepsin (Qian et al., 2007a; Lee et al., 2010), thermolysin (Yokoyama et al., 1991; Arihara et al., 2001), and alcalase (Chiang et al., 2006; Qian, 2007b; Yang et al., 2007). They cleave peptide bonds near to hydrophobic amino acid residues, resulting in peptides with the most favorable amino acid residues for antihypertensive activity at the C-terminal position (Otte et al., 2007a, Qian et al., 2007b).

Different strategies have been followed to increase antihypertensive activity. Pepsin treatment followed by digestion with pancreatin (Yang et al., 2003; Majumder and Wu, 2009; Escudero et al., 2010), corolase PP (Gómez-Ruiz et al., 2007) or trypsin (E.C. 3.4.21.4) with chymotrypsin (E.C. 3.4.21.1) (Li et al., 2002; Matsui et al., 2002; Gómez-Ruiz et al., 2007) has usually been employed to obtain smaller peptides with greater antihypertensive effects. Moreover, since these enzymes are present during gastrointestinal digestion, it is possible to assess whether these peptides will be inactivated during this process or not. Quiros et al. (2007a) attempted to promote the release of bioactive peptides from ovalbumin with chymotrypsin, trypsin, and pepsin using high hydrostatic pressures observing that antihypertensive effect of certain peptides improved when pressures of 200–400 MPa were employed. Another strategy for increasing antihypertensive activity was explored by Jia et al. (2010). They evaluated the effect of ultrasonic irradiation on the hydrolysis and the ACE inhibitory activity of defatted wheat germ protein (DWGP). Results suggested that this approach improved enzymatic hydrolysis by promoting the release of peptides. Moreover, some authors have demonstrated an increase in the antihypertensive activity of foodstuffs by the combination of bacterial fermentation and enzymatic digestion. Tonouchi et al. (2008) observed these results when using different enzymes to digest a Danish skim milk-cheese previously fermented with *Lactococcus*. Similarly, Hernández-Ledesma et al. (2004) found a higher number of antihypertensive peptides when a milk sample fermented with *Lactobacillus rhamnosus* was subjected to simulated gastrointestinal digestion. Chobert et al. (2005) compared the antihypertensive activity of peptides obtained from ovine milk by tryptic digestion and fermentation with different bacterial strains. Fermentation yielded higher ACE inhibitory activity than digestion, probably because fermentation yielded peptides with lower molecular masses (Chobert et al., 2005).

On some occasions, the foodstuffs contain antihypertensive peptides that are not encrypted in any protein and fermentation or digestion is not necessary. For example, few peptides, which exerted antihypertensive activity, were detected in garlic (*Allium sativum* L) (Suetsuna, 1998), in various kind of mushrooms (*Pholiota adiposa, Tricholoma giganteum*) (Hyoung et al., 2004; Koo et al., 2006), in soypaste (Shin et al., 2001), and in different kinds of cheeses (gouda, manchego, and varieties of Spanish and Swiss cheeses) (Saito et al., 2000; Gómez-Ruiz et al., 2002; Gómez-Ruiz et al., 2006; Meyer et al., 2009). These peptides can simply be extracted with water or alcohols like ethanol or methanol. With this in mind, it is possible to differentiate between processed and unprocessed products.

**Table 1** Commercially available milk products enriched with antihypertensive peptides (Sirtori et al., 2009; Ricci et al., 2010)

| Brand name | Company, country | Bioactive peptide |
|---|---|---|
| Ameal S ® | Calpis Co., Japan | VPP, IPP |
| BioZate ® | Davisco, USA | — |
| Calpis ® | Calpis Co., Japan | VPP, IPP |
| Casein DP ® | Kanebo Ltd., Japan | FFVAPFEVFGK |
| C12 peptide ® | DMV International, Holland | FFVAPFEVFGK |
| Danten ® | Danone, France | — |
| Evolus ® | Valio, Finland | VPP, IPP |

Unprocessed products are garlic or mushrooms while processed products comprised soypaste and cheese. The manufacture of these products involves the use of enzymes or bacterial organisms but, in no case, they are not added to release antihypertensive peptides.

The other way to release peptides without the addition of bacterial organisms or enzymes is autolyzation. Autolyzation involves the employment of proteolytic enzymes, which are already ingredients of foodstuffs. This approach was followed for the preparation of hydrolysates of bonito bowels (Fujii et al., 1993; Matsumura et al., 1993), pacific hake fish (Samaranayaka et al., 2010) or wheat bran (Nogata et al., 2009). A similar approach was also used when proteins of oyster and blue mussel were fermented without any addition of bacterial organism for six months at 20°C in salty conditions. In both cases, antihypertensive peptides were obtained after long-term fermentations (Je et al., 2005a; Je et al., 2005b).

## *DETERMINATION OF ACE INHIBITORY ACTIVITY OF FOOD PEPTIDES*

The general framework of the experimental investigation for the production, purification, and identification of antihypertensive peptides is presented in Figure 2. Work strategies commonly consist of the release of peptides, and the isolation, purification, identification, and determination of the amino acid sequence. After each step, the screening of ACE inhibitory activity is crucial to select the experimental conditions or fractions with the most potential antihypertensive abilities.

ACE inhibition activity is expressed using an $IC_{50}$ index, which represents the required concentration of a particular substance in order to inhibit 50% of the ACE activity. Different assays have been developed to determine the ACE inhibition value in vitro. Initially, the assays were based on the employment of Ang I or bradykinin as an ACE substrate and measurement of the generated product was done by radiochromatography, colorimetry or radioimmunoassays in the presence or absence of the inhibitor. Nevertheless, the problem related to the interferences produced by other peptidases, which were degradating substrates or products of ACE, led to erroneous results. Then, assays using artificial substrate started to play a considerable role since they were inexpensive, easy to obtain, not liable to be hydrolyzed by peptidases, and presented a higher dissociation constant for ACE (Meng and Oparil, 1996). Released compounds by the action of ACE could be quantified through a spectrophotometric (Holmquist et al., 1979), fluorometric (Alves et al., 2005), HPLC (Wu et al., 2002), CE (Van Dyck et al., 2003) or by a radiometric method (Rohzabach, 1978).

Nowadays, the most broadly spread method for the determination of ACE inhibition activity is that developed by Cushman and Cheung (1971). It is based on the reaction between hippuryl-L-histydyl-L-leucine (HHL) used as substrate and ACE and shows a subsequent formation of hippuric acid (HA). The ACE activity is directly related to the extent of HA liberated from

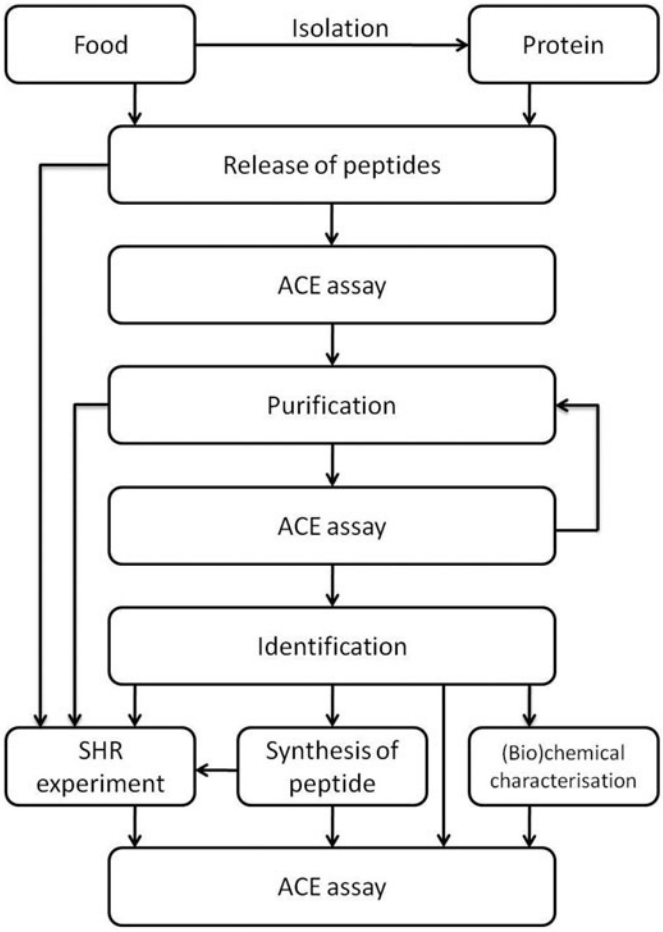


**Figure 2** Framework of production, purification, and identification of bioactive peptides with antihypertensive activity.

HHL. The extent of this reaction in the presence or absence of inhibitory peptides is evaluated by measuring the amount of formed HA from its absorbance at 228 nm. In this first approach ACE was acetone extracted from rabbit lung. Further modification of this procedure employed pure ACE from a rabbit's lung in place of their acetone extract (Nakamura et al., 1995; Vermeirssen et al., 2002). Despite the high selectivity of this reaction, the assay had low sensitivity since unhydrolyzed HHL was coextracted with HA (Meng and Oparil, 1996; Lopez-Fandiño et al., 2006). Furthermore, long incubation time (around 30 min) was required to obtain enough product amounts to be quantified. Therefore, numerous modifications appeared in the literature, and, as a consequence, the obtained $IC_{50}$ values differed significantly due to comparison amongst themselves not being possible (Kodera and Nio, 2006). In fact, the $IC_{50}$ of hydrolysates obtained by digestion of an insect protein with four different enzymes was determined by applying two different ACE assays. One method was based on the spectrophotometric measurement of FAPGG [2-furanacryloyl-phenylalanyl-glycyl-glycine], used as a substrate, while an HPLC method (which adopted DTG [dansyltriglycine] as a substrate) was employed in the second approach. When using the FAPGG method, $IC_{50}$

values were 3.935 ± 0.014 and 0.214 ± 0.179 mg/mL for the nonhydrolyzed and hydrolyzed extract, respectively. The HPLC method yielded $IC_{50}$ values of 22.465 ± 0.615 and 4.969 ± 0.622 mg/mL (with 50 $\mu$L of ACE extract) and 43.220 ± 12.66 and 1.253 ± 0.120 mg/mL (with 25 $\mu$L of ACE extract), respectively, for the nonhydrolyzed and hydrolyzed extract (Vercruysse et al., 2005).

As well as the in vitro determination of $IC_{50}$ values, additional experiments are very frequently included to confirm ACE inhibitory activity. Indeed, experiments using spontaneously hypertensive rats (SHR) have also been used for assaying ACE inhibition in living organisms. This type of experiment is usually focused on short and/or long-term administration studies. Nevertheless, results obtained by in vivo studies sometimes significantly differ from the results observed by in vitro assays. These differences could be justified by the bioavailability of peptides (Lopez-Fandiño et al., 2006; Ricci et al., 2010). As an example, a peptide ($\beta$-lactosin B, ALPM) derived from a commercial whey product that presented weak ACE inhibitory activity ($IC_{50} = 928$ $\mu$M), surprisingly showed a noticeable decrease of SBP after 8 h oral administration (2 mg/mL) to SHRs (Murakami et al., 2004). In other occasion, Fujita and Yoshikawa (1999) compared the ACE inhibitory activity of LKPNM and LKP peptides (obtained by digestion from dried bonito with thermolysin) with captopril using an in vitro and an in vivo study. The in vitro study yielded much lower ACE inhibitory activity for peptides while the in vivo study demonstrated that peptides were more effective for reducing BP than captopril (Fujita and Yoshikawa, 1999). The differences between the two approaches to assess ACE inhibitory activity also appeared when Yamamoto et al. (1999) purified and characterized a dipeptide from a yoghurt-like product. The $IC_{50}$ value of this dipeptide was estimated as 720 $\mu$M which would classify it as a peptide with moderate antihypertensive activity. However, the same peptide provoked similar in vivo antihypertensive activity to IPP and VPP, which are generally categorized as peptides with very high activity ($IC_{50}$ values, 5 and 9 $\mu$M, respectively) (Yamamoto et al., 1999).

In addition to the estimation of the $IC_{50}$ value, some authors also pay attention to additional measurements such as simulated gastrointestinal digestion or caco-2 cell monolayer transport. These experiments can yield information on the bioavailability of target compounds and will be discussed later. Additionally, the activity of peptides may be affected by factors such as the amino acid composition, hydrophobicity, size, stability, processing or the mechanism of action (Yamamoto et al., 2003). In fact, ACE inhibition activity is significantly influenced by the position of proline in the amino acid sequence, by protein hydrophobicity, and by the size of released peptides.

The following examples demonstrate this point. Different protein sources were hydrolyzed in the same conditions: soybean protein, wheat gluten, caseinate, and whey proteins. The $IC_{50}$ for these hydrolysates was 180, 340, 100, and 200 $\mu$g/mL, respectively. High antihypertensive activity of casein (100 $\mu$g/mL) could be attributed as much for their high hydrophobicity as for the high amount of encrypted prolines in its primary structure. Despite wheat gluten, also containing high amounts of proline residues but with lower hydrophobicity yielded a significantly high $IC_{50}$ value (340 $\mu$g/mL) (Kodera and Nio, 2006). In addition to the $IC_{50}$, another important parameter to take into account is the degree of hydrolysis (DH). This parameter is commonly calculated by the o-phtaldialdehyde (OPA) method (Chiang et al., 2006; Jiang et al., 2007). Nevertheless, other methods and techniques have also been employed for this purpose, such as SDS-PAGE (Jang and Lee, 2005), calculation of $\alpha$-amino nitrogen and total nitrogen, (Mao et al., 2007) or the calculation of the relative peak area in regard to whole protein (Chobert et al., 2005). Yak milk casein was hydrolyzed by alcalase at pH 8 and 55°C in times ranging from 0 to 340 min. It was observed that at 240 min of hydrolyzing, the ACE inhibitory activity reached the maximum level and DH was correlated with it. After this time, DH was too high and inhibition activity decreased due to the hydrolysis of small peptides with antihypertensive activity (Mao et al., 2007). However, when the same yak milk casein was hydrolyzed with various enzymes (trypsin, pepsin, alcalase, flavorzyme, papain, and neutrase) at their optimal pH and temperature and at different times till 12 h, the DH was not correlated with the ACE inhibition activity. ACE inhibition activity was the poorest when using flavourzyme despite its high DH. Inversely, the most promising antihypertensive activities were obtained with papain and neutrase that showed low DH (Jiang et al., 2007). Similar results were also observed when milk was fermented by 13 different strains of lactic acid bacteria (Nielsen et al., 2009) and when a soybean protein isolate was hydrolyzed by different enzymes (alcalase, flavourzyme, trypsin, chymotrypsin, and pepsin) (Chiang et al., 2006).

## *ISOLATION AND PURIFICATION OF BIOACTIVE PEPTIDES FROM FOODSTUFFS*

The purification of a hydrolysate showing antihypertensive activity is one of the most important steps in the framework presented in Figure 2. The purification pathway could significantly influence the number of identified peptides, their activity and characteristics (e.g., size of the peptide and their composition), and their properties. Generally, liquid chromatography (LC) is the most often employed technique. Different chromatographic modes can be selected from the base of the properties of ACE inhibitory peptides. After each chromatographic step, fractions with the highest in vitro ACE inhibitory activity are lyophilized and subjected to the next chromatographic step till pure peptide/s is/are obtained.

Table 2 summarizes the methods that have been employed for the release and purification of peptides with antihypertensive properties. Despite there being some general approaches that are more or less common in all procedures, the number of purification steps in each case depends on the complexity of the sample and the dynamic range and abundance of peptides (Gómez-Ruiz et al., 2004; De Simone et al., 2009).

