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Review

Vegetable foods: A cheap source of proteins and peptides with antihypertensive, antioxidant, and other less occurrence bioactivities

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ABSTRACT

Despite less explored than foods from animal origin, plant derived foods also contain biologically active proteins and peptides. Bioactive peptides can be present as an independent entity in the food or, more frequently, can be in a latent state as part of the sequence of a protein. Release from that protein requires protein hydrolysis by enzymatic digestion, fermentation or autolysis. Different methodologies have been used to test proteins and peptides bioactivities. Fractionation, separation, and identification techniques have also been employed for the isolation and identification of bioactive proteins or peptides. In this work, proteins and peptides from plant derived foods exerting antihypertensive, antioxidant, hypocholesterolemic, antithrombotic, and immunostimulating capacities or ability to reduce food intake have been reviewed.

Contents

1. Introduction

Many plant foods possess biological properties that make them to be considered as potential functional or health-promoting foods. Some of these properties are attributed to biologically active peptides and proteins. A protein or peptide is a functional ingredient if it has been successfully demonstrated its beneficial effect on one or more functions of the body beyond its nutritional effects, so that their effect is significant for health, in general, or enables to reduce the risk to suffer a disease [1]. Bioactive peptides and proteins can present diverse activities (antioxidant, antihypertensive, hypocholesterolemic, immunostimulating, etc.). Among peptides, it is possible to differentiate between bioactive peptides that are found naturally in foods as independent entities and those peptides obtained by the hydrolysis of a precursor protein, being in a latent state as part of the sequence of that protein. As a result of proteolytic processes occurring during *in vivo* digestion of proteins or as a result of its

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processing (fermentation, autolysis, *in vitro* digestion, etc.), these peptides can be released showing certain activities. Obviously, direct ingestion of functional peptides requires they were not susceptible to digestive proteases and peptidases, thus, retaining their biological function.

As a consequence of the interest for plant foods containing bioactive proteins and peptides is the development of transgenic foods expressing gens that accumulate high levels of them. This is the case of the hypocholesterolemic peptide lactostatin, derived from bovine milk β-lactoglobulin, which has been expressed in the glutelin fraction of transgenic rice [2,3]. Another examples are the modification of the α′-subunit of soybean β-conglycinin with three peptides (novokinin, Leu–Pro–Tyr–Pro–Arg, and rubiscolin) with hypotensive, hypocholesterol, and memory-enhancing activities, respectively, [4] and the introduction of a peptide from enterostatin, peptide produced in the intestine and having hypocholesterolemic activity, in soybean β-conglycinin α′-subunit [5]. On the other hand, chemical modification of peptides by amino acid substitution or by the introduction of bioactive fragments in an amino acid sequence has also been a common practice to increase food bioactivity [6].

In the search for new proteins and peptides with biological activities, bioinformatics constitutes an important tool. Indeed, bioinformatics enables the prediction of protein structure–function relationships, the identification of protein domains, and the computer simulation of proteolytic processes. All this information can be extracted from the vast number of biologically active peptides that have already been isolated. Structural motifs in active peptides serve as a source of information to be used in the search for new bioactive molecules. All this information is ordered in databases such as PepBank (http://pepbank.mgh.harvard.edu), EROP-Noscow (http://erop.inbi.ras.ru), BioPD (http://biopd.bjmu.edu), PeptideDB (http://www.peptides.be), APD (http://aps.unmc.edu/AP/main.php), BIOPEP (http://www.uwm.edu.pl/biochemia/index_en.php), etc. BIOPEP database contains information of proteins from 54 plants hydrolyzed with 21 endopeptidases. These data revealed that wheat gliadins were the most susceptible plant proteins for bioactive peptide release [7,8].

This work has been focused on bioactive proteins and peptides from plant derived foods and has revised antihypertensive and antioxidant activities in addition to other less frequent activities such as cholesterol reduction capability, immunomodulation, opioid activity, etc.

2. Antihypertensive peptides

Antihypertensivity is the main bioactivity found in plant food-derived peptides. Despite there are different biological pathways regulating blood pressure in living organisms, hypotensive peptides act mainly on the rennin-angiotensin system. The renin-angiotensin system starts by the conversion of angiotensinogen to the pre-hypertensive hormone angiotensin I (Asp–Arg–Val–Tyr–Ile–His–Pro–Phe–His–Leu) by the action of renin which is secreted by kidneys. Angiotensin I is further converted to angiotensin II (Asp–Arg–Val–Tyr–Ile–His–Pro–Phe), the active form of the hormone, by the action of angiotensin converting enzyme (ACE). Angiotensin II raises blood pressure by acting directly to blood vessels, symphatic nerves, and adrenal glands [9]. Moreover, ACE can also inactivate the antihypertensive vasodilator bradykinin. Potent synthetic ACE inhibitors are commercialized for the regulation of blood pressure. Nevertheless, these synthetic compounds yield side effects such as coughing, taste disturbances, and skin rushes. Food derived ACE inhibitor peptides could be an alternative to synthetic drugs since they do not present those after-effects.

There are different vegetable foods showing antihypertensive properties. Table 1 groups the research works focused to the determination of antihypertensive proteins and peptides from plant foods. They have been classified according to the way peptides and proteins have been obtained: peptides and proteins not encrypted in any parent molecule and peptides obtained by enzymatic hydrolysis, fermentation, gastrointestinal digestion, and autolysis. Accumulated information from all these research works has resulted in a clear relationship between peptide structure and ACE inhibitory potency. Both the type of amino acids and peptide sequences determine peptide activity. Most ACE inhibitors peptides present short sequences ranging from 2 to 12 amino acids. Best antihypertensive peptides contain hydrophobic amino acids such as Pro, especially, at C-terminal position or positively charged amino acids such as Lys or Arg at C-terminal position [76]. The spectrophotometric method described by Cushman and Cheung [77] is the most widely employed for measuring ACE *in vitro* inhibition. This method is based on the hydrolysis of hippuryl–histidyl–leucine (HHL) by ACE to yield hippuric acid (HA) and histidyl–leucine. The HA is extracted into ethyl acetate and quantified by measuring its absorbance at 228 nm. The inhibitory potency is expressed as the IC_{50} value which is defined as the concentration needed to inhibit 50% of the enzyme activity. Nevertheless, this method involves several tedious steps and ethyl acetate extraction of HA can also co-extract unhydrolyzed HHL which also absorbs at 228 nm, overestimating ACE activity. Alternative methods not comprising any extraction step have been proposed. Wu et al. [26,78] designed a rapid and sensitive method that used reversed-phase chromatography (RPC) for the separation of HHL and HA. Some years later, Li et al. [79] developed an extraction-free method based on the specific reaction of HA with benzene sulfonyl chloride in the presence of quinoline. More recently, other authors have proposed a rapid and sensitive method that simultaneously quantified HA and HHL by ultra-performance liquid chromatography–mass spectrometry (UPLC–MS) [80]. Nevertheless, this method has never been applied in the case of peptides but to synthetic drugs.

Peptides not obtained from a parent molecule and naturally occurring in foods have been observed in garlic, wakame seaweed, buckwheat, broccoli, and edible mushrooms. Extraction methods, in all cases, were very simple and consisted of protein extraction with water or an organic solvent such as MeOH. In most cases, a subsequent fractionation step was performed to finally isolate bioactive peptides. Most active peptides were obtained in wakame, garlic, and buckwheat. Indeed, seven different dipeptides were firstly isolated from garlic (*Allium sativum L*) by Suetsuna [10] despite some years before they could identified up to 16 peptides in a garlic extract [81]. The same authors identified ten peptides with antihypertensive properties from wakame (*Undaria pinnatifida*) [11]. Moreover, two tripeptides with sequences Gly–Pro–Pro and Tyr–Pro–Lys have also demonstrated hypotensive activity in buckwheat, a recognized functional food in China, Japan, and Poland [12], and in broccoli [13], respectively. Nevertheless, most naturally occurring peptides have been observed in mushrooms. Indeed, mushroom *Tricholoma giganteum* demonstrated native ACE inhibitory activity provided by a tripeptide that kept active after *in vitro* incubation with proteases [14]. Another mushroom that has also been studied for its content in peptides with antihypertensive activity is *Pleurotus cornucopiae*. Two peptides with IC_{50} values of 0.46 and 1.14 mg/mL were isolated and submitted to simulated gastrointestinal digestion. Fig. 1 shows the MS/MS spectra corresponding to these peptides and used for their sequence identification. Results suggested that purified peptides became stronger ACE inhibitors after gastrointestinal digestion [15]. Kokean et al. [16] evaluated the effect of cooking on antihypertensive properties of Hatakeshimeji

Table 1
Determination of antihypertensive proteins and peptides in vegetable foods.

Product	Objective	Sample preparation	Separation/fractionation	Peptide	Ref.
Peptides/proteins not encrypted in any parent molecule					
Garlic	Isolation and characterization of peptides with ACE inhibitory activities	Homogenization in water, precipitation with cold MeOH, and suspension in water	IEC; SEC; RPC (0–8% B (AcN+0.05% TFA) in 1 h, 1 mL/min, 220 nm)	Ser–Tyr (IC_{50}, 66.3 μM), Gly–Tyr (IC_{50}, 72.1 μM), Phe–Tyr (IC_{50}, 3.74 μM), Asn–Tyr (IC_{50}, 32.6 μM), Ser–Phe (IC_{50}, 130.2 μM), Gly–Phe (IC_{50}, 277.9 μM), and Asn–Phe (IC_{50}, 46.3 μM)	10
Wakame seaweed	Examination of ACE inhibitory activity of wakame and evaluation of *in vivo* activity	Extraction of peptides with hot water for 20 min and centrifugation	IEC; SEC; RPC (0–25% B (AcN+0.05% TFA) in 180 min, 1 mL/min, 220 nm); RPC (25% AcN in acetate buffer (pH 6.8), 0.5 mL/min). Sequence analysis by automatic Edman degradation	10 dipeptides being Tyr–His (IC_{50}, 5.1 μM), Lys–Tyr (IC_{50}, 7.7 μM), Phe–Tyr (IC_{50}, 3.7 μM), Ile–Tyr (IC_{50}, 2.7 μM) the most active	11
Buckwheat	To isolate ACE inhibitory substances from buckwheat	Protein extraction at pH 9 and protein precipitation at pH 4.5	IEC (water–amonium formate, 8 mL/min, 228 and 280 nm); SEC (water, 5 mL/min); SEC (10% MeOH, 8 mL/h); RPC (5–20% AcN, 3 mL/min). Identification by RPC–ESI–MS	Gly–Pro–Pro (IC_{50}, 6.25 μg/mL)	12
Broccoli	To examine the ACE inhibitory activity of broccoli and to isolate and identify the inhibitor	Extraction in water	IEC; SEC (8 mL/h); RPC (5–20% B (AcN), 3 mL/min, 210 nm)	Tyr–Pro–Lys (IC_{50}, 10.5 μg/mL)	13
Tricholoma giganteum mushroom	Extraction and characterization of a novel ACE inhibitory peptide	Mushroom extraction in water, EtOH, and MeOH	Ultrafiltration; SEC; RPC (0–100% B (AcN+0.1% TFA) by different C_{18} columns. LC–MS analysis and automatic Edman degradation	Gly–Glu–Pro (IC_{50}, 3200 μM)	14
Pleurotus cornucopiae mushroom	Characterization of a new ACE inhibitor from the fruiting body of *P. cornucopiae.*	Extraction in MeOH–water (30 °C, 24 h)	Ultrafiltration; SEC (0.5 mL/min); C_{18} and strong cation-exchange solid-phase extraction; RPC (5–25 % B (AcN+0.1% TFA))	Arg–Leu–Pro–Ser–Glu–Phe–Asp–Leu–Ser–Ala–Phe–Leu–Arg–Ala (IC_{50}, 52 μM), and Arg–Leu–Ser–Gly–Gln–Thr–Ile–Glu–Val–Thr–Se–Gu–Tyr–Leu–Phe–Arg–His (IC_{50}, 1079 μM)	15
Hatakeshimeji mushroom	To study the effect of deep-frying on the antihypertensive activity of this mushroom	Extraction in water (10 min) and acetone precipitation of proteins	SEC (0.5 mL/min)	–	16
Grifola frondosa	To isolate, identify, and purify peptides with ACE inhibitory activity	Extraction with water (100 °C, 150 min), MeOH, and EtOH (25 °C, 15 h)	IEC (10 mM Tris–HCl buffer (pH 8.0), 0–0.5 M NaCl, 0.5 mL/min); SEC (10 mM Tris–HCl buffer (pH 8.0), 6 mL/h); SEC (15% MeOH); IEC (imidazol–HCl buffer, pH 7.4; RPC (0–50% AcN)	–	17
Peptides obtained by enzymatic hydrolysis					
Mung bean	To isolate and identify peptides with ACE inhibitory activity	Suspension in water, centrifugation, and precipitation at pH 4.6. Alcalase digestion of pellet at 55 °C, pH 8, 2 h	Ultrafiltration; RPC (0–100% B (60% AcN+0.1% TFA) in 60 min, 1 mL/min, 220 nm); RPC (10–80% B (AcN+0.1% TFA) in 40 min). Peptide sequencing by MALDI-TOF	Lys–Asp–Tyr–Arg–Leu (IC_{50}, 26.5 μM), Val–Thr–Pro–Ala–Leu–Arg (IC_{50}, 82.4 μM), and Lys–Leu–Pro–Ala–Gly–Thr–Leu–Phe (IC_{50}, 13.4 μM)	18
Rice	Investigation of the antihypertensive effect of hydrolysed rice protein	Extraction with NaOH, centrifugation, and precipitation at pH 5.5. Alcalase digestion at 55 °C, pH 8, 2 h	SEC (20 mM acetate buffer (pH 4), 0.4 mL/min, 220 nm); RPC (0–60% B (AcN+0.1% TFA) in 60 min, 1 mL/min, 220 nm); RPC (10–30% B (AcN+0.1% TFA) in 40 min, 1 mL/min)	Thr–Gln–Val–Tyr (IC_{50}, 18.2 μM)	19
Chickpea (legumin fraction)	Determination of ACE inhibitory activity of chickpea	Differential dialysis, purification by IEC and SEC and alcalase hydrolysis at 50 °C, pH 7, 30 min	Ultrafiltration; RPC (0–30% B (AcN+0.1% TFA) in 60 min, 1 mL/min, 214 nm). Peptide sequencing by acid hydrolysis, amino acid derivatization, and RPC separation	Six peptides exhibiting antihypertensive activity were observed being Met–Asp–Phe–Leu–Ile (IC_{50}, 0.011 mg/mL) that showing the highest inhibition	20

