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ISOLATION AND CHARACTERIZATION OF PEPTIDES WITH ANTIHYPERTENSIVE ACTIVITY IN FOODTUFFS

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Abstract

Hypertension is one of the main causes of cardiovascular diseases. Different drugs have been employed in the treatment of hypertension being ACE inhibitors those showing the highest effectiveness. Synthetic drugs inhibiting ACE activity present high effectiveness but cause undesirable side effects. Recently, great attention was paid to peptides with antihypertensive activity. Antihypertensive peptides are naturally present in some foods and do not show adverse effect. Since hypertension is closely related to modern diet habits, the interest for this kind of foods is increasing. In most cases, antihypertensive peptides are encrypted in a parent protein from which they are released during gastrointestinal digestion or during food processing. In other occasions, antihypertensive peptides are added to certain foods to improve its functionality. There has been a great development of methodologies for the purification, isolation, and characterization of antihypertensive peptides in foods. Despite this wide literature, there is no revision work trying to summarize and compare the different strategies that have been employed. The aim of this work has been to review all the strategies employed with this purpose.

Keywords: ACE, chromatography, bioactivity, protein, enzyme

1. Introduction

Traditionally, the dietetic value of a protein was evaluated from its nutritional quality, mainly 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' has to show a potential beneficial health affect (Moller et al., 2008; Schrezenmeir et al., 2000). Bioactive peptides can be naturally occurring in foods but the most usual they are in parent proteins (Iwaniak and Minkiewicz, 2008; Korhonen and Pihlanto, 2003; Moller et al., 2008; Wang and de Mejia, 2005).

Several bioactive peptides from different origins such as milk (Hernandez-Ledesma et al., 2008; Madureira et al., 2010; Zimecki and Kruzel, 2007) and soybean (Wang and de Mejia, 2005) have been released, isolated, characterized, and briefly reviewed. Moreover, peptides showing numerous bioactivities such as antihypertensive, antilipemic, anticariogenic, antioxidative, antimicrobial, antiamnestic, opiate, antithrombotic, osteoprotective, vasodilatative, immunomodulating were thoroughly described (Iwaniak and Minkiewicz, 2008; Kitts and Weiler, 2003; Korhonen and Pihlanto, 2003; Wang and de Mejia, 2005). 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 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 and 70-76% of stroke 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 are unaware of their condition (Chockalingam, 2008). Hypertension can be treated with distinct medications such as nitrates, diuretics, β-blockers, α-adrenergic antagonist, vasodilators, dopamine agonists calcium channel blockers (CCBs), and angiotensin converting enzyme (ACE) inhibitors (Perez and Musini, 2008). Among them, ACE inhibitors (e.g. captopril) 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 provoked 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 which 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 (Lee et al. 2010; Pina and Roque, 2009).

Since hypertension is closely related to modern diet habits, interest in functional foods with antihypertensive activity is having a 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.

2. ACE and blood pressure (BP)

Several interacting biochemical pathways are associated to the control of blood pressure (BP) in living organisms being the *renin-angiotensin* system 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 removing of C-terminal dipeptide HL to Angiotensin II (Ang II- DRVYIHPF) by the action of angiotensin I converting enzyme (ACE; kinases II peptidyldipeptide hydrolase). Afterwards, Ang II is distributed in the blood until its inactivated by aminopeptidase A (E.C. 3.4.11.7) or N (E.C. 3.4.11.2) enzymes and its converted to Angiotensin III and IV (RVYIHPF and VYIHPF, respectively). Ang II peptide causes vasoconstriction by activation of AT1 receptor (AT1R) which leads to raise the BP. Furthermore, Ang II negatively affects to kidney retaining salts and water, causing raise of

extracellular fluid volume and, in a consequence, increasing BP (Chen et al., 2009; FitzGerald et al., 2004; Schmieder et al., 2007).

Moreover, ACE also removes a dipeptide from C-terminus of bradykinin (RPPGFSPFR) resulting in the inactivation of this vasodilator. Therefore, ACE inhibitors decrease 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 effective decrease of BP, most antihypertensive drugs employ this mechanism for the treatment of hypertension.

3. Release of antihypertensive peptides from foodstuffs

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

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 following 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 (Lopez-Fandino et al., 2006; Ricci et al., 2010; Yamamoto and Takano, 1999). In this case, advances have even enabled the development of commercial milk products enriched with antihypertensive peptides (**Table 1**) (Ricci et al., 2010; Sirtori et al., 2009).

In addition to milk and dairy products, several others 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, pork loin, bullfrog, porcine, 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 ways to *in vitro* release of antihypertensive peptides are enzymatic digestion (hydrolysis) and fermentation with bacterial organisms.

Regarding enzymatic digestion, the composition of hydrolysate depends on several parameters such as the enzyme to substrate ratio, hydrolysis time, pH and temperature of hydrolysis, etc. but it mostly depends on the kind of proteolytic enzyme. Most commonly used enzymes are pepsin (Lee et al., 2010; Qian et al., 2007a), thermolysin (Arihara et al., 2001; Yokoyama et al., 1991), and alcalase (Chiang et al., 2006; Qian, Z.J., 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 (Escudero et al., 2010; Majumder and Wu, 2009; Yang et al., 2003), corolase PP (Gomez-Ruiz et al., 2007) or trypsin (E.C. 3.4.21.4) with chymotrypsin (E.C. 3.4.21.1) (Gomez-Ruiz et al., 2007; Matsui et al., 2002; Li et al., 2002) 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 that these peptides will not be inactivated during this process. 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 antihypertensive activity of foodstuffs by the combination of bacterial fermentation and enzymatic digestion. Tonouchi et al. (2008) observed these results when digesting with different enzymes a Danish skim milk-cheese previously fermented with *Lactococcus*. Similarly, Hernandez-Ledesma et al. (2004) found a higher number of antihypertensive peptides when a milk sample fermented with *Lactobacillus rhamnosus* was submitted 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).

In some occasions, the foodstuffs contain antihypertensive peptides that are not encrypted in any protein, not being necessary any fermentation of digestion. 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 kind of cheeses (gouda, manchego and varieties of Spanish and Swiss cheeses) (Gomez-Ruiz et al. 2002; Gomez-Ruiz et al., 2006; Meyer, et al., 2009; Saito et al., 2000). These peptides can simply be extracted with water or alcohols like ethanol or methanol. At this regard, it is possible to differentiate between processed and unprocessed products. 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 added to release antihypertensive peptides.

The other way to release peptides without addition of bacterial organisms or enzymes is autolyzation. Autolyzation involves the employment of proteolytic enzymes which already are ingredients of foodstuffs. This approach was followed for the preparation of hydrolyzates 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 6 months at 20°C in salty conditions. In both cases, antihypertensive peptides were obtained from hydrolysate after long-term fermentation (Je et al. 2005a; Je et al., 2005b).

4. Determination of ACE inhibitory activity of food peptides

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

ACE inhibition activity is expressed using IC_{50} index which is the concentration required of a particular substance to inhibit 50% of the ACE activity. Different assays have been developed to determinate the ACE inhibition value *in vitro*. First assays underwent with errors related to peptidases interferences. Then, assays using artificial substrate started to play considerable role since they were inexpensive, easy to obtain, not liable to be hydrolyzed by peptidases and presented higher dissociation constant for ACE (Meng and Oparil, 1996). Released compounds by the action of ACE could be quantified by 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 the subsequent formation of hippuric acid (HA). The ACE activity is directly related to the extent of HA liberated from 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 firstly acetone extracted from rabbit lung. Further modification of this procedure employed pure ACE from rabbit 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 co-extracted with HA (Meng and Oparil, 1996; Lopez-Fandino et al., 2006). Furthermore, long incubation times (around 30 min) were required to obtain enough product amounts to be quantified. Hence, numerous modifications appeared in the literature, as a consequence obtained IC_{50} values differed significantly among them being not possible their comparison (Kodera and Nio, 2006). In fact, the IC_{50} of hydrolysates obtained by digestion of an insect protein with four different enzymes were determined by applying two different ACE assays. One method was based on the spectrophotometric measurement of FAPGG [2-furanacryloyl-phenylalanyl-glycyl-glycine] used as substrate while an HPLC method which adopted DTG [dansyltriglycine] as substrate was employed in the second approach. When using 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 µL of ACE extract) and 43.220 ± 12.66 and 1.253 ± 0.120

mg/mL (with 25 µL of ACE extract), respectively, for the nonhydrolyzed and hydrolyzed extract (Vercruysse et al., 2005).

In addition to the *in vitro* determination of the 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 kind of experiments is usually focused on short and/or long-term administration studies. Nevertheless, results obtained by *in vivo* studies significantly differ from the results observed by *in vitro* assays. These differences could be justified by the bioavailability of peptides (Lopez-Fandino et al., 2006; Ricci et al., 2010). In fact, a peptide (β- lactosin B, ALPM) derived from a commercial whey product that presented weak ACE inhibitory activity (IC_{50}=928 µM) showed a noticeable decrease of SBP after 8 h oral administration (2 mg/mL) to SHRs (Murakami et al., 2004). 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 two approaches to assess the ACE inhibitory activity appeared also when Yamamoto et al. (1999) purified and characterized a dipeptide from a yoghurt-like product. The IC_{50} value of the dipeptide was estimated at 720 µM which would classify it as a peptide with moderate antihypertensive activity. However, the same peptide provoked similar *in vivo* antihypertensive activity as IPP and VPP which are generally categorized as peptides with very high activity (IC_{50} values, 5 and 9 µM, respectively) (Yamamoto et al., 1999).

In addition to the estimation of IC_{50} value, some authors pay also attention to additional measurements such as simulated gastrointestinal digestion or caco-2 cell monolayer transport.

