Bioactive peptides from vegetable food matrices: research trends and novel biotechnologies for synthesis and recovery

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Abstract

Currently, the interest for health-promoting functional foods, dietary supplements and pharmaceutical preparations containing bioactive peptides deriving from food proteins, is increasing. Despite the large literature concerning peptides derived from animal proteins, only recently the scientific community investigated the possibility to obtain bioactive peptides from vegetable sources, also discovering novel functional features. In this article, the functional effects of vegetable-derived peptides, including antihypertensive, antioxidant, antitumoral, antiproliferative, hypocholesterolemic, antinflammatory activities, are described. The novel biotechnologies for the release of bioactive peptides from vegetable matrices, including microbial fermentation and the use of microbial enzymes are investigated. Moreover, the modern technologies for their recovery, purification, and analysis, are reviewed and discussed.

Keywords: bioactive peptides, plants, vegetable, fermentation, enzymes, synthesis, recovery

1. Introduction

Proteins are important macronutrients of foods, serving as source of energy and amino acids, that contribute to growth and maintenance of the body (Shahidi & Zhong, 2008). Beside the nutritional role, proteins are responsible for various physicochemical and sensory properties of foods, and may act as functional and health-promoting ingredients (Shahidi & Zhong, 2008). Many of the physiological and functional properties of proteins are attributed to biologically active peptides which are often encrypted in the native sequence (Gobbetti, Minervini, & Rizzello, 2007; Shahidi & Zhong, 2008). Biogenic or bioactive peptides can be produced from the protein precursor by digestive enzymes (gastrointestinal digestion), during food processing (ripening, fermentation, cooking), storage, or by *in vitro* hydrolysis by proteolytic enzymes (Carrasco-Castilla, Hernandez-Alvarez, Jimenez-Martınez, Gutierrez-Lopez, & Davila-Ortiz, 2012).

Bioactive peptides mainly contain 3-20 amino acid units, but in some cases the size is larger (Shahidi & Zhong, 2008), and they can be considered as components of functional foods which may exert regulatory activities in the human organism, irrespective of their nutritive functions (Gobbetti et al., 2007). *In vitro* and some *in vivo* studies show a large spectrum of biological functions attributed to bioactive peptides: opioid like, mineral-binding, immunomodulatory, antimicrobial, antioxidative, antithrombotic, hypocholesterolemic, antihypertensive (Coda, Rizzello, Pinto, & Gobbetti, 2012), and antitumoral (Rizzello, Nionelli, Coda, & Gobbetti, 2012; Rizzello et al., 2015a).

The interest for health-promoting functional foods, dietary supplements and pharmaceutical preparations containing bioactive peptides is markedly increasing (Coda et al., 2012). Numerous studies were performed on bioactive peptides derived from animal proteins, especially from caseins, which appear to be proteins with high functional potential. More recently, the scientific community investigated the possibility to obtain bioactive peptides from plants. In particular, the identification of bioactive peptides deriving from vegetable food proteins follows the growing interest of the scientific community and public opinion towards vegetable foods, due to their higher sustainability with respect to animal foods and the increased consumer requirements of healthy and balanced diets.

Cereals (supplying half the world's protein needs) and legumes are the main target of this research, being both rich sources of proteins with a complementary spectrum of amino acids (García, Puchalska, Esteve, & Marina, 2013; Malaguti et al., 2014). Nevertheless bioactive peptides were found in many other vegetables (pseudocereals, algae, edible fungi, garlic, ginkgo biloba seeds, curcuma, sesame, peanut, alfalfa, spinach, sunflower, hempseeds, tubers, cocoa beans and others) as the consequence of fermentation, enzymatic hydrolysis, but also not encrypted in any parent molecule (García et al., 2013).