**Table 2** Purification of peptides with antihypertensive activity

| Source of peptide | Release of peptides | Employed enzyme(s) or bacterial strains | Purification steps | Ref. |
|---|---|---|---|---|
| | | **Milk and dairy products** | | |
| Milk | Fermentation/ digestion | Several bacterial strains or *Lactbacillus rhamnosus* and pepsin, corolase PP | UF: $M_{WCO}$ 3 kDa<br>RPC: Widepore C18, 250 × 4.6 mm; 0–45% B in 60 min; A: 0.037% TFA; B: ACN + 0.027% TFA; 0.8 mL/min; UV detection (220 nm) | Hernández-Ledesma et al., 2004 |
| Milk | Fermentation | Strains *Enterococcus faecalis* | Centrifugation (20,000 g, 10 min, 10°C) / filtration (Whatman no. 40)<br>RPC: Widepore C18, 250 × 4.6 mm; 0–45% B in 60 min; A: 0.037% TFA; B: ACN + 0.027% TFA; 0.8 mL/min; MS detection | Quiros et al., 2006 |
| Milk | Fermentation | Strains *Enterococcus faecalis* | Extraction followed by centrifugation (20000 g, 10 min, 10°C) and filtration (Whatman no. 40)<br>UF: $M_{WCO}$ 3 kDa<br>RPC: Prep Nova Pak® HR C18, 300 × 7.8 mm; 0–35% B in 70 min; A: 0.1% TFA; B: ACN + 0.08% TFA; 4 mL/min; UV detection (214 nm)<br>RPC: Prep Nova Pak® HR C18, 300 × 7.8 mm; 20–35% B in 40 min; A: 0.1% TFA; B: ACN + 0.08% TFA; 4 mL/min; UV detection (214 nm) | Quiros et al., 2007b |
| Goat milk* | Digestion | Alcalase | RPC: Resource RP; linear gradient of 0.05% TFA and 84% ACN + 0.05% TFA, 40 min; 1 mL/min; UV detection (220 nm)<br>Caco-2 cell<br>RPC: Zorbax 5 C18, 2.1 × 250 mm; 0–30% in 60 min; 30–80% in 10 min; A: 0.05% TFA; B: ACN + 0.04% TFA; 350 $\mu$L/min; UV detection (220 nm) | Geerlings et al., 2006 |
| Ovine milk | Simulated gastrointestinal digestion | Pepsin, trypsin with chymotrypsin or pepsin and corolase PP | UF: Centrifugation/ filtration (Whatman no. 40); $M_{WCO}$ 3 kDa<br>RPC: Prep Nova Pak® HR C18, 300 × 7.8 mm; 0–40% B in 70 min; A: 0.1% TFA; B: ACN + 0.08% TFA; 4 mL/min; UV detection (214 nm)<br>CE: bare fused silica capillary, 76 cm × 50 $\mu$m; 0.9 M HFo, pH = 2.0; 18 kV; MS detection | Gómez-Ruiz et al., 2007 |
| Ovine $\beta$-lactoglobulin from skimmed milk | Digestion | Trypsin | RPC: Nucleosil C18, 250 × 4 mm; 10–100% B in 23 min; A: 0.11% TFA; B: 80% ACN + 0.09% TFA; 0.6 mL/min | Chobert et al., 2005 |
| Yak milk casein | Digestion | Trypsin, pepsin, alcalase, flavourzyme, papain or neutrase | UF: $M_{WCO}$ 10 kDa and 6 kDa<br>SEC: Sephadex G- 25, 26 × 800 mm; elution with water; 0.6 mL/min; UV detection (215 nm);<br>RPC: Shim-pack PREP-ODS C18, 20 × 250 mm; 10–60% B in 35 min; A: 0.05% TFA; B: ACN + 0.05% TFA; 6 mL/min; UV detection (215 nm)<br>RPC: C18, 4.6 × 250 mm; 20–50% B in 20 min; A: 0.05% TFA; B: ACN + 0.05% TFA; 1 mL/min; UV detection (215 nm) | Jiang et al., 2007 |
| Yak milk casein | Digestion | Alcalase | UF: $M_{WCO}$ 10 kDa and 6 kDa<br>IEC: DE-52, 1.6 × 30 cm; 0–0.4 mM NaCl in 5 mM PBS, pH 8.0; 24 mL/h; UV detection (220 nm)<br>SEC: Sephadex G-25, 2.5 × 100 cm; elution with water; 16 mL/h; UV detection (220 nm)<br>RPC: Zorbax Eclipse XDB-C18, 10 × 400 mm; 0–60% B (B: ACN + 0.1% TFA) in 45 min; 1 mL/min; UV detection (214 nm)<br>RPC: Zorbax Eclipse XDB-C18, 2.1 × 150 mm; A: 0.1% TFA; B: ACN + 0.1% TFA; 0.4 mL/min; UV detection (214 nm) | Mao et al., 2007 |

*(Continued on next page)*

**Table 2** Purification of peptides with antihypertensive activity *(Continued)*

| Source of peptide | Release of peptides | Employed enzyme(s) or bacterial strains | Purification steps | Ref. |
|---|---|---|---|---|
| Casein | Fermentation | *Lactobacillus helveticus CP790* | RPC: $\mu$-Bondashere C18, 3.9 × 150 mm; 0–40% B in 60 min; A: 0.1% TFA; B: ACN + 0.1% TFA; 1 mL/min; UV detection (215 nm)<br>RPC: $\mu$-Bondashere C18, 3.9 × 150 mm; 90–30% B in 60 min; A: 0.1% TFA; B: ACN + 0.1% TFA; 1 mL/min; UV detection (215 nm) | Maeno et al., 1996 |
| Skimmed milk | Fermentation | Strains *Lactobacillus helveticus JCM1004* | RPC: YMC-Pack ODS-AP-303, 4.6 × 250 mm; 0–100% B; A: 10% ACN + 0.1% TFA; B: 90% ACN + 0.8% TFA; 1 mL/min; UV detection (215 nm)<br>RPC: ODS-100S, 3.9 × 150 mm; 0–40% B in 40 min, 40–70% B in 28 min, 70–100% B in 22 min; A: 10% ACN + 0.1% TFA; B: 90% ACN + 0.8% TFA; 1 mL/min; UV detection (215 nm)<br>RPC: $\mu$Bondasphere C18, 3.9 × 150 mm; 0–30% B in 45 min, 30–65% B in 25 min, 65–100% B in 20 min; A: 10% ACN + 0.1% TFA; B: 90% ACN + 0.8% TFA; 1 mL/min; UV detection (215 nm) | Pan et al., 2005 |
| Sheep milk yoghurt | Fermentation | Different bacterial cultures | RPC: Nucleosil C18, 250 × 4 mm; 0% B in 10 min; 0–80% B in 80 min; 100% B in 10 min; A: 0.1% TFA; B: 60% ACN + 0.09% TFA; 0.8 mL/min; UV detection (214 nm)<br>RPC: Nucleosil C18, 250 × 4 mm; 0% B in 10 min; 0–80% B in 80 min; 100% B in 10 min; A: 0.1% TFA; B: 60% ACN + 0.09% TFA; 0.2 mL/min; UV detection (278 nm) | Papadimitriou et al., 2007 |
| Yoghurt | Fermentation | Different bacterial cultures | RPC: C-18 monomeric, 250 × 4.6 mm; 0–100% B in 90 min; A: 0.1% TFA; B: 90% ACN + 0.1% TFA; 0.75 mL/min; UV detection (214 nm)<br>CE: Coated capillary, 50 cm × 50 $\mu$m; 30 mM $Na_3BO_3$ and 17 mM PBS, pH = 8.2; 15 kV, 30 min; 20°C; UV detection (214 nm) | Donkor et al., 2007 |
| Yoghurt- like product | Fermentation | *Lactobacillus helveticus CPN4* | Extraction in Sep-pak C-18 cartridges; elution with different ACN ratios 10–50% B (ACN + 0.1% TFA)<br>RPC: $\mu$-Bondasphere C18, 3.9 × 150 mm; 100–60% in 60 min; A: 0.1% TFA; B: ACN + 0.1% TFA; 1 mL/min; UV detection (215 nm)<br>RPC: $\mu$-Bondasphere C18, 3.9 × 150 mm; 5–20% B in 60 min; A: 0.1% TFA; B: ACN + 0.1% TFA; 1 mL/min; UV detection (215 nm) | Yamamoto et al., 1999 |
| Caprine Kefir* | Fermentation | Different bacteria strains | Centrifugation/ filtration/ UF<br>12,000 × g, 10 min, 5°C/ filter Whatman no. 40/ $M_{WCO}$ 3 kDa<br>RPC: 0–30% B in 70 min; A: 0.1% TFA; B: ACN + 0.08% TFA; UV detection (214 nm)<br>RPC: 8–20% B in 45 min; A: 0.1% TFA; B: ACN + 0.08% TFA; UV detection (214 nm) | Quiros et al., 2005 |
| Cheese | Fermentation/ digestion | *Lactococcus starter culture* (MM100)/ protease N amino, Umamizyme and Flavourzyme | Dialysis: Molecular porous membrane tubing (Spectra/Por 3; $M_{WCO}$ 3.5) against water; 48 h; 4°C<br>RPC: YMC-Pack R&D ODS, 20 × 250 mm; 0–70% B (B: ACN + 0.1% TFA) in 50 min; 7.5 mL/min; UV detection (214 nm)<br>RPC: TSK-gel ODS 80Ts, 20 × 250 mm; 0–30% B (B: ACN + 0.1% TFA) in 50 min; 7.5 mL/min; UV detection (214 nm)<br>SEC: Superdex Peptide HR 10/30, 10 × 300 mm; elution with water + 0.1% TFA; 0.5 mL/min; UV detection (214 nm)<br>RPC: CAPCELL PAK C18 MG, 4.6 × 250 mm; elution with 12% ACN + 0.1% TFA; 0.4 mL/min; UV detection (214 nm) | Tonouchi et al., 2008 |

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**Table 2** Purification of peptides with antihypertensive activity *(Continued)*

| Source of peptide | Release of peptides | Employed enzyme(s) or bacterial strains | Purification steps | Ref. |
|---|---|---|---|---|
| Gouda cheese | Extraction | ———— | Hydrophobic chromatography in Wakogel LP-40c18 resin; 15–90% ACN<br>RPC: Superiorex ODS, 4.6 × 150 mm; 0–100% B in 30 min; A: 10% ACN + 0.05% TFA; B: 60% ACN + 0.05% TFA; 0.5 mL/min; UV detection (214 nm)<br>SEC: Superdex Peptide HR 10/30, 1 × 30 cm; elution with 0.1% TFA; 0.5 mL/min; UV detection (214 nm)<br>CE: Coated capillary, 24 cm × 25 $\mu$m; BGE: 0.1 M PBS, pH = 2.5; 10 kV, 15 min; UV detection (200 nm) | Saito et al., 2000 |
| Several Spanish cheeses | Extraction | ———— | UF: $M_{WCO}$ 1 kDa<br>RPC: Hi-Pore C18, 250 × 4.6 mm; 0–40% B in 60 min; 40–70% B in 5 min; A: 0.037% TFA; B: ACN + 0.027% TFA; 0.8 mL/min; UV detection (214 nm) | Gómez-Ruiz et al., 2006 |
| Manchego cheese | Fermentation/ ripening | Different bacterial strains | Extraction/ centrifugation/ filtration/ centrifugation/ filtration<br>UF: $M_{WCO}$ 3 kDa<br>CEC: HiLoadt 26/10 SP Sepharose Fast Flow; 0% B in 20 min; 0- 30% B in 40 min; 30–100% B in 10 min; 100% B in 10 min; A: 10 mM HFo; B: 5M $NH_4OH$; 5 mL/min; UV detection (280 nm)<br>RPC:C18 Prep-Nova Pak HR, 300 × 7.8 mm; 5-50% B in 60 min; 60- 100% B in 10 min; 100% B in 10 min; A: 0.1% TFA; B: 80% ACN + 0.08% TFA; 6 mL/min; UV detection (214, 280 nm)<br>RPC: Hi-Pore C18, 250 × 4.6 mm; 10- 25% B in 30 min or 18–23% B in 25 min or 20–24% B in 25 min; A: 0.1% TFA; B: 80% ACN + 0.08% TFA; 0.8 mL/min; UV detection (214, 280 nm) | Gómez-Ruiz et al., 2002 |
| Manchego cheese | Fermentation | Several bacterial strains | UF: $M_{WCO}$ 3 kDa<br>RPC: Widepore C18, 250 × 4.6 mm; 0–40% A in 60 min; A: 0.037% TFA; B: 80% ACN + 0.027% TFA; 0.8 mL/min; UV detection (220 nm) | Gómez-Ruiz et al., 2004 |
| Enzyme-modified cheese | Digestion | Neutrase, *Lactobacillus casei* enzyme or Debitrase® DBP20 | RPC: Delta Pak C18, 30 × 150 mm; 20–40% B in 15 min; 40–60% B in 15 min; 60–100% B in 5 min; 100–20% B in 35 min; A: 0.1% TFA; B: 40% ACN + 0.08% TFA; 0.5 mL/min; UV detection (214 nm) | Haileselassie et al., 1999 |
| Cheese whey protein | Digestion | Trypsin, proteinase K, actinase E, thermolysin, papain, chymotrypsin or pepsin | Hydrophobic chromatography in LiChropep RP-18 resin (25–40 $\mu$m); 0–90% MetOH<br>Hydrophobic chromatography in LiChropep RP-18 resin (25–40 $\mu$m); 30–42% MetOH<br>RPC: Superiorex ODS, 4.6 × 150 mm; 0–100% B in 30 min; 100% B in 10 min; A: 10% ACN + 0.05% TFA; B: 60% ACN + 0.05% TFA; 0.5 mL/min; UV detection (214 nm)<br>SEC: Superdex peptide HR 10/30, 1 × 30 cm; elution with 0.1% TFA; 0.5 mL/min; UV detection (214 nm)<br>CE: Coated capillary, 24 cm × 25 $\mu$m; 0.1 M PBS, pH = 2.5; 10 kV; UV detection (200 nm) | Abubakar et al., 1998 |