Cowpea	To determine ACE inhibitory activity in cowpea hydrolysates	Cowpea flour was extracted with water at pH 11, proteins were precipited at pH 4.5 and hydrolyzed with alcalase (pH 8.0, 50 °C, 90 min), flavourzyme (pH 7.0, 50 °C, 90 min), and pepsin (pH 2, 37 °C, 45 min)-pancreatin (pH 7.5, 37 °C, 45 min)	Ultrafiltration	–	21
Peanut	To explore ACE antihypertensive properties of peanuts	Defatting with hexane, hydrolysis with alcalase (pH 7.5, 60°C, 20 min) and pepsin (pH 1.9, 37 °C, 20 min)-pancreatin (pH 7.5, 37 °C, 20 min)	RPC (0–70% B (AcN–TFA) in 75 min, 1 mL/min, 210 nm)	–	22
Amaranth	To obtain ACE inhibitory peptide fractions from amaranth albumin and globulin	Extracts were hydrolyzed with alcalase at 50 °C, pH 7.4, 5 min	SEC (phosphate buffer, 0.4 mL/min, 214 nm); RPC (0–30% B (AcN+0.1% TFA) in 60 min, 2 mL/min, 214 nm)	–	23
Amaranth	To study the hydrolytic release of encrypted peptides from storage proteins	Defating with hexane, flour suspension at pH 9 and protein precipitation at pH 5. Hydrolysis with alcalase and pronase, papain, trypsin or chymotrypsin	–	–	24
Amaranth	To evaluate the antihypertensive activity of amaranth glutelins	Glutelins were extracted by removing of albumin with distilled water, globulins with phosphate buffer+NaCl and EDTA, and prolamins with aqueous EtOH. Solid residue was digested with trypsin at 37 °C, 10 h	RPC (60–80% B (AcN–water–0.1% formic acid), 400 nL/min)	–	25
Soybean	Investigation of the *in vivo* effect of ACE inihibitory peptides in soybean	Soybean meal solution was digested with alcalase at 50 °C, pH 9, 12 h	Two SEC fractionation steps using Sephadex G-15 and Sephadex G-25 columns; two RPC fractionations using two different C_{18} columns. Peptide sequencing using an automatic sequencer	Asp–Leu–Pro (IC_{50}, 4.8 μM) and Asp–Gly (IC_{50}, 12.3 μM)	26
Soybean glycinin	To investigate ACE inhibitory properties of enzymatic hydrolysates of glycinin	Glycinin extraction in water+β-mercaptoethanol, centrifugation, and precipitation with $MgCl_2$. Digestion with protease P, trypsin or chymotrypsin at 37 °C, pH 8.2, 18 h	RPC (0–35% B (water–TFA–AcN), 0.7 mL/min, 230 nm); RPC (0–50% B (50 mM NH_4Ac/AcN); RPC separation as in first step	Val–Leu–Ile–Val–Pro (IC_{50}, 1.69 μM)	27
Soybean whey proteins	Evaluation of antihypertensive activity of soybean whey proteins	Digestion with protease S	SEC fractionation with a Sephadex G-25 column; RPC	Val–Ala–Pro, Val–Lys–Pro, Val–Thr–Pro	28
Soybean whey protein	Investigation of antihypertensive effect of peptides from soybean whey proteins	Thermolysin and protease S digestion	SEC; RPC	Leu–Ala–Pro, Leu–Asn, Leu–His–Pro, Leu–Lys–Pro, Val–Thr–Thy, Leu–Thy–Gln–Ala, Thy–Glu–Ala–Pro, Thy–Gln–Ala–Pro	29
Soybean	Investigation of the conditions to produce soybean hydrolysates with a high ACE inhibitory activity and a bland taste	Digestion with protease M at 40 °C, pH 4.5. Digestion with orientase 90 N at 40 °C, pH 7.5	–	–	30
Soybean protein isolate	Identification of novel ACE inhibitory peptides	Digestion with protease D3 at 37–40 °C, pH 4.5, 24–48 h	SEC; RPC (0–50% B (AcN+0.1% TFA) in 50 min, 0.75 mL/min). Peptide sequencing by ESI–MS	Eight peptides were identified being Asp–Trip–Gly–Pro–Leu–Val the most active (IC_{50}, 21 μM)	31
Soybean fermented products (natto and tempeh) and soybean hydrolysate	Evaluation of biological activities of soybean fermented or hydrolyzed products submitted to digestion with endoproteases	Samples were dissolved in ammonium bicarbonate and individually digested with pronase, trypsin, Glu C protease, plasma proteases, and kidney membrane proteases	RPC (5–75% AcN in 70 min, 1 mL/min, 206 and 280 nm). Separated peptides were infused by MS for peptide identification	–	32
Soybean	Isolation of ACE inhibitory peptides derived from soybean	Pepsin hydrolysis at 37 °C, pH 2, 24 h. Filtration and centrifugation	IEC; two different SEC; RPC (0–16% B (AcN+0.05% TFA) in 60 min, 1 mL/min, 220 nm). Automatic peptide sequencing	Tyr–Leu–Ala–Gly–Asn–Gln (IC_{50}, 14 μM), Phe–Phe–Leu (IC_{50}, 37 μM), Ile–Tyr–Leu–Leu (IC_{50}, 42 μM), Val–	33

Table 1 (*continued*)

Product	Objective	Sample preparation	Separation/fractionation	Peptide	Ref.
				Met–Asp–Lys–Pro–Gln–Gly (IC_{50}, 39 μM), and Ile–Ala (IC_{50}, 153 μM)	
Rice koji	Manufacture of a peptide with hypotensive properties	Pepsin digestion	–	Val–Ala–Asn–Asp (IC_{50}, 411 ppm)	34
Wakame	Identification of antihypertensive peptides	Digestion with pepsin at 45 °C, pH 2, 5 h	Dyalization; IEC; SEC; RPC (0–25% B (AcN+0.05% TFA) in 2 h, 1 mL/min, 220 nm)	Ala–Ile–Tyr–Lys (IC_{50}, 213 μM), Tyr–Lys–Tyr–Tyr (IC_{50}, 64.2 μM), Lys–Phe–Tyr–Gly (IC_{50}, 90.5 μM), and Tyr–Asn–Lys–Leu (IC_{50}, 21 μM)	35
Wakame	Isolation and identification of the ACE inhibitory peptides from Protease S digest of wakame	Digestion with protease S. Extraction with butanol by several times	RPC (0–35% B (water–AcN–0.1% TFA) in 140 min, 30 mL/min, 220 nm); RPC (0–20% B (50 mM NH_4Ac (pH 10)/AcN, 5:95, v/v) in 50 min, 1 mL/min, 220 nm); RPC (0–20% B (50 mM NH_4Ac (pH 10)/AcN, 5:95, v/v) in 30 min, 0.5 mL/min); RPC (0–30% B (AcN+0.07% TFA) in 40 min, 1 mL/min, 220 nm)	Val–Tyr (IC_{50}, 35.2 μM); Ile–Tyr (IC_{50}, 6.1 μM), Ala–Trp (IC_{50}, 18.8 μM), Phe–Tyr (IC_{50}, 42.3 μM), Val–Trp (IC_{50}, 3.3 μM), Ile–Trp (IC_{50}, 1.5 μM), and Leu–Trp (IC_{50}, 23.6 μM)	36
Chlorella vulgaris and *Spirulina platensis* algae	To isolate and characterize ACE inhibitory peptides from two microalgae	Pepsin digestion at 45 °C, pH 2, 5 h	IEC; SEC (30 mL/h); RPC (0–16% B (AcN+0.05% TFA) in 2 h, 1 mL/min, 220 nm)	Ile–Val–Val–Glu, Ala–Phe–Leu, Phe–Ala–Leu, Ala–Glu–Leu, Val–Val–Pro–Pro–Ala *(C. vulgaris)* and Ile–Ala–Glu, Phe–Ala–Leu, Ala–Glu–Leu, Ile–Ala–Pro–Gly, Val–Ala–Phe (*S. platensis)*	37
Yam tuber protein dioscorin	To investigate the antihypertensive activity of yam tuber protein dioscorin and its hydrolysate	Pepsin digestion at 37 °C, pH 2, and different times	SEC (20 mM Tris–HCl buffer (pH 7.9), 30 mL/h, 230 nm)	–	38
Potato	To evaluate the ACE inhibitory activity of proteins from potato and by-products	Hydrolysis with alcalase (pH 7, 55 °C, 5 h), neutrase (pH 7, 50 °C, 5 h), and esperase (pH 7, 55 °C, 5 h)	Ultrafiltration, RPC (2–60% B (AcN–water–0.05% TFA) in 55 min, 1 mL/min, 214 nm)	–	39
Native α-zein (maize standard)	Isolation and identification of ACE inhibitory peptides	α-zein digestion with thermolysin at 65 °C, pH 8	IEC, SEC, RPC (10–60% B (AcN+0.1% TFA)), IEC. Peptide sequencing by Edman degradation	14 peptides were isolated being the most active Leu–Arg–Pro (IC_{50}, 0.27 μM), Leu–Ser–Pro (IC_{50}, 1.7 μM), and Leu–Gln–Pro (IC_{50}, 1.9 μM)	40
Urea denatured α-zein (from corn gluten meal)	Isolation and identification of ACE inhibitory peptides in urea denatured α-zein	Extraction of α-zein from corn gluten meal with 70% EtOH and thermolysin digestion at 37 °C, 3 h	RPC (0–30% B (AcN+0.1% TFA)). Peptide sequencing by manual Edman degradation	36 peptides were purified	41
Maize lines	To develop an analytical method to evaluate the content of three antihypertensive peptides in different maize lines	Extraction of α-zeins with EtOH, purification by precipitation with acetone+β-mercaptoethanol, thermolysin digestion (pH 8.5, 6 h, 50 °C)	RPC (3–97% B (AcN+20 mM acetic acid) in 12 min, 0.4 mL/min, 40 °C, 210 nm)	Leu–Gln–Pro (IC_{50}, 2.0 μM), Leu–Ser–Pro (IC_{50}, 1.7 μM), and Leu–Arg–Pro (IC_{50}, 0.29 μM)	42
Corn gluten	Preparation of an hydrolysate of corn gluten containing powerful ACE inhibitory activity	Starch digestion with termamyl, filtration, thermal denaturation (80–100 °C, 30 min), hydrolysis with flavourzyme, protamax, proleather, protease A, aroase AP-10, pescalase	–	–	43
Corn gluten meal	Isolation and characterization of an ACE inhibitory peptide	Starch digestion with α-amylase, suspension in Na_2SO_3 at 90 °C for 15 min, alcalase digestion at 60 °C, pH 8, 5 h	Ultrafiltration; SEC; RPC (0–40% B (AcN+0.1% TFA) at 3 mL/min, 220 nm). Peptide sequencing by LC/MS	Ala–Tyr (IC_{50}, 14.2 μM)	44
Sesame	Isolation of antihypertensive peptides	Commercial thermolysin digested sesame was dissolved in 10% EtOH	SEC; RPC (5–40% B (AcN+0.1% TFA) in 80 min, 10 mL/min); RPC in a C_{30} column (4 mL/min and 210 nm); RPC in a phenyl column (4 mL/min, 210 nm). Peptide sequencing by MS	Leu–Val–Tyr (IC_{50}, 1.80 μM), Leu–Ser–Ala (IC_{50}, 7.81 μM), Leu–Gln–Pro (IC_{50}, 1.04 μM), Leu–Lys–Tyr (IC_{50}, 0.78 μM), Ile–Val–Tyr (IC_{50}, 14.74 μM) Val–Ile–Tyr (IC_{50},	45

				4.50 μM), and Met–Leu–Pro–Ala–Tyr (IC_{50}, 1.58 μM)	
Flaxseed	To determine the ability of flaxseed hydrolysate to inhibit ACE activity	Thermolysin digestion (37 °C, pH 7.5, 5 h) followed by papain (37 °C, pH 6.5, 5 h), ficin (37 °C, pH 7.0, 5 h), alcalase (50 °C, pH 8.0, 5 h), and pronase digestion (40 °C, pH 7.4, 5 h)	Adsorption onto activated carbon; SEC (50 mM phosphate buffer+0.15 M NaCl (pH 7), 0.75 mL/min, 214 nm)	–	46
Wheat gliadin	Exploration of ACE inhibiting activity of peptides obtained by hydrolysis of wheat gliadin with different enzymes	Gliadin extraction with 70% EtOH. Protein was dissolved with citric acid and hydrolysed at 45 °C, 5 h, with different enzymes (pepsin, molsin F, rapidase, orientase 5A, protease M, pepsin–protease M)	IEC (0–5 M NaCl); SEC (eluted with water); RPC (0–30% B (AcN+0.1% TFA), 1 mL/min)	Ile–Ala–Pro (IC_{50}, 2.7 μM)	47
Lentils and beans	Preparation of proteases from different plants and tuna, application to the hydrolysis of lentils and beans, and evaluation of ACE inhibitory activity of hydrolysates	Digestions were performed at 37 °C, pH 7.0, 24 h	–	–	48
Brassica carinata	To study the biological activity of hydrolysates	Immobilized trypsin, chymotrypsin, and carboxypeptidase A	SEC (NH_4HCO_3, 10 mL/h)	–	49
Apricot almond	To investigate *in vitro* and *in vivo* the ACE inhibitory activity of apricot almond hydrolysate	Simultaneous digestion with neutrase and N120P protease	SEC; RPC	–	50
Rice dregs	Optimization of a hydrolysis method for the production of rice dreg antihypertensive peptides	Trypsin hydrolysis	SEC		51
Rapeseed	To investigate rapeseed protein as a source of new peptides having ACE inhibitory activity	Digestion with trypsin, pancreatin, thermolysin, and subtilisin at 37 °C, pH 7.5, 5 h. Digestion with pepsin at 37 °C, pH 2, 5 h	RPC (0–40% B (AcN+0.1% TFA) in 40 min, 10 mL/min); RPC (0–40% B (AcN+0.1% TFA) in 40 min, 5 mL/min); RPC (0–40% B (AcN+0.1% TFA) in 40 min, 1 mL/min)	Ile–Tyr, Arg–Ile–Tyr, Val–Trp, and Val–Trp–Ile–Ser	52
Wheat germ	Isolation of antihypertensive peptides from wheat germ hydrolysate	Hydrolysis with *Bacillus licheniformis* alkaline protease (8 h) after α-amylase treatment (3 h)	SEC, IEC (0.1 M NH_4OH)	Ile–Val–Tyr (IC_{50}=0.48 μM)	53
Corn germ	To study suitable enzymatic conditions leading to the formation of ACE inhibitory peptides	Enzymatic hydrolysis with trypsin, thermolysin, GC 106, and flavourzyme at 45 °C, 4 h	Ultrafiltration		54
Peptides obtined by fermentation					
Miso paste	Study of the effect on antihypertensive capacity of adding casein to miso paste	–	–	–	55
Soybean sauce	Development of a peptide-enriched soybean sauce with higher ACE antihypertensive activity	–	Dyalisis; RPC (0–100% B (AcN+0.1% TFA) in 25 h, 45 mL/min); RPC (0–100% B (70% AcN+0.1% TFA) in 90 min, 5 mL/min; RPC (0–100% B (70% AcN+0.1% TFA) in 90 min, 5 mL/min). NMR; RPC–MS	Ala–Trp (IC_{50}, 10 μg/mL), Gly–Trp (IC_{50}, 30 μg/mL), Ala–Tyr (IC_{50}, 48 μg/mL), Ser–Tyr (IC_{50}, 67 μg/mL), Gly–Tyr (IC_{50}, 30 μg/mL), Ala–Phe (IC_{50}, 190 μg/mL), Val–Pro (IC_{50}, 480 μg/mL), Ala–Ile (IC_{50}, 690 μg/mL), Val–Gly (IC_{50}, 1100 μg/mL), and nicotianamine (IC_{50}, 0.62 μg/mL)	56
Soybean sauce	Evaluation of ACE inhibitory activity of a salt-free soybean sauce	Reversed-phase solid extraction (AcN+0.1% TFA)	RPC (5–35% B (AcN+0.1% TFA) in 65 min, 0.4 mL/min, 220 nm); RPC (5–35% B (AcN+0.1% TFA) in 65 min, 0.3 mL/min, 220 nm)	Ala–Phe (IC_{50}, 165.3 μM) and Ile–Phe (IC_{50}, 65.8 μM)	57
Fermented soybean	Isolation and identification of ACE inhibitors by accelerated fermentation of soybean	Incubation with *Aspergillus oryzae*	Ultrafiltration; FPLC (0–100% B (20 mM acetate buffer+1 M NaCl, pH 4) in 40 min, 5 mL/min, 214 nm); SEC; RPC (0–100% B (40% AcN+0.1% TFA) in 40 min, 1 mL/min, 214 nm). Peptide sequencing by Edman degradation	Leu–Val–Gln–Gly–Ser (IC_{50}, 43.7 μM)	58
Tofoyu			RPC (0–70% EtOH); RPC (0–40% (AcN+0.05% TFA) in 80 min, 0.5 mL/min, 220 nm).	Ile–Phe–Leu (IC_{50}, 44.8 μM) and Trp–Leu (IC_{50}, 29.9 μM)	59