Overall, sequences showing the same bioactivities can be released from native proteins deriving from vegetable or animal matrices. For example, the multi-functional dipeptide VY can be released through the hydrolysis of vegetable (brewed sake) or milk proteins (Saito, Wanezaki, Kawato, & Imayasu, 1994; Tagliazucchi, Shiamsia, & Conte, 2016). The ACE-inhibitory dipeptide AI was isolated from a soy sauce and pinto bean proteins as well as from milk after gastro-intestinal digestion (Nakahara et al., 2010; Tagliazucchi, Martini, Bellesia, & Conte, 2015; Tagliazucchi et al., 2016). The antihypertensive peptide SY was identified in protein hydrolysates of soybean, garlic, cereals, milk, pork sarcoplasmic and chicken proteins (García et al., 2013; Castellano, Aristoy, Sentandreu, Vignolo, & Toldrá, 2013; Iwaniak & Dziuba, 2009; Weimann, Meisel, & Erhardt, 2009); the peptide YPFVV, isolated from an hydrolysate of soy β-conglycinin showed high similarity with the human casomorphin-5 (YPFVE) (Ohinata, Agui, & Yoshikawa, 2007); the bioactive peptide soymorphin-5 shares the same sequence (YPF), the same bioactivity (opioid agonist) and the same mechanism of action (interaction with μ-receptors) of human and bovine casomorphins.

In this article, the recent advances in the biotechnologies for release, recovery, purification, and characterization of bioactive peptides from vegetable food matrices, are described. The *in vivo* release of bioactive peptides during gastrointestinal digestion, has not been reviewed.

2. Release of bioactive peptides by microbial fermentation

Several microorganisms are able to produce enzymes, including proteases, and to release them into the extracellular medium during growth, leading to proteolysis and peptide production. Biotechnologies including the use of specific microorganisms, are typically based on fermentation processes allowed to proceed for a period ranging from a few hours to several days, depending on the type of fermenting microorganism and the desired peptide product. At the end of fermentation, the product can be used directly or collected after purification.

Among microorganisms involved in food biotechnologies, lactic acid bacteria are recognized as the most useful in bioactive peptides enrichment, thanks to the large adaptability to different environments and animal or vegetable matrices, the safety, and, especially, the efficient proteolytic system of which they are provided. Indeed, proteinase activity and a large portfolio of peptidases are considered the pre-requisites to liberate bioactive peptides from proteins (De Angelis et al., 2006; Gobbetti et al., 2007). Lactic acid bacteria belong to a microbial group having a large variability, derived from the evolution in a wide range of environments. Their large variability is very suitable for the selection of strains to be used for different biotechnology processes. An increasing number of scientific articles (Coda et al., 2011; Coda et al., 2012; Rizzello, Cassone, Di Cagno, & Gobbetti, 2008; Rizzello et al., 2012) described the use of lactic acid bacteria isolated from sourdough for the release of bioactive peptides in vegetable matrices. Sourdough is the natural starter traditionally used for making baked goods. It was reported that fermentative and proteolytic activities of sourdough lactic acid bacteria determined not only the sensory, technology and nutritional characteristics, but also the functional features of the resulting baked goods (Gobbetti, De Angelis, Corsetti, & Di Cagno, 2005). Besides the microbial agents, also the fermentation conditions typical of the sourdough technology (Coda et al., 2012; Rizzello et al., 2008; Rizzello et al., 2012) were adopted and optimized to promote the peptides release in a wide range of food matrices. A description of the main antihypertensive, antioxidant and anticancer peptides released by fermentation processes of vegetal matrices is reported below. Moreover, in Table 1 a list of bioactive peptides identified from plant matrices specifically fermented by lactic acid bacteria is also reported.

2.1 Antihypertensive peptides

Bioactive peptides having Angiotensin I-Converting Enzyme (ACE)-inhibitory properties, may be used for preventing hypertension as well as for other therapeutic purposes (Rizzello et al, 2008). Within the enzyme cascade of the renin-angiotensin system, ACE removes histidyl-leucine from angiotensin I to form the physiologically active octapeptide angiotensin II, one of the most potent known vasoconstrictors. Therefore, a rationale for treating hypertension would be to administer drugs or natural compounds which selectively inhibit ACE. Although most of the studies on ACE-inhibitory peptides referred to synthesis occurring in dairy products, some antihypertensive sequences have been recently isolated from plant proteins. Although many experiments were carried out on enzymatic hydrolysates (see below), the production of ACE-inhibitory peptides through microbial fermentation has been also investigated (Rizzello et al., 2008). A pool of selected sourdough lactobacilli having specific proteinase and peptidase activities towards cereal proteins, was successfully used for releasing ACE-inhibitory peptides during a long-time sourdough fermentation (Rizzello et al., 2007; Rizzello et al., 2008). In particular, high activity was found fermenting flours under semi-liquid conditions and, especially, by using wholemeal wheat flour. The addition of commercial proteases to the starters did not increase the ACE-inhibitory activity of the fermented matrices, since they caused a too extensive proteolysis of the active sequences (Rizzello et al., 2008). Fourteen ACE-inhibitory peptides (IC_{50} ranging from 0.19 to 0.54 mg/mL) were identified from the fermented wholemeal wheat. Almost all contained the well-known antihypertensive epitope VAP (Rizzello et al., 2008). Previously, the epitope VAP was found either as antihypertensive peptide or as encrypted epitope of ACE-inhibitory peptides from milk proteins (Gobbetti et al., 2007). Anti-ACE peptides deriving from legume proteins were also isolated, as reported by Jakubczyk, Karaś, Baraniak, & Pietrzak (2013), that used a *L. plantarum* strain to ferment and hydrolyze pea flour proteins. The active and purified peptide fraction obtained from the pea proteins hydrolysates had an IC_{50} of 64.04 µg/ml (Jakubczyk et al., 2013). Antihypertensive peptides were also obtained in soybean products (soymilk, soy-yogurt,