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**Table 2** Purification of peptides with antihypertensive activity *(Continued)*

| Source of peptide | Release of peptides | Employed enzyme(s) or bacterial strains | Purification steps | Ref. |
|---|---|---|---|---|
| Commercial whey product* | Not shown | Not shown | Extraction with Wakogel LP-40C18 resin (20–40 $\mu$m); elution with 90% EtOH<br>SEC: Sephadex G-15, 2.6 × 90 cm; 0.05% TFA; 0.5 mL/min; UV detection (214 nm)<br>RPC: Wakosil-II 5C18, 4.6 × 150 mm; 0–80% B in 15 min; A: 10% ACN + 0.05% TFA; B: 60% ACN + 0.05% TFA; 0.5 mL/min; UV detection (214 nm)<br>CE: Coated capillary, 17 cm × 25 $\mu$m; 0.1 M PBS, pH = 2.5; 10 kV, 15 min; 20°C; UV detection (200 nm) | Murakami et al., 2004 |
| Sodium caseinate | Fermentation | *Lactobacillus (Lb.) helveticus NCC 2765* | SEC: Superdex 75 HR 10/30; 50 mM $NH_4Ac$ (pH 7) for 65 min; 0.5 mL/min; UV detection (215, 280 nm)<br>RPC: C8 208TP54; 0% B in 5 min, 0–50% B in 60 min, 50–100% B in 1 min, 100% B in 4 min; A: 0.05% TFA; B: 80% ACN + 0.045% TFA; 0.8 mL/min; UV detection (215 nm) | Robert et al., 2004 |
| $\alpha$-lactalbumin[1] and $\beta$-casein[2] | Digestion | Thermolysin | SEC: Superdex$^{TM}$ 30 prep grade, 2.6 × 61 cm; 0.1M $NH_4HCO_3$, pH 8.0; 2.5 mL/min; UV detection (280 nm)<br>RPC: Nucleosil 300-S C18, 4.6 × 250 mm; [1]0–80% B (10–90 min); [2]30–55% B (90 min) or [2]2–40% B (80 min); A: 0.1% TFA; B: 60% ACN + 0.1% TFA; 1 mL/min; UV detection (210 and 280 nm)<br>RPC: Nucleosil 300-S C18, 4.6 × 250 mm; [1] 20–45% B (80 min) or [1]20–50% B (80 min); A: 0.1% TFA; B: 60% ACN + 0.1% TFA; 1 mL/min; UV detection (210 and 280 nm) | Otte et al., 2007b |
| | | | **Plant origin products** | |
| Soybean | Digestion | Pepsin | IEC: Dowex 50 W, 45 × 200 mm; elution with 5% $NH_4OH$<br>SEC: Sephadex G-25, 26 × 1400 mm; water; 30 mL/min<br>IEC: Sephadex C-25, 20 × 500 mm; 0–3% NaCl; 30 mL/min<br>RPC: Develosil ODS-5, 4.6 × 250 mm; 0–16% B (B: ACN + 0.05% TFA) in 60 min; 1 mL/min; UV detection (220 nm) | Chen et al., 2002 |
| Soybean | Fermentation | *Aspergillus oryzae* | UF: $M_{WCO}$ 3, 10, 30 kDa<br>CEC: HiPreP 16/10 SP FF, 16 × 100 mm; 0–100% B in 40 min: A: 10 mM NaAc, pH 4.0; B: 20 mM NaAc (pH 4.0) in 1 M NaCl; 5 mL/min; UV detection (214 nm)<br>Desalination: Cellulose dialysis membrane, $M_{WCO}$ 100<br>SEC: Superdex Peptide 10/300 GL, 10 × 300 mm; elution with 30% ACN in 40 min; 0.36 mL/min; UV detection (214 nm)<br>RPC: $\mu$Bondapak$^{TM}$ C18, 4.6 × 300 mm; 0–100% B in 40 min; A: 0.1% TFA; B: 40% ACN + 0.1% TFA; 1 mL/min; UV detection (214 nm)<br>RPC: $\mu$Bondapak$^{TM}$ C18, 4.6 × 300 mm; 0–100% B in 40 min; A: 25% ACN + 0.1% TFA; B: 35% ACN + 0.1% TFA; 1 mL/min; UV detection (214 nm) | Rho et al., 2009 |
| Soybean protein | Digestion | Mature D3 protease | Desalination by electrodialyzation<br>SEC: Superdex Peptide HR 10/300; elution with 0.05% TFA; 0.5 mL/min; UV detection (215 nm)<br>RPC: Cosmosil 5C18 AR 4.6/250; 0–50% B in 50 min; A: 0.05% TFA; B: ACN + 0.065% TFA; 0.75 mL/min; UV detection (215 nm) | Kodera and Nio, 2006 |
| Glycinin from soybean* | Digestion | Protease P, trypsin, chymotrypsin or ginger protease | RPC: C-18 Shimpak, 250 × 4.6 mm; 0–35% B; A: 0.1% TFA; B: 70% ACN + 0.05% TFA; 0.7 mL/min; UV detection (230 nm) | Gouda et al., 2006 |

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**Table 2** Purification of peptides with antihypertensive activity *(Continued)*

| Source of peptide | Release of peptides | Employed enzyme(s) or bacterial strains | Purification steps | Ref. |
|---|---|---|---|---|
| | | | RPC: C-18 Shimpak, 250 × 4.6 mm; 0–100% B; A: 50 mM $NH_4Ac$; B: 50 mM $NH_4Ac$/ ACN (50:50); UV detection (230 nm)<br>RPC: C-18 Shimpak, 250 × 4.6 mm; 0–35% B; A: 0.1% TFA; B: 70% ACN + 0.05% TFA; 0.7 mL/min; UV detection (230 nm) | |
| Steamed soybean mixed with roasted wheat | Fermentation | *Tane koji* rich in *A. sojae* | Desalination by electrodialyzing<br>RPC: SP-120–40/60-ODS-B, 150 × 1000 mm; 0–100% B in 25 h; water- 0.1% TFA-ACN; 45 mL/min; UV detection (220 nm)<br>RPC: Cosmosil-5C18-ARII, 20 × 250 mm; 0–100% B in 90 min; water- 0.1% TFA-ACN; 5 mL/min; UV detection (220 nm)<br>RPC: C30 Develosil RPAQUEOUS-AR, 20 × 250 mm; 0–100% B in 90 min; water-ACN; 5 mL/min; UV detection (220 nm) | Nakahara et al., 2010 |
| Wheat bran* | Autolysis | Endogenous proteases | RPC: LiChroprep RP-18, 2.5 × 25 cm; 10–95% EtOH<br>IEC: AG MP-1 resin, 3 × 20 cm<br>SEC: Superdex 75HR, 10 × 30 cm; elution with 30% ACN + 0.1% TFA; 0.5 mL/min; UV detection (220 nm)<br>RPC: Jupiter C4, 10 × 250 mm; 0–35% ACN + 0.1% TFA in 30 min; 4 mL/min<br>RPC: Jupiter C18, 10 × 250 mm; 0–35% ACN + 0.1% TFA in 30 min; 4 mL/min | Nogata et al., 2009 |
| Wheat gliandin | Digestion | Pepsin and protease M | IEC: SP-Toyopearl 550C, 2.6 × 40 cm; 0–0.5M NaCl in 5 mM NaAc, pH 3.5; 1 mL/min; UV detection (220 nm)<br>SEC: Bio-gel P-2, 1.6 × 100 cm; elution with water; 0.33 mL/min; UV detection (220 nm)<br>RPC: TSK-GEL ODS 120T, 4.6 × 250 mm; 0–30% ACN + 0.01% TFA; 1 mL/min; UV detection (220 nm) | Motoi and Kodama, 2003 |
| White wheat, wholemeal wheat, rye flours* | Fermentation | Different sourdoughs | Extraction with 30 mL 50 mM Tris-HCl, pH 8.8 at 4°C and centrifugation at 20,000g, 20 min<br>RP-FPLC: 5% B 16 min; 5–46% B 46 min; A: 0.05% TFA; B: ACN + 0.05%; 1 mL/min; UV detection (214 nm) | Rizzello et al., 2008 |
| Rice* | Digestion | Alcalase | Desalination with an ion exchange resin<br>SEC: Sephadex G-15, 1.8 × 60 cm; 20 mM NaAc, pH 4.0; 0.4 mL/min; UV detection (220 nm)<br>RPC: Sephasil Peptide C18 ST 4.6/250, 4.6 × 250 mm; 0-60% B (B: ACN + 0.1% TFA) in 60 min; 1 mL/min; UV detection (220 nm)<br>RPC: Sephasil Peptide C2/C18 ST 4.6/250, 4.6 × 250 mm; 10–30% B (B: ACN + 0.1% TFA) in 40 min; 1 mL/min; UV detection (220 nm) | Li et al., 2007 |
| Rice | Fermentation | *Monascus* strains | Separation in SEPABEADS SP825 and elution with different EtOH percentages: 10–70%<br>SEC: Sephadex G-25, 1.2 × 142.5 cm; elution with water; UV detection (220 nm)<br>RPC: Cosmosil 5 C18-AR-300; 0–50% B (B: ACN + 0.05% TFA) in 50 min; 0.5 mL/min; UV detection (220 nm)<br>RPC: Cosmosil 5 C18-AR-300 or Cosmosil 5Ph-AR-300; ACN + 0.05% TFA; 0.25 mL/min; UV detection (220 nm) | Kuba et al., 2009 |
| Mushroom *Tricholoma giganteum* | Extraction | ———— | UF: $M_{WCO}$ 5 kDa<br>SEC: Sephadex G-25, 3.0 × 35 cm; elution with water; 12 mL/min<br>RPC: $\mu$Bondapack C18; 0–100% B; A: 0.1% TFA; B: ACN | Hyoung et al., 2004 |

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**Table 2** Purification of peptides with antihypertensive activity *(Continued)*

| Source of peptide | Release of peptides | Employed enzyme(s) or bacterial strains | Purification steps | Ref. |
|---|---|---|---|---|
| | | | RPC: $\mu$Bondapack C18; 0- 100% B; A: 0.1% TFA; B: ACN<br>RPC: Nova-pak C18; 0- 100% B; A: 0.1% TFA; B: ACN | |
| Mushroom *Pholiota adiposa** | Extraction | ———— | UF: $M_{WCO}$ 5 kDa<br>SEC: Sephadex G-25, 3.0 × 80 cm; elution with water; 24 mL/min<br>RPC: $\mu$Bondapack C18; 0–100% B; A: 0.1% TFA; B: ACN<br>RPC: $\mu$Vydac protein/peptide 218Tp; 0–100% B; A: 0.1% TFA; B: ACN | Koo et al., 2006 |
| $\alpha$-zein* | Digestion | Thermolysin | DEAE-Tyopearl 650 M, 2.6 × 100 cm; 0–0.3 M NaCl in 5mM Tris-HCl (pH 8); 3 mL/min; UV detection (254 nm)<br>Desalination with Sephadex LH-20, 1.6 × 100 cm<br>RPC: C-18 Capcellpak, 1.5 × 25 cm; 10–60% B (B: ACN + 0.1% TFA); 8 mL/min; UV detection (210 nm)<br>RPC: C-18 Capcellpak, 1.5 × 25 cm; ACN + 0.1% TFA; 8 mL/min; UV detection (210 nm)<br>CEC: Senshupak SCN-1251, 0.46 × 25 cm; 20 mM/pH 4.0–50 mM/pH 6.3 $NH_4Ac$<br>SP-Toyopearl 650 M, 2.6 × 100 cm; 20 mM/pH 4.0-50 mM/pH 6.3 $NH_4Ac$; 3 mL/min; UV detection (254 nm)<br>RPC: C-18 Capcellpak, 1.5 × 25 cm; 10–60% B (B: ACN + 0.1% TFA); 8 mL/min; UV detection (210 nm)<br>RPC: C-18 Capcellpak, 1.5 × 25 cm; 5–30% B (ACN + 0.1% TFA); UV detection (210 nm) | Miyoshi et al., 1991 |
| Urea denaturated Z19 $\alpha$-zein | Digestion | Thermolysin | RPC: YMC-GEL C4, 4.6 × 110 mm; 0 -30% B (B: ACN + 0.1% TFA); 1 mL/min; UV detection (220 nm)<br>RPC: YMC-GEL C18, 4.6 × 250 mm; 0–15% or 0–25% B (B: ACN + 0.1% TFA); 1 mL/min; UV detection (220 nm) | Yano et al., 1996 |
| Urea denaturated total $\alpha$-zein | | | RPC: YMC-GEL C18, 4.6 × 250 mm; 0–30% B (B: ACN + 0.1% TFA); 1 mL/min; UV detection (220 nm) | |
| Corn gluten meal* | Digestion | Protamex, neutrase, alcalase or trypsin | UF: $M_{WCO}$ 5 kDa<br>SEC: Bio-Rad P-2, 700 × 15 mm; 2 mM PBS, pH 8.0; 0.25 mL/min; UV detection (220 nm)<br>RPC: $\mu$-Bondapak C 18, 300 × 7.8 mm; 0–40% B (B: ACN + 0.1% TFA); 3 mL/min; UV detection (220 nm) | Yang et al., 2007 |
| Spinach Rubisco | Simulated gastrointestinal digestion | Pepsin and pancreatin | RPC: Cosmosil 5C18-AR-II, 20 × 250 mm; 1%/min B (B: ACN + 0.1% TFA); 10 mL/min; UV detection (230 nm)<br>RPC: 5PE-MS, 4.6 × 250 mm; 1%/min B (B: ACN + 0.1% TFA); 1 mL/min; UV detection (230 nm)<br>RPC: Cosmosil 5 CN-R, 4.6 × 250 mm; 1%/min B (B: ACN + 0.1% TFA); 1 mL/min; UV detection (230 nm)<br>RLC: 5NPE, 4.6 × 150 mm; 1%/min B (B: ACN + 0.1% TFA); 1 mL/min; UV detection (230 nm) | Yang et al., 2003 |
| Amaranth (*Amaranthus hypochondriacus*) grain | Digestion | Alcalase | SEC: Sephadex G-200, 1.4 × 29 cm; 0.4 M NaCl + 20 mM 2-MER; 0.4 mL/min; UV detection (280 nm)<br>SEC: Sephadex G-15, 1.4 × 29 cm; 0.4 M NaCl + 20 mM 2-MER; 0.4 mL/min; UV detection (214 nm)<br>RPC: Nucleosil 100 C18 RP, 4.6 × 250 mm; 0–30% B (B: ACN + 0.1% TFA) in 60 min; 2 mL/min; UV detection (214 nm) | Tovar-Perez et al., 2009 |
| Buckwheat | Digestion | Pepsin, chymotrypsin, trypsin | SEC: Superdex Peptide HR 10/30, 1 × 30 cm; elution with 30% ACN + 0.1% TFA; 0.3 mL/min; UV detection (220 nm)<br>RPC: Cosmosil 5Ph, 4.6 × 250 mm; 5–35% B (B: ACN + 0.1% TFA) in 30 min; 0.3 mL/min; UV detection (220 nm) | Li et al., 2002 |