Table 1 (*continued*)

Product	Objective	Sample preparation	Separation/fractionation	Peptide	Ref.
	Isolation and characterization of ACE inhibitor peptides from a fermented soybean product	Suspension in water, centrifugation to take the supernatant, and filtration	Peptide sequencing by automatic Edman degradation		
Douchi	Exploration of ACE inhibitory activity	Suspension in water, centrifugation, and supernatant filtration	SEC; RPC (0–60% B (AcN+0.1% TFA) in 60 min, 1 mL/min, 220 nm)	–	60
Korean fermented soybean paste	Isolation and identification of ACE inhibitory peptides	–	RPC (2–65% B in 55 min, 5 mL/min, 214 nm); IEC (0.01 M succinate buffer (pH 4.3)/AcN (80:20, v/v), 4 mL/min); RPC (0.05% TFA in AcN, 5 mL/min, 214 nm); IEC (20 mM Tris–HCl buffer+125 mM NaCl, pH 7.5, 1 mL/min). Automatic peptide sequencing	His–His–Leu (IC_{50}, 2.2 μg/mL)	61
Sourdough fermented products from wheat and rye	Investigation of the synthesis of ACE inhibitory peptides and γ-aminobutyric acid during sourdough fermentation of different cereals	Wheat and rye flours were fermented with different *Lactobacillus* strains. An aliquot of dough (7.5 g) was diluted in Tris–HCl buffer (pH 8.8) and centrifuged	RPC (5–46% B (water–0.05% TFA–AcN) in 48 min, 1 mL/min). Peptide identification by nanoLC–ESI–MS/MS	14 peptides were identified in wheat ($IC_{50,}$ 0.19–0.54 mg/mL)	62
Alfalfa	Hydrolysis of an alfalfa concentrate and characterization of ACE inhibitory peptides in the hydrolysate	Industrial hydrolysis with a protease mixture consisting of *Bacillus licheniformis*	SEC (30% AcN+0.1% TFA, 0.2 mL/min, 226 nm); RPC (0–47% B (AcN+0.1% TFA) in 70 min, 226 nm). Peptide identification by ESI–MS	Val–Trp (IC_{50}, 3.6 μM)	63
Rice-koji	Selection of most favorable *Monascus* strains to obtain red-mold rice with high ACE inhibitory activity, isolation and identification of peptides, and *in vitro* characterization	Rice was cultivated with 24 different *Monascus* strains at 30 °C for 7 days. Resulting red-mold-rice was extracted with water, centrifuged, and filtrated	IEC; SEC; RPC (0–50% B (AcN+0.05% TFA) in 50 min, 0.5 mL/min, 220 nm); RPC at the same conditions but 0.25 mL/min	Ile–Tyr (IC_{50}, 4.0 μM), Val–Val–Tyr (IC_{50}, 22.0 μM), Val–Phe (IC_{50}, 49.7 μM), and Val–Trp (IC_{50}, 3.1 μM)	64
Wine	Fraction and purification of hypotensive peptides in wine	Concentration	RPC (0–90% EtOH); SEC; RPC (0–50% AcN)	Leu–Ile–Pro–Pro–Gly–Val–Pro–Tyr (IC_{50}, 17.5 μM), Tyr–Tyr–Ala–Pro–Phe–Asp–Gly–Ile–Leu (IC_{50}, 83.0 μM), Tyr–Tyr–Ala–Pro–Phe (IC_{50}, 26.4 μM), Se–Trp–Ser–Phe (IC_{50}, 76.3 μM), Trp–Val–Pro–Ser–Val–Tyr IC_{50}, 25.7 μM), and Ala–Trp–Pro–Phe (IC_{50}, 18.3 μM)	65
Sake and sake less	*In vivo* evaluation of antihypertensive effects of sake peptides	–	–	–	66
Peptides obtained by gastrointestinal digestion					
Sake and sake less	Investigation of resistance of antihypertensive peptides to hydrolysis with digestive enzymes	Pepsin digestion (pH 2, 3 h, 37 °C) followed by pancreatin digestion (pH 8, 20 h, 37 °C)	RPC (5–60% B (AcN+0.1% TFA), 1 mL/min); SEC (water, 2 mL/min)	Val–Trp, Tyr–Trp, Val–Trp–Tyr, His–Tyr, Val–Tyr, Tyr–Gly–Gly–Tyr, Phe–Trp–Asn, Ile–Tyr–Pro–Arg–Tyr, and Arg–Phe	67
Soybean protein isolate (SPI)	Investigation of ACE inhibitory activity in pepsin–pancreatin digested SPI	SPI was solubilised in water at pH 2.0, digested with pepsin (37 °C, 1 h), and digested with pancreatin (pH 7.5, 37 °C, 2 h)	IEC (0.2–2 M NaCl, 2.5 mL/min, 280 nm); ultrafiltration; RPC (2–100 % B (AcN+0.05% TFA) in 80 min, 0.5 mL/min, 214 nm); SEC (30% AcN+0.05% TFA, 0.2 mL/min, 214 nm)	–	68
Spinach	To test spinach rubisco for ACE inhibitory peptides	Pepsin digestion (pH 2, 37 °C, 5 h); pancreatin digestion (pH 7.5, 37 °C, 5 h)	Two RPC separations using C_{18} columns and water–0.1% TFA–AcN gradients, 230 nm, flow-rates of 10 and 1 mL/min, respectively. Further purification in ciano and phenyl columns	Met–Arg–Trp–Arg–Asp (IC_{50}, 2.1 μM), Met–Arg–Trp (IC_{50}, 0.6 μM), Leu–Arg–Ile–Pro–Val–Ala (IC_{50}, 0.38 μM), and Ile–Arg–Tyr–Lys–Pro–Arg–Gly (IC_{50}, 4.2 μM)	69
Buckwheat	Evaluation of production of antihypertensive peptides during buckwheat gastrointestinal digestion and isolation of peptides	Heat-treated ground buckwheat was dissolved in water and submitted to hydrolysis with pepsin (pH 1.2, 4 h,	SEC (30% AcN+0.1% TFA, 0.3 mL/min, 220 nm, 35 °C), two RPC separation (5–35% B (AcN+0.1% TFA) in 30 min, 0.3 mL/min, 220 nm)	Eleven peptides were identified being the following the most potent ones: Tyr–Gln–Tyr (IC_{50}, 4 μM), Val–	70

		37 °C), chymotrypsin, and trypsin (pH 6.8, 4 h, 37 °C)		Lys (IC_{50}, 13 μM), and Pro–Ser–Tyr (IC_{50}, 16 μM)	
Pea	To assess *in vitro* ACE inhibitory activity of pea	Digestion by pepsin (pH 2, 2 h, 37 °C), trypsin, and chymotrypsin (pH 6.5, 2.5 h, 37 °C)	–	–	71
Sunflower	Production of ACE inhibitory peptides during digestion of sunflower protein isolates	Pepsin digestion (pH 2, 37 °C, 180 min) followed by pancreatin digestion (pH 7, 37 °C, 180 min)	Ultrafiltration, SEC (ammonium bicarbonate (pH 9.1), 10 mL/h), preparative RPC (0–30% B (AcN+0.1% TFA) in 50 min, 1 mL/min, 30 °C, 215 nm), analytical RPC (10–30% B (AcN+0.1% TFA) in 50 min, 1 mL/min, 30 °C, 215 nm)	Phe–Val–Asn–Pro–Gln–Ala–Gly–Ser	72
Hempseed	To determine antihypertensive properties in hemp seed hydrolysates	Consecutive treatment with pepsin (2 h) and pancreatin (4 h)	Ultrafiltration	–	73
Peptides obtained by autolysis					
Wheat bran	Examination of ACE inhibitory activity by autolysis of different parts of wheat	Milled samples were defatting for 3 times with *n*-hexane, suspended in citrate–phosphate buffer (pH 3.2), and autolyzed at 40 °C for 12 h	RPC (EtOH); SEC (30% can+0.1% TFA), 0.5 mL/min, 220 nm); RPC (0–35% B (can+0.1% TFA) in 30 min, 4 mL/min). Automatic protein sequencing	Leu–Gln–Pro (IC_{50}, 2.2 μM), Ile–Gln–Pro (IC_{50}, 3.8 μM), Leu–Arg–Pro (IC_{50}, 0.21 μM), Val–Tyr (IC_{50}, 21 μM), Ile–Tyr (IC_{50}, 3.4 μM), and Thr–Phe (IC_{50}, 18 μM)	74
Cacao beans	To explore antihypertensive properties of cacao extracts	Fat removal with hexane, extraction with chloroform, and polyphenol removal. Autolysis in acetic acid (pH 3.5) or acetate buffer (pH 5.2) at 50 °C for 16 h	–	–	75

IEC-Ion Exchange Chromatography; SEC-Size Exclusion Chromatography; RPC-Reversed Phase Chromatography; ESI–MS-electrospray mass spectrometry; AcN–Acetonitrile; MeOH–methanol; EtOH–ethanol; TFA–trifluoroacetic acid.

mushroom. Despite the molecular mass of proteins in the fruit body was lowered by deep-frying with cooking oil, antihypertensive capacity was not lost. Furthermore, Choi et al. [17] investigated the ACE inhibitory activity of ten edible mushrooms observing the higher hypotensive effect in *Grifola frondosa*.

Nevertheless, the main approach to produce peptides is enzymatic hydrolysis. Different enzymes have been employed being alcalase (commercial name of Subtilisin Carlsberg endopeptidase) and pepsin the most popular ones. It is also important to highlight the nonspecific protease thermolysin that yielded some of the most hypotensive peptides. Regarding foods, most active peptides were found in legumes (chickpea and soybean), wakame seaweed, cereals (maize and wheat), and sesame.

Li et al. [18,19] employed alcalase for the hydrolysis of mungbean and rice proteins and could isolate three antihypertensive peptides in the case of mungbean and one in the case of rice. Moreover, they also demonstrated the *in vivo* effect of the peptide produced from rice protein using spontaneously hypertensive rats (SHRs). Chickpea, cowpea, peanut, and amaranth have also been treated with alcalase for the production of ACE inhibiting peptides [20–23]. Nevertheless, the first work predicting antihypertensive properties in amaranth was based on the results obtained by the analysis of amaranth sequences using BIOPEP database. In fact, the *in silico* analysis of amaranth seeds [82] revealed the presence of antihypertensive peptides in globulin 11S and glutelin protein fractions but it could not specify their sequences. Later, Vecchi et al. predicted the presence of antihypertensive peptides Ala–Leu–Glu–Pro (IC_{50}=6.32 mM) and Val–Ile–Lys–Pro (IC_{50}=0.18 mM) in amaranth 11S globulin by means of an *in-silico* based peptide library screening method [83]. A more comprehensive study was recently published by Fritz et al. [24] who studied the hydrolytic release of antihypertensive peptides from storage proteins of amaranth by *in vitro* assay using alcalase in addition to other four different enzymes (pronase, papain, trypsin, and chymotrypsin) and by *in vivo* and *ex vivo* assays. Furthermore, Barba de la Rosa et al. [25] explored the inhibitory ACE activity of the amaranth glutelin fraction using trypsin digestion and highlighting its high potential as nutraceutical.