soy sauce) through fermentation with lactic acid bacteria (*Lactobacillus casei, L. acidophilus, L. bulgaricus, Streptococcus thermophilus)*, but also using *Bacillus natto, B. subtilis, Bifidobacterium longum* as starters (Singh, Vij, & Hati, 2014). Antihypertensive peptides also showing anti-thrombotic, surface tension and antioxidant properties were isolated in the traditional soy-fermented foods *natto* and *tempeh* (Gibbs, Zougman, Masse, & Mulligan, 2003). More recently, it was demonstrated that lactic acid bacteria allowed the release of antihypertensive peptides during *Phaseolus vulgaris* (navy bean) fermentation (Rui et al., 2015). Moreover, it was demonstrated that fermented tropical legumes (Bambara groundnut, locust bean, soybean) are able to reduce the hypertension state, together with the diabetes-induced dyslipidemia, of rats (Ademiluyi & Oboh, 2015).

Peptide fractions having ACE-inhibitory activity were purified also in fermented alcoholic beverages, including red and white wines (Pozo-Bayòn, Alcaíde, Polo, & Pueyo, 2005) and sake (Saito et al., 1994).

2.2 Antioxidant peptides

Recently, the interest for antioxidant peptides deriving from food proteins has increased, according to the recognized role in the prevention mechanisms of the oxidative stresses associated with numerous degenerative aging diseases (e.g., cancer and atherosclerosis) (Adebiyi, Adebiyi, Yamashita, Ogawa, & Muramoto, 2009). Moreover, the application of the antioxidants in food industry is also related to the ability to retard food discoloration and deterioration, which occur as the consequence of oxidative processes (Rizzello et al., 2007). Thanks to the consumers request for healthy foods, natural antioxidants gained the attention of food manufacturers as alternative to the synthetic ones (Minervini et al., 2003).

Plants are known for antioxidant properties mostly because of their polyphenolic compounds, but recently the antioxidant properties of vegetable proteins and peptides have been increasingly explored both in *in vitro* and *in vivo* studies (García et al., 2013). Biologically active peptides with potential

antioxidant activity were obtained from many animal and plant proteins (Coda et al., 2012). They were isolated from peanut kernels, rice bran, sunflower protein, alfalfa leaf protein, corn gluten meal, frog skin, yam, egg-yolk protein, milk-kefir and soymilk kefir, mushrooms, mackerel, curry leaves, cotton leaf worm, casein, algae protein waste, wheat, and buckwheat (Sarmadi & Ismail, 2010). It was argued that antioxidant peptides act as inhibitors of lipid peroxidation, direct scavengers of free radicals and/or as agents to chelate transition metal ions that catalyze the generation of radical species (Shimada, Fujikawa, Yahara, & Nakamura, 1992). Usually, antioxidant peptides are constituted by 2-20 amino acidic residues, and have molecular masses less than 6.0 kDa (Coda et al., 2012). The antioxidant activity seems to be strongly correlated to the amino acid composition, conformation and hydrophobicity. A pool of selected lactic acid bacteria including ten strains, previously selected based on proteinase and peptidase activities (Coda et al., 2012), was used to ferment various flours with the aim to release antioxidant peptides. Fermentation by selected lactic acid bacteria was allowed for long time and with semi-liquid conditions, which are indispensable to fully exploit microbial proteolysis (Rizzello, Nionelli, Coda, De Angelis, & Gobbetti, 2010). It was found that the radical scavenging activity of soluble extracts from fermented flours was significantly higher than that of unfermented controls. Among different flours obtained from cereals, pseudo-cereals and legumes, the highest activity was found for whole wheat, spelt, rye, and kamut sourdoughs. The purified active peptides, having the size from 8 to 57 amino acid residues, were resistant to further hydrolysis by digestive enzymes. Almost all their sequences shared compositional features which are typical of other antioxidant peptides (Coda et al., 2012) and showed *ex vivo* antioxidant activity which was comparable to that of α-tocopherol on mouse fibroblasts artificially subjected to oxidative stress.