These experiments can yield information on bioavailability of target compounds and will be discuss later. Additionally, the activity of peptides may be affected by factors such us stability, processing or 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 size of released peptides. Next examples can show these facts. Different protein sources were hydrolyzed in the same conditions: soybean protein, wheat gluten, caseinate, and whey proteins. IC_{50} for these hydrolyzates were 180, 340, 100, and 200 µg/mL, respectively. High antihypertensive activity of casein (100 µg/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 contain high amount of proline residues, its lower hydrophobicity caused a significant increase of its IC_{50} value (340 µg/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) neverthless other methods and techniques have also been employed for this purpose as SDS-PAGE (Jang and Lee, 2005), calculation of α-amino nitrogen and total nitrogen (Mao et al., 2007) or calculation of 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 0, 60, 120, 180, 240, 300, and 340 min. It was observed that at 240 min of hydrolyzing, 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, flavourzyme, 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 which 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).

5. Isolation and purification of bioactive peptides from foodstuffs

The purification of a hydrolyzate showing antihypertensive activity is one of the most important steps in the framework presented in **Figure 2**. The purification path could significantly influence the number of identified peptides, their activity and characteristic (e.g. size of the peptide and their composition), and their properties. Generally, Liquid Chromatography (LC) is the most employed technique. Different chromatographic modes can be selected on 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 are obtained.

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

Generally, first step in the purification is a separation based on peptide size using either ultrafiltration (UF) or size-exclusion chromatography (SEC). UF is a low-pressure technique where 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 removal of solvent, 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 being the first purification step in many cases. Despite membranes with Mw in the range 1-30 kDa have been tried, smaller cut-off membranes are preferred. As example, an hydrolyzate 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. On the base of ACE inhibition activity, the fraction containing molecules smaller than 1 kDa was 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; Samaranayaka et al., 2010; Zhang et al., 2009). In fact, results obtained when a pacific hake protein hydrolysate was ultrafiltrated through membranes of 10, 3, and 1 Mw cut-off 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 non-aqueous solution system) is also very used for the purification of peptides. SEC tends to be used at the beginning of the purification path similarly as 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 Mw ranging from 100 to 7000 like Peptide 10/300 GL column (Hatanaka et al., 2009; Rho et al., 2009; Majumder and Wu, 2009) and Peptide HR 10/30 column (Abubakar et al., 1998; Cheng et al., 2009; Matsui et al., 2002; Li et al., 2002; Saito et al., 2000; Tonouchi et al., 2008) were mostly chosen. Regarding Sephadex columns, most used were Sephadex G-25 (Mw range, 1000-5000) and Sephadex G-15 (Mw ≤ 1500). Other less used Sephadex columns are G-50 (Mw range, 1500–30000) (Je et al. 2005a), G-75 (Mw range, 30000–80000) (Je et al., 2005b), and G-200 (Mw range, 5000–250000) (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 observing signals corresponding to molecules of 4.70 and 0.55 kDa for the albumin hydrolysate and signal corresponding to Mw of 7.50, 4.70, 0.55, and 0.40 kDa for the globulin hydrolysate (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 acid whose pI's are between 5-7, both cation exchange (CEC) and anion exchange (AEC) can be employed. When AEC is used, pH tends to be around 7.5 and binding peptides are negatively charged, while in CEC pH is maintained at acidic level (4.0) to retain positively charged peptides. AEC purification methods focus rather on column with weak ion-exchange ligands as DEAE (Lee

et al., 2010; Qian et al., 2007a) or DE (Katayama et al., 2008; Katayama et al., 2007; Mao et al., 2007) 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 constant pH (Herraiz, 1997). CEC with isocratic elution has also been possible by using of sodium succinate buffer in 20% ACN (Shin et al., 2001), 2 N NH_4OH (Suetsuna, 1998; Suetsuna et al., 2004), ammonia solution (Chen et al. 2002; Suetsuna and Nakano, 2000) or ammonium carbonate buffer (Majumder and Wu, 2009).

Reserved-Phase Chromatography (RPC) is the dominate 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 (Chobert et al., 2005; Fujita et al., 2000; Gouda et al., 2006; Lee et al., 2006ab; Maeno et al., 1996; Pan et al., 2005; Papadimitriou et al., 2007; Yano et al., 1996; Yokoyama et al., 1991).

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 wide range of selectivity by 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 prefers 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 standing a wide range of pHs have also been employed.

Mobile phases consist of mixtures of water with and organic modifier being acetonitrile followed by alcohols such us methanol, ethanol or isopronanol as the most popular options

(Neville, 2004). Gradient elution by the increasing concentration of the organic modifier is the most usual. Mobile phases are usually set up at acidic pH. Moreover, the addition of ion-pairing agents is also very usual to increase hydrophobicity of peptides by complexing 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, 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 performed by UV absorption at the following wavelengths: 210-220 nm (corresponding to the absorption of peptide bonds), and/or 254 and 280 nm (specific absorption of aromatic amino acids as tryptophan, phenylalanine and tyrosine) (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., 2005ab; 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).

6. Identification and characterization of bioactive peptides from foodstuffs

Isolated and purified peptides possesing the most potential antihypertensive activity at the end of the framework (see **Figure 2**) are identified and characterized. Characterization mostly involved 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 all 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 fully automated using special automated protein/peptide sequencers (Gouda et al., 2006; Kuba et al., 2009; Lee et al., 2006ab; Papadimitriou et al., 2007; Rho et al., 2009). 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).

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 post-translational 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 previously to the MS analysis. In fact, a CE-IT-MS system has enabled the identification of 28 different peptides from an ovine casein hydrolysate. Neverthless, the separation of peptides by CE requires special attention to avoid peptides are adsorbed on the wall of the capillary (Gomez-Ruiz et al., 2007).

Antihypertensive peptides are mainly short peptides with 2-12 amino acid residues. In fact, active sites of ACE cannot accommodate big molecules (Murray and FitzGerald, 2007).

Amino acid composition is determined by the chemical hydrolysis of peptides and 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 (Murakami et al., 2004; Saito et al., 2000).

Although full relationship between structure and ACE inhibitory properties of antihypertensive peptides is still not established, it is important to highlight some common features for antihypertensive peptides. In addition to low molecular weight and short sequences, antihypertensive peptides contain a significant amount of hydrophobic amino acids especially at C-terminal position (Meisel, 1997). The presence at C-terminal of proline (P) or positive charge of lysine (K) (ε- amino group) or arginine (R) (guanidine group) enhances the potency of antihypertensive peptides (Hernandez-Ledesma et al., 2008; Kitts, and Weiler, 2003; Meisel, 1997; Murray and FitzGerald, 2007). This fact could be related with 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 *in vitro* antihypertensive activity of isolated peptide does not involve the activity *in vivo*. In fact, orally administered peptide need to reach the target cardiovascular system in an active form. Before that, 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 by the action of pepsin in acidic conditions. Following, peptides are digested in the luminal phase of small intestine at alkaline pH by the action of pancreatic proteases like trypsin, α-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 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 stability of purified antihypertensive peptides against gastrointestinal enzymatic digestion can be carried out. Pure peptide can be submitted to a simulated gastrointestinal digestion using different enzyme systems. 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 monolayers format express a variety of intestinal enzymes and transporters and have been employed as a model of intestine epithelium (Lopez-Fandino et al., 2006). Geerling et al. purified three peptides (TGPIPN, SLPQ, and SQPK) from goat milk hydrolysate with similar IC_{50} values. All peptides were subjected to the Caco-2 monolayer experiment but only TGPIPN was found to pass intact in small amount. Nevertheless, intake of goat milk hydrolysate by SHR for 12 weeks had resulted in a decrease of SBP (Geerlings et al., 2006). The reason for this disagree could be that Caco-2 model is tighter than intestinal mammalian tissue and some molecules which do not show sufficient absorption in the model can exert *in vivo* effect (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 where *'Pro-drug type'* peptides are transformed to true inhibitor by ACE or gastrointestinal proteases (Li et al., 2004; Vermeirssen, et al., 2004). True inhibitor type (Tonouchi et al., 2008; Yang et al., 2007; Zhao et al., 2009), substrate type (Katayama et al., 2007; Katayama et al., 2008), and pro-drug 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 inhibition mode of peptides. Overall pattern for ACE inhibition was investigated by the incubation of inhibitory peptides with different concentrations of HHL and the measurement 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 antihypertensive peptide where substrate or competitive inhibitors containing at all positions 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), glycinin from soybean (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 ovotransfferin hydrolysates (Lee et al,. 2006b).

7. Quantification of peptides with antihypertensive activity

Since first discover of antihypertensive peptide from foodstuff, studies in the area of ACE inhibitory peptides were mainly focused on the area of isolation, purification, identification, and characterization of these peptides. In last year new trend can be observed where additional quantitative analysis of particular peptides with high IC_{50} value is added. This fact can be related to variety of causes. First of all recent rapid development of functional foods which contain antihypertensive peptides require established standardized methodologies for quantification of peptides including stability studies in complex biological matrices. Continuously due to the high dosage dependent of some antihypertensive activity, the quantification of peptide in functional food to assess the safety, activity of the final product and health claims is essential (Contreras et al., 2008; Gilani et al., 2008). The amount of encrypted peptide in different crops varieties or amount of released peptides in slightly different conditions of hydrolysate preparation like time of storage (Papadimitriou et al., 2007) or supporting of hydrolysis by high pressure (Quiros et al., 2007a) can fluctuate drastically. Moreover considering that functional food become more widespread (including genetically modified organism) and probably in a future will be a crucial diet supplement for hypertensive population, cited information are necessary for regulatory agencies to expand policy and regulations of adding particular peptides to commercial foodstuff (Contreras et al., 2008; Gilani et al., 2008).