Alcalase has also been employed in the digestion of soybean. Wu and coworkers developed a high-performance liquid chromatography (HPLC) method for the *in vitro* determination of soybean antihypertensive activity and also tested its *in vivo* activity. They identified two peptides that yielded the highest antihypertensive activities: Asp–Leu–Pro and Asp–Gly [26,78,84]. Among soybean proteins, glycinin or 11S globulin constitutes a potent source of antihypertensive peptides. Some authors explored the antihypertensivity of peptides released from soybean glycinin using proteases of varied specificity and observing the greatest activity when using protease P [27]. Another soybean protein fraction that has also been explored for its antihypertensive effect is the whey. This is the residue remaining after isoelectric precipitation of 11S and 7S globulins. Its digestion using protease S enzyme resulted in three peptides (Val–Ala–Pro, Val–Lys–Pro, and Val–Thr–Pro) that after oral administration to SHRs produced a significant reduction in blood pressure [28]. Thermolysin was also applied to digest soybean whey proteins releasing the peptide Leu–Ala–Pro with *in vivo* demonstrated antihypertensive capacity [29]. Other enzymes that have been employed for the digestion of whole soybean are protease M and orientase 90 N. In this case, authors observed an increasing inhibitory effect with time up to 10 h [30]. Kodera and Nio [31] employed a protease from the germinated cotyledons of soybean, for the first time, for the hydrolysis of a soybean protein isolate. They identified a novel active peptide with IC_{50} of 21 μM in soybean. On the other hand, Gibbs et al. [32] demonstrated a range of biological activities in fermented products natto and tempeh and hydrolyzed soybean

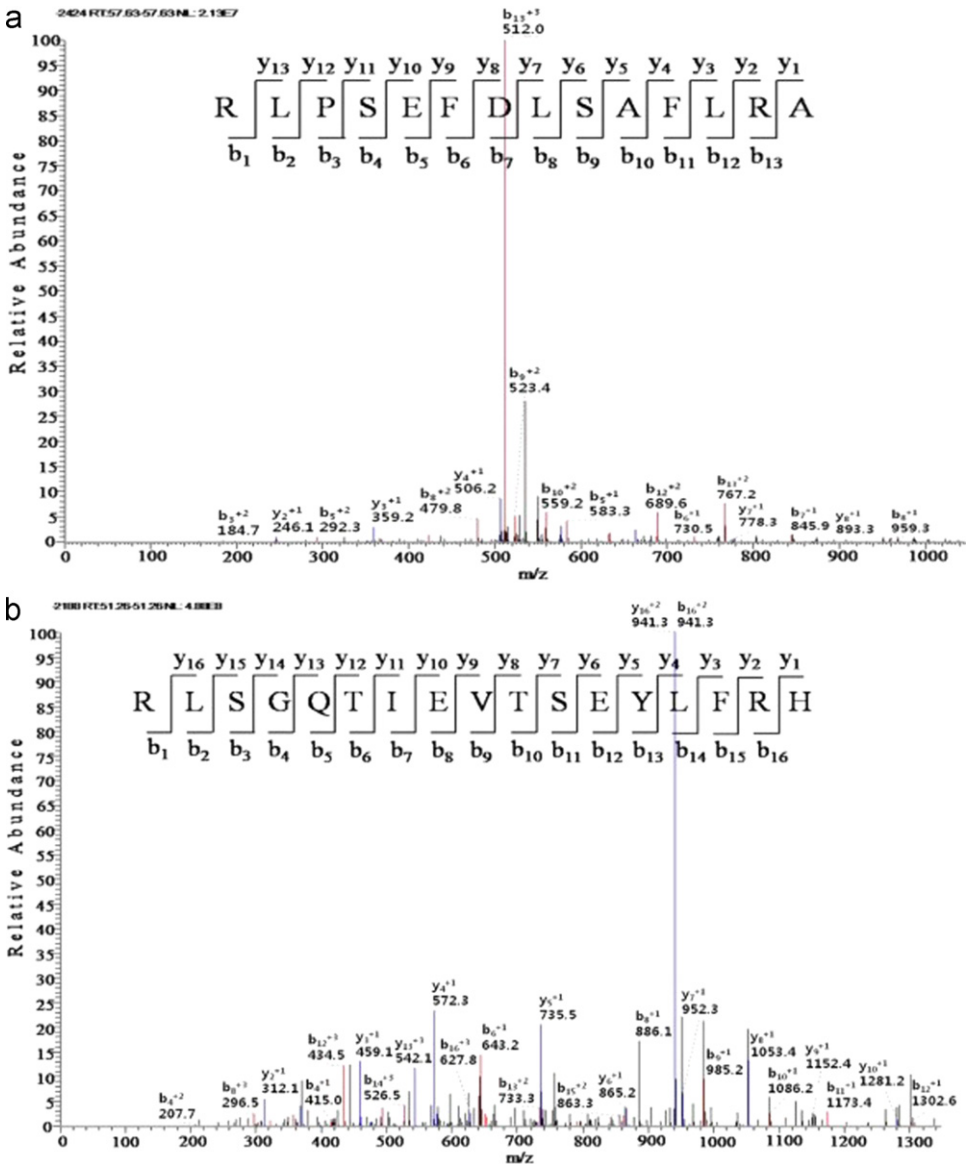

Fig. 1. MS/MS spectra corresponding to two antihypertensive peptides from *Pleurotus cornucopiae* mushroom and their identification. With permission from Ref. [15].

submitted to digestion with different endoproteases (pronase, trypsin, Glu C protease, plasma protesases and kidney membrane proteases). Most biological activities were observed in peptides derived from glycinin. Moreover, ACE inhibitors were also obtained from the pronase and kidney membrane digestion of natto. Other enzyme that has been employed for the production of antihypertensive peptides is pepsin [33]. Five different peptides with IC_{50} values ranging from 14 to 153 μM were identified. This pepsin hydrolyzed soybean also demonstrated *in vivo* antihypertensive activity on SHRs [85,86]. On the other hand, some authors have focused their works to reduce the cost of conventional batch-type enzymatic hydrolysis. In fact, enzymatic hydrolysis in its conventional format requires the use of large amounts of enzymes that cannot be reused. In this regard, Chiang et al. [87] proposed the use of a continuous membrane reactor to overcome these problems and tried this proposal for the hydrolysis of soybean. They screened different enzymes and ultrafiltration membranes observing that the peptides with the highest ACE inhibitory activity were obtained by digestion with alcalase.

Pepsin has also been employed for the digestion of rice-koji which is the main ingredient of sake. Rice-koji is rice fermented with *Aspergilluss spp*. Its pepsin digestion yielded the peptide Val–Ala–Asn–Asp that showed hypotensive properties [34]. This enzyme was also used in the case of wakame observing four tetrapeptides with antihypertensive activity as low as 21 μM [35]. Two years later, another authors used the enzyme protease S "Amano" for the digestion of this seaweed resulting in a greater number of antihypertensive peptides [36]. Some of these peptides were not produced during digestion but were naturally occurring in the seaweed [11]. Nevertheless, wakame is not the only seaweed that has been studied for its antihypertensive effects. Indeed, Suetsuna et al. observed that peptic digests of alga *Porphya yezoensis*, [88]. *Hizikia fusiformis,* [89] *Chrorella vulgaris*, and *Spirulina platensis* [37] lowered blood pressure on SHRs.

Pepsin was also employed for the digestion of the yam tuber protein, dioscorin. It was found that the ACE inhibitory activity of pepsin hydrolysate increased from 51.32%, obtained with the native protein, to 75%, obtained after 32 h pepsin hydrolysis [38]. *In vivo* studies on SHRs with dioscorin and with its peptic hydrolysate demonstrated that its short-term administration resulted in a reduction in blood pressure with both native protein and hydrolysate. Nevertheless, its long-term administration

yielded a higher reduction in the case of the pepsin hydrolysate [90]. Despite this result, it is important to highlight that this is one of the few documented cases in which the direct administration of a native protein exhibited antihypertensive activity *in vivo*. Moreover, *in vivo* studies with humans have confirmed that the intake of an instant food containing 140 mg of dioscorin over 5 weeks had a regulating effect on human blood pressure [91]. Another tuber exerting antihypertensive properties is potato [39]. In this case, authors observed a higher ACE inhibition in hydrolyzed potato proteins than in native potato proteins.

Maize has also been extensively studied for its content in antihypertensive peptides. The maize protein γ-zein has been reported as having strong ACE inhibitory activity due to the peptide Leu–Pro–Pro but it was not easily released [92]. Another zein fraction that has also resulted in hydrolysates having *in vivo* ACE inhibitory activity is α-zeins. In fact, Maruyama and coworkers used different enzymes (thermolysin, trypsin, α-chymotrypsin, subtilisin, papain, and pepsin) for the hydrolysis of α-zeins observing the highest activity with thermolysin. The same authors, next, isolated 14 different ACE inhibitory peptides from the thermolysin digest of α-zeins. However, about 8% of α-zeins remained insoluble using this methodology and next efforts were aimed to increase its solubilization [40,92]. Indeed, Yano et al. described an improved method using urea for the solubilization of α-zeins and thermolysin digestion that enabled the isolation of 36 peptides, most of them showing antihypertensive activity [41]. More recently, Puchalska et al. [42] have developed an analytical method for estimating the content of three peptides with extremely high antihypertensive capacity. Leu–Gln–Pro (IC_{50}=2.0 μM), Leu–Ser–Pro (IC_{50}=1.7 μM), and Leu–Arg–Pro (IC_{50}=0.29 μM) released by thermolysin digestion of α-zeins were estimated in different maize crops observing clear differences among maize lines. Other proteases were tried by Kim et al. [43] for hydrolyzing corn gluten observing higher antihypertensive activity when using flavourzyme enzyme. Moreover, alcalase has also been employed in the case of maize observing a peptide (Ala–Tyr) with an IC_{50} of 14.2 μM. This peptide was orally administrated to SHRs to confirm its activity [44] Later on, Lin et al. [93] developed a pilot-scale production to prepare corn gluten meal with high ACE inhibitory properties. For that purpose, a corn protein isolate was obtained through heat treatment of corn gluten meal. Two step enzymatic hydrolysis and multistage separation were applied. Again, the peptide Ala–Tyr was identified as one major ACE-inhibitory peptide. Similarly, Huang et al. [94] used an enzymatic membrane reactor to produce corn peptides with potent ACE activity. A hydrolysate with IC_{50}=0.29 mg protein/mL that was intragastrically administrated to SHRs revealed the greatest antihypertensive effects.

Thermolysin has been extensively employed not only in the case of maize but also for sesame and flaxseed. Sesame is a potent source of antihypertensive peptides that has been widely studied in Asian countries [95]. A commercial thermolysin sesame hydrolysate exhibited *in vivo* ACE inhibitory activity being possible the isolation of six representative peptides for this activity exerting IC_{50} values from 0.78 to 14.74 μM [45]. In the case of flaxseed, the thermolysin digestion was followed by digestion with another four different enzymes: papain, ficin, alcalase, and pronase [46]. Hydrolysis of flaxseed with thermolysin and pronase resulted in a product with antihypertensive properties in addition to other functionalities that was suitable for patient with liver diseases. The combination of different proteases for protein digestion is not an isolated case in flaxseed but it has been a common practice in the search for antihypertensive peptides. Motoi and Kodama [47] tried different acid proteases (pepsin, molsin F, rapidase, orientase 5 A, protease M and a mixture pepsin–protease M) for the hydrolysis of wheat gliadin observing the highest percentage of ACE inhibition with the mixture pepsin–protease M. Nazif and El-Sayed [48] isolated protease enzymes from different plants (wheat, maize, and barley leaves, grass, pea pods and beans) and from tuna. The most active ones (protease from maize leaves and from tuna peel) were employed for the hydrolysis of lentils and beans. The hydrolysates showed a potent ACE inhibitory activity. Sequential hydrolysis with immovilized trypsin, chymotrypsin, and carboxypeptidase A on glioxyl-agarose supports was also evaluated for the hydrolysis of *Brassica carinata*. Obtained peptides yielded different functionalities including angiotensin inhibiting activity suggesting the consumption of *B. carinata* for the treatment of heart and related diseases [49].

Moreover, food residues can also be rich sources of bioactive peptides. As example, apricot almond showed antihypertensive properties when hydrolyzing with Neutrase and N120P proteases [50]. Moreover, this activity was kept after gastrointestinal digestion with pepsin–trypsin and results were confirmed by *in vivo* assays on SHRs. Another example is rice dreg. This residue constitutes an interesting protein source since it contains a high protein content (65%). In order to use this residue to obtain high added value substances, some authors have optimized its hydrolysis with trypsin based on its ACE inhibitory activity. For that purpose, an experimental design methodology, response surface methodology (RSM), has been employed. Twelve experiments were designed for the optimization of pH, temperature, enzyme: substrate ratio, water: rice dreg ratio, and hydrolysis time. Best conditions enabled an inhibitory activity of 85.27% [51]. The same methodology was also employed by the same research group observing that the ACE inhibitory activity of tryptic hydrolysed rice dreg proteins was significant using an *in vivo* assay on SHRs [96]. Another example of food residue that has been studied for its hypotensive activity is rapeseed meal. Rapeseed meal is a by-product obtained from the oil removal of rapeseed and contains approximately 40% proteins. Four different ACE inhibitory peptides have been isolated from subtilin digest of rapeseed protein. Moreover, despite all peptides presented significantly lower *in vitro* antihypertensive activity than synthethic captopril, *in vivo* assays showed only a slightly lower activity than captopril indicating that ACE inhibitors from rapeseeds possessed higher *in vivo* activities than those observed by *in vitro* analysis. This result was attributed to a higher affinity of peptides to tissue and a slower elimination than captopril. Furthermore, subtilisin digest of rapeseed proteins was stable under enzymatic digestion [52]. On the other hand, germ is often, along with bran, a by-product in the production of refined grain products. Nevertheless, wheat germ has high protein content and has been of interest as a source of bioactive peptides. Indeed, a main peptide, Ile–Val–Tyr with IC_{50} of 0.48 μM, resulted in hypotensive effects when administrating to SHRs. Moreover, this peptide was metabolized in rats and human plasma by aminopeptidase to form the subsequent ACE inhibitor Val–Tyr [53,97]. Similarly, corn germ has also been a target food for the exploration of new bioactive peptides [54]. In this work, proteins from corn germ were hydrolyzed with trypsin, thermolysin, GC 106, and flavourzyme being this the only enzyme not enabling to show antihypertensive activity in the hydrolyzate.

Another mechanism for obtaining bioactive peptides is fermentation. Special attention has been addressed to fermented soybean. In fact, consumption of soybean products is hindered by the presence of unpleasant off-flavors and gastrointestinal disorders to consumers. Alleviation of these undesirable side effects is possible by its fermentation with different strains. A result of such fermentation is also the production of peptides. Different authors have focused their research works to the study of antihypertensive activity in soybean fermented with different strains and under different conditions [58,98,99]. Results showed that the exhibition of ACE inhibitory activity was strain-specific,

was influenced by the environmental conditions, and that not all released peptides expressed *in vitro* ACE inhibition.