Recently, it was reported that antioxidant peptides (together with anti-ACE peptides) were released also *by Oenococcus oenis, B. subtilis, B. pumilus,* and *L. plantarum* during grape must (Apud, Vaquero, Rollan, Stivala, & Fernández, 2013), rapeseed/flaxseed (He et al., 2012; Pihlanto, Johansson, & Mäkinen, 2012), rice protein (Dei Più, Tassoni, Serrazanetti, Ferri, Babini, Tagliazucchi, & Gianotti, 2014), and soybean (Singh et al., 2014) fermentation, respectively. *In vitro* and *in vivo*

evidences of the antioxidant activities of peptides isolated from *douchi* (a traditional Chinese salt-fermented soybean food) were also reported (Wang et al., 2008), semi-fermented cacao seeds (Preza et al., 2010), and commercial fermented mushrooms *Ganoderma lucidum* (Sun, He, & Xie, 2004) and Abalone (Li et al., 2012). In particular, antioxidant peptides from cacao beans showed antitumor activity against murine lymphoma L5178Y in BALB/c mice after a 15-days treatment with an oral dose of 25 mg/kg/day, while the *G. lucidum* Peptide (GLP) showed scavenging activity toward hydroxyl radicals produced in a deoxyribose system with an IC_{50} value of 25 µg/mL, and effectively quenched superoxide radical anion produced by pyrogallol autoxidation in a dose-dependent manner.

2.3 Anticancer peptides

It was emphasized that proteins, peptides and amino acids might be implicated at various levels in the prevention of different types of cancer (Shahidi & Zhong, 2008). Lunasin is a 43-amino acid peptide having molecular weight of 5 kDa. It corresponds to the small subunit peptide (Gm2S-1) of 2S soy albumin (Galvez, Chen, Macasieb, & de Lumen, 2001). Lunasin contains 9 aspartic acid residues at the C-terminus, a tripeptide arginine-glycine-aspartic acid cell adhesion motif and a predicted helix whose structure is similar to the conserved region of chromatin-binding proteins (Galvez et al., 2001). The amino acid sequence of lunasin peptide from soybean is deposited at the National Center for Biotechnology Information (NCBI) database with the accession number AAP62458. During *in vitro* assays, lunasin showed an inhibitory effect against the core histone acetylation of mammalian cells (Rizzello et al., 2012), suggesting an involvement in the chromatin modification, the process implicated in cell-cycle control and suppression of carcinogenesis (de Lumen, 2005). Recently, *in vivo* trials were performed with the aim to investigate the bioactivity and the bioavailability of lunasin after oral administration (Gonzalez de Mejia & Dia, 2010) and during food processing (Jeong, Jeong, Hsieh, Hernández-Ledesma, & de Lumen, 2010).

Originally isolated from soybean, lunasin was also found in barley, wheat, amaranth, rye, and *Solanum nigrum* L. (Rizzello et al., 2012). The high costs for chemical synthesis of lunasin limit its