In the majority quantification of selected antihypertensive peptide was done by the use of mass spectrometry with previous HPLC separation, however some other attempts also can be found in the literature. The quantification of particular peptides which posses antihypertensive activity was made on the standard calibration curve of corresponding synthetic peptide injected to LC-MS system. By this methodology the concentration of seven dipeptides in the wakame (Sato et al., 2002) and eight dipeptides in salmon muscle (Enari et al., 2008) hydrolysates was 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 mass spectrum obtained with the synthetic peptide was identical to that observed in 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. Developed method was validated by the determination of repeatability, reproducibility, linearity, and recovery. Calibration was performed base on the peak area of the precursor and it adducts in the MS experiments and on peak area of the most abundant product ions after fragmentation of precursor in MS/MS analysis. Limits of detection and quantification determined by MS/MS were 7 μg/mL and 25 μg/mL, respectively (Quiros et al., 2006). Similarly, method for determination of LKPNM antihypertensive peptide in bonito muscle hydrolysate was evaluated by use of HPLC-MS and HPLC-MS/MS system. Validation of method by measuring specificity, linearity, accuracy, precision and reproducibility was presented (Curtis et al., 2002). Next, quantification of nine antihypertensive dipeptides in fermented soybean seasoning and soybean sauce 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 chesses with different ripening time (Meyer et al., 2009) using HPLC-MS^3 and PPPP as internal standard revealed that large variations were obtain among individual loaves from various producers and high concentration of both peptides often occurred in long-term ripened cheeses produced from raw milk. The same peptides were quantified in miso paste by LC-MS using also internal standard methodology, whereas 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 quantification of IY, VY and IVY in Protease N treated Royal Jelly (Tokunaga et al., 2004).

8. Conclusions

The role of antihypertensive peptides derived from foodstuff becomes increasingly appreciated since hypertension is a grave problem, especially, in highly-developed countries. Possibility of partly substitution and support of synthetic ACE inhibitor treatment or prevention and mitigation of hypertension by functional food consisting antihypertensive peptides, can be significant. The knowledge about ACE inhibitory peptides in last year improved such as the specification of their biochemistry, bioavailability, properties or mechanism of inhibition. As well, the number of identified peptides with certain ACE inhibition activity from various sources noteworthy increased. However due to the high interest in supplementation of food by some peptides the development of standardized methodologies for isolation, purification and identification is needed. Next, selection of appropriate source of protein with suitable releasing technique is crucial in production of antihypertensive peptides. The most frequently involve technique is enzyme digestion where the use of biomolecules with low specificity or their combination to produce small antihypertensive peptides is essential. The techniques such as fermentation, autolysis or simple extraction in a case of naturally presented antihypertensive peptides was also found in the literature as an alternative. Also the varieties of ACE assays to evaluate IC_{50} value is presented where the need of standardized method for measuring antihypertensive activity should be underline since significant differences between used methods appears. The purification path for separation of antihypertensive peptides is a difficult task. Even if the number of employed steps generally depends on the complexity of hydrolysate, some general

features in purification can be extracted. For production of peptides with probably the highest ACE inhibitory activity as a first step UF or SEC is mainly in use. Both techniques are on the base of partial exclusion of molecules by the mass. Since peptides with antihypertensive activity are small molecules which posses from 2- 12 amino acid residues, overall the technique should use suitable conditions to remove bigger molecules and in the same time not exclude peptides of interest. Next IEC, where as much CEC as AEC frequently was used in the purification path, where the first one with much higher repeatability. The most powerful technique RP-HPLC appears both at finalization of purification path of peptide as also as the unique used technique. Since antihypertensive peptides contain significant amount of hydrophobic amino acid and the standard conditions for separation of peptides are mainly in use, it is quite easy to predict where high potent peptides should elute. Some others methods as desalination, liquid-liquid extraction, solid phase extraction or capillary electrophoresis also randomly appears in purification of antihypertensive peptides. Furthermore for identification mainly two methods namely, mass spectrometry or Edman degradation method appeared, where the first one in last time gain special attention since is quick, reliable and some additional information as accurate molecular weight can be obtain in the same time. After identification frequently additional (bio)chemical tests for antihypertensive peptides are made since habitually ACE inhibition activity *in vitro* does not provide *in vivo* activity. Namely, gastrointestinal digestion, Caco-2 monolayer, preincubation with ACE or inhibition mode are employed to check inter alia the bioavailability of peptide. Following the quantitative analysis of some particular peptides starts to play important role since future trend of functional food supplemented with ACE inhibitory peptides can be observe. In this area standard calibration curve with synthetic analogs measured by HPLC-MS system domain, however UV detection also randomly was employed.

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Figure 1. Scheme of Renin- Angiotensine system.

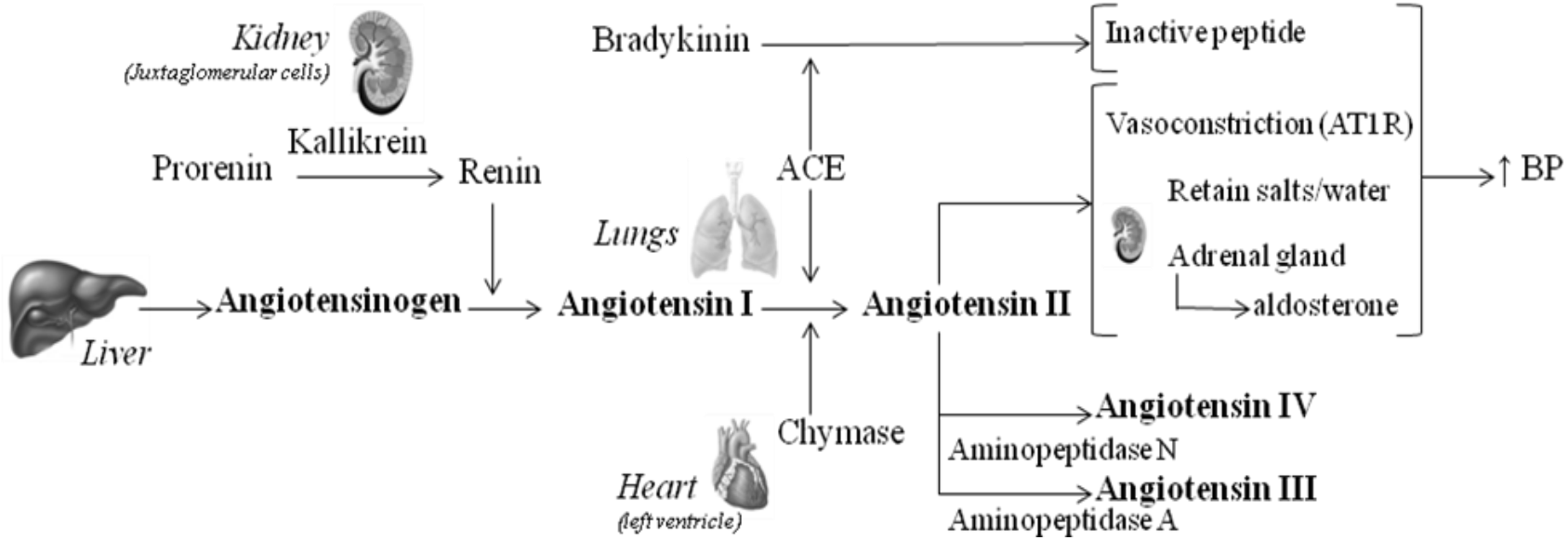

Figure 2. Framework of production, purification and identification of bioactive peptides with antihypertensive activity.