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**Table 2** Purification of peptides with antihypertensive activity *(Continued)*

| Source of peptide | Release of peptides | Employed enzyme(s) or bacterial strains | Purification steps | Ref. |
|---|---|---|---|---|
| | | | RPC: Cosmosil 5C18-ARII, 4.6 × 250 mm; 5–35% B (B: ACN + 0.1% TFA) in 60 min; 0.3 mL/min; UV detection (220 nm) | |
| Garlic (*Allium sativum* L*)* | Extraction | ———— | CEC: Dowex 50WX4, 2.5 × 30 cm; elution with 2 N $NH_4OH$<br>SEC: Sephadex G-25, 2.5 × 150 cm; elution with 0.1 M PBS, pH 7.0; 30 mL/min<br>RPC: Develosil ODS-5, 4.6 × 150 cm; 0–8% B (B: ACN + 0.05% TFA) in 1 h; 1 mL/min; UV detection (220 nm) | Suetsuna, 1998 |
| Mung bean protein | Digestion | Alcalase | UF: $M_{WCO}$ 6 kDa<br>SEC: Sephadex G-15, 1.8 × 60 cm; elution with 20 mM NaAc, pH 4.0; 0.4 mL/min; UV detection (220 nm)<br>RPC: Sephasil Peptide C18 ST 4.6/250, 4.6 × 250 mm; 0–100% B in 60 min; A: 0.1% TFA; B: 60% ACN + 0.1% TFA; 1 mL/min; UV detection (220 nm)<br>RPC: Sephasil Peptide C18 ST 4.6/250, 4.6 × 250 mm; 10–80% B in 40 min; A: 0.1% TFA; B: 60% ACN + 0.1% TFA; 1 mL/min; UV detection (220 nm) | Li et al., 2006 |
| Sesame protein hydrolysate | Hydrolysis | Thermolysin | SEC: Bio-Gel P-2, 15 × 820 mm; elution with 10% EtOH; 0.18 mL/min; UV detection (210 nm)<br>RPC.: Develosil ODS-10, 20 × 250 mm; 5% B in 20 min; 5–40% B in 60 min; B, ACN + 1% TFA; 10 mL/min; UV detection (210 nm)<br>RPC: Develosil C-30-UG-5, 10 × 250 mm; ACN + 1% TFA; 4 mL/min; UV detection (210 nm)<br>RPC: Develosil Ph-UG-5, 10 × 250 mm; 6% ACN + 1% TFA; 4 mL/min; UV detection (210 nm) | Nakano et al., 2006 |
| Alfalfa white protein* | Hydrolysation at pilot plant scale by Delvolase® in enzymatic membrane reactor | | SEC: Superdex Peptide HR 10/300, 10 × 300 mm; elution with 30% ACN + 0.1% TFA; 0.2 mL/min; UV detection (226 nm)<br>RPC: C18, 4.6 × 250 mm; 0–28% B in 50 min, 28–47% B in 20 min; B, ACN + 0.1% TFA; UV detection (226 nm) | Kapel et al., 2006 |
| | | | **Meat and chicken** | |
| Chicken bone | Digestion | Pepsin | SEC: TSK gel G2000SWXL, 7.8 × 300 mm; elution with 0.2 M PBS, pH 7.0; 1 mL/min; UV detection (225 nm)<br>RPC: Inertsil ODS-2;0–35% B (B: ACN + 0.1% TFA); 1 mL/min; UV detection (225 nm)<br>RPC: Inertsil ODS-2; 8–14% B (B: ACN + 0.1% TFA); 0.5 mL/min; UV detection (225 nm)<br>RPC: Cosmosil 5PE-MSl; elution with 10% ACN and 5% ACN; 0.5 mL/min; UV detection (215 nm) | Nakade et al., 2008 |
| Chicken leg | Digestion | *Aspergillus oryzae* protease, protease FP/ protease A amano G/ protease N, pepsin and trypsin/ chymotrypsin | UF: $M_{WCO}$ 3 kDa<br>RPC: C18 ODS, 22 × 250 mm and 4.6 × 250 mm; 8–40% B in 40 min or 8–40% B in 64 min (B: ACN + 0.1% TFA); 1 mL/min; UV detection (220 nm) | Saiga et al., 2008 |
| Chicken leg bone | Digestion | Alcalase | UF: $M_{WCO}$ 5 kDa<br>SEC: Superdex$^{TM}$ Peptide HR 10/30, 10 × 300 mm; elution with water; 0.5 mL/min; UV detection (220 nm) | Cheng et al., 2009 |

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**Table 2** Purification of peptides with antihypertensive activity *(Continued)*

| Source of peptide | Release of peptides | Employed enzyme(s) or bacterial strains | Purification steps | Ref. |
|---|---|---|---|---|
| Chicken muscle[1] and ovalbumin[2] | Digestion | Thermolysin[1] and pepsin, trypsin, chymotrypsin or thermolysin[2] | RPC: Cosmosil 5C18-AR, 20 × 250 mm; 0–50% B (B: ACN + 0.1% TFA) in 50 min; 10 mL/min; UV detection (215[1] or 230[2] nm)<br>RPC: Cosmosil 5 Ph, 4.6 × 250 mm; 0–40% B (B: ACN + 0.1% TFA) in 40 min; 1 mL/min<br>RPC: Cosmosil 5CN-R, 4.6 × 250 mm; 0–40% B (B: ACN + 0.1% TFA) in 40 min; 1 mL/min<br>RPC: Cosmosil 5C18-AR, 20 × 250 mm; 0–40% B (B: ACN + 10 mM PBS, pH 7.0) in 40 min; 1 mL/min | |
| Porcine skeletal muscle | Digestion | Trypsin, $\alpha$-chymotrypsin, pronase E, proteinase K, thermolysin, ficin, papain or pepsin | RPC: CAPCELL PAK C18 UG120, 4.6 × 150 mm; 0–100% B; A: 0.1% TFA; B: ACN + 0.1% TFA; 1 mL/min; UV detection (215 nm)<br>RPC: CAPCELL PAK C18 UG120, 4.6 × 150 mm; 0–100% B; A: 0.015% $NH_4OH$; B: ACN + 0.015% $NH_4OH$; 1 mL/min; UV detection (215 nm) | Arihara et al., 2001 |
| Porcine skeletal muscle troponin | Digestion | Pepsin | AEC: DE53, 16 × 150 mm; 0–300 mM NaCl in 20 mM Tris-acetate, pH 7.5; 1.13 mL/min; UV detection (215 nm)<br>Desalination: Sep-Pak Plus C18; elution with 50% ACN<br>RPC: Cosmosil 5C18 ARII, 4.5 × 150 mm; 1–80% B (B: ACN + 0.1% TFA); 0.5 mL/min; UV detection (215 nm)<br>RPC: Cosmosil 5C18 ARII, 4.5 × 150 mm; elution with 12 or 16% ACN + 0.1% TFA); 0.5 mL/min; UV detection (215 nm)<br>SEC: TSK-gel G2000SWXL, 7.8 × 300 mm; elution with 20 mM PBS, pH 7.0; 0.5 mL/min; UV detection (215 nm)<br>RPC: Cosmosil 5PEMS, 4.6 × 250 mm; elution with 12 or 15% ACN + 0.1% TFA; 1 mL/min; UV detection (215 nm); | Katayama et al., 2008 |
| Porcine hemoglobin | Digestion | Pepsin, trypsin or papain | SEC: Sephadex LH-20, 2.6 × 90 cm; elution with 30% MetOH; 0.5 mL/min; UV detection (280 nm)<br>RPC: Hypersil BDS C18, 4.6 × 250 mm; 0% B in 5 min; 0–50% B in 40 min; 100% B in 10 min; A: 10% ACN + 0.1% TFA; B: 90% ACN + 0.1% TFA; 1 mL/min; UV detection (215 nm)<br>RPC: Hypersil BDS C18, 4.6 × 250 mm; 0% B in 3 min; 0–40% B in 15 min; 100% B in 10 min; A: 10% ACN + 0.1% TFA; B: 90% ACN + 0.1% TFA; 1 mL/min; UV detection (215 nm) | Yu et al., 2006 |
| Porcine myosin B | Digestion | Pepsin | SEC: Superdex$^{TM}$ 30, 1.6 × 90 cm; elution with 20 mM NaAc (pH 7.0) + 150 mM NaCl; 0.45 mL/min<br>Desalination: SEP-PAK Plus C18; elution with 50% ACN<br>RPC: Inertsil ODS-2, 4.6 × 250 mm; 1–80% B and:1–50% B (ACN + 0.1% TFA); 0.5 mL/min; UV detection (225 nm)<br>RPC: Comosil 5PE-MS, 4.6 × 250 mm; elution with ACN at different proportions and flow-rates (0.1–0.5 mL/min); UV detection (225 nm) | Muguruma et al., 2009 |
| Pork loin | Digestion | Pepsin | AEC: DE53, 16 × 150 mm; 0–300 mM NaCl in 20 mM Tris-acetate, pH 7.5; 1.13 mL/min; UV detection (215 nm)<br>Desalination with Sep-Pak Plus C18; elution with 50% ACN<br>RPC: Cosmosil 5C18 AR-II, 4.5 × 150 mm; 1–80% B (B: ACN + 0.1% TFA); 0.5 mL/min; UV detection (215 nm) | Katayama et al., 2007 |

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**Table 2** Purification of peptides with antihypertensive activity *(Continued)*

| Source of peptide | Release of peptides | Employed enzyme(s) or bacterial strains | Purification steps | Ref. |
|---|---|---|---|---|
| | | | RPC: Cosmosil 5C18 AR-II, 4.5 × 150 mm; elution with 12% ACN + 0.1% TFA; 0.5 mL/min; UV detection (215 nm)<br>SEC: TSK-gel G2000 SWXL, 7.8 × 300 mm; elution with 20 mM PBS, pH 7.0; 0.5 mL/min; UV detection (215 nm)<br>RPC: Cosmosil 5PE-MS, 4.6 × 250 mm; elution with 12% ACN + 0.1% TFA; 1 mL/min; UV detection (215 nm) | |
| Pork meat | Simulated gastrointestinal digestion | Pepsin and pancreatin | RPC: Symmetry C18, 4.6 × 250 mm; 1% B in 5 min and 1–100% B in 80 min; water-0.1–0.085% TFA-ACN; 0.8 mL/min; | Escudero et al., 2010 |
| Bullfrog (*Rana catesbeiana* Shaw) muscle | Digestion | Alcalase, α-chymotrypsin, neutrase, papain, pepsin or trypsin | IEC: HiPrep 16/10 CM FF; 0–2 M NaCl in 20 mM PBS, pH 4.0; 62 mL/ h; UV detection (215 nm)<br>RPC: Primesphere 10 C18, 10 × 250 mm; 0–35% B (B: ACN + 0.1% TFA) in 35 min; 1.2 mL/ min; UV detection (215 nm)<br>RPC: SynChropak RP-P-100, 4.6 × 250 mm; elution with 15% ACN + 0.1% TFA in 20 min; 1.2 mL/min; UV detection (215 nm) | Qian et al., 2007b |
| Beef rump | Digestion | Thermolysin, proteinase A or protease type XIII and their combination | UF: $M_{WCO}$ 10 kDa<br>SEC: Sephadex G-25, 2.6 cm × 1 m; elution eith 20 mM PBS, pH 7.4; 1.6 mL/min<br>RPC: C18, 25 × 0.46 cm; 0% B in 10 min, 0–65% B in 20 min, 100% B in 10 min; A: 0.1% TFA; B: ACN + 0.07% TFA; 0.8 mL/min; UV detection (214 nm) | Jang and Lee, 2005 |
| Bovine $\alpha_{S2}$- casein | Digestion | Trypsin | RPC: XTerra C18, 4.6 × 250 mm; 1.6% B in 3 min; 1.6–40% B in 87 min (B:ACN + 0.1% TFA); 1 mL/min; UV detection (210–300 nm) | Tauzin et al., 2002 |
| Bovine lactoferrin | Digestion | Pepsin or trypsin and chymotrypsin | RPC: Capcell PAK C18, 4.6 × 150 mm; 0–45% B in 25 min; A: 0.1% TFA; B: ACN + 0.1% TFA; 1 mL/min; UV detection (230 nm) | Lee et al., 2006a; |
| Hen ovotransfferin | | | RPC: TSK gel ODS 80-Ts, 4.6 × 150 mm; 0–45% B in 25 min; A: 0.1% TFA; B: ACN + 0.1% TFA; 1 mL/min; UV detection (230 nm) | Lee et al., 2006b |
| | | | **Seafood** | |
| Antarctic krill (*Euphausia superba*) tail meat | Digestion | Thermoase PC10F | CEC: HiPrep 16/10 SP XL, 16 × 100 mm; 0–1 M NaCl + 26.5 mM HFo; 2 mL/min; UV detection (214 nm)<br>SEC: Superdex Peptide 10/300 GL, 10 × 300 mm; 0.9 mL/min; UV detection (214 nm);<br>RPC: ODS-80TM, 4.6 × 75 mm; 0- 40% ACN + 0.1% TFA; 0.5 mL/min; UV detection (214 nm)<br>RPC: μRPC C2/C18 SC 2.1/10, 2.1 × 100 mm; 7–13% ACN in 35 min; UV detection (214 nm) | Hatanaka et al., 2009 |
| Blue mussel (*Mytilus edulis*) | Fermentation | Salty conditions for 6 months | SEC: Sephadex G-75, 2.5 × 90 cm; elution with 50 mM PBS, pH 7.0; 60 mL/h; UV detection (280 nm)<br>IEC: SP-Sephadex C-25, 2.5 × 45 cm; 0–1 M NaCl in 20 mM NaAc, pH 4.0; UV detection (280 nm)<br>RPC: Nucleosil 100-7 ODS C18, 10 × 250 mm; 0–40% B (B: ACN + 0.1% TFA); 2 mL/min; UV detection (215 nm)<br>RPC: Nucleosil 100-7 ODS C18, 10 × 250 mm; 0–25% B (B: ACN + 0.1% TFA); 2 ml/min; UV detection (215 nm) | Je et al., 2005b |

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**Table 2** Purification of peptides with antihypertensive activity *(Continued)*

| Source of peptide | Release of peptides | Employed enzyme(s) or bacterial strains | Purification steps | Ref. |
|---|---|---|---|---|
| Marine shrimp *(Acetes chinensis)* | Fermentation | *Labtobacillus fermentum SM 605* | UF: $M_{WCO}$ 3 kDa<br>SEC: Sephadex G-15, 1.6 × 80 cm; elution with water; 25 mL/min; UV detection (220 nm)<br>RPC: HIQ sil C18-10, 4.6 × 250 mm; 0–50% B (B: MetOH + 0.1% TFA) in 50 min; 0.8 mL/min; UV detection (214 nm) | Wang et al., 2008b |
| Gelatin of sea cucumber | Digestion | Bromelain and alcalase | UF: $M_{WCO}$ 1, 5, 10 kDa<br>IEC: SP Sephadex C-25, 16 × 300 mm; 0–0.15 M NaCl in 20 mM NaAc (pH 4); 0.4 mL/min; UV detection (220 nm)<br>SEC: Sephadex G-15, 16 × 300 mm; elution with water; 0.3 mL/min; UV detection (220 nm)<br>RPC.: Zorbax C18, 1 × 250 mm; 0–10% ACN in 15 min; 0.8 mL/min; UV detection (220 nm)<br>RPC: Zorbax SB C18, 4.6 × 250 mm; 0–10% ACN in 10 min; 0.8 mL/min; UV detection (220 nm) | Zhao et al., 2007 |
| Sea cucumber (*Acaudina molpadioidea*) | Digestion | Bromelain and alcalase | UF: Mwco 2 kDa<br>SEC: Sephadex G-25, 1.6 × 30 cm; elution with water; 0.6 mL/min; UV detection (220 nm)<br>IEC: SP Sephadex C-25, 2.6 × 30 cm; 0–1 M NaCl in 20 mM NaAc, pH 4.0; 0.6 mL/min; UV detection (220 nm)<br>SEC: Sephadex G-25, 1.6 × 100 cm<br>RPC: Zorbax C18, 9.4 × 250 mm; 5–40% B (B: ACN + 0.1% TFA) in 40 min; 0.8 mL/min; UV detection (220 nm) | Zhao et al., 2009 |
| Oyster* | Fermentation | Salty conditions for 6 months | IEC: SP-Sephadex C-25, 4.0 × 40 cm; 0–2 M NaCl in 20 mM NaAc, pH 4.0; 60 mL/h; UV detection (215 nm)<br>Desalination by electrodialyzation<br>SEC: Sephadex G-50, 2.5 × 98 cm; elution with water; 60 mL/h; UV detection (215 nm)<br>SEC: elution with water; 60 mL/min; UV detection (215 nm)<br>RPC: Nucleosil 100-3 ODS C18; 0–11% B (B: ACN + 0.1% TFA); 1 mL/min; UV detection (215 nm) | Je et al., 2005a |
| Oyster (*Crassostrea talienwhanensis crosse*) | Digestion | Pepsin | Filtration: $M_{wco}$ 10 kDa<br>SEC: Sephadex LH-20, 2.7 × 80 cm; elution with 30% MetOH; 0.5 mL/min; UV detection (280 nm)<br>RPC: Hypersil BDS C18, 4.6 × 210 mm; 0–100% B in 40 min; 100% B in 10 min; A: 0.1% TFA; B: ACN; 1 mL/min; UV detection (215 nm) | Wang et al., 2008a |
| Wakame (*Undaria pinnatifida*) | Digestion | Pepsin | Dialysis against water in cellulose tubing for 2 days<br>IEC: Dowex 50W, 2.6 × 20 cm; elution with 3.7% $NH_4OH$<br>SEC: Sephadex C-25, 2 × 50 cm; elution with 1.5% NaCl; 70 mL/h<br>RPC: Develosil C18 ODS-5, 4.6 × 250 cm; 0–25% B (B: ACN + 0.05% TFA) in 2 h; 1 mL/min; UV detection (220 nm) | Suetsuna and Nakano, 2000 |
| Wakame (*Undaria pinnatitida)* | Digestion | Protease S "amano" | Extraction with 1-butanol<br>RPC: $\mu$Bondasphere C18, 300 × 30 mm; 0–35% B in 140 min; A: 0.1% TFA; B: ACN + 0.07% TFA; 30 mL/min; UV detection (220 nm)<br>RPC: XTerraRP18, 150 × 4.6 mm; 0% B in 10 min, 0–20% B in 40 min; A: 50 mM $NH_4Ac$ (pH 10) + 1% ACN; B: 50 mM $NH_4Ac$ (pH 10) + 95% ACN; 1 mL/min; UV detection (220 nm) | Sato et al., 2002 |