application in chemo-preventive and nutritional treatments (Dia, Wanga, Oh, de Lumen, & Gonzalez de Mejia, 2009). In the quest for readily available natural sources of lunasin, its identification and purification from different vegetable sources is deserving a marked interest. The potential of lactic acid bacteria to release lunasin during fermentation of cereal and non-conventional (pseudocereals and legumes) flours was investigated (Rizzello et al., 2012). Different lactic acid bacteria strains, selected on the basis of specific peptidase activities among a large number of isolates, were used as starters to ferment wholemeal wheat, soybean, barley, amaranth and rye flours. Compared to untreated flours, the concentration of lunasin increased up to 2-4 times during fermentation. In particular, *Lactobacillus curvatus* SAL33 and *Lactobacillus brevis* AM7, characterized by high non-specific aminopeptidase activity, released the highest concentrations of lunasin in all the flours. Besides the presence of the entire lunasin sequence, fragments containing the immunoreactive epitope RGDDDDDDDDD were also found in fermented flours, as confirmed by mass spectra analyses (Rizzello et al., 2012). Recently, flours obtained from Italian legume varieties belonging to *Phaseolus vulgaris*, *Cicer arietinum*, *Lathyrus sativus*, *Lens culinaris* and *Pisum sativum* species were chemically subjected to fermentation with *L. plantarum* C48 and *L. brevis* AM7, strains selected on the basis of different peptidase activities (Rizzello et al., 2015a). Although western blot analysis, using an anti-lunasin primary antibody, showed the absence of lunasin, other immunoreactive polypeptides were found. It was observed that the number and the concentration of lunasin-like polypeptides increased during fermentation, as the consequence of the proteolysis of the native proteins carried out by the selected lactic acid bacteria. Nine different lunasin-like polypeptides, having similarity to lunasin, were identified and characterized (Rizzello et al., 2015a). A marked inhibitory effect on the proliferation of human adenocarcinoma Caco-2 cells was observed using extracts from fermented legume doughs (up to 70%).

2.4 Antifungal peptides

In recent years, bio-preservation (the use of microorganisms and/or their metabolites to prevent spoilage and to extend the shelf life of foods) has gained interest due to the increasing demand for more natural and preservative-free foods. Lactic acid bacteria are considered as useful bio-preservative organisms because of their capacity of synthesizing or releasing various antimicrobial and antifungal molecules. Synergistic activities between different compounds synthesized or released during sourdough fermentation, such as organic acids and peptides, can be responsible for the overall antifungal effect. The molecular understanding of the mechanism of action of antifungal peptides is still lacking, although the ability to form membrane pores, or to alter cytoplasmic membrane septum formation, such as the inhibition of cell-wall/nucleic acid/enzyme synthesis, were hypothesized.

Different peptides with antifungal activity were identified in the water-soluble extracts of wheat flour fermented with *L. brevis* AM7 (Coda et al., 2008) and *L. plantarum* 1A7 (Coda et al., 2011), as the results of the proteolytic activity on the native wheat proteins. Recently, the antifungal activity of wheat germ fermented with two autochthonous lactic acid bacteria, previously selected for their technology properties, (*L. plantarum* LB1 and *L. rossiae* LB5) was investigated (Rizzello, Cassone, Coda, & Gobbetti, 2011). The activity was attributed to a mixture of organic acids and peptides which were synthesized or released during fermentation. Four peptides, encrypted in wheat proteins were identified, and the activity confirmed after chemical synthesis (Rizzello et al., 2011). Overall, all the peptides produced by lactic acid bacteria in wheat-based matrices were characterized by a large inhibitory spectrum against species that commonly contaminate baked goods and bakeries (fungi belonging to the genera *Penicillium*, *Aspergillus*, *Monilia*, *Mucor*, *Endomyces*, *Cladosporium*, *Fusarium* and *Rhizopus*) (Coda et al., 2008; Coda et al., 2011; Rizzello et al., 2011), allowed a long-storage of breads (at least 21-28 days) and behaved as the calcium propionate. Recently,

3. Synthesis of bioactive peptides by enzymatic hydrolysis

The most common way to produce bioactive peptides is the use of single or multiple specific or unspecific proteases. Compared to microorganisms, enzymes generally require less time to generate

a similar degree of hydrolysis and their reaction can be controlled giving reproducible molecular weight profiles and peptide composition. Additionally, enzymes present substrate specificity which allows the development of protein hydrolysates with well-defined chemical and nutritional characteristics (Castro et al., 2011). In food industry, the use of enzymes is preferred to other processes also for the lack of residual organic solvents or toxic chemicals in the final products (Carrasco-Castilla et al., 2012).