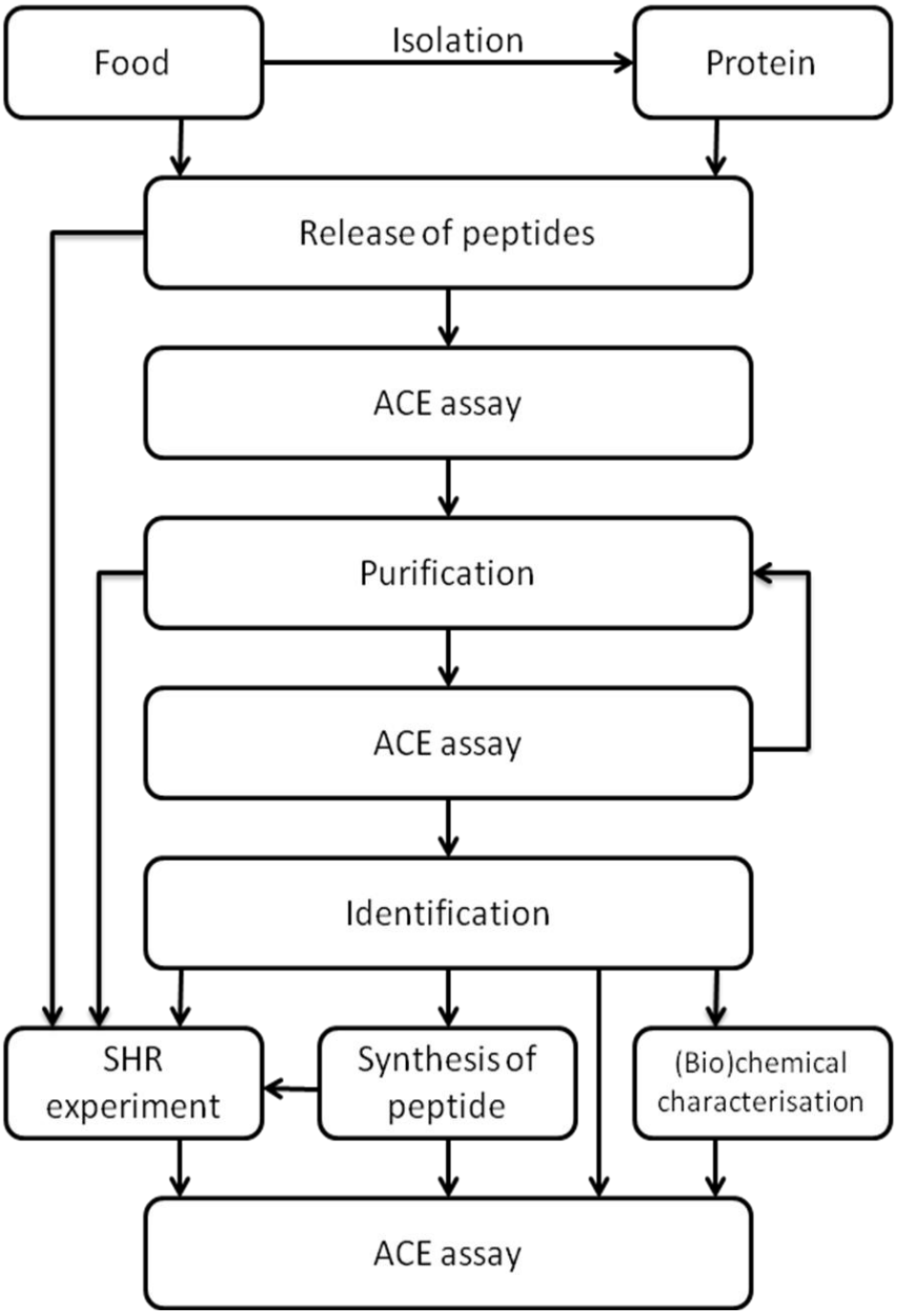

Figure 3. Chromatograms (A,C) and mass spectra (B, D) obtained by LC-MS and corresponding to FY: (A,B) synthetic FY; (C,D) FY in the hydrolysate of wakame. LC conditions: Column, Xterra MS C18, 150 x 2.1 mm; gradient, 3-20% in 40 min; mobile phases, A: water + 0.05% TFA; B: ACN+0.05% TFA; flow-rate, 0.2 mL/min; MS conditions: cone voltage: +30V; 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).

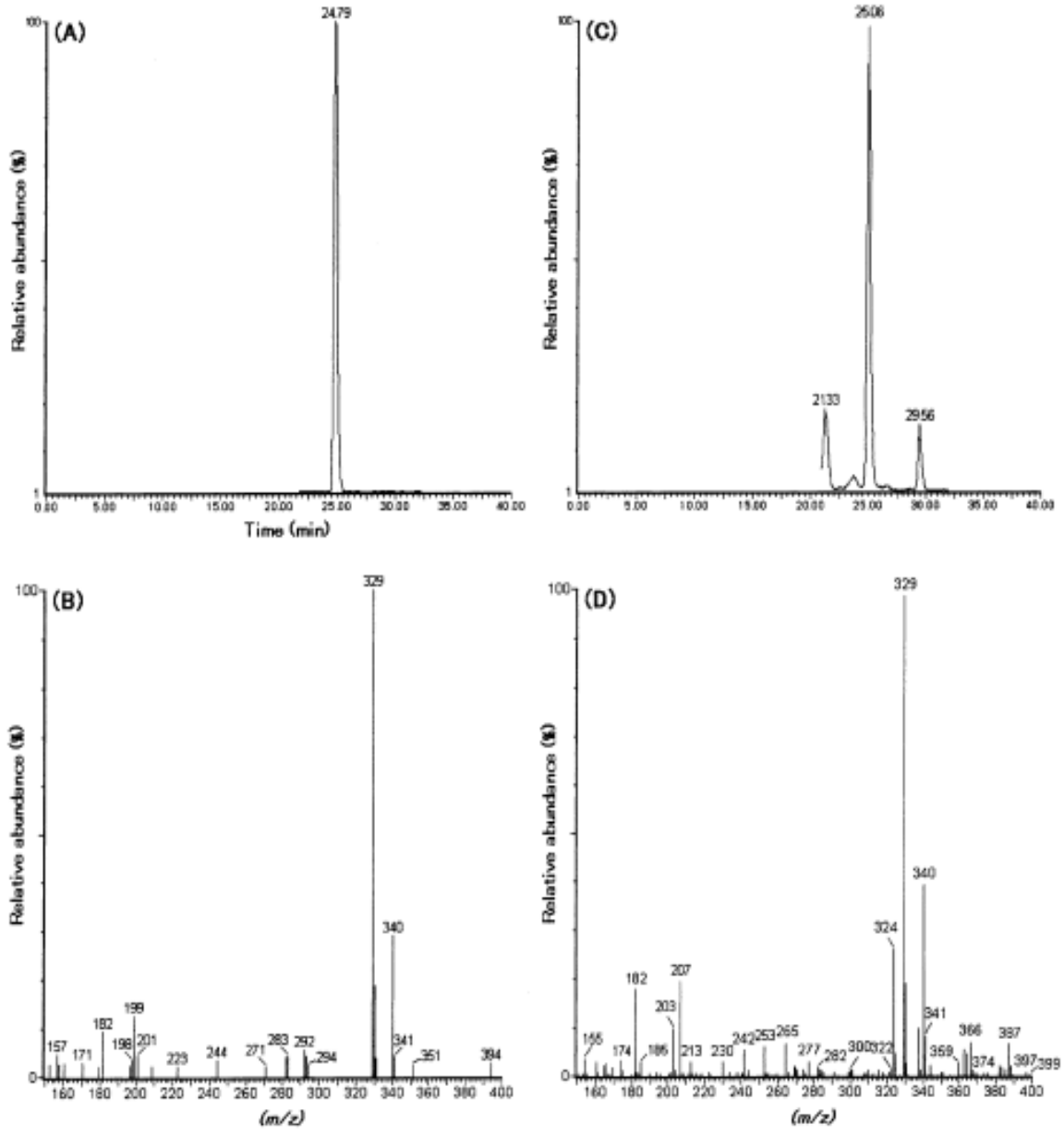

Table 1. Commercialy available milk products enriched with antihypertensive peptides (Ricci et al., 2010; Sirtori et al., 2009).

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

1 **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 or sequentially fermentation and digestion	Several bacterial strains or *Lactbacillus rhamnosus* and pepsin, corolase PP	UF: M_{WCO} 3 kDa RPC: Widepore C18, 250 x 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)	Hernandez-Ledesma et al., 2004
Milk	Fermentation	Strains *Enterococcus faecalis*	Centrifugation (20,000g, 10 min, 10°C) / filtration (Whatman no. 40) RPC: Widepore C18, 250 x 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 (20000g, 10 min, 10˚C) and filtration (Whatman no. 40) UF: M_{WCO} 3 kDa RPC: Prep Nova Pak® HR C18, 300 x 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) RPC: Prep Nova Pak® HR C18, 300 x 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) Caco-2 cell RPC: Zorbax 5 C18, 2.1 x 250 mm; 0-30% in 60 min; 30-80% in 10 min; A: 0.05% TFA; B: ACN + 0.04% TFA; 350 µL/min; UV detection (220 nm)	Greelings 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 RPC: Prep Nova Pak® HR C18, 300 x 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) CE: bare fused silica capillary, 76 cm x 50 µm; 0.9 M HFo, pH=2.0; 18 kV; MS detection	Gomez-Ruiz et al., 2007
Ovine β-lactoglobulin from skimmed milk	Digestion	Trypsin	RPC: Nucleosil C18, 250 x 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 SEC: Sephadex G- 25, 26 x 800 mm; elution with water; 0.6 mL/min; UV detection (215 nm); RPC: Shim-pack PREP-ODS C18, 20 x 250 mm; 10-60% B in 35 min; A: 0.05% TFA; B: ACN + 0.05% TFA; 6 mL/min; UV detection (215 nm) RPC: C18, 4.6 x 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 IEC: DE-52, 1.6 x 30 cm; 0-0.4 mM NaCl in 5 mM PBS, pH 8.0; 24 mL/h; UV detection (220 nm) SEC: Sephadex G-25, 2.5 x 100 cm; elution with water; 16 mL/h; UV detection (220 nm) RPC: Zorbax Eclipse XDB-C18, 10 x 400 mm; 0–60% B (B: ACN + 0.1% TFA) in 45 min; 1 mL/min; UV detection (214 nm) RPC: Zorbax Eclipse XDB-C18, 2.1 x 150 mm; A: 0.1% TFA; B: ACN + 0.1% TFA; 0.4 mL/min; UV detection (214 nm)	Mao et al., 2007