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**Table 2** Purification of peptides with antihypertensive activity *(Continued)*

| Source of peptide | Release of peptides | Employed enzyme(s) or bacterial strains | Purification steps | Ref. |
|---|---|---|---|---|
| | | | RPC: [1]C.: ODP50-4D, 150 × 4.6 mm; 0–20% B in 30 min; A: 50 mM $NH_4OH$ (pH 10) + 1% ACN; B: 50 mM, $NH_4OH$ $NH_4OH$ (pH 10) + 95% ACN; 0.5 mL/min; UV detection (220 nm); [2]C.: XTerra RP18, 150 × 4.6 mm; 0–30% B in 40 min; A: 0.1% TFA/0.07% TFA in ACN (99/1); B: 0.1% TFA/0.07% TFA in ACN (5/95); 1 mL/min; UV detection (220 nm) | |
| Wakame (*Undaria pinnatifida*) | Extraction | ———— | Dialysis against water (10 L) in cellulose tubular membrane (90 cm)<br>IEC: Dowex 50W, 45 × 450 mm; elution with $NH_4OH$<br>SEC: Sephadex G-25, 2.6 × 140 cm; elution with 0.1 M PBS, pH 7.0; 30 mL/min<br>RPC: Develosil ODS-5, 4.6 × 250 mm; 0–25% B (B: ACN + 0.05% TFA) in 180 min; 1 mL/min; UV detection (220 nm)<br>RPC: Asahipack CG-320HQ, 7.6 × 300 mm; elution with 25% ACN in 50 mM $NH_4Ac$, pH 6.8; 0.5 mL/min | Suetsuna et al., 2004 |
| | | | **Fish** | |
| Tuna dark muscle | Digestion | Alcalase, neutrase, pepsin, papain, α-chymotrypsin or trypsin | UF: $M_{WCO}$ 3 kDa<br>IEC: HiPrep 16/10 DEAE FF; 0–2 M NaCl in NaAc (pH 4); 2.0 mL/min; UV detection (280 nm)<br>RPC: ODS C18 Primesphere 10, 20 × 250 mm; 0–50% B (B: ACN + 0.1% TFA) in 55 min; 2.0 mL/min; UV detection (215 nm)<br>RPC: Synchropak RPP-100, 4.6 × 250 mm; elution with 20% ACN + 0.1% TFA; 1 mL/min; UV detection (215 nm) | Qian et al., 2007a |
| Tuna | Digestion | Alcalase, α-chymotrypsin, papain pepsin, neutrase or trypsin | UF: $M_{WCO}$ 1, 5, 10 kDa<br>IEC: Hiprep 16/10 DEAE FF; 0–2 M NaCl in 20 mM NaAc, pH 4.0; 62 mL/h; UV detection (280 nm)<br>RPC: Primesphere 10 C18, 20 × 250 mm; 0–50% B (B: ACN + 0.1% TFA) in 20 min; 2 mL/min; UV detection (215 nm)<br>RPC: Synchropak RPP-100, 4.6 × 250 mm; elution with 15% ACN + 0.1% TFA; 1.2 mL/min; UV detection (215 nm) | Lee et al., 2010 |
| Upstream chum salmon muscle | Digestion | Thermolysin | RPC: ODS, Comsosil 140,C18- OPN, 44 × 370; elution with 10, 25, 50, 99.5% EtOH<br>SEC: Sephadex G-25, 16 × 650 mm; elution with water<br>RPC: Mightysil RP-18, 4.6 × 250 mm; elution with 10% ACN + 0.1% TFA; 1 mL/min; UV detection (220 nm) | Ono et al., 2003 |
| Salmon muscle | Digestion | Papain | Extraction with 1-butanol<br>Separation in silica gel; PSQ 100B, 1380 × 100 mm; elution with $CHCl_3$: MetOH: water: $CH_3COOH$ (65:25:4:1, 31 L)<br>Separation in silica gel, 400 × 80 mm; elution with $CHCl_3$:2-propanol:water: $CH_3COOH$<br>IEC: Amberlite CG50-type, 400 × 80 mm; elution with water; water:MetOH; MetOH; MetOH:2M HCl<br>RPC: XTerra MS C18, 100 × 4.6 mm; 5–30% B (B: ACN + 0.1% HFo) in 30 min; 0.2 mL/min<br>Methylation: 10% sodium methoxide in MetOH (50 mL); 16 h, RT; refluxing (4.5 h)<br>Separation in Silica gel 60, 600 × 20 mm; elution with $CHCl_3$:2-propanol at different ratios | Enari et al., 2008 |
| Dried bonito | Digestion | Pepsin, chymotrypsin, trypsin, thermolysin | RPC: YMC-Pack ODS-AQ, SH-343-5, 20 × 250 mm; 1–41% B (B: ACN + 0.1% TFA) in 40 min; 10 mL/min; UV detection (230 nm) | Yokoyama et al., 1991 |

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**Table 2** Purification of peptides with antihypertensive activity *(Continued)*

| Source of peptide | Release of peptides | Employed enzyme(s) or bacterial strains | Purification steps | Ref. |
|---|---|---|---|---|
| | | | RPC: Cosmosil 5Ph, 4.6 × 250 mm; 0–40% B (B: ACN + 0.1% TFA) in 40 min; 1 mL/min; UV detection (215 nm)<br>RPC: Cosmosil 5CN-R, 4.6 × 250 mm; 0–40% B (B: ACN + 0.1% TFA) in 40 min; 1 mL/min; UV detection (215 nm)<br>RPC: Cosmosil 5C18, 4.6 × 150 mm; 0–40% B (B: ACN + 0.1% TFA) in 40 min; 1 mL/min; UV detection (215 nm) | |
| Bonito bowels | Autolysis | Endogenous proteases | UF: $M_{wco}$ 6 kDa<br>Purification with Sep-Pak Plus C18; elution with 15% ACN<br>Purification with Toyopak IC-SP M; elution with 10 mM PBS, pH 9.0<br>RPC: RP-18(e), 100 mm × 250 mm; 0–30% B (B: ACN + 0.05% TFA); 4 mL/min; UV detection (210 nm)<br>RPC: RP-18(e), 4 mm × 250 mm; 0–30% B (B: ACN + 0.05% TFA); 1 mL/min; UV detection (210 nm)<br>SEC: Asahipak GS-220 and GS-320, 7.6 mm × 500 mm; 50 mM $NH_4Ac$; 1 mL/min; UV detection (210 nm)<br>IEC: SP-2SW, 4.6 mm × 250 mm; 0–0.5 M NaCl in 20 mM PBS, pH 6.0; UV detection (210 nm)<br>RPC: RP-18(e), 4 mm × 250 mm; elution with 7% ACN + 0.05% TFA; UV detection (210 nm) | Matsumura et al., 1993 |
| Alaska pollack (*Theragra chalcogramma*) skin | Digestion | Alcalase, pronase E and collagenase | SEC: Sephadex G-25, 2.5 × 90 cm; elution with water; 0.5 mL/min; UV detection (220, 280 nm)<br>CEC: SP-Sephadex C-25, 2.5 × 45 cm; 0–1 M NaCl in 20 mM NaAc, pH 4.0; 2 mL/min; UV detection (220, 280 nm)<br>SEC: Sephadex G-15; elution with water; 0.5 mL/min; UV detection (220, 280 nm)<br>RPC: ODS C18; 10–50% B in 40 min; A: 0.1% TFA; B: ACN + 0.1% TFA; 2 mL/min; UV detection (215 nm)<br>CE: Coated capillary, 24 cm × 25 $\mu$m; 0.1 M PBS, pH = 2.5; 10 kV; UV detection (200 nm) | Byun and Kim, 2001 |
| Yellowfin sole frame | Digestion | $\alpha$-chymotrypsin | UF: $M_{WCO}$ 5, 10, 30 kDa<br>IEC: SP-Sephadex C-25, 35 × 350 mm; 0–2 M NaCl in 20 mM NaAc, pH 4.0; 1 mL/min; UV detection (215 and 280 nm)<br>SEC: OHpak SB-803 HQ, 8.0 × 300 mm; 20 mM NaAc, pH 4.0; 1 mL/min; UV detection (215 nm)<br>RPC: SP Nucleosil 100- 7 C18, 1 × 250 mm; 0–19% B (B: ACN + 0.1% TFA) in 40 min; 2.0 mL/min; UV detection (215 nm)<br>RPC: Zorbax SB C18, 4.6 × 250 mm; 0–19% B (B: ACN + 0.1% TFA) in 30 min; 0.5 mL/min; UV detection (215 nm) | Jung et al., 2006 |
| Pacific hake fish | Autolysis | Endogenous proteases | UF: $M_{WCO}$ 1, 3, 10 kDa<br>RPC: Jupiter C12 Proteo 90 Å, 250 × 4.6 mm; 0–25% B in 25 min and 25–80% B in 5 min; water- 0.05% TFA-ACN; 1 mL/min; UV detection (214 nm) | Samaranayaka et al., 2010 |
| | | **Processed products and others** | | |
| Fermented soybean paste | Extraction | ———— | RPC: JAIGEL-A-343-10, 250 × 20 mm; 98% B in 5 min; 96% B in 20 min; 65% B in 30 min; 5 mL/min; B, ACN; UV detection (214 nm)<br>IEC: JAIGEL-ES-502CP, 20 × 100 mm; elution with 0.01M sodium succinate buffer (pH 4.3) in 20% ACN; 4 mL/min; UV detection (214 nm)<br>RPC: JAIGELODS-A-343-10, 250 × 20 mm; elution with 0.05% TFA/ACN (95:5, v/v); 5 mL/min; UV detection (214 nm) | Shin et al., 2001 |

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**Table 2** Purification of peptides with antihypertensive activity *(Continued)*

| Source of peptide | Release of peptides | Employed enzyme(s) or bacterial strains | Purification steps | Ref. |
|---|---|---|---|---|
| | | | IEC: Shodex Asahipak ES-2502N-7C, 100 × 7.6 mm; elution with 20 mM Tris-HCl (pH 7.5) + 125 mM NaCl; 1 mL/min; UV detection (214 nm) | |
| Salt-free soy sauce | Fermentation | *Aspergillus oryzae* | Extraction: Sep-Pak Plus C18; elution with 35% ACN + 0.1% TFA<br>RPC: Cosmosil 5C18-ARII, 4.6 × 250 mm; 5–35% B (B: ACN + 0.1% TFA) in 65 min; 0.4 mL/min; UV detection (220 nm)<br>RPC: Cosmosil 5C18-AR300, 4.6 × 250 mm; 5–35% B (B: ACN + 0.1% TFA) in 65 min; 0.3 mL/min; UV detection (220 nm) | Zhu et al., 2008 |
| Douchi | Fermentation and ripening | *Aspergillus egypticus* culture | Extraction/centrifugation (3,000g, 10 min)/filtration<br>SEC: Sephadex- G25, 10 × 750 mm; elution with PBS; 0.2 mL/min; UV detection (220 and 280 nm)<br>RPC: Vydac 218TP54; 0–60% B (B: ACN + 0.1% TFA) in 60 min; 1 mL/min; UV detection (220 nm) | Zhang et al., 2006 |
| Miso paste with addition of casein | Digestion | Porcine pepsin A | UF: $M_{WCO}$ 3 kDa<br>RPC: Prep Nova Paks HR C18, 300 × 7.8 mm; 0- 21% B in 30 min, 21- 35% B in 40 min, 35–70% B in 5 min; A: 0.1% TFA; B: ACN + 0.08% TFA; 4 mL/min; UV detection (214 nm) | Contreras et al., 2009 |
| Royal jelly[b] | Digestion | Pepsin, chymotrypsin, trypsin | SEC: Superdex Peptide HR 10/30, 1 × 30 cm; elution with 30% ACN + 0.1% TFA; 0.3 mL/min; UV detection (220 nm)<br>RPC: Cosmosil 5Ph, 4.6 × 250 mm; [a]5–35% B (B: ACN + 0.1% TFA) in 30 min and [b]1–40% B (B: ACN + 0.1% TFA) in 60 min; 0.3 mL/min; UV detection (220 nm)<br>RPC: Cosmosil 5C18-ARII, 4.6 × 250 mm; 5–35% B (B: ACN + 0.1% TFA) in 60 min; 0.3 mL/min; UV detection (220 nm) | Li et al., 2002<br>Matsui et al., 2002 |
| Egg | Simulated gastrointestinal digestion | Pepsin and pancreatin | UF: $M_{WCO}$ 3 kDa<br>CEC: HiPreP 16/10 SP FF, 16 × 100 mm; Eq.: 10 mM $NH_4Ac$ pH 4; Elution: 0.5 M $NH_4HCO_3$; 5 mL/min<br>SEC: Superdex peptide 10/300GL, 10 × 300–310 mm | Majumder and Wu, 2009 |

*- indicates papers in which not all data were shown

$M_{WCO}$ = membrane with molecular weight cutoffs; Eq. = equilibration; UF = ultrafiltration; IEC = Ion Exchange Chromatography; RPC = Reserved-Phase High Performance Liquid Chromatography; FPLC = Fast Protein Liquid Chromatography; CEC = Cation Exchange Chromatography; AEC = Anion Exchange Chromatography; SEC = Size Exclusion Chromatography; ACN = acetonitryl; TFA = trifluoroacetic acid; MetOH = methanol; EtoH-ethanol; RT = room temperature; PBS = phosphate buffer solution.