There are different fermented soybean products that have been studied for their hypotensive effects: miso paste, soybean sauce, tofuyo, douchi, etc. In most of these cases, the release of peptides with capability for reducing blood pressure has been demonstrated. An exception was miso paste in whose case, the analysis of peptides revealed a low hypotensive activity. Nevertheless, the addition of casein during the fermentation process enabled to increase miso antihypertensive activity [55]. In the case of soybean sauce, some authors have developed an enriched peptide soybean sauce by modifying the process of soybean sauce brewing. This enrichment resulted in a product with an ACE inhibitory activity (IC_{50}=454 μg/mL) greater than that of the regular soybean sauce (IC_{50}=1620 μg/mL) [56]. On the other hand, taking into account that many fermented soybean products contain a large amount of salts causing hypertension, different authors have targeted their efforts to the production of salt free fermented products. At this regard, Zhu et al. [57] prepared a salt-free soybean sauce using *Aspergillus oryzae* and investigated its hypotensive effects. Moreover, fermentation processes used to be very time consuming and in order to reduce fermentation time, acceleration of fermentation at high temperatures has been attempted. Rho et al. [58] observed that fermentation at 45 °C was beneficial for obtaining a potent ACE inhibitory peptide from soybean.

On the other hand, tofoyu is a traditional fermented tofu from Japan with *in vitro* ACE inhibitory activity. It contains two ACE inhibitors that were identified as Ile–Phe–Leu (IC_{50}=44.8 μM) and Trp–Leu (IC_{50}=29.9 μM) [59]. Moreover, the inhibitory activity of tofoyu was confirmed by *in vivo* analysis [100]. The fermented soybean product douchi has also been investigated for its hypotensive activity which was improved after digestion by gastrointestinal proteases [60]. A Korean fermented soybean paste is another soybean product exerting hypotensive activity. Shin et al. purified the peptide responsible for such activity and evaluated *in vivo* its activity using SHRs [61].

Sourdough fermentation is extensively employed for improving the flavor and structure of many cereal-based baked products. Nevertheless, this fermentation process also results in products with enhanced nutritional value and higher level of bioactive peptides. Rizello et al. [62] reported the results obtained when using selected strains of lactic acid bacteria for sourdough fermentation of wheat and rye. In addition to a higher level of antihypertensive peptides, the use of lactic acid bacteria enabled the production of γ-aminobutyric acid, a nonprotein amino acid with a high antihypertensive effect. Other cereals demonstrating ACE inhibitory activity after fermentation is alfalfa [63] and rice-koji [64,101]. Spiritual drinks have also been studied for their content in hypotensive peptides. In fact, Takayanagi and Yokotsuka [65] could isolate six different peptides with IC_{50} values ranging from 17.5 to 83.0 μM in red wine. Furthermore, Saito et al. [66] isolated nine peptides that were capable of inhibiting ACE enzyme from sake and sake lees. Most peptides contained Tyr or Trp at the C-terminus. Two of them were isolated and submitted to digestion with pepsin and pancreatin observing that one of the peptides lost its activity. This result was confirmed by the results obtained by *in vivo* digestion on SHRs [67].

Testing peptide behavior when submitting to digestion by gastrointestinal enzymes is very common [14,15]. Indeed, ACE inhibitory peptides only exert an antihypertensive effect *in vivo* if they reach the cardiovascular system in an active form. Therefore, after oral administration, they need to resist gastrointestinal digestion by proteases and brush border peptidases and they have to be absorbed through the intestinal wall keeping their activity. Degradation of peptides could take place during this process with their subsequent inactivation. Although animal and human studies are the best way to assess the efficacy of bioactive peptides, simulated assays are more rapid and inexpensive. Gastrointestinal digestion is usually simulated by sequentially digesting proteins with pepsin (pH 2, 37 °C) and pancreatin (pH 7.5, 37 °C) and, in some occasions, digestion with chymotrypsin and trypsin is also performed (pH 6.5–6.8, 37 °C). Transport through intestinal epithelial is determined by the evaluation of the permeability of peptides through human adenocarcinoma colon cancer (caco-2) cell monolayers. Caco-2 cells are a representative model of human intestinal epithelial cells and can be related to the extent of peptide absorption.

Some authors have also employed the gastrointestinal digestion to demonstrate hypotensive activity in some plant foods such as soybean, [60,68] spinach, [69] buckwheat, [70] pea, [71] sunflower, [72] and hemp seed [73].

On the other hand, some plant products contain proteolytic enzymes that can result in autolysis reactions and peptide production. As example, mature wheat seeds contain proteolytic enzymes that can produce proteinous amino acids and γ-aminobutyric acid, a non-protein amino acid with high antihypertensive effect, by autolysis [74]. Nogata explored the production of ACE inhibitory peptides during the autolysis of the whole grain and different parts of wheat (shorts, bran, and red dog). Shorts exhibited the strongest inhibitory activity followed by bran, red dog, and the whole grain. The level of inhibitory activity rose with increasing the temperature up to 40 °C and autolysis time up to 12 h. Cacao beans also contains peptidases (aspartic endoprotease) that can cleave proteins under optimal conditions. Indeed, cacao beans autolysated at pH 3.5 showed angiotensin converting enzyme inhibitory activity [75].

Finally, *in-silico* techniques have also been employed for the production of ACE inhibiting peptides [102]. In fact, artificial neural networks and quantitative structure-activity relationship (QSAR) modeling have been employed to develop statistical computer models capable of identifying ACE-inhibitory peptides [103]. For that purpose, modeled proteins are *in-silico* proteolyzed with enzymes using special softwares and peptide sequence activities are then modelized. This technique has enabled to predict the existence of more potent antihypertensive peptides in soybean, canola, barley, oat, and pea when using thermolysin than when employing other enzymes reported in literature.

3. Antioxidant peptides

Reactive oxygen species formed as a consequence of respiration in aerobic organisms can react with DNA, membrane lipids, and proteins. Oxidative stress is produced by an unbalance between oxidizing species and natural antioxidants and has been associated with aging, cell apoptosis, and severe diseases such as cancer. Different studies have observed an inverse association between intake of antioxidants and mortality. Antioxidant compounds can be present in foods and their search is of great interest not only for health reasons but also due to their relation with organoleptic food properties. In fact, radical mediated lipid oxidation in foods produces rancid flavors, food discoloration, and toxic undesirable compounds leading to quality deterioration and shortened shelf life. The addition of synthetic antioxidants in food processing is a common practice to avoid these problems. Nevertheless, the use of synthetic antioxidants and antibacterial peptides is limited by their carcinogenicity and health hazards [104,105]. Consequently, many researchers are searching for natural antioxidants in foods that may protect the body and foodstuffs from free radicals and retard their deterioration. Table 2 shows the works focused to the determination of proteins and peptides in vegetable foods. As in the case of antihypertensive

Table 2
Determination of antioxidant proteins and peptides in vegetable foodstuffs.

Product	Objective	Sample preparation	Separation/fractionation	Peptide/protein	Ref.
Peptides/proteins not encrypted in any parent molecule					
Ginkgo biloba seeds	Separation and characterization of a protein with antioxidant properties from *Ginkgo biloba* albumin	Ammonium sulfate salt fractionation	IEC (triethanolamine buffer (pH 8.0)-NaCl, 0.2 mL/min); SEC (triethanolamine buffer (pH 8.0)	Gab protein (29.25 kDa)	106
Curcuma comosa	To investigate whether any of the antioxidant activity of *Curcuma comosa* can be attributed to proteins	Protein extraction overnight in a buffer (20 mM Tris–HCl, pH 7.4, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM Mn Cl_2, 0.15 M NaCl), centrifugation, precipitation in NH_4SO_4, dissolved in water, dialysis against 20 mM Tris–HCl (pH 7.4)	IEC (20 mM Tris–HCl (pH 7.4)+0.5 M NaCl)	Superoxide dismutase homologue	107
Apium graveolens	To estimate the antioxidant activity of peptides from celery seeds 5 kDa peptide with partial sequence: Ala–Asp–Asn–Ala–Ala–Arg–Pro–Val–Arg–Glu–Thr–Asp–Ala–Val–Pro	Peptide extraction using a phosphate buffer (pH 7.4) containing KCl, EDTA, and PVP. Centrifugation and peptide precipitation with $(NH_4)_2SO_4$ 108	IEC (12 M NH_4Ac (pH 9), 0.5 mL/min); IEC (0–1 M NaCl with 0.05 M NH_4Ac (pH 6), 0.5 mL/min); SEC (water, 60 mL/min)		
Yam tuber	To investigate the antioxidant activity of the major yam tuber storage protein, dioscorin	–	IEC (50 mM Tris–HCl (pH 8.3)+150 mM NaCl)	Dioscorin (32 kDa)	109
Australian beer	To determine whether thiol-containing polypeptides in beer could play a role in beer ageing	–	Ultrafiltration; SEC (Tris–HCl buffer (pH 7.4), 1 mL/min, 280 nm). Peptide sequencing of trypsin digested proteins by ESI–MS/MS	LTP1 protein (10 kDa)	110
Peptides obtained by enzymatic hydrolysis					
Soybean proteins and wheat gluten	To prepare a peptide fraction with a high antioxidant activity by autofocusing from commercial hydrolysates	–	Soybean and wheat gluten commercial hydrolysates were fractionated by autofocusing at 500 V for 24 h into ten fractions	–	111
Cowpea	To determine ACE inhibitory activity in cowpea hydrolysates	Cowpea flour was extracted with water at pH 11, proteins were precipited at pH 4.5 and hydrolyzed with alcalase (pH 8.0, 50 °C, 90 min), flavourzyme (pH 7.0, 50 °C, 90 min), pepsin (pH 2, 37 °C, 45 min)-pancreatin (pH 7.5, 37 °C, 45 min)	Ultrafiltration	–	21
African yam bean seed	To evaluate the antioxidant activity of African yam bean seed hydrolysates	Protein extraction at pH 10, centrifugation and pH adjustment to 5.0 to precipitate proteins. Alcalase digestion at pH 9.0, 50 °C, 4 h	–	–	112
Chinese yam tuber dioscorin	Evaluation of antioxidant properties in peptic hydrolysates	Pepsin digestion	SEC (20 mM Tris–HCl buffer, pH 7.9, 30 mL/min, 210 nm)	Dioscorin	113
Potato	To evaluate antioxidant activity of proteins from potato and by-products	Hydrolysis with alcalase (pH 7, 55 °C, 5 h), neutrase (pH 7, 50 °C, 5 h) and esperase (pH 7, 55 °C, 5 h)	Ultrafiltration; RPC (2–60% B (AcN–water–0.05% TFA) in 55 min, 1 mL/min, 214 nm)	–	39
Hempseed	To evaluate the antioxidant activity of peptides from hydrolyzed hempseed protein isolate	Alkaline extraction for 70 min at 40 °C followed by isoelectric precipitation (pH 5) and alcalase hydrolysis (pH 9.4, 50 °C, 1 h)	Adsorption on macroporous resin at 2 mL/min and desorption with 75% EtOH at 1 mL/min; SECTION (1 mL/min, 220 nm); preparative RPC (10–100 %B (TFA+AcN) in 25 min, 3 mL/min, 220 nm); RPC (10–100 %B (TFA+AcN) in 25 min, 1 mL/min, 220 nm); MALDI-TOF MS/MS	Asn–His–Ala–Val and His–Val–Arg–Glu–Thr–Ala–Leu–Val	114

Table 2 (*continued*)

Product	Objective	Sample preparation	Separation/fractionation	Peptide/protein	Ref.
Flaxseed	To obtain a flaxseed protein hydrolysate with functional properties	Flaxseed suspension in Na_2CO_3 (pH 7.5, 37 °C); hydrolysis with thermolysin (pH 7.5, 5 h) and one of the following proteases: pronase (pH 7.4, 5 h), ficin (pH 7.0, 5 h), alcalase (pH 8.0, 5 h) or papain (pH 6.5, 5 h)	Adsorption on active carbon; SEC (phosphate buffer+NaCl, 0.75 mL/min, 214 nm)	–	115
Amaranth	To analyse proteins and peptides with antioxidant properties	Protein isolate was obtained by extraction at pH 9 and isoelectric precipitation at pH 5. Protein fractions were obtained by sequential extraction and isoelectric precipitation. Fractions were hydrolysed with alcalase at pH 10 and 37 °C	SEC (phosphate buffer (pH 8.0), 280 nm)	–	116
Rapeseed	To evaluate the antioxidant activity of rapeseed peptides	Albumin was extracted in saline solution, centrifuged, and dissolved in water. Albumin was digested with alcalase (1 h) and flavourzyme (2 h) at pH 8 and 50 °C	SEC (0.5 mL/min)	–	117
Sunflower	To investigate the effects of enzymes and sunflower meals on the physicochemical properties of hydrolysates	Hydrolysis with fungal protease (pH 8.0, 25 °C), trypsin (pH 8.1, 37 °C) or papain (pH 7.0, 25 °C) for 80 min		–	118
Oat Flour	To investigate antioxidant properties of oat proteins	Proteins were extracted with NaOH (pH 9.5), centrifuged, and precipited with HCl (pH 4). Trypsin hydrolysis was performed at pH 8.0 and 37 °C (20 h) while alcalase hydrolysis was at pH 8.0 and 50 °C (4 h)	Ultrafiltration	–	119
Wheat	To evaluate the impacts of oxidative modification in wheat peptides after exposure to heat or lipid peroxidation	Synthetic wheat peptides were mixed with malandialdehyde and incubated at 25 °C, 24 h	–	–	120
Corn gluten meal	To identify antioxidant properties of corn peptides using different antioxidant mechanisms	Corn gluten meal was pretreated with α-amylase and saccharification enzyme to relase carotenoids and starch and extract proteins. Alcalase hydrolysis at pH 8.0, 65 °C for 2 h	–	–	121
Barley hordein	To produce antioxidant peptides from barley hordein	Barley hordein extraction. Hydrolysis with alcalase (pH 8, 50 °C), flavourzyme (pH 7, 50 °C), and pepsin (pH 2, 37 °C). Hydrolysis time was varied from 0 to4 h	–	–	122
Rice bran	To assess the antioxidant activity of enzymatic hydrolysates derived from rice bran	Rice bran defatting with hexane, followed by protein precipitation at pH 4. Phytate free rice bran protein (PFRBP) was obtained by removal of phytate at acid pH. PFRBP was hydrolysed with different proteases (M, N, S, and P) and pepsin	RPC (0–80% EtOH); preparative RPC (10–40% B (AcN+0.1% TFA) in 20 min, 3 mL/min, 40 °C, 250 nm); RPC (0–100% B (AcN+6 mM HCl), 1 mL/min); RPC (0–100% B (AcN+0.1% TFA)). MALDI-TOFMS analysis	Ala–Ile–Arg–Gln–Gly–Asp–Val–Phe, Val–Leu–Glu–Ala–Asn–Pro–Arg–Ser–Phe, and Tyr–Phe–Pro–Val–Gly–Gly–Asp–Arg–Pro–Glu–Ser–Phe	123
Peptides obtined by fermentation					
Natto and tempeh	To evaluate the presence of bioactive peptides after treatment with proteases	Natto and tempeh were dissolved in ammonium bicarbonate and next hydrolyzed with commercial proteases (pronase and chymotrypsin), kidney	RPC (5–75% B (AcN) in 70 min, 1 mL/min, 206 and 280 nm); MS analysis of infused samples	–	32