Casein	Fermentation	*Lactobacillus helveticus CP790*	RPC: µ-Bondashere C18, 3.9 x 150 mm; 0-40% B in 60 min; A: 0.1% TFA; B: ACN + 0.1% TFA; 1 mL/min; UV detection (215 nm) RPC: µ-Bondashere C18, 3.9 x 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 x 250 mm; 0-100% B; A: 10% ACN + 0.1% TFA; B: 90% ACN + 0.8% TFA; 1 mL/min; UV detection (215 nm) RPC: ODS-100S, 3.9 x 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) RPC: µBondasphere C18, 3.9 x 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 x 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) RPC: Nucleosil C18, 250 x 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 x 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) CE: Coated capillary, 50 cm x 50 µm; 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) RPC: µ-Bondasphere C18, 3.9 x 150 mm; 100-60% in 60 min; A: 0.1% TFA; B: ACN + 0.1% TFA; 1 mL/min; UV detection (215 nm) RPC: µ-Bondasphere C18, 3.9 x 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 12,000 x g, 10 min, 5°C/ filter Whatman no. 40/ M_{WCO} 3 kDa RPC: 0-30% B in 70 min; A: 0.1% TFA; B: ACN + 0.08% TFA; UV detection (214 nm) 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 RPC: YMC-Pack R&D ODS, 20 x 250 mm; 0-70% B (B: ACN + 0.1% TFA) in 50 min; 7.5 mL/min; UV detection (214 nm) RPC: TSK-gel ODS 80Ts, 20 x 250 mm; 0-30% B (B: ACN + 0.1% TFA) in 50 min; 7.5 mL/min; UV detection (214 nm) SEC: Superdex Peptide HR 10/30, 10 x 300 mm; elution with water + 0.1% TFA; 0.5 mL/min; UV detection (214 nm) RPC: CAPCELL PAK C18 MG, 4.6 x 250 mm; elution with 12% ACN + 0.1% TFA; 0.4 mL/min; UV detection (214 nm)	Tonouchi et al., 2008
Gouda cheese	Extraction	----------	Hydrophobic chromatography in Wakogel LP-40c18 resin; 15-90% ACN RPC: Superiorex ODS, 4.6 x 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) SEC: Superdex Peptide HR 10/30, 1 x 30 cm; elution with 0.1% TFA; 0.5 mL/min; UV detection (214 nm) CE: Coated capillary, 24 cm x 25 µm; 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 RPC: Hi-Pore C18, 250 x 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)	Gomez-Ruiz et al., 2006
Manchego cheese	Fermentation /ripening	Different bacterial strains	Extraction/ centrifugation/ filtration/ centrifugation/ filtration UF: M_{WCO} 3 kDa	Gomez-Ruiz et al., 2002

			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: 10mM HFo; B: 5M NH_4OH; 5 mL/min; UV detection (280 nm) RPC:C18 Prep-Nova Pak HR, 300 x 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) RPC: Hi-Pore C18, 250 x 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)	
Manchego cheese	Fermentation	Several bacterial strains	UF: M_{WCO} 3 kDa RPC: Widepore C18, 250 x 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)	Gomez-Ruiz et al., 2004
Enzyme –modified cheese	Digestion	Neutrase, Lactobacillus casei enzyme or Debitrase® DBP20	RPC: Delta Pak C18, 30 x 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 µm); 0-90% MetOH Hydrophobic chromatography in LiChropep RP-18 resin (25-40 µm); 30-42% MetOH RPC: Superiorex ODS, 4.6 x 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) SEC: Superdex peptide HR 10/30, 1 x 30 cm; elution with 0.1% TFA; 0.5 mL/min; UV detection (214 nm) CE: Coated capillary, 24 cm x 25 µm; 0.1 M PBS, pH=2.5; 10 kV; UV detection (200 nm)	Abubakar et al., 1998
Commercial whey product*	Not shown	Not shown	Extraction with Wakogel LP-40C18 resin (20-40 µm); elution with 90% EtOH SEC: Sephadex G-15, 2.6 x 90 cm; 0.05% TFA; 0.5 mL/min; UV detection (214 nm) RPC: Wakosil-II 5C18, 4.6 x 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) CE: Coated capillary, 17 cm x 25 µm; 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) 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
α-lactalbumin[1] and β-casein[2]	Digestion	Thermolysin	SEC: Superdex™ 30 prep grade, 2.6 x 61 cm; 0.1M NH_4HCO_3, pH 8.0; 2.5 mL/min; UV detection (280 nm) RPC: Nucleosil 300-S C18, 4.6 x 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) RPC: Nucleosil 300-S C18, 4.6 x 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				
Soybean	Digestion	Pepsin	IEC: Dowex 50 W, 45 x 200 mm; elution with 5 % NH_4OH SEC: Sephadex G-25, 26 x 1400 mm; water; 30 mL/min IEC: Sephadex C-25, 20 x 500 mm; 0- 3% NaCl; 30 mL/min RPC: Develosil ODS-5, 4.6 x 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 CEC: HiPreP 16/10 SP FF, 16 x 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) Desalination: Cellulose dialysis membrane, M_{WCO} 100	Rho et al., 2009

			SEC: Superdex Peptide 10/300 GL, 10 x 300 mm; elution with 30% ACN in 40 min; 0.36 mL/min; UV detection (214 nm) RPC: μBondapak™ C18, 4.6 x 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) RPC: μBondapak™ C18, 4.6 x 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)	
Soybean protein	Digestion	Mature D3 protease	Desalination by electrodialyzation SEC: Superdex Peptide HR 10/300; elution with 0.05 % TFA; 0.5 mL/min; UV detection (215 nm) 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 x 4.6 mm; 0-35% B; A: 0.1% TFA; B: 70% ACN + 0.05% TFA; 0.7 mL/min; UV detection (230 nm) RPC: C-18 Shimpak, 250 x 4.6 mm; 0-100% B; A: 50 mM NH_4Ac; B: 50 mM NH_4Ac/ ACN (50:50); UV detection (230 nm) RPC: C-18 Shimpak, 250 x 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
Steamed soybean mixed with roasted wheat	Fermentation	*Tane koji* rich in *A. sojae*	Desalination by electrodialyzing RPC: SP-120-40/60-ODS-B, 150 x 1000 mm; 0-100% B in 25 h; water- 0.1% TFA-ACN; 45 mL/min; UV detection (220 nm) RPC: Cosmosil-5C18-ARII, 20 x 250 mm; 0-100% B in 90 min; water- 0.1% TFA-ACN; 5 mL/min; UV detection (220 nm) RPC: C30 Develosil RPAQUEOUS-AR, 20 x 250 mm; 0-100% B in 90 min; water-ACN; 5 mL/min; UV detection (220 nm)	Nakahara et al., 2010
Wheat bran*	Autolyzation	Endogenous proteases	RPC: LiChroprep RP-18, 2.5 x 25 cm; 10-95% EtOH IEC: AG MP-1 resin, 3 x 20 cm SEC: Superdex 75HR, 10 x 30 cm; elution with 30% ACN + 0.1% TFA; 0.5 mL/min; UV detection (220 nm) RPC: Jupiter C4, 10 x 250 mm; 0-35% ACN + 0.1% TFA in 30 min; 4 mL/min RPC: Jupiter C18, 10 x 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 x 40 cm; 0-0.5M NaCl in 5 mM NaAc, pH 3.5; 1 mL/min; UV detection (220 nm) SEC: Bio-gel P-2, 1.6 x 100 cm; elution with water; 0.33 mL/min; UV detection (220 nm) RPC: TSK-GEL ODS 120T, 4.6 x 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 20,000*g*, 20 min 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 SEC: Sephadex G-15, 1.8 x 60 cm; 20 mM NaAc, pH 4.0; 0.4 mL/min; UV detection (220 nm) RPC: Sephasil Peptide C18 ST 4.6/250, 4.6 x 250 mm; 0- 60% B (B: ACN + 0.1% TFA) in 60 min; 1 mL/min; UV detection (220 nm) RPC: Sephasil Peptide C2/C18 ST 4.6/250, 4.6 x 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% SEC: Sephadex G-25, 1.2 x 142.5 cm; elution with water; UV detection (220 nm) 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) 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
α-zein*	Digestion	Thermolysin	DEAE-Tyopearl 650 M, 2.6 x 100 cm; 0-0.3 M NaCl in 5mM Tris-HCl (pH 8); 3 mL/min; UV detection (254 nm) Desalination with Sephadex LH-20, 1.6 x 100 cm RPC: C-18 Capcellpak, 1.5 x 25 cm; 10-60% B (B: ACN + 0.1% TFA); 8 mL/min; UV detection (210 nm) RPC: C-18 Capcellpak, 1.5 x 25 cm; ACN + 0.1% TFA; 8 mL/min; UV detection (210 nm)	Miyoshi et al., 1991