Generally, the first step in the purification process is separation based on the size of the peptide using either ultrafiltration (UF) or size-exclusion chromatography (SEC). UF is a low-pressure technique where the solution is processed through a semipermeable membrane and molecules are isolated by molecule size. Moreover, UF also enables the concentration and enrichment of fractions by the removal of the solvent. What is more, it is quite easy to use, it does not require special equipment, and can be used at cold room temperature (Schratter, 2004). UF enables the separation of small antihypertensive peptides from bigger molecules, such as unproteolyzed proteins and other interferences, thus in many cases it is the first step toward purification. Despite the fact that membranes with Mw in the range of 1–30 kDa have been tried, smaller cut-off membranes are preferred. For example, a hydrolysate of sea cucumber gelatin was subjected to UF using membranes with cut-offs of 10, 5, and 1 kDa observing $IC_{50}$ values of 0.72, 0.47, and 0.35 mg/mL, respectively. Based on the ACE inhibition activity, the fraction containing molecules smaller than 1 kDa were purified (Zhao et al., 2007). However, very low Mw cut-off membranes can sometimes result in a loss of activity (Miguel et al., 2009; Rho et al., 2009; Zhang et al., 2009; Samaranayaka et al., 2010). In fact, results obtained when a pacific hake protein hydrolysate was ultrafiltrated through membranes cut off at 10, 3, and 1 Mw indicated that the fraction with the highest ACE inhibition activity was that obtained when the hydrolysate passed through the 3 kDa cut-off membrane (Samaranayaka et al., 2010).

SEC (also known as Gel-Filtration Chromatography (GFC) when an aqueous solution system is used and Gel-Permeation

Chromatography (GPC) with a nonaqueous solution system) is also frequently used for the purification of peptides. SEC tends to be used at the beginning of the purification path, as is UF, as well as in the middle of protocols for removing interferences. SEC is quick, easy to use, universal, and compatible with physiological conditions. SEC is also useful for estimating the Mw range or for desalting. Nevertheless, the separation of a target peptide from a closely related peptide mixture is practically impossible and additional SEC separations using stationary phases with different pore diameters are needed (Sewald and Jakubke, 2009). Among SEC columns, porous silica base TSK-gel SW (Katayama et al., 2007; Katayama et al., 2008; Nakade et al., 2008) and polyhydroxymethacrylate base OHpak (Jung et al., 2006) are preferred. For low and medium pressure SEC, dextran base Sephadex or agarose/dextran base Superdex columns are mainly employed. Among Superdex columns, those with an Mw ranging from 100 to 7000 like the Peptide 10/300 GL column (Hatanaka et al., 2009; Majumder and Wu, 2009; Rho et al., 2009) and the Peptide HR 10/30 column (Abubakar et al., 1998; Saito et al., 2000; Li et al., 2002; Matsui et al., 2002; Tonouchi et al., 2008; Cheng et al., 2009) were mostly chosen. Regarding Sephadex columns, the ones most commonly used were Sephadex G-25 (Mw range, 1000–5000) and Sephadex G-15 (Mw $\leq$ 1500). Other less used Sephadex columns are G-50 (Mw range, 1500–30,000) (Je et al., 2005a), G-75 (Mw range, 30,000–80,000) (Je et al., 2005b), and G-200 (Mw range, 5000–250,000) (Tovar-Perez et al., 2009). Tovar-Perez et al. (2009) purified alcalase aramanth albumin and globulin protein hydrolysates using sequentially Sephadex G-200 and G-15 columns. Albumin hydrolysate eluted in 18 h in one broad peak ($M_r$ < 1.35 kDa) using the G-200 column while globulin hydrolysate eluted in 5 h in two separated fractions. Afterwards, fractions were individually separated in a G-15 column. The peaks corresponding to the albumin hydrolysate were observed at Mw of 4.70 and 0.55 kDa and peaks corresponding to the globulin hydrolysate were observed at Mw of 7.50, 4.70, 0.55, and 0.40 kDa (Tovar-Perez et al., 2009).

An alternative and complementary chromatographic mode for the purification of ACE inhibitory peptides is ion exchange chromatography (IEC). IEC is mainly employed as a further purification step after or between UF or SEC purification. Cation exchange resins with negatively charged groups like sulfopropyl (SP), methyl sulfonate (S), and carboxymethyl (CM) and anion exchange resins positively charged with quaternary ammonium (Q), quaternary aminoethyl (QAE) or DEAE (diethylaminoethyl) are mostly employed (Selkirk, 2004). Since antihypertensive peptides contain mainly hydrophobic amino acids, whose pIs are between 5 –and 7, both cation exchange (CEC) and anion exchange (AEC) can be employed. When AEC is used, the pH tends to be around 7.5 and binding peptides are negatively charged, while in CEC the pH is maintained at acidic level (4.0) to retain positively charged peptides. AEC purification methods focus more on columns with weak ion-exchange ligands, such as DEAE (Qian et al., 2007a; Lee et al., 2010) or DE (Katayama et al., 2007; Mao et al., 2007; Katayama et al., 2008), while CEC methods mainly prefer strong ion-exchange ligands as SP. In both cases, peptides are eluted by increasing the eluent ion strength using NaCl gradients at a constant pH (Herraiz, 1997). CEC with isocratic elution has also been possible through the use of a sodium succinate buffer in 20% ACN (Shin et al., 2001), an ammonia solution (Suetsuna, 1998; Suetsuna and Nakano, 2000; Chen et al., 2002; Suetsuna et al., 2004) or an ammonium carbonate buffer (Majumder and Wu, 2009).

Reserved-phase chromatography (RPC) is the dominant technique in the purification of peptides with antihypertensive activity (Herraiz, 1997). Generally, RPC is employed at the end of the purification protocol after UF, SEC or IEC separations. However, there are also examples in which this has been the only technique employed in the purification (Yokoyama et al., 1991; Maeno et al., 1996; Yano et al., 1996; Fujita et al., 2000; Chobert et al., 2005; Pan et al., 2005; Gouda et al., 2006; Lee et al., 2006a, 2006b; Papadimitriou et al., 2007).

There is a large number of RPC columns that can be used in the separation and purification of peptides where column support, bonded phase, pore size, particle size, and column dimension should be taken into consideration. Porous silica-based supports are the first choice since they offer good mechanical stability and a wide range of selectivity through the bonding of different phases. $C_4$-$C_{12}$ phases are typically used with high hydrophobic samples like large peptides and small hydrophilic proteins, while $C_{18}$ phases prefer slightly more hydrophilic analytes and are the perfect choice for small peptides. Moreover, phases such as cyano, hexyl, phenyl, hexyl/phenyl, perfluorinated are also available (Neville, 2004). Alternatively, polymeric reserved phases such as polystyrene divinylbenzene, withstand a wide range of pHs and have also been employed.

Mobile phases consist of mixtures of water with an organic modifier being acetonitrile and alcohols such as methanol, ethanol or isopropanol (Neville, 2004) the most popular options. Gradient elution by the increased concentration of the organic modifier is the option most commonly used. Mobile phases are usually prepared at acidic pH. Moreover, the addition of ion-pairing agents is also very useful to increase hydrophobicity of peptides, as it creates a pair with positively charged peptides. Trifluoroacetic acid (TFA) is usually the first choice because it is transparent to UV light, does not block amino groups (therefore, derivatization of peptides is possible), is highly volatile (therefore, it is easy to remove by lyophylization), and easily miscible with most organic mobile phases. Other ion-pairing agents like acetic acid, formic acid, phosphoric acid, heptafluorobutyric acid (HFBA) or quaternary ammonium salts can be alternatives to TFA.

Online detection during purification was mainly performed using UV absorption at 210–220 nm (absorbance wavelength of peptide bonds). Moreover, in some cases wavelengths of 254 nm, where phenylalanine residues absorbs, and 275–280 nm, where aromatic residues (tyrosine, tryptophan) absorb, are also employed (Herraiz, 1997).

Moreover, additional steps involving liquid–liquid extraction, desalination or dialysis are also employed for the

purification of antihypertensive peptides. Desalination of samples is usually conducted by electrodialysis (Je et al., 2005a, 2005b; Kodera and Nio, 2006; Nakahara et al., 2010) or by solid-phase extraction (Katayama et al., 2007; Katayama et al., 2008; Muguruma et al., 2009).

### *IDENTIFICATION AND CHARACTERIZATION OF BIOACTIVE PEPTIDES FROM FOODSTUFFS*

Isolated and purified peptides possessing the most potential antihypertensive activity at the end of the framework (see Figure 2) are next identified and characterized. Characterization mostly involves the determination of the amino acid sequence and the $IC_{50}$ value. Moreover, in some cases, additional information like the amino acid composition, molecular weight, molecular weight distribution, peptide content, molecular structure, and purity are also determined. Table 3 summarizes the peptides that have been identified from foodstuffs and the kind of characterization that has been performed.

Amino acid sequence determination can be carried out by mass spectrometry (MS) or by Edman degradation sequencing. Edman degradation is based on the sequential elimination of N-terminal amino acids by chemical procedures. However, this method is time consuming and requires highly purified samples (free of salts, detergents, and nonvolatile additives such as urea). Edman degradation can be performed manually or it can be fully automated using special automated protein/peptide sequencers (Gouda et al., 2006; Lee et al., 2006a, 2006b; Papadimitriou et al., 2007; Kuba et al., 2009; Rho et al., 2009).

Mass spectrometry (MS) is a powerful technique widely employed for the characterization of bioactive peptides. In addition to the amino acid sequence, MS can also yield accurate information on molecular masses, peptide purity or posttranslational modifications, etc. (Herraiz, 1997). MALDI (matrix assisted laser desorption and ionization), ESI (electrospray ionization) and, less frequently, FAB (Fast Atom Bombardment) have been the ionization sources employed. LC and, less frequently, capillary electrophoresis (CE) are sometimes needed previous to the MS analysis. An alternative technique to determine molecular structure is NMR. NMR has been used for tripeptides of salmon muscle hydrolysate ($^1$H NMR) (Enari et al., 2008) and dipeptides of steamed soybean mixed with roasted wheat hydrolysate ($^1$H NMR and $^{13}$C NMR) (Nakahara et al., 2010).

Another strategy for peptide characterization is to determine its amino acid composition. This is determined by the chemical hydrolysis of peptides and the amino acid analysis using an automatic analyzer. Other options for the amino acid analysis have been peptide hydrolysis with HCl and phenol, followed by RPC separation and UV detection (Saito et al., 2000; Murakami et al., 2004).

Although a full relationship between structure and the ACE inhibitory properties of antihypertensive peptides has still not been established, it is important to highlight some common features for antihypertensive peptides. In addition to low molecular weight and short sequences (2–12 amino acid residues (Murray and FitzGerald, 2007)), antihypertensive peptides contain a significant amount of hydrophobic amino acids especially at C-terminal position (Meisel, 1997). The presence at C-terminal position of proline (P) or a positive charge of lysine (K) ($\varepsilon$- amino group) or arginine (R) (guanidine group) enhances the potency of antihypertensive peptides (Meisel, 1997; Kitts and Weiler, 2003; Murray and FitzGerald, 2007; Hernández-Ledesma et al., 2008). This fact could be related to the bioavailability of antihypertensive peptides since it has been demonstrated that peptides including proline at the end of the sequence are particularly resistant to in vivo proteolysis (Quiros et al., 2008).

Nevertheless, the presence of in vitro antihypertensive activity of isolated peptides does not involve the activity in vivo. In fact, orally administered peptides need to reach the target cardiovascular system in an active form. Before reaching the cardiovascular system, however, orally delivered peptides have to resist the gastrointestinal tract digestion and be transported in bioactive form (Vermeirssen et al., 2004). Primary digestion of peptides starts in the stomach with the action of pepsin in acidic conditions. Following that, peptides are digested in the luminal phase of the small intestine at an alkaline pH by the action of pancreatic proteases like trypsin, $\alpha$-chymotrypsin, elastase, and carboxypeptidase A and B (Vermeirssen et al., 2004). Next, peptides resisting gastrointestinal digestion are subjected to the intestinal brush border membrane where a variety of peptidases can further hydrolyze the ACE inhibitory peptide. Generally, peptides resisting this step can be transported to the blood circulation (Pihlanto-Leppala, 2000).

In order to demonstrate peptide bioavailability, additional (bio)chemical characterization is needed. Several measurements of the stability of the purified antihypertensive peptides against gastrointestinal enzymatic digestion can be carried out. The pure peptide can be submitted to a simulated gastrointestinal digestion using different enzyme systems. A combination of trypsin–chymotrypsin (Rizzello et al., 2008), pepsin-pancreatin (Robert et al., 2004), pepsin–corolase PP (Quiros et al., 2005), pepsin–trypsin (Koo et al., 2006) or pepsin-trypsin-protease N (Hyoung et al., 2004) enzymes have been employed for this purpose. This procedure has been assayed with peptides isolated from sea cucumber (Zhao et al., 2009), rice (Kuba et al., 2009), oyster (Wang et al., 2008a), porcine hemoglobin (Yu et al., 2006), and wakame (Sato et al., 2002) hydrolysates. Resistance to intestinal digestion can also be demonstrated by the use of a model system such as Caco-2 cells. Caco-2 cells in a monolayers format display a variety of intestinal enzymes and transporters and have been employed as a model of intestine epithelium (Lopez-Fandiño et al., 2006). Geerlings et al. (2006) purified three peptides (TGPIPN, SLPQ, and SQPK) from goat milk hydrolysate, which all had similar $IC_{50}$ values. All peptides were subjected to the Caco-2 monolayer experiment but only TGPIPN was found to pass through the Caco-2-monolayer intact (albeit in a small quantity). Therefore, the intake of goat milk