		membrane proteases or plasma proteases. Peptides were extracted with AcN+TFA			
Douchi	To evaluate the antioxidant properties of Douchi	–	–	–	124
Rice wine	To evaluate the antioxidant activity of peptides found in rice wine	Alcohol removal under vacuum at 35 °C	SEC (NH_4Ac buffer, 0.3 mL/min, 214 and 280 nm); RPC (0–60% B (TFA in AcN) in 60 min, 15 mL/min); RPC (5–80% B (AcN))	Ile–His–His, Val–Val–His(Asn), Leu–Val–Pro, and Leu (Val)–Lys–Arg–Pro	125
Cereal flours (wheat, spelt, rye, kamut, oat, rice, barley, and maize)	Investigating the *in vitro* and *ex vivo* antioxidant potential of different cereal flours fermented with lactic acid bacteria	Fermentation with 10 different lactic acid bacteria at 37 °C for 24 h	Ultrafiltration; RP-FPLC (5–46% B (AcN+0.05% TFA) in 16–62 min, 1 mL/min, 214 nm); nanoLC–MS/MS	25 peptides	126
Cacao beans	To evaluate the presence of antitumor activity and to find a correlation between antitumor and antioxidative activities	Proteins from unfermented and semifermented cacao seeds were precipited with acetone. Protein fractions were obtained by extraction with 10 mM Tris–HCl (albumin), 0.5 M NaCl (globulin), 70% EtOH (prolamin), and 0.1 M NaOH (glutelin)	SECTION (1 mL/min, 18 °C, 280 nm)		127
Commercial fermented mushroom *Ganoderma lucidum*	To evaluate the antioxidant activity	–	Ultrafiltration; SEC (254 nm)	–	128
Abalone mushroom	Isolation of a polysaccharide-peptide in the fruit body of *P. abalones.*	Protein extraction and precipitation	Different SEC separations	–	129
Peptides obtained by gastrointestinal digestion					
Soybean	To evaluate the antioxidant activity of soybean protein hydrolysate	Pepsin digestion at pH 2, 37 °C, and 60 min followed by pancreatin digestion at pH 7.5, 37 °C, and 60 min	Ultrafiltration		130
Hempseed	To determine the potential liberation of antioxidant peptides during oral consumption of hempseed	Protein solubilization at pH 10, 37 °C for 2 h and centrifugation. Supernatant is adjusted to pH 5 to precipitate proteins. Pepsin hydrolysis at pH 2, 37 °C for 2 h. Pancreatin digestion at pH 7.5, 37 °C for 4 h	–	–	131
Brassica carinata	To evaluate the antioxidant activity	Sequential hydrolysis with immobilized trypsin, chymotrypsin, and carboxypeptidase A	SEC (50 mM NH_4HCO_3, 10 mL/min)		49
Peptides obtained by autolysis					
Cacao beans	To explore antioxidant properties of cacao extracts	Fat removal with hexane, extraction with chloroform, and polyphenol removal. Autolysis in acetic acid (pH 3.5) or acetate buffer (pH 5.2) at 50 °C for 16 h	–	–	75
Wheat germ	To investigate an economical method for the production of antioxidant peptides from wheat germ	Active proteolysis was obtained at pH 4.39, 45 °C for 35 and 270 min	Ultrafiltration (3, 10, 50, and 100 kDa); SECTION (1 mL/min, 280 nm); RPC (0–50 % B (AcN+0.1% TFA) in 15 min; 6 mL/min); RPC (0–50 % B (AcN+0.1% TFA) in 15 min; 0.5 mL/min). Peptide identification by LC–ESI–MS/MS	Val–His–His–His	132

DEAE: diethylaminoethanol; IEC: Ion exchange chromatography; LC–MS/MS: Liquid chromatography/tandem mass spectrometry; EDTA: ethylendiaminetetraacetic acid; PVP:Poly vinyl pyrrolidone; SEC: Size exclusión chromatography.

activity, antioxidant proteins and peptides can be naturally observed in vegetable foods or can be encrypted in a parent protein from which it can be released by enzymatic hydrolysis, fermentation, gastrointestinal digestion or autolysis. Nevertheless, unlike antihypertensive peptides, most works focused on investigating the presence of antioxidant peptides does not yield an amino acid sequence and are more interested in evaluating antioxidant activity as a whole. There are different mechanisms to evaluate antioxidant capacity being those grouped in Table 3 the most used in the case of proteins and peptides from plant foods. Most methodologies are based on the determination of the damage induced in a probe by an oxidant and the inhibition or reduction of such damage in presence of a potential antioxidant. This inhibition is proportional to the antioxidant capacity of the tested compound or sample. The different used methodologies differ in the oxidant agent, the probe, the instrumental technique employed, etc. Most common methodologies evaluate the inhibition of lipid oxidation by the measurement of oxidation products, the ability to reduce ferric ion, and the scavenging activity of tested compounds on free radicals (i.e., superoxide, hydroxyl, and DPPH (1,1-diphenyl-2-picrylhydrazyl) radicals).

Different factors seem to affect to the antioxidant activity of hydrolysates from plant proteins: molecular weight, hydrolysate concentration, degree of hydrolysis, and amino acid composition. Antioxidant peptides used to present a large amount of hydrophobic amino acids such as Leu, Ala, and Phe. Nevertheless, unlike antihypertensive activity, antioxidant properties are also usual in proteins and not only limited to peptides. Indeed, a new antioxidant protein (G4b) with 29.25 kDa has been purified from *Ginkgo biloba* seed albumin [106]. The protein consisted of two peptides with similar molecular weights linked by a disulfide bond. Another example of a protein exerting antioxidant properties was found in the food herb *Curcuma comosa*. The crude protein extract from the plant rhizomes showed antioxidant properties that were attributed to a main protein of 18 kDa, as determined by SDS-PAGE, that corresponded, according to the LC–MS/MS data, to a Cu/Zn superoxide dismutase homologue [107]. Peptides naturally present in celery seeds have also been investigated by their antioxidant properties [108]. A main peptide of 5 kDa yielded the highest antioxidant activity. Another example of a protein with scavenging activity against DPPH and hydroxyl radicals is dioscorin from Japanese yam tubers (*Dioscorea batatas* Decne). Dioscorin is one of the few examples of a native protein with antihypertensive capacity. This protein with 32 kDa could capture both DPPH and hydroxyl radicals in a concentration dependent manner [109]. Nevertheless, dioscorin proteins from different yam tubers differ in amino acid composition and secondary structure which led Liu and coworkers to think that this protein could also exert different antioxidant properties depending on the yam species. Therefore, these authors compared the antioxidant capacity of Chinese and Japanese yam dioscorins observing that they exhibited different scavenging activities against DPPH and hydroxyl radicals. Moreover, dioscorin from Chinese yam was pepsin digested observing that smaller molecular weight fractions still had antioxidant activities [113]. On the other hand, Wu et al. [110] found that barley peptides played an important role in beer flavor stability. In fact, they studied three different Australian beers, one fresh and two beers aged at different conditions (aged for 12 weeks at 30 °C and aged at 20 °C for 5 years (vintage)). Characteristic Australian lager flavor was kept in the fresh and vintage beers but was lost in the aged beer. Surprisingly, these two beers presented a 10 kDa protein with high thiol content. This protein, which was identified as a lipid transfer protein (LTP1), showed antioxidant activity.

Like in the case of antihypertensive peptides, most usual method for obtaining antioxidant peptides is enzymatic hydrolysis being alcalase the most employed enzyme. Soybean proteins and peptides have been evaluated as antioxidants [133] Park et al. [111] demonstrated a significant reduction of lipid oxidation in the presence of soybean proteins and peptides. This research group also proposed preparative isoelectric focusing as an alternative to traditional preparative HPLC using organic solvents. They prepared peptide fractions with a high antioxidant activity from soybean protein and wheat gluten hydrolysates. The acidic fraction from soybean protein hydrolysate showed strong DPPH radical scavenging activity while basic fractions were strong suppressor of linoleic acid oxidation [111]. Moreover, Yang et al. [85] demonstrated the *in vivo* antioxidant capacity of pepsin hydrolyzed soybean [85]. Another legume that has been investigated for its antioxidant properties is cowpea *Vigna unguiculata* [21]. Authors observed *in vitro* antioxidant activity in cowpea hydrolyzed with alcalase, flavourzyme, and pepsin–pancreatin. On the other hand, yam bean has also been hydrolyzed to investigate the antioxidant capacity of resulting peptides. Indeed, Ajiloba et al. [112] produced an alcalase hydrolysate from African yam bean seeds and studied its antioxidant properties related to peptide size. Those peptides with <1 kDa molecular weight showed significant better ferric reducing power and higher DPPH and hydroxyl radical scavenging activities than higher molecular weight peptides. Another tuber showing antioxidant properties is potato (*Solanum tuberosum*) [39]. Authors demonstrated that potato by-products were also a potent source of bioactive compounds.

On the other hand, Lu et al. [114] proposed hempseed proteins as a source of antioxidant peptides. They could isolate two peptides from the alkaline hydrolysate of this seed and demonstrated their protective effects against cell death and oxidative apoptosis. Fig. 2 shows the morphological characteristics of PC12 cells after hydrogen peroxide treatments in absence or presence of hempseed antioxidant peptides. When exposed to hydrogen peroxide, the number of cells was reduced (see Fig. 2b) while this effect was attenuated when cells were preincubated with hempseed peptides (see Fig. 2c). Flaxseed has also been investigated for its functionality. Udenigwe and Aluko [115] evaluated the antioxidant activity of peptides obtained from the hydrolysis of a flaxseed protein extract. For that purpose, thermolysin in addition to other enzyme (pronase, ficin, alcalase or papain) were employed observing the best antioxidant capacity with thermolysin and pronase enzymes. Moreover, this peptide extract possessed a high level of branched-chain amino acids (Leu, Ile, and Val) and a low level of aromatic amino acids (Tyr and Phe) which made it useful for the treatment of patients with liver disease.

Tironi and Añón [116] demonstrated the presence of proteins and peptides with antioxidant properties in amaranth protein fractions and alcalase hydrolysates. Moreover, the digestion of rapeseed proteins with alcalase and flavourzyme resulted in a hydrolysate showing *in vitro* antioxidant properties and preventing malondialdehyde production in rat liver and autohemolisis of red blood cells which are produced as a consequence of oxidative damage [117]. Another oilseed that has been studied for its antioxidant activity is sunflower[118]. Different sunflower meals were digested with different enzymes (papain, fungal protease, and trypsin) observing that physicochemical properties were significantly affected by the enzyme. In the case of antioxidant properties, the highest activity was observed in the hydrolysate obtained with the fungal protease.

Cereal proteins like those present in oat, wheat, corn, barley, and rice have also been investigated for their antioxidant capacity. Trypsin and alcalase enzymes were used to prepare oat protein hydrolysates. Free radical scavenging properties were observed in both oat hydrolysates and fractions obtained by ultrafiltration (<2 kDa, 2–10 kDa, >10 kDa). The alcalase fraction of 2 kDa yielded the highest radical scavenging activity on DPPH assay while trypsin digestion had better ferrous ion-chelating properties

Table 3
In vitro methods employed for the evaluation of antioxidant activity in proteins and peptides from vegetable foods.