			CEC: Senshupak SCN-1251, 0.46 x 25 cm; 20 mM/pH 4.0- 50 mM/pH 6.3 NH_4Ac SP-Toyopearl 650 M, 2.6 x 100 cm; 20 mM/pH 4.0- 50 mM/pH 6.3 NH_4Ac; 3 mL/min; UV detection (254 nm) RPC: C-18 Capcellpak, 1.5 x 25 cm; 10-60% B (B: ACN + 0.1% TFA); 8 mL/min; UV detection (210 nm) RPC: C-18 Capcellpak, 1.5 x 25 cm; 5-30% B (ACN + 0.1% TFA); UV detection (210 nm)	
Urea denaturated Z19 α-zein	Digestion	Thermolysin	RPC: YMC-GEL C4, 4.6 x 110 mm; 0 -30% B (B: ACN + 0.1% TFA); 1 mL/min; UV detection (220 nm) RPC: YMC-GEL C18, 4.6 x 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 α-zein			RPC: YMC-GEL C18, 4.6 x 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 SEC: Bio-Rad P-2, 700 x 15 mm; 2 mM PBS, pH 8.0; 0.25 mL/min; UV detection (220 nm) RPC: μ-Bondapak C 18, 300 x 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 x 250 mm; 1%/min B (B: ACN + 0.1% TFA); 10 mL/min; UV detection (230 nm) RPC: 5PE-MS, 4.6 x 250 mm; 1%/min B (B: ACN + 0.1% TFA); 1 mL/min; UV detection (230 nm) RPC: Cosmosil 5 CN-R, 4.6 x 250 mm; 1%/min B (B: ACN + 0.1% TFA); 1 mL/min; UV detection (230 nm) RLC: 5NPE, 4.6 x 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 x 29 cm; 0.4 M NaCl + 20 mM 2-MER; 0.4 mL/min; UV detection (280 nm) SEC: Sephadex G-15, 1.4 x 29 cm; 0.4 M NaCl + 20 mM 2-MER; 0.4 mL/min; UV detection (214 nm) RPC: Nucleosil 100 C18 RP, 4.6 x 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
Mung bean protein	Digestion	Alcalase	UF: M_{WCO} 6 kDa SEC: Sephadex G-15, 1.8 x 60 cm; elution with 20 mM NaAc, pH 4.0; 0.4 mL/min; UV detection (220 nm) RPC: Sephasil Peptide C18 ST 4.6/250, 4.6 x 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) RPC: Sephasil Peptide C18 ST 4.6/250, 4.6 x 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 x 820 mm; elution with 10% EtOH; 0.18 mL/min; UV detection (210 nm) RPC.: Develosil ODS-10, 20 x 250 mm; 5% B in 20 min; 5-40% B in 60 min; B, ACN + 1% TFA; 10 mL/min; UV detection (210 nm) RPC: Develosil C-30-UG-5, 10 x 250 mm; ACN + 1% TFA; 4 mL/min; UV detection (210 nm) RPC: Develosil Ph-UG-5, 10 x 250 mm; 6% ACN + 1% TFA; 4 mL/min; UV detection (210 nm)	Nakano et al., 2006
Alfalfa white protein*	Hydrolyzation at pilot plant scale by Delvolase® in enzymatic membrane reactor		SEC: Superdex Peptide HR 10/300, 10 x 300 mm; elution with 30% ACN + 0.1% TFA; 0.2 mL/min; UV detection (226 nm) RPC: C18, 4.6 x 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
ANIMAL ORIGIN				
Chicken bone	Digestion	Pepsin	SEC: TSK gel G2000SWXL, 7.8 x 300 mm; elution with 0.2 M PBS, pH 7.0; 1 mL/min; UV detection (225 nm) RPC: Inertsil ODS-2;0–35% B (B: ACN + 0.1% TFA); 1 mL/min; UV detection (225 nm) RPC: Inertsil ODS-2; 8-14% B (B: ACN + 0.1% TFA); 0.5 mL/min; UV detection (225 nm) 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 RPC: C18 ODS, 22 x 250 mm and 4.6 x 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 SEC: Superdex™ Peptide HR 10/30, 10 x 300 mm; elution with water; 0.5 mL/min; UV detection (220 nm)	Cheng et al., 2009
Chicken muscle[1] and ovalbumin[2]	Digestion	Thermolysin[1] and pepsin, trypsin, chymotrypsin or thermolysin[2]	RPC: Cosmosil 5C18-AR, 20 x 250 mm; 0-50% B (B: ACN + 0.1% TFA) in 50 min; 10 mL/min; UV detection (215[1] or 230[2] nm) RPC: Cosmosil 5 Ph, 4.6 x 250 mm; 0-40% B (B: ACN + 0.1% TFA) in 40 min; 1 mL/min RPC: Cosmosil 5CN-R, 4.6 x 250 mm; 0-40% B (B: ACN + 0.1% TFA) in 40 min; 1 mL/min RPC: Cosmosil 5C18-AR, 20 x 250 mm; 0-40% B (B: ACN + 10 mM PBS, pH 7.0) in 40 min; 1 mL/min	Fujita et al., 2000
Porcine skeletal muscle	Digestion	Trypsin, α-chymotrypsin, pronase E, proteinase K, thermolysin, ficin, papain or pepsin	RPC: CAPCELL PAK C18 UG120, 4.6 x 150 mm; 0-100% B; A: 0.1% TFA; B: ACN + 0.1% TFA; 1 mL/min; UV detection (215 nm) RPC: CAPCELL PAK C18 UG120, 4.6 x 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 x 150 mm; 0–300 mM NaCl in 20 mM Tris-acetate, pH 7.5; 1.13 mL/min; UV detection (215 nm) Desalination: Sep-Pak Plus C18; elution with 50% ACN RPC: Cosmosil 5C18 ARII, 4.5 x 150 mm; 1–80% B (B: ACN + 0.1% TFA); 0.5 mL/min; UV detection (215 nm) RPC: Cosmosil 5C18 ARII, 4.5 x 150 mm; elution with 12 or 16% ACN + 0.1% TFA); 0.5 mL/min; UV detection (215 nm) SEC: TSK-gel G2000SWXL, 7.8 x 300 mm; elution with 20 mM PBS, pH 7.0; 0.5 mL/min; UV detection (215 nm) RPC: Cosmosil 5PEMS, 4.6 x 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 x 90 cm; elution with 30% MetOH; 0.5 mL/min; UV detection (280 nm) RPC: Hypersil BDS C18, 4.6 x 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) RPC: Hypersil BDS C18, 4.6 x 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™ 30, 1.6 x 90 cm; elution with 20 mM NaAc (pH 7.0) + 150 mM NaCl; 0.45 mL/min Desalination: SEP-PAK Plus C18; elution with 50% ACN RPC: Inertsil ODS-2, 4.6 x 250 mm; 1–80% B and:1-50% B (ACN + 0.1% TFA); 0.5 mL/min; UV detection (225 nm) RPC: Comosil 5PE-MS, 4.6 x 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 x 150 mm; 0- 300 mM NaCl in 20 mM Tris-acetate, pH 7.5; 1.13 mL/min; UV detection (215 nm) Desalination with Sep-Pak Plus C18; elution with 50% ACN RPC: Cosmosil 5C18 AR-II, 4.5 x 150 mm; 1-80% B (B: ACN + 0.1% TFA); 0.5 mL/min; UV detection (215 nm) RPC: Cosmosil 5C18 AR-II , 4.5 x 150 mm; elution with 12% ACN + 0.1% TFA; 0.5 mL/min; UV detection (215 nm) SEC: TSK-gel G2000 SWXL, 7.8 x 300 mm; elution with 20 mM PBS, pH 7.0; 0.5 mL/min; UV detection (215 nm)	Katayama et al., 2007

			RPC: Cosmosil 5PE-MS, 4.6 x 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 x 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) RPC: Primesphere 10 C18, 10 x 250 mm; 0–35% B (B: ACN + 0.1% TFA) in 35 min; 1.2 mL/ min; UV detection (215 nm) RPC: SynChropak RP-P-100, 4.6 x 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 SEC: Sephadex G-25, 2.6 cm x 1 m; elution eith 20 mM PBS, pH 7.4; 1.6 mL/min RPC: C18, 25 x 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 α_{S2}- casein	Digestion	Trypsin	RPC: XTerra C18, 4.6 x 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 x 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 x 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 x 100 mm; 0-1 M NaCl + 26.5 mM HFo; 2 mL/min; UV detection (214 nm) SEC: Superdex Peptide 10/300 GL, 10 x 300 mm; 0.9 mL/min; UV detection (214 nm); RPC: ODS-80TM, 4.6 x 75 mm; 0- 40% ACN + 0.1% TFA; 0.5 mL/min; UV detection (214 nm) RPC: μRPC C2/C18 SC 2.1/10, 2.1 x 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 x 90 cm; elution with 50 mM PBS, pH 7.0; 60 mL/h; UV detection (280 nm) IEC: SP-Sephadex C-25, 2.5 x 45 cm; 0-1 M NaCl in 20 mM NaAc, pH 4.0; UV detection (280 nm) RPC: Nucleosil 100-7 ODS C18, 10 x 250 mm; 0-40% B (B: ACN + 0.1% TFA); 2 mL/min; UV detection (215 nm) RPC: Nucleosil 100-7 ODS C18, 10 x 250 mm; 0-25% B (B: ACN + 0.1% TFA); 2 ml/min; UV detection (215 nm)	Je et al., 2005b
Marine shrimp *(Acetes chinensis)*	Fermentation	*Labtobacillus fermentum SM 605*	UF: M_{WCO} 3 kDa SEC: Sephadex G-15, 1.6 x 80 cm; elution with water; 25 mL/min; UV detection (220 nm) RPC: HIQ sil C18-10, 4.6 x 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 IEC: SP Sephadex C-25, 16 x 300 mm; 0-0.15 M NaCl in 20 mM NaAc (pH 4); 0.4 mL/min; UV detection (220 nm) SEC: Sephadex G-15, 16 x 300 mm; elution with water; 0.3 mL/min; UV detection (220 nm) RPC.: Zorbax C18, 1 x 250 mm; 0-10% ACN in 15 min; 0.8 mL/min; UV detection (220 nm) RPC: Zorbax SB C18, 4.6 x 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 SEC: Sephadex G-25, 1.6 x 30 cm; elution with water; 0.6 mL/min; UV detection (220 nm)	Zhao et al., 2009
			IEC: SP Sephadex C-25, 2.6 x 30 cm; 0–1 M NaCl in 20 mM NaAc, pH 4.0; 0.6 mL/min; UV detection (220 nm) SEC: Sephadex G-25, 1.6 x 100 cm	