**Table 3** Characterization of purified peptides with antihypertensive activity

| Source of peptide(s) | Identified peptide(s) | $IC_{50}$ [units] | Characterization | Ref. |
|---|---|---|---|---|
| Goat milk | TGPIPN, SLPQ, SQPK | 316, 330, 354 [$\mu$M] | Edman degradation sequencing | Geerlings et al., 2006 |
| Bonito bowels | YRPY, GHF, VRP, IKP, LRP, IRP | 320, 1100, 2.2, 2.5, 1.0, 1.8 [$\mu$M] | Automated protein sequencing by Edman degradation | Matsumura et al., 1993 |
| Yoghurt | 8 peptides | 1.56–12.41 [$\mu$g/mL] | Automated protein/peptide sequencing by Edman degradation | Donkor et al., 2007 |
| Beef rump | VLAQYK | 23.2 [$\mu$g/mL] | Peptide sequencing by Edman degradation | Jang and Lee, 2005 |
| Spinach Rubisco | MRWRD, MRW, LRIPVA, IAYKPAG | 2.1, 0.6, 0.38, 4.2 [$\mu$M] | Automated protein sequencing by Edman degradation | Yang et al., 2003 |
| Cheese whey protein | VYPFPG, GKP, IPA, FP, VYP, TPVVVPPFLQP | 221, 352, 141, 315, 288, 749 [$\mu$M] | Automated protein sequencing by Edman degradation | Abubakar et al., 1998 |
| Rice | IY, VVY, VF, VW | 4.0, 22.0, 49.7, 3.1 [$\mu$M] | Gas/liquid phase protein sequencing by Edman degradation | Kuba et al., 2009 |
| Porcine myosin B | KRVIQY, VKAGF | 6.1, 20.3 [$\mu$M] or 4.9, 10.6 [$\mu$g/mL] | Protein sequencing | Muguruma et al., 2009 |
| Porcine skeletal muscle troponin | EKERERQ, KRQKYDI | 552.5, 26.2 [$\mu$M] | Protein sequencing | Katayama et al., 2008 |
| Salt-free soy sauce | AF, FI, IF | 165, NI, 65.8 [$\mu$mol/L] | Protein sequencing | Zhu et al., 2008 |
| Chicken bone | YYRA | 33.9 [$\mu$g/mL] | Protein sequencing | Nakade et al., 2008 |
| Pork loin | VKKVLGNP | 28.5 [$\mu$M] | Protein sequencing | Katayama et al., 2007 |
| Casein | 10 peptides | 22-> 1000 [$\mu$M] | Protein sequencing | Maeno et al., 1996 |
| Dried bonito | 8 peptides | 3.7–62 [$\mu$M] | Protein sequencing | Yokoyama et al., 1991 |
| Chicken muscle | LKA, LKP, LAP, IKW, FQKPKR, FKGRYYP, IVGRPRHQG | 0.32–14 [$\mu$M] | Protein sequencing | Fujita et al., 2000 |
| Ovalbumin | FGRCVSP, ERKIKVYL, FFGRCVSP, LW, FCF, NIFYCP | 0.4–15 [$\mu$M] | | |
| Bovine lactoferrin | LRPVAA | 4.14 [$\mu$M] | Gas-phase sequencing | Lee et al., 2006a |
| Hen ovotransferrin | KVREGTTY | 102.8 [$\mu$M] | Gas-phase sequencing | Lee et al., 2006b |
| Sheep milk yoghurt | 12 peptides | ———— | Liquid-phase protein/peptide sequencing | Papadimitriou et al., 2007 |
| Miso paste with casein | RYLGY; AYFYPEL; YQKFPQY | 0.71, 6.58, 20.08 [$\mu$M] | Amino acid sequencing by RP-HPLC-MS/MS | Contreras et al., 2009 |
| Caprine Kefir | PYVRYL, LVYPFTGPIPN | 2.4, 27.9 [$\mu$M] | Amino acid sequencing by RP-HPLC-MS/MS | Quiros et al., 2005 |
| Milk | LHLPLP | ———— | Amino acid sequencing by HPLC-MS/MS | Quiros et al., 2006 |
| Manchego cheese | 75 peptides | 13.4–> 1000 [$\mu$M] | Amino acid sequencing by HPLC-MS/MS | Gómez-Ruiz et al., 2004 |
| Milk | 40 peptides* | ———— | Amino acid sequencing by HPLC-MS/MS | Hernández-Ledesma et al., 2004 |
| White wheat, wholemeal wheat, rye flours | 14 peptides | 0.19–0.45 [mg/mL] | Amino acid sequencing by nano LC-ESI-MS/MS | Rizzello et al., 2008 |
| Ovine $\beta$-lactoglobulin from skimmed milk | 21 peptides** | 30–71.2 [%] | Amino acid sequencing by LC-MS/MS | Chobert et al., 2005 |
| Fermented milk | 27 peptides | ———— | Amino acid sequencing by LC-MS/MS | Nielsen et al., 2009 |
| Soybean protein | 8 peptides | 21- > 10000 [$\mu$M] | Amino acid sequencing by ESI-MS/MS | Kodera and Nio, 2006 |
| Sodium caseinate | 21 peptides | 39- > 1000 [$\mu$M] and 15–650 [$\mu$M] | Amino acid sequencing by ESI- MS/MS | Robert et al., 2004 |

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**Table 3** Characterization of purified peptides with antihypertensive activity *(Continued)*

| Source of peptide(s) | Identified peptide(s) | $IC_{50}$ [units] | Characterization | Ref. |
|---|---|---|---|---|
| Marine shrimp *(Acetes chinensis)* | DP, GTG, ST | 2.15, 5.54, 4.03 [$\mu$M] | Mw determination and amino acid sequencing by ESI-MS/MS | Wang et al., 2008b |
| Yak milk casein | YQKFPQY, LPQNIPPL, SKVLPVPQK, LPYPYY, FLPYPYY | ———— | Mw determination and amino acid sequencing by ESI-MS/MS | Jiang et al., 2007 |
| Egg | VDF, LPF, MPF, YTAGV, ERYPI, IPF, TTI | 6.59–27.38 [$\mu$M] | Amino acid sequencing by LC-ESI- MS/MS | Majumder and Wu, 2009 |
| Several Spanish cheeses | 41 major peptides** | 113.1–2419.4 [$\mu$M] | Amino acid sequencing by RPC and off-line MS/MS | Gómez-Ruiz et al., 2006 |
| Ovine milk | IAK,VR, EKDERF, KDERF, YIPIQY, LPYPY | 10.0–848.0 [$\mu$M] | Amino acid sequencing by CE-ESI-IT-MS | Gómez-Ruiz et al., 2007 |
| Milk | 8 peptides | 5.2- >1500 [$\mu$M] | Amino acid sequencing by ESI-Q-IT-MS | Quiros et al., 2007a, 2007b |
| Porcine hemoglobin | LGFPTTKTYFPHF, VVYPWT | 4.92, 6.02 [$\mu$M] | Amino acid sequencing by MALDI-TOF/MS and ESI-MS/MS | Yu et al., 2006 |
| Enzyme –modified cheese | 13 peptides | ———— | Mw and amino acid sequencing by API-MS | Haileselassie et al. 1999 |
| Salmon muscle | 20 di- and tri-peptides | Dipeptides: 1.2-86% Tripeptides: 7.5-59% | Amino acid sequencing by LC-ESI-MS and $^1$H NMR | Enari et al., 2008 |
| Chicken leg bone | GAVGPSG, AVKQPAVVYP, AATENM, DMSVF, EGGPKP, ANSSIL, AITAKL, IGNTLI, NLAPFL, EIAKLM | ———— | Amino acid sequencing by LC/MS/MS | Cheng et al., 2009 |
| Gelatin of sea cucumber | ———— | 0.0142 [mg/mL] | Hydrolysis/derivatization/Automatic amino acid analysis, Mw distribution by ESI-IT-MS | Zhao et al., 2007 |
| Corn gluten meal | AY | 14.2 [$\mu$M] | Amino acid sequencing by HPLC-ESI-MS and determination of amino acid composition by hydrolysis/OPA derivatization/fluorescence detection | Yang et al., 2007 |
| Rice | TQVY | 18.2 [$\mu$M] | Determination of amino acid composition by hydrolysis/OPA derivatization/automatic amino acid analysis and amino acid sequencing by MALDI-TOF-MS/MS | Li et al., 2007 |
| Garlic (*Allium sativum* L) | SY, GY, FY, NY, SF, GF, NF | 66.3, 72.1, 3.74, 32.6, 130.2, 277.9, 46.3 [$\mu$M] | Determination of amino acid composition by hydrolysis/amino acid analysis and automatic protein sequencing by Edman degradation and FAB-MS | Suetsuna et al., 1998 |
| Wakame (*Undaria pinnatifida*) | AIYK, YKYY, KFYG, YNKL | 213, 64.2, 90.5, 21 [$\mu$M] | Determination of amino acid composition by hydrolysis/amino acid analysis and automated protein sequencing by Edman degradation and FAB-MS | Suetsuna and Nakano, 2000 |
| $\alpha$-zein | Among 3 with high activity: LRP, LSP, LQP | 0.29, 1.7, 2.0 [$\mu$M] | Determination of amino acid composition by hydrolysis/amino acid analysis and automated protein sequencing by Edman degradation and FAB-MS | Miyoshi et al., 1991 |
| Soybean | IA, YLAGNQ, FFL, IYLL, VMDKPQG | 153, 14, 37, 42, 39 [$\mu$M] | Determination of amino acid composition by hydrolysis/amino acid analysis and automated protein sequencing by Edman degradation | Chen et al., 2002 |
| Wheat gliadin | IAP | 2.7 [$\mu$M] | Determination of amino acid composition by hydrolysis/amino acid analysis and automated protein sequencing by Edman degradation | Motoi and Kodama, 2003 |
| Royal jelly | FY, KF, IF, IVY, IMY, DGL, TKY, LTF, FNF, AVL, GLY | 1.67–930 [$\mu$M] | Determination of amino acid composition by hydrolysis/amino acid analysis and automatic protein sequencing by Edman degradation | Matsui et al., 2002 |
| Yoghurt- like product | YP | 720 [$\mu$M] | Determination of amino acid composition by hydrolysis/amino acid analysis and automated protein sequencing by Edman degradation | Yamamoto et al., 1999 |

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**Table 3** Characterization of purified peptides with antihypertensive activity *(Continued)*

| Source of peptide(s) | Identified peptide(s) | $IC_{50}$ [units] | Characterization | Ref. |
|---|---|---|---|---|
| Buckwheat | VK, FY, AY, LF, YV, YQ, YQY, PSY, LGI, ITF, INSQ | 4–628 [$\mu$M] | Determination of amino acid composition by HPLC and automated protein sequencing by Edman degradation | Li et al., 2002 |
| Urea denaturated Z19 $\alpha$-zein | 17 peptides** | 1.9- 57 [$\mu$M] | Determination of amino acid composition and automated protein sequencing by Edman degradation | Yano et al., 1996 |
| Urea denaturated total $\alpha$-zein | 27 peptides** | 3.9- 100 [$\mu$M] | | |
| Mung bean protein | KDYRL, VTPALR, KLPAGTLF | 26.5, 82.4, 13.4 [$\mu$M] | Determination of amino acid composition by hydolysis/derivatization/automatic amino acid analysis, determination of Mw and amino acid sequencing by MALDI-TOF MS | Li et al., 2006 |
| Oyster | ———— | 0.0874 [mg/mL] | Mw determination by SEC | Je et al., 2005a |
| Yak milk casein | PPEIN, PLPLL | 0.29, 0.25 [mg/mL] | Mw determination by LC-MS and amino acid sequencing by LC-ESI-MS/MS | Mao et al., 2007 |
| Soybean | LVQGS | 22 [$\mu$g/mL] (43.7 [$\mu$M]) | Mw determination by MALDI-TOF-MS and liquid-phase peptide sequencing by Edman degradation | Rho et al., 2009 |
| Porcine skeletal muscle | MNPPK, ITTNP | 945.5, 549 [$\mu$M] | Mw determination by FAB-MS and automated protein sequencing by Edman degradation | Arihara et al., 2001 |
| Manchego cheese | 22 peptides | 23.7- 100 [%] | Mw determination and amino acid sequencing by ESI-MS/MS | Gómez-Ruiz et al., 2002 |
| Alfalfa white protein | VW | 1.1 [$\mu$M] | Mw determination and amino acid sequencing by ESI-MS | Kapel et al., 2006 |
| Tuna dark muscle | WPEAAELMMEVDP | 21.6 [$\mu$M] | Mw determination and amino acid sequencing by ESI-MS | Qian et al., 2007a |
| Bullfrog (*Rana catesbeiana* Shaw) muscle | GAAELPCSADWW | 0.95 [$\mu$M] | Mw determination and amino acid sequencing by ESI-MS | Qian et al., 2007b |
| Sea cucumber (*Acaudina molpadioidea*) | MEGAQEAQGD | 15.9 [$\mu$M] | Mw determination and amino acid sequencing by (nano) ESI-MS/MS | Zhao et al., 2009 |
| Tuna | GDLGKTTTVSNWSPPKY KDTP | 11.28 [$\mu$M] | Mw determination and amino acid sequencing by ESI-Q-TOF-MS | Lee et al., 2010 |
| Pork meat | ER, EPR, PER, KLP, AGLP, GPR, NVR, PGR, VGPR, RPR, PAGPR, PAGPVG | 382- >1000 [$\mu$M] | Amino acid identification by nano LC-ESI-MS/MS | Escudero et al., 2010 |
| | MMVPI, IGGSI, KAPVA, PTPVP, YPGIA, NIIPA, MYPGIA, VIPEL, INDPF, VLPEI | 46.56- >1000 [$\mu$M] | Amino acid sequencing and Mw determination by MALDI-TOF/TOF | |
| Wheat bran | LQP, IQP, LRP, VY, IY, TF | 2.2, 3.8, 0.21, 21, 3.4, 18 [$\mu$M] | Mw determination by MALDI-TOF-MS and automatic protein sequencing-HPLC | Nogata et al., 2009 |
| *Pholiota adiposa* | GEGGP | 254 [$\mu$M] | Mw determination by MALDI-MS and automated protein sequencing by Edman degradation | Koo et al., 2006 |
| Yellowfin sole frame | MIFPGAGGPEL | 28.7 [$\mu$g/mlL | Mw determination by SEC and automated protein sequencing by Edman degradation | Jung et al., 2006 |
| Blue mussel (*Mytilus edulis*) | EVMAGNLYPG | 19.34 [$\mu$g/mL] | Mw determination by SEC and automated protein sequencing by Edman degradation | Je et al., 2005b |
| Oyster (*Crassostrea talienwhanensis Crosse*) | VVYPWTQRF | 66 [$\mu$mol/L] | Mw determination by LC-MS (LC-APCI-QQQ-MS) and automated protein sequencing by Edman degradation | Wang et al., 2008a |

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**Table 3** Characterization of purified peptides with antihypertensive activity *(Continued)*

| Source of peptide(s) | Identified peptide(s) | $IC_{50}$ [units] | Characterization | Ref. |
|---|---|---|---|---|
| Chicken leg | GA(Hyp)GLHypGP, GA(Hyp)GPAGPGGI(Hyp) GERG, GL(Hyp)GSRGE RGL(Hyp)G, GI(Hyp) GERGPVGPSG | 29.4, 45.6, 60.8, 43.4 [$\mu$M] | Mw determination by LC-ESI-Q-MS and protein sequencing | Saiga et al., 2008 |
| Skimmed milk | VPP, IPP | 9.13, 5.15 [$\mu$M] | Determination of peptide content, amino acid content by hydrolysis/amino acid analysis and automated protein sequencing by Edman degradation | Pan et al., 2005 |
| Steamed soybean mixed with roasted wheat | AW, GW, AY, SY, GY, AF, VP, AI, VG | 10–1100 [$\mu$g/mL] | Amino acid analysis and determination of molecular structure by $^{1}$H NMR; $^{13}$C NMR; LC-MS | Nakahara et al., 2010 |
| Antarctic krill (*Euphausia superba*) tail meat | VW, LKY, ITRY, VFER | 12.9, 10.1, 236.9, 152.8 [$\mu$M] or 2.75, 4.26, 130.7, 84 [$\mu$g/mL] | Mw determination by SEC, quantitation of peptides by UPLC-ESI-MS, and protein sequencing | Hatanaka et al., 2009 |
| $\alpha$-lactalbumin and $\beta$-casein | 9 peptides | 1->76 [$\mu$M] | Amino acid sequencing by LC-MS/MS and automatic Edman degradation | Otte et al., 2007b |
| Glycinin from soybean | VLIVP | 1.69 [$\mu$M] | Determination of amino acid composition by hydrolysis/derivatization/HPLC, Mw by MALDI-TOF, and gas- phase protein sequencing by Edman degradation | Gouda et al., 2006 |
| Alaska pollack (*Theragra chalcogramma*) skin | GPM, GPL | 17.13, 2.65 [$\mu$M] | Determination of Mw distribution by SEC, amino acid composition by hydrolysis/amino acid analysis, and automated protein sequencing by Edman degradation | Byun and Kim, 2001 |
| Cheese | LQP, MAP | 3.4, 0.8 [$\mu$M] | Determination of Mw by LC/MSD, amino acid composition by hydrolysation/inspissation/amino acid analysis, and automated protein sequencing by Edman degradation | Tonouchi et al., 2008 |
| Gouda cheese | RPKHPIKHQ, RPKHPIKHQGLPQ, YPFPGPIPN, MPFPKYPVQPF | 13.4, –, 14.8,- - [$\mu$M] | Determination of Mw by FAB-MS, amino acid composition by hydrolysis/HPLC and protein sequencing | Saito et al., 2000 |
| Sesame protein hydrolysate | LVY, LSA, LQP, LKY, IVY, VIY, MLPAY | 0.33–5.80 [$\mu$g/ mL] | Determination of amino acid composition by hydrolysis/amino acid analysis, peptide content by LC/MS/MS and protein sequencing by TOF-MS/MS | Nakano et al., 2006 |
| Wakame (*Undaria pinnatifida*) | 10 dipeptides YH, KW, KY, KF, FY, VW, VF, IY, IW, VY | 2.7- 43.7 [$\mu$mol/l] | Determination of Mw by FAB-MS, amino acid composition by hydrolysis/amino acid analysis, and automated protein sequencing by Edman degradation | Suetsuna et al., 2004 |
| Commercial whey product | ALPM | 928 [$\mu$M] | Determination of Mw by FAB-MS, amino acid composition by hydrolysis/HPLC, and protein sequencing by automatic Edman degradation | Murakami et al., 2004 |
| Mushroom *Tricholoma giganteum* | GEP | 0.04 [mg] | Determination of Mw by LC-MS, amino acid composition by hydrolysis/fluorometric analysis, and automated protein sequencing by Edman degradation | Hyoung et al., 2004 |
| Fermented soybean paste | HHL | 2.2 [$\mu$g/mL] | Determination of Mw by SEC, amino acid composition by HPLC, protein sequencing | Shin et al., 2001 |
| Upstream chum salmon muscle | WA, VW, WM, MW, IW, LW | 2.5- 277.3 [$\mu$M] | Determination of Mw by ESI-MS, amino acid composition by hydrolysis/amino acid analysis and automated protein sequencing by Edman degradation | Ono et al., 2003 |