Assay	Methodology	Ref.
Inhibition of linoleic acid autoxidation (ferric thiocyanate method)	It measures primary oxidation products formed as a consequence of lipid oxidation. The procedure consists of mixing a linoleic acid emulsion with the tested compound. After an incubation time for lipid oxidation, resultant lipid oxidation products are determined. For that purpose, EtOH, ammonium thiocyanate, and $FeCl_2$ are added and the absorbance of the formed red $Fe(SCN)^{2+}$ is measured at 500 nm	46, 111–113, 116, 119, 121, 123, 126, 130
Inhibition of linoleic acid autoxidation (thiobarbituric acid method)	It measures secondary oxidation products formed as a consequence of lipid oxidation. The procedure consists of mixing with SDS, acetic acid, and TBA (thiobarbituric acid), heating at 100 °C for 60 min, cooling and centrifuging. The absorbance of upper layer is measured at 532 nm. This absorbance is related to the secondary oxidation products formed	120
β-carotene blenching test	It measures the capability of a potential antioxidant to inhibit the oxidation of β-carotene induced by radicals generated in the oxidation of linoleic acid. β-carotene solution absorbs at 470–492 nm and this absorbance decreases as a consequence of its oxidation. For that purpose, β-carotene is mixed with linoleic acid, Tween 40, and H_2O_2 to induce linoleic acid oxidation	49, 75, 125
Scavenging activity on 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals	It measures the scavenging capacity of tested compounds on DPPH free radicals. The procedure consists of mixing the peptide with DPPH in ethanol or methanol, incubation under light protection at room temperature, and measurement of DPPH absorbance at 515–517 nm. Unpon reduction, the DPPH color fade and this color decrease is proportional to antioxidant concentration	46, 106, 107, 109–114, 119–122, 124–126, 132
Scavenging effect on superoxide radicals (O_2^-)	Superoxide radicals are generated from autoxidation of pyrogallol at basic pH. The tested peptide is mixed with pyrogallol inhibiting its autoxidation and superoxide radicals formation. The rate of formation of O_2^- is measured at 320–420 nm or by an aminophthalhydrazide chemiluminescence system	46, 106, 122, 128
Scavenging effect on hydroxyl radicals (OH)	There are different systems to investigate the hydroxy radical scavenging activity but the most used is the $FeSO_4$–Phenanthroline–H_2O_2 system. Hydroxyl radicals are generated from $FeSO_4$ and H_2O_2, and detected by their ability to hydroxylate 1,10-phenantroline-Fe^{2+} to 1,10-phenantroline-Fe^{3+} with the subsequence reduction in absorbance at 536 nm. The presence of an antioxidant compound will inhibit that oxydation and absorbance will increase. Other systems are $CuSO_4$–phenanthroline-vitamin C–H_2O_2 using chemiluminescence detection, $FeSO_4$-5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO)–H_2O_2, $FeCl_3$-2-deoxy-D-ribose-Vitamin C–H_2O_2 using detection at 532 nm after addition of TBA (thiobarbituric acid)	46, 106, 109, 112– 114, 117, 121, 128, 132
Superoxide dismutase activity	It determines the inhibition of nitroblue tetrazolium (NBT) reduction mediated by riboflavin or a non-enzymatically generated superoxide radical. It consists of mixing the antioxidant molecule with riboflavin and NBT, incubation, and measurement of NBT absorbance at 560 nm. The lower is the absorbance, the higher is the scavenging capacity of tested compunds	107, 114, 117
Ferric ion reducing antioxidant power/metal chelating activity (Fe(II) chelating activity)	The method is based on the reduction of a Fe^{3+} complex to Fe^{2+} complex by an antioxidant compound (reductant). It consists of mixing a Fe^{3+} complex such as potasium ferricyanide ($K_3Fe(CN)_6$) or 2,4,6-tripyridyl-*s*-triazine with the tested peptide and $FeCl_3$. Antioxidant is capable of reducing Fe^{3+} to Fe^{2+} and the signal of Fe^{2+} formation is proportional to antioxidant capacity	75, 112–114, 117, 120, 121, 128, 132
	The method is based on the reduction of Fe^{3+}-ferrozine (3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-4′,4″-disulfonic acid sodium salt) complex to Fe^{2+}-ferrozine complex by antioxidants and it requires the addition of $FeCl_2$ solution. Ferrozine-Fe^{2+} is a pink chromophore whose signal can be measured at 562 nm	112, 119, 122, 124
Cu(II) chelating activity	A mixture of catechol and Cu(II) yields in a substance absorbing at 632 nm. The addition of a metal chelator to this mixture results in the disruption of this molecule and disappearance of such absorbance. The assay consists of mixing the cathecol with $CuSO_4$ and the tested compunds, incubation, and absorbance measurement at 632 nm	132
Saccharomyces Cerevisiae-based antioxidant assay	It measures the capacity of tested compounds to restore yeast growth after incubation of yeast with an oxidant compound such as hydrogen peroxide, cumene hydroperoxide, linoleic acid 13-hydroperoxide, menadione, peroxynitrite, etc	110
ORAC method	It used fluorescein as probe and APPA (2,2'-azobis (2-methylpropionamidine)dihydrochloride) as radical initiator generated by termal decomposition at 37 °C. Generated radicals oxidize probe and rate of oxidation inhibition due to the presence of a tested compound (potential antioxidant) is evaluated by the measurement of fluorescein at λ_{EXC} of 485 nm and λ_{EMI} of 538 nm. The antioxidant capacity of tested compound is directly related to intact fluorescein concentration	119, 127
TEAC method	ABTS (2,2'-aziino-bis(3-ethylbenzthiazoline-6-sulphonic acid) absorbing at 734/690/420 nm is used as probe and oxidant. $ABTS^+$ radicals is produced by the reaction of ABTS with potassium persulfate and are capable of abstracting electrons from a tested compound (potential antioxidant) and decoloring. The assay measures the decrease in ABTS absorbance at 734/690/420 nm in the presence of tested compound and in comparison with standards	21, 39, 108, 116, 124, 127
TRAP	Incubation with luminol, addition of ABAP (2,2'-Azo-bis(*a*-amidinopropane), and fluorescence detection	39

[119]. Park et al. [111] investigated the antioxidant capacity of wheat gluten hydrolysates observing the same behavior previously detected in soybean. In fact, acidic fractions yielded strong DPPH radical scavenging activity while basic fractions were strong suppressors of linoleic acid oxidation. On the other hand, other authors investigated the stability of antioxidation properties of wheat peptides obtained by enzymatic hydrolysis with alcalase. They reported that wheat peptides lose their surface hydrophobicity and reducing power after exposure to heat or lipid peroxidation. These modifications also lead to gradual formation of

aggregates in wheat peptides and induced more reactive oxygen species *in vivo* [120]. Moreover, corn gluten meal digested with alcalase has also resulted in peptides with antioxidant activity. Authors observed the highest antioxidant activity in peptides with 500–1500 Da [121]. On the other hand, barley is a cereal with 13% protein content. The effect of hydrolysis of its main protein, hordein, with different proteases (alcalase, flavourzyme, and pepsin) and hydrolysis times on its antioxidant activity has been studied. Flavourzyme hydrolyzed barley faster into small- and medium-size peptides while alcalase and pepsin resulted into medium- and large-peptides. Authors concluded that barley hydrolysates, particularly those produced with flavourzyme and alcalase, showed the highest antioxidant properties [122]. The antioxidant capacity of rice bran proteins has also been explored. Phytate free and crude rice bran protein extracts were analysed observing, as expected, higher antioxidant capacity in the crude extract due to the presence of phytate. Nevertheless, when phytate free extracts were hydrolyzed with different enzymes this antioxidant capacity increased being possible to identify three main peptides as responsible of such capacity[123].

Peptides produced during fermentation have also yielded antioxidant properties [125]. The ferric thiocyanate method has been employed for the screening of antioxidant peptides in two fermented soybean products, natto and tempeh [32]. Authors found two peptides with antioxidant activity in every soybean product that, in addition, were able to resist digestion by kidney membrane proteases and pronase. Another fermented soybean product that has been target in this kind of studies is Douchi, a traditional salt-fermented soybean food also exerting antihypertensive properties [124]. *In vitro* and *in vivo* studies were carried out observing that superoxide dismutase activity in liver and kidney, catalase activity in liver, and glutathione peroxidase activity in kidney increased when rats were fed with Douchi. Nevertheless, it was not possible to confirm that this antioxidant capacity was only due to soybean peptides but to a combination of peptides and isoflavones. On the other hand, rice wine was separated into 60 fractions and antioxidant capacity was measured in each one using the DPPH method and measuring the lipid peroxidation inhibition capacity. Those fractions showing the best antioxidant properties were repurified and four main peptides were finally identified as responsible for antioxidant capacity in rice wine [125]. Moreover, a pool of different lactic acid bacteria (10 strains) have been used in the fermentation of various cereal flours (wheat, kamut, spelt, rye, barley, maize, oat, and rice) with the aim of synthesizing antioxidant peptides. Twenty-five different peptides of 5–57 amino acids were identified as responsible of antioxidant activity observed by *in vitro* and *ex vivo* studies. None of these peptides seemed to be affected by sequential *in vitro* treatments with digestive enzymes [126] Preza et al. [127] explored the antitumor activity of cacao seeds (unfermented and semifermented) and looked for a correlation between antitumor and antioxidative activities. Moreover, albumin, globulin, and glutelin fractions were also extracted and tested. Antitumor activity was only observed in the semifermented albumin fraction and free radical scavenging capacity was observed mainly in the glutelin and albumin fractions, the last one in a lower rate. Therefore, no direct correlation between antioxidant and antitumor activities was found. Other authors [128] confirmed the presence of a peptide in fermented *Ganoderma lucidum* mushroom behaving as the main responsible for its antioxidant activity. Antioxidant properties of this peptide were demonstrated in food lipids and animal biological systems and on different biological free radicals. Moreover, a polysaccharide-peptide complex with antioxidant properties was purified and identified by FTIR, ^{13}C NMR, and ^{1}H NMR from the abalone mushroom (*Pleurotus abalonus*) [129].

Gastrointestinal digestion has also been employed to check the bioavailability of bioactive peptides such as in the case of dioscorin from Chinese yam [113] or the peptides isolated from fermented cereals [126]. Moreover, the investigation of the formation of bioactive peptides by gastrointestinal digestion of plant products is also possible. Indeed, soybean hydrolyzed with pepsin and pancreatin has been tested for their antioxidant capacity. Linoleic acid oxidation was inhibited by all soybean hydrolysate fractions and by the whole hydrolysate. Nevertheless, the fraction with the highest molecular weights (5–10 kDa) resulted in a lower inhibition activity than the fraction with the lowest molecular weight (0–5 kDa) [130]. Other authors explored the antioxidant capacity of peptides obtained by gastrointestinal digestion of hempseed. Whole hydrolysate and fractions with different sizes ($>$ 1, 1–3, 3–5, and 5–10 kDa) were analyzed observing that fractions resulted in significant improvements in ferric reducing power and DPPH and hydroxyl radical scavenging activities but decreased the metal chelation capacity [131]. Furthermore, *Brassica carinate* seed proteins were also investigated observing they were a good source of bioactive peptides after hydrolysis with digestive enzymes [49].

Another procedure that has resulted in antioxidant peptides is autolysis. Indeed, cacao beans autolyzed at pH 5.2 (optimum pH

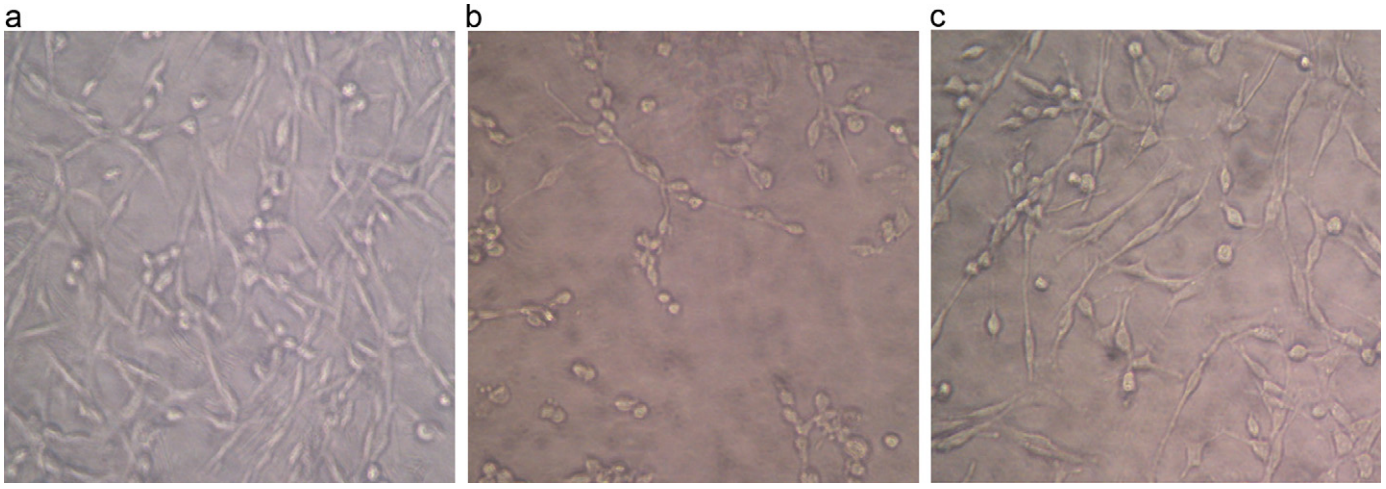

Fig. 2. Morphological characteristics of PC12 cells after hydrogen peroxide treatment in the absence and presence of antioxidant peptides from hempseed: (a) no H_2O_2 treatment; (b) treated with 150 μM H_2O_2 for 2 h; (c) treated with antioxidant peptides at 50 μg/mL for 2 h before exposure to 150 μM H_2O_2. With permission from Ref. [114].

of carboxypeptidase) showed highest antioxidant activity than the extract autolyzed at pH 3.5 (optimum pH of aspartic endoprotease) [75]. Moreover, a by-product of wheat, the wheat germ, has also resulted interesting for its antioxidant properties [132]. Indeed, wheat germ is a valuable source of proteins for humans because of its high content in essential amino acids. A cheap methodology based on hydrolysis with endogenous peptidases at optimized conditions resulted in a peptide with high reducing power, hydroxyl radical scavenging activity, and Cu(II) chelating ability.

4. Other bioactive proteins and peptides

4.1. Opioid agonist and antagonist

Opioids are chemical substances with opiate-like activity. There are peptides with similar structure to endogenous opioid peptides (endorphins and enkephalins) that have been termed exorphins. Exorphins can interact with opioid receptors of the δ, μ, and K-type. Most of them present an N-terminal Tyr residue linked to another aromatic residue in third or fourth position from the N-terminus. Moreover, they used to have a Pro residue in second position. On the other hand, there are also peptides yielding activities that are antagonist to endogenous opioids. It has not been found any peptide derived from vegetal foods with antiopioid activity unlike foods of animal origin in whose case there are examples of peptides with this antagonistic activity.

The most important opioid peptide in soybean is derived from the β-subunit of β-conglycinin. This subunit has the human β-casomorphin-4 sequence (Tyr–Pro–Phe–Val) which is derived from human β-casein and that yields high opioid activity. Longer peptides (soymorphin-5 (Tyr–Pro–Phe–Val–Val), soymorphin-6 (Tyr–Pro–Phe–Val–Val), and soymorphin-7 (Tyr–Pro–Phe–Val–Val–Asn–Ala)) derived from this sequence and synthetically obtained, presented higher opioid activity than human β-casomorphin-4. Moreover, soymorphin-5 has also shown capacity to improve glucose and lipid metabolisms [134]. Opioid activity has been associated with emotional behavior and Ohinata et al. [135] could demonstrate this fact in the case of soymorphins.

Another vegetable food that has been related to opioid activity is wheat. Zioudrou et al. [136] isolated peptides with opioid activity, for the first time, from pepsin digest of wheat gluten. More recently, Fanciulli et al. [137] demonstrated the highest opioid capacity in gluten wheat was from the peptide GE-B5 (Tyr–Gly–Gly–Trp–Leu). Other opioid peptides that have been obtained from wheat by sequential hydrolysis with pepsin (pH 2) and thermolysin (pH 7) were: Gly–Tyr–Tyr–Pro, Gly–Tyr–Tyr–Pro–Thr, Gly–Tyr–Tyr–Pro–Thr–Ser, Tyr–Pro–Ile–Ser–Leu, Tyr–Gly–Gly–Trp–Leu, and Tyr–Gly–Gly–Trp [138].

4.2. Peptides with capacity to suppress food intake and gastric emptying

Dietary peptides increasing satiety beyond that attributable to their energy content are of specific interest against obesity. These peptides promote the release of cholecystokinin (CCK) which inhibits food intake and gastric emptying. CCK is produced by the so-called "I-cells" found in the central nervous system and in the gastrointestinal tract. In the brain, CCK acts as a neurotransmitter, while in the gastrointestinal tract, CCK is localized in both secretion and neural systems and is secreted by cells in the duodenal and jejuna mucosa. Nishi et al. attributed this ability to induce satiety to peptides rich in Arg residues such as the β-subunit 51–63 (Val–Arg–Ile–Arg–Leu–Leu–Gln–Arg–Phe–Asn–Lys–Arg–Ser) sequence of the soybean β-conglycinin [139–141]. Nevertheless, it is uncertain whether such peptides could stand trypsin gastrointestinal digestion. On the other hand, it has been demonstrated that opioid agonists can also reduce food intake in animals. Indeed, the soybean peptide soymorphin in addition of being an opioid agonist showed food intake suppression capacity after oral administration in fasted mice [142]. In addition to yellow soybean, black soybean has also demonstrated capacity to reduce food intake [143].