			RPC: Zorbax C18, 9.4 x 250 mm; 5–40% B (B: ACN + 0.1% TFA) in 40 min; 0.8 mL/min; UV detection (220 nm)	
Oyster*	Fermentation	Salty conditions for 6 months	IEC: SP-Sephadex C-25, 4.0 x 40 cm; 0-2 M NaCl in 20 mM NaAc, pH 4.0; 60 mL/h; UV detection (215 nm) Desalination by electrodialyzation SEC: Sephadex G-50, 2.5 x 98 cm; elution with water; 60 mL/h; UV detection (215 nm) SEC: elution with water; 60 mL/min; UV detection (215 nm) 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 SEC: Sephadex LH-20, 2.7 x 80 cm; elution with 30% MetOH; 0.5 mL/min; UV detection (280 nm) RPC: Hypersil BDS C18, 4.6 x 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 IEC: Dowex 50W, 2.6 x 20 cm; elution with 3.7% NH_4OH SEC: Sephadex C-25, 2 x 50 cm; elution with 1.5% NaCl; 70 mL/h RPC: Develosil C18 ODS-5, 4.6 x 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 RPC: µBondasphere C18, 300 x 30 mm; 0-35% B in 140 min; A: 0.1% TFA; B: ACN + 0.07% TFA; 30 mL/min; UV detection (220 nm) RPC: XTerraRP18, 150 x 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) RPC: ^{1}C.: ODP50-4D, 150 x 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 x 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)	Sato et al., 2002
Wakame (*Undaria pinnatifida*)	Extraction	----------	Dialysis against water (10 L) in cellulose tubular membrane (90 cm) IEC: Dowex 50W, 45 x 450 mm; elution with NH_4OH SEC: Sephadex G-25, 2.6 x 140 cm; elution with 0.1 M PBS, pH 7.0; 30 mL/min RPC: Develosil ODS-5, 4.6 x 250 mm; 0-25% B (B: ACN + 0.05% TFA) in 180 min; 1 mL/min; UV detection (220 nm) RPC: Asahipack CG-320HQ, 7.6 x 300 mm; elution with 25 % ACN in 50 mM NH_4Ac, pH 6.8; 0.5 mL/min	Suetsuna et al., 2004
			FISHES	
Tuna dark muscle	Digestion	Alcalase, neutrase, pepsin, papain, α-chymotrypsin or trypsin	UF: M_{WCO} 3 kDa IEC: HiPrep 16/10 DEAE FF; 0–2 M NaCl in NaAc (pH 4); 2.0 mL/min; UV detection (280 nm) RPC: ODS C18 Primesphere 10, 20 x 250 mm; 0–50% B (B: ACN + 0.1% TFA) in 55 min; 2.0 mL/min; UV detection (215 nm) RPC: Synchropak RPP-100, 4.6 x 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 IEC: Hiprep 16/10 DEAE FF; 0–2 M NaCl in 20 mM NaAc, pH 4.0; 62 mL/h; UV detection (280 nm) RPC: Primesphere 10 C18, 20 x 250 mm; 0–50% B (B: ACN + 0.1% TFA) in 20 min; 2 mL/min; UV detection (215 nm) RPC: Synchropak RPP-100, 4.6 x 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 x 370; elution with 10, 25, 50, 99.5% EtOH SEC: Sephadex G-25, 16 x 650 mm; elution with water RPC: Mightysil RP-18, 4.6 x 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	Enari et al.,

			Separation in silica gel; PSQ 100B, 1380 x 100 mm; elution with $CHCl_3$: MetOH: water: CH_3COOH (65:25:4:1, 31 L) Separation in silica gel, 400 x 80 mm; elution with $CHCl_3$:2-propanol:water: CH_3COOH IEC: Amberlite CG50-type, 400 x 80 mm; elution with water; water:MetOH; MetOH; MetOH:2M HCl RPC: XTerra MS C18, 100 x 4.6 mm; 5–30% B (B: ACN + 0.1% HFo) in 30 min; 0.2 mL/min Methylation: 10% sodium methoxide in MetOH (50 mL); 16 h, RT; refluxing (4.5 h) Separation in Silica gel 60, 600 x 20 mm; elution with $CHCl_3$:2-propanol at different ratios	2008
Dried bonito	Digestion	Pepsin, chymotrypsin, trypsin, thermolysin	RPC: YMC-Pack ODS-AQ, SH-343-5, 20 x 250 mm; 1-41% B (B: ACN + 0.1% TFA) in 40 min; 10 mL/min; UV detection (230 nm) RPC: Cosmosil 5Ph, 4.6 x 250 mm; 0-40% B (B: ACN + 0.1 % TFA) in 40 min; 1 mL/min; UV detection (215 nm) RPC: Cosmosil 5CN-R, 4.6 x 250 mm; 0-40% B (B: ACN + 0.1 % TFA) in 40 min; 1 mL/min; UV detection (215 nm) RPC: Cosmosil 5C18, 4.6 x 150 mm; 0-40% B (B: ACN + 0.1 % TFA) in 40 min; 1 mL/min; UV detection (215 nm)	Yokoyama et al., 1991
Bonito bowels	Autolyzed	Endogenous proteases	UF: M_{wco} 6 kDa Purification with Sep-Pak Plus C18; elution with 15% ACN Purification with Toyopak IC-SP M; elution with 10 mM PBS, pH 9.0 RPC: RP-18(e), 100 mm x 250 mm; 0-30% B (B: ACN + 0.05% TFA); 4 mL/min; UV detection (210 nm) RPC: RP-18(e), 4 mm x 250 mm; 0-30% B (B: ACN + 0.05% TFA); 1 mL/min; UV detection (210 nm) SEC: Asahipak GS-220 and GS-320, 7.6 mm x 500 mm; 50 mM NH_4Ac; 1 mL/min; UV detection (210 nm) IEC: SP-2SW, 4.6 mm x 250 mm; 0-0.5 M NaCl in 20 mM PBS, pH 6.0; UV detection (210 nm) RPC: RP-18(e), 4 mm x 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 x 90 cm; elution with water; 0.5 mL/min; UV detection (220, 280 nm) CEC: SP-Sephadex C-25, 2.5 x 45 cm; 0-1 M NaCl in 20 mM NaAc, pH 4.0; 2 mL/min; UV detection (220, 280 nm) SEC: Sephadex G-15; elution with water; 0.5 mL/min; UV detection (220, 280 nm) 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) CE: Coated capillary, 24 cm x 25 μm; 0.1 M PBS, pH=2.5; 10 kV; UV detection (200 nm)	Byun and Kim, 2001
Yellowfin sole frame	Digestion	α-chymotrypsin	UF: M_{WCO} 5, 10, 30 kDa IEC: SP-Sephadex C-25, 35 x 350 mm; 0-2 M NaCl in 20 mM NaAc, pH 4.0; 1 mL/min; UV detection (215 and 280 nm) SEC: OHpak SB-803 HQ, 8.0 x 300 mm; 20 mM NaAc, pH 4.0; 1 mL/min; UV detection (215 nm) RPC: SP Nucleosil 100- 7 C18, 1 x 250 mm; 0-19% B (B: ACN + 0.1% TFA) in 40 min; 2.0 mL/min; UV detection (215 nm) RPC: Zorbax SB C18, 4.6 x 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	Autolyzation	Endogenous proteases	UF: M_{WCO} 1, 3, 10 kDa RPC: Jupiter C12 Proteo 90 Å, 250 x 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)	Samanayaka et al., 2010
PROCEED PRODUCTS AND OTHERS				
Mushroom *Tricholoma giganteum*	Extraction	----------	UF: M_{WCO} 5 kDa SEC: Sephadex G-25, 3.0 x 35 cm; elution with water; 12 mL/min RPC: μBondapack C18; 0-100 % B; A: 0.1% TFA; B: ACN RPC: μBondapack C18; 0- 100% B; A: 0.1% TFA; B: ACN RPC: Nova-pak C18; 0- 100% B; A: 0.1% TFA; B: ACN	Hyoung et al., 2004
Mushroom *Pholiota adiposa**	Extraction	----------	UF: M_{WCO} 5 kDa SEC: Sephadex G-25, 3.0 x 80 cm; elution with water; 24 mL/min RPC: μBondapack C18; 0-100% B; A: 0.1% TFA; B: ACN	Koo et al., 2006

			RPC: μVydac protein/peptide 218Tp; 0-100% B; A: 0.1% TFA; B: ACN	
Fermented soybean paste	Extraction	----------	RPC: JAIGEL-A-343-10, 250 x 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) IEC: JAIGEL-ES-502CP, 20 x 100 mm; elution with 0.01M sodium succinate buffer (pH 4.3) in 20% ACN; 4 mL/min; UV detection (214 nm) RPC: JAIGELODS-A-343-10, 250 x 20 mm; elution with 0.05% TFA/ACN (95:5, v/v); 5 mL/min; UV detection (214 nm) IEC: Shodex Asahipak ES-2502N-7C, 100 x 7.6 mm; elution with 20 mM Tris-HCl (pH 7.5) + 125 mM NaCl; 1 mL/min; UV detection (214 nm)	Shin et al., 2001
Salt-free soy sauce	Fermentation	*Aspergillus oryzae*	Extraction: Sep-Pak Plus C18; elution with 35% ACN + 0.1% TFA RPC: Cosmosil 5C18-ARII, 4.6 x 250 mm; 5–35% B (B: ACN + 0.1% TFA) in 65 min; 0.4 mL/min; UV detection (220 nm) RPC: Cosmosil 5C18-AR300, 4.6 x 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 SEC: Sephadex- G25, 10 x 750 mm; elution with PBS; 0.2 mL/min; UV detection (220 and 280 nm) 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 RPC: Prep Nova Paks HR C18, 300 x 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
Buckwheat[a]	Digestion	Pepsin, chymotrypsin and trypsin	SEC: Superdex Peptide HR 10/30, 1 x 30 cm; elution with 30% ACN + 0.1% TFA; 0.3 mL/min; UV detection (220 nm)	Li et al., 2002
Royal jelly[b]			RPC: Cosmosil 5Ph, 4.6 x 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) RPC: Cosmosil 5C18-ARII, 4.6 x 250 mm; 5-35% B (B: ACN + 0.1% TFA) in 60 min; 0.3 mL/min; UV detection (220 nm)	Matsui et al., 2002
Garlic (*Allium sativum L)*	Extraction	----------	CEC: Dowex 50WX4, 2.5 x 30 cm; elution with 2 N NH_4OH SEC: Sephadex G-25, 2.5 x 150 cm; elution with 0.1 M PBS, pH 7.0; 30 mL/min RPC: Develosil ODS-5, 4.6 x 150 cm; 0-8% B (B: ACN + 0.05% TFA) in 1 h; 1 mL/min; UV detection (220 nm)	Suetsuna, 1998
Egg	Simulated gastrointestinal digestion	Pepsin and pancreatin	UF: M_{WCO} 3 kDa CEC: HiPreP 16/10 SP FF, 16 x 100 mm; Eq.: 10 mM NH_4Ac pH 4; Elution: 0.5 M NH_4HCO_3; 5 mL/min SEC: Superdex peptide 10/300GL, 10 x 300-310 mm	Majumder and Wu, 2009

*- indicate articles in which not all data was shown

Abbreviations:

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.