The enzymatic process is based on the use of single or different combinations of proteinases like pepsin, trypsin, chymotrypsin, bromelain, papain, alcalase, neutrase, flavourzyme and many others. The critical hydrolysis parameters (temperature, pH, aqueous or buffered solution), must be optimized for each protein substrate and each selected enzyme or combination of enzymes, and must be maintained during proteolysis to ensure efficient release of peptides. The hydrolysis duration has a substantial inverse relationship with peptide size, but in most cases a plateau is reached during which further increases in hydrolysis time do not produce any effect on peptide size or activity (Aluko, 2015). Hydrolysis can be performed basically in three ways (but with many variations available in the literature): i) under traditional batch conditions, ii) using immobilized enzymes or iii) by ultrafiltration membranes. Batch enzymatic hydrolysis methods have certain disadvantages such as the relatively high cost of enzymes, the inherent inefficiency (resulting in low yields and productivity), and the generation of secondary metabolites due to enzymatic autolysis. These limitations can be overcome separating the enzyme and product during the reaction using a two-phase system, one phase containing the enzyme and the other containing the product. The enzyme is imprisoned within its phase allowing its re-use or continuous use, but preventing it from contaminating the product; other molecules, including the reactants, are able to move freely between the two phases. As compared to soluble enzymes, immobilized proteases allow the process to be performed in milder and more controlled conditions, prevent the generation of secondary metabolites originating from autolysis of enzymes and do not need to be inactivated by heat or acidification, which may be damaging for the product. Finally, immobilized enzymes can be easily recovered and

reused, decreasing the cost of the entire process (Pedroche et al., 2004). Enzymatic hydrolysis in continuous enzyme membrane reactor integrates enzymatic hydrolysis, product separation and catalyst recovery into a single operation. Basically, the reaction mixture made of substrate and enzyme are pumped continuously from a reaction vessel to a membrane filter where only small fractions can pass through out and are collected as permeate, while large particles such as large polypeptides, unhydrolysed substrate and enzyme are recycled back to the hydrolysis tank. This system allows faster reactions, higher yields, cleaner products and lower operating costs (due mainly to the membranes and their replacement at regular intervals), which are advantageous in industrial-scale production (Fan, Bai, Zhu, Yang & Zhang, 2014; Bhat, Kumar & Bhat, 2015).

A large number of hydrolysates and peptides were obtained by enzymatic hydrolysis of vegetable matrices, changing nutritional, bioactive and functional properties of proteins, which include improved digestibility, biological activities, sensory qualities (such as texture or taste), or reduction in allergenic compounds (Tavano, 2013). Table 2 summarizes the bioactive peptides (described in the next paragraphs), obtained by the enzymatic hydrolysis of vegetable food protein sources reported up to date in the literature. In many cases, hydrolysates contained a large number of bioactive peptides. Table 2 reports, in those cases, only the peptides with the highest activity.

3.1 Antihypertensive peptides

As mentioned before, peptides obtained by the proteolysis of food proteins can act as antihypertensive agents modulating the physiological mechanisms of blood pressure regulation (Aluko, 2015). Wheat is one of the vegetable sources of antihypertensive peptides. An ACE-inhibitory peptide, IAP, was isolated from wheat gliadin hydrolysate prepared with acid protease (Motoi & Kodama, 2003) while a wheat germ hydrolysate obtained with an alcaline protease produced the ACE-inhibitory tripeptide IVY (Matsui, Li & Osajima, 1999). Nogata, Nagamine, Yanaka, & Ohta, (2009) discovered six ACE-inhibitory peptides LQP, IQP, LRP, VY, IY, and TF that were produced by autolysis reactions from wheat milling by-products that probably involved aspartic proteases. Extensive hydrolysis of

sunflower protein by pepsin and pancreatin led to the release of the ACE-inhibitory peptide FVNPQAGS (Megías et al., 2004). Li, Qu, Wan, & You (2007) hydrolyzed rice proteins with alcalase obtaining a strong ACE-inhibitory peptide, TQVY, which was able to decrease the blood pressure in spontaneously hypertensive rats after single oral administration. Peptides RF and IHRF were released in the chymotrypsin digestate of rice glutelin (Kagebayashi et al., 2012; Kontani et al., 2014). In particular, IHRF, corresponding to rice glutelin (155-158) had vasorelaxing activity in the mesenteric artery of spontaneous hypertensive rats (SHRs). Orally administered, IHRF lowered systolic blood pressure in SHRs and its antihypertensive activity was more potent and long-lasting than that of RF (Kontani et al., 2014). Two peptides (VNP and VWP) were obtained by hydrolysis of rice proteins with alcalase and trypsin (Chen et al., 2013). These peptides were both competitive ACE-inhibitors, and stable against ACE and gastrointestinal proteases, pepsin and chymotrypsin (Chen et al., 2013). Furthermore, single oral administration of these tripeptides in SHRs significantly decreased the systolic blood pressure with antihypertensive effects lasting for about 8 hours (Chen et al., 2013). Many antihypertensive peptides have been obtained by enzymatic hydrolysis of proteins from rapeseed, a plant that has been known by human civilization for about 3000 years. Peptides IY, VW, RIY, VWIS (Marczak et al., 2003) and LY, TF, RALP (He et al., 2013a) were released treating rapeseed proteins with alcalase while peptide GHS was obtained with a combination of pepsin and pancreatin (He et al., 2013b). The hydrolysis with thermolysin of a maize endosperm protein called α-zein led to the release of antihypertensive tripeptides such as LAY, LQP, LRP, and LSP. The activity of LQP was verified on SHRs whose blood pressure decreased by 15 mmHg after a 30 mg/kg intravenous injection (Miyoshi et al., 1991). The three antihypertensive peptides (LQP, LRP, and LSP) were found in different maize varieties (Puchalska, Marina, & García, 2012). The content of LRP peptide was very low regardless of the maize variety. LQP and LSP peptides, presenting higher activity compared to the most known and studied VPP and IPP peptides, were detected in all maize varieties. Significant differences in the content of LQP and LSP were observed among different maize lines (Puchalska et al. 2012). A corn gluten hydrolysate prepared by Pescalase (a serine protease from