Another plant food exerting capacity to suppress food intake is rapeseed. In fact, peptide Arg–Ile–Tyr from rapeseed that has been previously reported as an antihypertensive peptide exerted capacity to suppress food intake via stimulation of CCK release [144].

4.3. Immunomodulating peptides

Immodulation is the manipulation of immune system by increasing (immunostimulation) or decreasing (immunosuppression) the magnitude of the immune response.

Soymetide (Met–Ile–Thr–Leu–Ala–Ile–Phe–Val–Asn–Lys–Phe–Gly-Arg) is a peptide derived from residues 173–185 of the soybean β-conglycinin α′-subunit that is released by trypsin digestion of soybean proteins. This peptide has a specific binding site on human blood phagocytic cells stimulating bacterial death by phagocytosis. Soymetide has been quantified in different soybean dairy-like products (powdered milks and infant formulas). For that purpose, a capillary-HPLC method using a fused-core technology column was employed to separate soybean from other tryptic peptides. Soymetide contents in soybean foodstuffs ranged from 0.28 to 1.85 mg/g protein [145]. Nevertheless, soymetide shows weak affinity for the *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) receptor [146]. Indeed, peptides binding fMLP receptors contribute to a rapid response to bacterial infection and to activate immune system. Different soymetide derivatives have been synthesized by deleting or replacing amino acid residues in order to increase this affinity to fMLP receptors [147,148]. Soymetide-9 (Met–Ile–Thr–Leu–Ala–Ile–Phe–Val–Asn) exhibited the highest affinity to fMLP receptors as well as the strongest phagocytosis-stimulating activity *in vitro*. However, soymetide-4 (Met–Ile–Thr–Leu) showed higher immunostimulating activity *in vivo*. This fact could be explained taking into account that longer peptides are not absorbed as well by the digestive system as smaller peptides. Moreover, soymetide-4 may be released in the intestine following soybean digestion. On the other hand, this peptide has also demonstrated to suppress alopecia induced by cancer chemotherapy [149,150]. Despite most studies have been centered on soymetide, it has also been documented the presence of another immunomodulating peptide in the A_{1a} subunit of the glycinin fraction of soybean. This peptide is also released by tryptic digestion and its sequence is His–Cys–Gln–Arg–Pro–Arg [151].

A rice peptide called oryzatensin has also demonstrated ability to stimulate immune system [152]. This peptide (Gly–Tyr–Pro–Met–Tyr–Pro–Leu–Pro–Arg) was isolated from the tryptic digest of rice proteins and it was demonstrated its resistance to pepsin and pancreatin digestions. Oryzatensin exhibited significant phagocytosis-promoting activity above 1 μM.

On the other hand, mushrooms, in general, are widely known for their immunomodulatory activities partly derived from proteins working alone or jointly with β-glucans [153]. Specifically, fungal immunomodulatory proteins (FIP), mostly identified as lectins, have demonstrated to play an important role in the regulation of immune cell growth and in the inactivation of macrophages and lymphocites. As an example, mushroom *Flammulina velutipes*, a common oriental food, contains a FIP protein that has been proposed to fight against immunoprophylaxis in allergic diseases [154]. Moreover, since most edible mushrooms are cooked or processed before its consumption,

there have been many works focused to assess the immunomodulatory activity remaining after these treatments. Chang et al. [155] demonstrated that the immunostimulating capacity of mushrooms *Agaricus bisporus* and *Auricularia polytricha* was kept when they were submitted to different industrial treatments: heating, freezing, acid, alkali, and dehydratation. Other authors studied the influence of different processing conditions on the functions of two proteins (FVE and LZ8) from the popular mushrooms Enoki (*Flammulia velutipes*) and Reishi (*Ganoderma lucidum*), respectively. They concluded that these mushrooms presented a good thermal/freezing resistance and acid tolerance and a moderate resistance to alkali and dehydration treatments [156].

Another example of mushroom with immunomodulating properties is *Pleurotus citrinopileatus*. This mushroom has recently become in Easter Asia a popular delicacy due to its health beneficial effects and taste. Among health promoting activities are immunomodulation that was related with protein PCP-3A. This protein stimulated the growth of human mononuclear cells and $CD4^+$ T lymphocites increasing the secretion of cytokines TNF-α (tumor necrosis factor), IL-2 (interleukin), and IFN-γ (interferon) that, subsequently, inhibited the growth of leukemic cells [157].

Chinese yam tubers (*Dioscorea alata* cv. Taining No. 1) have already been reported in this review for its antihypertensive and antioxidant activities. Moreover, this tuber, and more specifically, its storage protein dioscorin, exerted immunomodulating activity and this effect remained after *in vivo* digestion [158,159].

4.4. Antithrombotic peptides

Some peptides have also shown capacity to inhibit platelet aggregation and thrombosis. Most of this activity has been studied and found in dairy peptides and not much work has been performed in the case of vegetable food. Two peptides (Asp–Glu–Glu and Ser–Ser–Gly–Glu) has been purified and identified as inhibitors of platelet aggregation in soybean protein hydrolysates manufactured by successive enzymatic digestions with endopeptidase Promod 278 and exopeptidase Promod 279 [160]. Moreover, Gibbs et al. [32] observed anti-thrombotic activity in the kidney membrane hydrolysate of natto. The peptide responsible of such activity presented homology to hirutonin, a previously described synthetic thrombin inhibitor.

4.5. Hypocholesterolemic peptides

Hypocholesterolemic properties of soybean have been assessed by numerous clinical studies and even the US Food and Drug Administration approved the health claim "25 g of soybean protein per day may reduce the risk of heart disease". Moreover, this reduction is greater with soybean hydrolysates than with non hydrolyzed soybean proteins [161]. Different enzymes have been employed for hydrolyzing soybean proteins to produce hypocholesterolemic peptides (trypsin, pepsin, neutrase, alcalase, etc.). The tryptic digestion of the soybean glycinin fraction has enabled the isolation of the peptide Leu–Pro–Tyr–Pro which showed high hypocholesterolemic activity. Kwon et al. [162] investigated the effect of modifying this peptide on its hypocholesterolemic activity. They observed that this activity was lost when the hydrophobic amino acid Leu was substituted by the polar amino acid Ser while the C-terminus Pro was not so critic to keep the hypocholesterolemic activity. On the other hand, the use of pepsin has also resulted in peptides with cholesterol lowering activity. Indeed, Pak et al. [163] isolated a peptide from soybean glycinin pepsin hydrolysate with capability to bind bile acids shielding them from reabsorption and stimulating the transformation of cholesterol in blood plasma. It had a molecular weight of 755.2 Da and its amino acid sequence was Ile–Ala–Val–Pro–Gly–Glu–Val–Ala. Based on this sequence, six different peptides keeping the amino acid sequence Ile–Ala–Val–Pro were synthesized and that yielding the higher hypocholesterolemic effect was subjected to further investigation on its mechanism of action [164]. Moreover, two years later, Zhong et al. [165] compared the hypocholesterolemic activity of hydrolysates obtained from soybean with two different enzymes, a neutral and an alkaline protease. *In vitro* and *in vivo* assays demonstrated the highest cholesterol reducing capacity of the alcalase hydrolysate at 18% hydrolysis degree and its *in vitro* stability to gastrointestinal protease digestion. This hydrolyzed extract was then fractionated by gradient ethanol elution from a macroporous adsorption resin and separated by Sephadex G-15 and RP-HPLC (see Fig. 3). The responsible of this strong cholesterol lowering effect was a peptide with sequence Try–Gly–Ala–Pro–Ser–Leu [166]. Other researchers used a protease from *Bacillus amyloliquefaciens* to hydrolyze soybean observing that it strongly stimulated transcription of low density lipoprotein receptors (LDL-R). These receptors play an

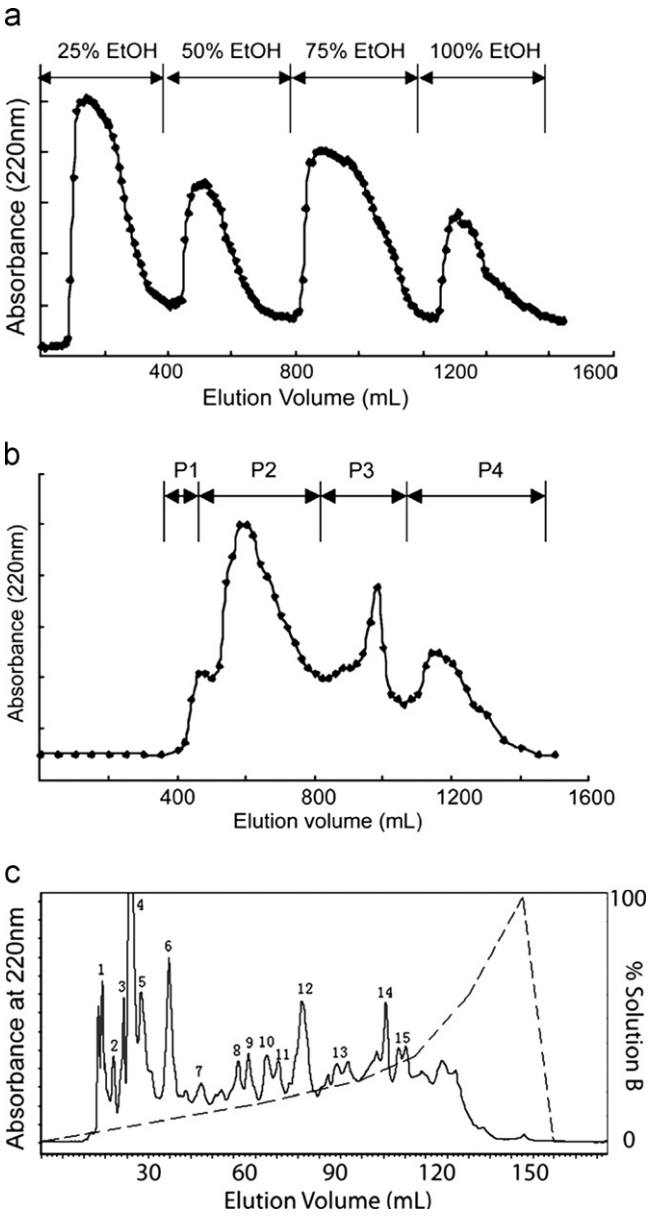

Fig. 3. Fractionation of a novel hypocholesterolemic peptide derived from soybean alcalase hydrolyzated. (A) Separation of hydrolysate from a non-polar resin using an ethanol gradient; (B) Gel filtration profile of the 75% ethanol fraction obtained with a Sephadex G-15 column; (C) Chromatograph corresponding to P2 fraction obtained on a semi-preparative RP-HPLC column. With permission from Ref. [166].

important role in the reduction of plasma LDL cholesterol. The responsible for such capacity was the peptide Phe–Val–Val–Asn–Ala–Thr–Ser–Asn [167].

In addition to hydrolysis, fermented soybean products such as tofoyu have also shown hypocholesterolemic effects [100]. Nevertheless, all these studies have been performed using yellow soybeans and not much attention has been paid to black soybean (*Rhynchosia volubilis* Lour.). Rho et al. [143] investigated the effect of black soybean hydrolysates on the lipidic composition by an *in vivo* assay suggesting this legume as a potent source of nutraceutical components for its hypolipidaemic effects.

As well as soybean, there are other legumes that have also proven to be useful for hypercholesterol treatment. Lupinus has demonstrated by *in vivo* studies its capacity to reduce cholesterol and lupin β-conglutin has been proposed as the protein responsible for this activity [168]. On the other hand, it is known the cholesterol lowering capacity of rice bran, but it was not demonstrated the effect of its proteins and hydrolysates. Very recently, the group of Zhang [169] investigated the potential of rice bran proteins hydrolysates to reduce cholesterol. They hydrolyzed rice bran with different proteases (alcalase, neutrase, papain, and trypsin) observing the highest activity in the alcalase hydrolysates.

The hypocholesterolemic activity of potato hydrolysates has also been explored by *in vivo* assays [170]. Two different potato hydrolysates differing in the hydrolysis time (16 h hydrolysis time and 25 h hydrolysis time) were compared. Results suggested a higher hypocholesterolemic activity in the 16 h hydrolysate.

Finally, Pedroche et al. [49] demonstrated that *B. carinata* hydrolysed with immobilized digestive proteases (trypsin, chymotrypsin, and carboxypeptidase A) resulted in an extract with ability to reduce micellar cholesterol.

5. Conclusions

There are proteins and peptides in plant derived foods with biological activities. Some bioactive proteins and peptides are naturally occurring in plant foods but in most cases bioactive peptides are in a latent state as part of the sequence of a protein and have to be released. Among releasing mechanisms, most usual is enzymatic digestion using enzymes as alcalase and pepsin. Most explored capacities have been ACE inhibition capacity and antioxidant capacity although there are also examples of peptides and proteins derived from plant foods with opioid, antithrombotic, immunomodulating, and hypocholesterolemic capacity or with ability to suppress food intake. Determination of such capabilities is mostly performed by the use of *in vitro* methodologies. Antihypertensive activity is always evaluated by the inhibition of the ACE induced hydrolysis of hyppuryl–histidyl–leucine to hippuric acid. Antioxidant activity can be evaluated by different methods being the inhibition of lipid oxidation, the determination of the reduction capability of ferric ion, and the scavenging capacity of free radicals the most usual. Among plant foods, soybean has been the most explored one probably due to its high protein content, its known health benefits, and its extensive use all over the world. Nevertheless, bioinformatics revelaed that the most susceptible plant protein to release bioactive peptides was wheat gliadin. Most bioactive peptides consisted of short sequences containing hydrophobic amino acids. Despite the number of works devoted to the search of bioactive proteins and peptides was more extensive in foods of animal origin than in foods of plant origin, the revised bibliography demonstrated that the former constitute an alternative and cheap source of bioactive proteins and peptides. Taking into account, as well, that the variety of plant foods is higher than the variety of foods of animal origin, it is possible to consider plant foods as an universe of potentially containing health-promoting proteins and peptides that have been very little explored.

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