IEC = Ion Exchange Chromatography; SEC = Size Exclusion Chromatography; MSD = mass selected detector; NI = no inhibition; OPA = O-phthalaldehyde; (*)- in the paper authors did not show which peptides are antihypertensive although optimization of the fermentation procedure in order to obtain them was based on ACE inhibitory activity; (**)- among identified peptides only for selected the ACE inhibitory activity were measured; (***)- in review one letter abbreviations for amino acids were adopted, however due to the lack of abbreviation for a nonprotein amino acid in this system, three letter abbreviation was used: hydroxyproline (Hyp).

hydrolysate by SHR over 12 weeks had resulted in a decrease of SBP (Geerlings et al., 2006). Nevertheless, it is important to highlight that this model could yield erroneous conclusions since the Caco-2 model is tighter than intestinal mammalian tissue. Therefore, some molecules exerting in vivo activity could not show sufficient absorption in this model (Vermeirssen et al., 2005).

Furthermore, since ACE cleaves the C-terminal of oligopeptides with wide specificity, antihypertensive peptides reaching the cardiovascular system also need to resist ACE action. In relation to this fact, peptides can be divided into three groups: inhibitor type, substrate type, and pro-drug type (Fujita et al., 2000). "*Inhibitor type*" peptides are not affected when they are preincubated with ACE. "*Substrate type*" peptides show a decrease in activity when they are exposed to ACE whereas "*Prodrug type*" peptides are transformed to a true inhibitor by ACE or gastrointestinal proteases (Li et al., 2004; Vermeirssen et al., 2004). True inhibitor type (Yang et al., 2007; Tonouchi et al., 2008; Zhao et al., 2009), substrate type (Katayama et al., 2007; Katayama et al., 2008), and prodrug inhibitor (Lee et al., 2006c) peptides have been found in different hydrolysates.

One of the attempts to understand the inhibition site and to explore the inhibition mechanism of antihypertensive peptides is the measurement of the inhibition mode of peptides. The overall pattern for ACE inhibition was investigated by the incubation of inhibitory peptides with different concentrations of HHL and by measuring of the ACE inhibitory activity. The majority of antihypertensive peptides inhibit ACE following a competitive mode although noncompetitive inhibition has also been found (Li et al., 2004). Structure-activity correlation is influenced by the three C-terminal residues of the antihypertensive peptide where the substrate or competitive inhibitors, which constantly contain hydrophobic (aromatic or branched-side chains) residues, are preferred. However, the most favorable are aromatic amino acid residues and proline (Li et al., 2006). Competitive ACE inhibitor peptides have been found inter alia in porcine skeletal muscle troponin (Katayama et al., 2008), soybean glycinin (Gouda et al., 2006), *Pholiota adiposa* (Koo et al., 2006), oyster (Je et al., 2005a), mushroom *Tricholoma giganteum* (Hyoung et al., 2004), and porcine hemoglobin (Yu et al., 2006) hydrolysates. Noncompetitive ACE inhibitor peptides have been found in oyster (Wang et al., 2008a), tuna dark muscle (Qian et al., 2007a), bullfrog muscle (Qian et al., 2007b), pork loin (Katayama et al., 2007), bovine lactoferrin (Lee et al., 2006a), and hen ovotransferrin hydrolysates (Lee et al., 2006b).

## *QUANTIFICATION OF PEPTIDES WITH ANTIHYPERTENSIVE ACTIVITY*

Since the first discovery of antihypertensive peptides from foodstuffs, studies in the area of ACE inhibitory peptides were mainly focused on isolation, purification, identification, and characterization of these peptides. In the last years, a new trend has been the quantitative analysis of particular peptides with high $IC_{50}$ values. In fact, the recent rapid development of functional foods, which contain antihypertensive peptides, requires established standardized methodologies for the quantification of peptides including stability studies in complex biological matrices. In this case, quantitative determination of antihypertensive peptides is essential to assess safety, product activity, and healthy claims (Contreras et al., 2008; Gilani et al., 2008). Furthermore, the amount of encrypted peptides could vary within crop varieties and the amount of released peptides could depend on hydrolysis and storage conditions (Papadimitriou et al., 2007; Quiros et al., 2007a). Moreover, considering that functional foods could become widespread, quantitative information will be essential to establish regulations controlling the addition of antihypertensive peptides to commercial foodstuffs (Contreras et al., 2008; Gilani et al., 2008).

Generally, quantification of selected antihypertensive peptides is carried out through MS with previous HPLC separation; however, some other attempts can also be found in literature. The quantification of particular peptides, which possess antihypertensive activity, was made on the standard calibration curve of corresponding synthetic peptides injected into the LC-MS system. Through this methodology, the concentration of seven dipeptides in wakame (Sato et al., 2002) and eight dipeptides in salmon muscle (Enari et al., 2008) hydrolysates were estimated. Figure 3 shows the chromatograms and the mass spectra corresponding to the antihypertensive peptide FY in a synthetic standard and in the hydrolysate of wakame. Since the mass spectrum obtained with the synthetic peptide was identical to that observed in the hydrolysate, this was used for the quantitation of the peptide in wakame by LC-MS (Sato et al., 2002). A similar approach has also been used for the determination of three peptides in goat milk hydrolysate (Geerlings et al., 2006). Quantification of LHLPLP peptide in fermented milk has been performed by HPLC-MS and HPLC-MS/MS. The developed method was validated by the determination of repeatability, reproducibility, linearity, and recovery. Calibration was performed based on the peak areas of the precursor and its adducts in the MS experiments and on the peak area of the most abundant product ions after precursor fragmentation by MS/MS analysis. The limits of detection and quantification determined by MS/MS were 7 $\mu$g/mL and 25 $\mu$g/mL, respectively (Quiros et al., 2006). Similarly, the LKPNM antihypertensive peptide was determined in bonito muscle hydrolysates by HPLC-MS and HPLC-MS/MS. Validation of the method by measuring specificity, linearity, accuracy, precision, and reproducibility was also presented (Curtis et al., 2002). Next, the quantification of nine antihypertensive dipeptides in fermented soybean seasonings and soybean sauces was performed by LC-MS/MS (Nakahara et al., 2010). A comparative study of the concentration of IPP and VPP in Swiss cheeses and non-Swiss cheeses (Butikofer et al., 2007) and in cheeses with different ripening degrees (Meyer et al., 2009) using HPLC-$MS^3$ and PPPP as an internal standard revealed that there were large variations among individual loaves from various producers. Moreover, high concentrations of both peptides

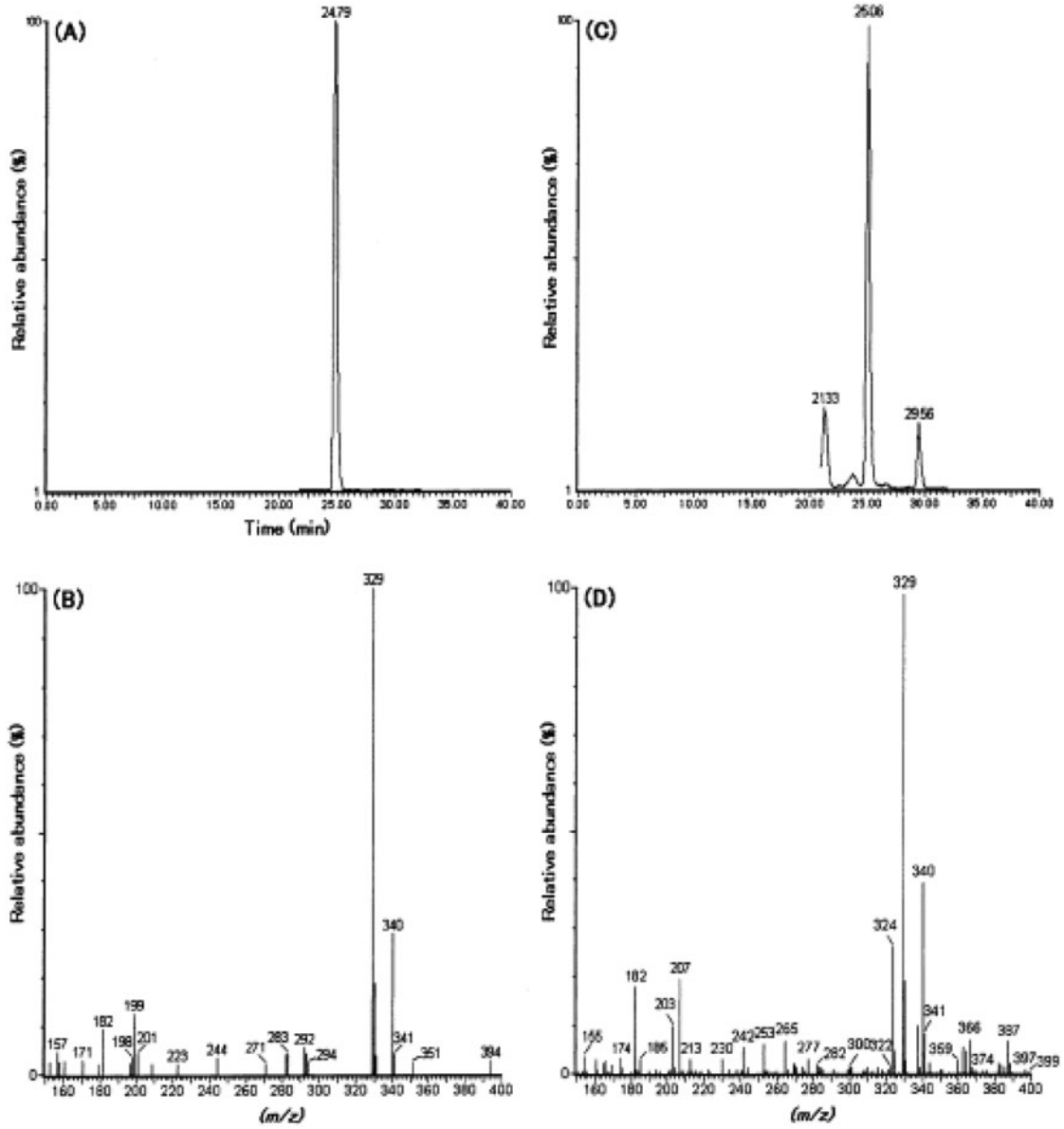


**Figure 3** Chromatograms (A, C) and mass spectra (B, D) obtained by LC-MS and corresponding to FY: (A, B) synthetic FY; (C, D) FY in the hydrolysate of wakame. LC conditions: Column, Xterra MS C18, 150 × 2.1 mm; gradient, 3–20% in 40 min; mobile phases, A: water + 0.05% TFA; B: ACN+0.05% TFA; flow-rate, 0.2 mL/min; MS conditions: cone voltage: +30 V; capillary voltage: 3 kV; desolvation temp.: 300°C; source block temp.: 100°C; desolvation gas flow: 350 L/min; cone gas flow: 50 L/min (Sato et al., 2002).

often occurred in these cheeses produced from raw milk that was matured over a long period of time. The same peptides were also quantified in miso paste by LC-MS using internal standard methodology. In this case, isotopes ($^{13}C_5$)Val($^{13}C_5$)Pro-Pro and Ile-($^{13}C_5$)Pro-Pro were involved (Inoue et al., 2009). HPLC with UV detection has also been employed for the quantitation of antihypertensive peptides in foodstuffs. Yamamoto et al. (1999) used it for the quantitation of YP in yoghurt-like products using a synthetic peptide for the calibration (Yamamoto et al., 1999). The same methodology was employed in the quantification of IY, VY, and IVY in Protease N-treated Royal Jelly (Tokunaga et al., 2004).

Moreover, targeted peptides are usually presented in highly complex matrices and at a low concentration. These complex matrices could compromise the determination of these minor components. Multiple reaction monitoring (MRM) assays could be the technique of choice in these cases. Nevertheless, only one work in which antihypertensive peptides were quantified in rat plasma after their administration was found (Nakashima et al., 2011). To our knowledge, there is no work that had used this methodology to quantify antihypertensive peptides in foodstuffs. Since the MRM assay offers reliable quantification for low abundance analytes in complex matrices, it can be a potential tool for overcoming these future challenges.

## *CONCLUSIONS*

The role of antihypertensive peptides derived from foodstuffs becomes increasingly appreciated since hypertension is a serious problem, especially in highly developed countries. The knowledge about ACE inhibitory peptides in the last years improved at the same rate as the specifications of their biochemistry, bioavailability, properties, and mechanisms of inhibition. In addition to this, the number of identified peptides with certain ACE inhibition activity from various sources increased considerably. Antihypertensive peptides usually contain 2–12 amino acids and significant amounts of hydrophobic residues. Second, the selection of an appropriate source of protein with a suitable releasing technique is crucial in the production of antihypertensive peptides. The most frequently involved technique is enzymatic digestion, where the use of enzymes with low specificity is essential. Techniques such as fermentation, autolysis or simple extraction in the case of naturally presented antihypertensive peptides were also found in literature to be an alternative. Different ACE assays have been employed to evaluate $IC_{50}$ values. Nevertheless, the significant differences among obtained results demand a standardized method for measuring antihypertensive activity. Purification paths generally depend on the complexity of the hydrolysate although some common features can be extracted. UF or SEC are commonly used as a first-stage purification. IEC, as much CEC as AEC, is also frequently used in the purification path. RP-HPLC can be used both at the end of the purification stage or as the only technique used in the purification of peptides. Some other methods, such as desalination, liquid–liquid extraction, solid phase extraction or capillary electrophoresis, also randomly appear in the purification paths of antihypertensive peptides. Peptide identification has been perfomed by Edman degradation despite MS is now the preferred technique. Antihypertensive peptide characterization also includes (bio)chemical tests to assess bioactivity. Namely, gastrointestinal digestion, Caco-2 monolayer, preincubation with ACE or inhibition mode are employed to check inter alia the bioavailability of peptides. Quantitative analysis of some targeted peptides is becoming more usual being HPLC-MS the preferred technique for this purpose.

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