1 **Table 3. Characterization of purified peptides with antihypertensive activitiy**

Source of peptide(s)	Identified peptide(s)	IC_{50} [units]	Characterization	Ref.
Goat milk	TGPIPN, SLPQ, SQPK	316, 330, 354 [µM]	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 [µM]	Automated protein sequencing by Edman degradation	Matsumura et al., 1993
Yoghurt	8 peptides	1.56- 12.41 [µg/mL]	Automated protein/peptide sequencing by Edman degradation	Donkor et al., 2007
Beef rump	VLAQYK	23.2 [µg/mL]	Peptide sequencing by Edman degradation	Jang and Lee, 2005
Spinach Rubisco	MRWRD, MRW, LRIPVA, IAYKPAG	2.1, 0.6, 0.38, 4.2 [µM]	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 [µM]	Automated protein sequencing by Edman degradation	Abubakar et al., 1998
Rice	IY, VVY, VF, VW	4.0, 22.0, 49.7, 3.1 [µM]	Gas/liquid phase protein sequencing by Edman degradation	Kuba et al., 2009
Porcine myosin B	KRVIQY, VKAGF	6.1, 20.3 [µM] or 4.9, 10.6 [µg/mL]	Protein sequencing	Muguruma et al., 2009
Porcine skeletal muscle troponin	EKERERQ, KRQKYDI	552.5, 26.2 [µM]	Protein sequencing	Katayama et al., 2008
Salt-free soy sauce	AF, FI, IF	165, NI, 65.8 [µmol/L]	Protein sequencing	Zhu et al., 2008
Chicken bone	YYRA	33.9 [µg/mL]	Protein sequencing	Nakade et al., 2008
Pork loin	VKKVLGNP	28.5 [µM]	Protein sequencing	Katayama et al., 2007
Casein	10 peptides	22-> 1000 [µM]	Protein sequencing	Maeno et al., 1996
Dried bonito	8 peptides	3.7- 62 [µM]	Protein sequencing	Yokoyama et al., 1991
Chicken muscle	LKA, LKP, LAP, IKW, FQKPKR, FKGRYYP, IVGRPRHQG	0.32- 14 [µM]	Protein sequencing	Fujita et al., 2000
Ovalbumin	FGRCVSP, ERKIKVYL, FFGRCVSP, LW, FCF, NIFYCP	0.4- 15 [µM]		
Bovine lactoferrin	LRPVAA	4.14 [µM]	Gas-phase sequencing	Lee et al., 2006a
Hen ovotransferrin	KVREGTTY	102.8 [µM]	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 [μM]	Amino acid sequencing by RP-HPLC-MS/MS	Contreras et al., 2009
Caprine Kefir	PYVRYL, LVYPFTGPIPN	2.4, 27.9 [μM]	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 [μM]	Amino acid sequencing by HPLC-MS/MS	Gomez-Ruiz et el., 2004
Milk	40 peptides *	----------	Amino acid sequencing by HPLC-MS/MS	Hernandez-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 β-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 [μM]	Amino acid sequencing by ESI-MS/MS	Kodera and Nio, 2006
Sodium caseinate	21 peptides	39- > 1000 [μM] and 15-650 [μM]	Amino acid sequencing by ESI- MS/MS	Robert et al., 2004
Marine shrimp *(acetes chinensis)*	DP, GTG, ST	2.15, 5.54, 4.03 [μM]	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 [μM]	Amino acid sequencing by LC-ESI- MS/MS	Majumder and Wu, 2009
Several Spanish cheeses	41 major peptides**	113.1- 2419.4 [μM]	Amino acid sequencing by RPC and off-line MS/MS	Gomez-Ruiz et al., 2006
Ovine milk	IAK,VR, EKDERF, KDERF, YIPIQY, LPYPY	10.0- 848.0 [μM]	Amino acid sequencing by CE-ESI-IT-MS	Gomez-Ruiz et al., 2007
Milk	8 peptides	5.2- >1500 [μM]	Amino acid sequencing by ESI-Q-IT-MS	Quiros et al., 2007
Porcine hemoglobin	LGFPTTKTYFPHF, VVYPWT	4.92, 6.02 [μM]	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, AVKQPAVVTrYP, 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 [μM]	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 [μM]	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 [μM]	Determination of amino acid composition by hydrolysis/amino acid analysis and automatic protein sequencing by Edman degradation and FAB-MS	Sutsuna et al., 1998
Wakame (*Undaria pinnatifida*)	AIYK, YKYY, KFYG, YNKL	213, 64.2, 90.5, 21 [μM]	Determination of amino acid composition by hydrolysis/amino acid analysis and automated protein sequencing by Edman degradation and FAB-MS	Suetsuna and Nakano, 2000
α-zein	Among 3 with high activity: LRP, LSP, LQP	0.29, 1.7, 2.0 [μM]	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 [μM]	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 [μM]	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 [μM]	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 [μM]	Determination of amino acid composition by hydrolysis/amino acid analysis and automated protein sequencing by Edman degradation	Yamamoto et al., 1999
Buckwheat	VK, FY, AY, LF, YV, YQ, YQY, PSY, LGI, ITF, INSQ	4-628 [μM]	Determination of amino acid composition by HPLC and automated protein sequencing by Edman degradation	Li et al., 2002
Urea denaturated Z19 α-zein	17 peptides**	1.9- 57 [μM]	Determination of amino acid composition and automated protein sequencing by Edman degradation	Yano et al., 1996
Urea denaturated total α-zein	27 peptides**	3.9- 100 [μM]		
Mung bean protein	KDYRL, VTPALR, KLPAGTLF	26.5, 82.4, 13.4 [μM]	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 [μg/mL] (43.7 [μM])	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 [μM]	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	Gomez-Ruiz et al., 2002
Alfalfa white protein	VW	1.1 [μM]	Mw determination and amino acid sequencing by ESI-MS	Kapel et al., 2006
Tuna dark muscle	WPEAAELMMEVDP	21.6 [μM]	Mw determination and amino acid sequencing by ESI-MS	Qian et al., 2007a
Bullfrog (*Rana catesbeiana Shaw*) muscle	GAAELPCSADWW	0.95 [μM]	Mw determination and amino acid sequencing by ESI-MS	Qian et al., 2007b
Sea cucumber (*Acaudina molpadioidea*)	MEGAQEAQGD	15.9 [μM]	Mw determination and amino acid sequencing by (nano) ESI-MS/MS	Zhao et al., 2009
Tuna	GDLGKTTTVSNWSPPKYKDTP	11.28 [μM]	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 [μM]	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 [μM]	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 [μM]	Mw determination by MALDI-TOF-MS and automatic protein sequencing-HPLC	Nogata et al., 2009
Pholiota adiposa	GEGGP	254 [μM]	Mw determination by MALDI-MS and automated protein sequencing by Edman degradation	Koo et al., 2006
Yellowfin sole frame	MIFPGAGGPEL	28.7 [μg/mlL	Mw determination by SEC and automated protein sequencing by Edman degradation	Jung et al., 2006
Blue mussel *(Mytilus edulis)*	EVMAGNLYPG	19.34 [μg/mL]	Mw determination by SEC and automated protein sequencing by Edman degradation	Je et al., 2005b
Oyster (*Crassostrea talienwhanensis Crosse*)	VVYPWTQRF	66 [μmol/L]	Mw determination by LC-MS (LC-APCI-QQQ-MS) and automated protein sequencing by Edman degradation	Wang et al., 2008a
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 [μM]	Mw determination by LC-ESI-Q-MS and protein sequencing	Saiga et al., 2008

Skimmed milk	VPP, IPP	9.13, 5.15 [µM]	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 [µg/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 [µM] or 2.75, 4.26, 130.7, 84 [µg/mL]	Mw determination by SEC,quantitation of peptides by UPLC-ESI-MS, and protein sequencing	Hatanaka et al., 2009
α-lactalbumin and β-casein	9 peptides	1->76 [µM]	Amino acid sequencing by LC-MS/MS and automatic Edman degradation	Otte et al., 2007b
Glycinin from soybean	VLIVP	1.69 [µM]	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 [µM]	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 [µM]	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,- - [µM]	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 [µg/ 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 [µmol/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 [µM]	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 [µg/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 [µM]	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

Abbreviations:

IEC-Ion Exchange Chromatography; SEC- Size Exclusion Chromatography; MSD- mass selected detector; NI- no inhibition; OPA- O-phthalaldehyde;

(*)- in article authors did not show which peptides are antihypertensive although optimization of fermentation procedure in order to obtain them was on a base of 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 non-protein amino acid in this system, three letter abbreviation was used: hydroxyproline (Hyp).