Bacillus licheniformis) was the source of the ACE-inhibitory peptide PSGQYY, composed of an hydrophobic amino acid at the amino terminal, a basic amino acid residue at the center, and a carboxyl terminal tyrosine. A dose of 30 mg per kg body weight antagonized the rat's pressor response to angiotensin I, lowering the blood pressure (Suh et al., 1999). Corn gluten meal was hydrolyzed by alcalase after starch removal and the ACE-inhibitory peptide AY was isolated. The activity of this peptide was not affected by preincubation with ACE and persisted after oral administration to SHRs. A maximal reduction of systolic blood pressure of 9.5 mmHg was observed 2 h after oral administration of AY at doses of 50 mg/kg (Yang, Tao, Liu & Liu, 2007). Enzymatic hydrolysates of soybean glycinin were recognized as sources of ACE-inhibitors. Among them, the high activity of the protease P glycinin hydrolysate resulted related to the peptide VLIVP which was a competitive inhibitor of ACE and resistant to digestion by proteases of the gastrointestinal tract (Mallikarjun et al., 2006). Its sequence corresponds to Val397-Pro401 of the glycinin subunit G2 (Swiss Prot: P04405) of soybean (Mallikarjun et al., 2006). Hydrolysis of soybean glycinin by acid proteinase from *Monascus purpureus* led to the release of the peptides SPYP and WL, while the hydrolysis of soybean β-conglycinin with the same enzyme produced two other antihypertensive peptides, LAIPVNKP and LPHF (Kuba, Tana, Tawata & Yasuda, 2005). The further hydrolysis of SPYP peptide *in vitro* by pepsin, chymotrypsin and trypsin enhanced its ACE-inhibitory activity (Kuba et al., 2005). Digestion of soybean with pepsin revealed activity against ACE due to the release of the peptides IA, YLAGNQ, FFL, IYLL, and VMDKPQG (Chen, Okada, Muramoto, Suetsuna & Yang, 2002). Their antihypertensive activity was confirmed on SHRs which were fed by saline solutions of peptidic fraction powders (0.9% w/v; 2.0 g per kg body weight). A significant reduction of blood pressure (17.5 mm Hg) was observed after 2 h, and continued for 6 h following oral administration (Chen et al., 2002). The ACE-inhibitory peptides DLP and DG were identified in soy protein alkaline hydrolysate, obtained with alcalase (Wu & Ding, 2002). The antihypertensive peptides YVVFK, PNNKPFQ, NWGPLV, and IPPGVPYWT were obtained from soybean protein isolates using a recombinant protease D3, a cathepsin L-like protease derived from germinating soybean cotyledons,

which was expressed and isolated from *Escherichia coli* (Kodera & Nio, 2006). Antihypertensive activity was investigated in SHRs and the dose-dependent antihypertensive effect was confirmed, with systolic blood pressure significantly reduced after the oral administration of doses exceeding 100 mg/kg. Yang, Marczak, Yokoo, Usui & Yoshikawa (2003) isolated four active peptides (MRWRD, MRW, LRIPVA, and IAYKPAG) in pepsin-pancreatin digests of the spinach Rubisco protein which inhibited ACE. The antihypertensive effect of these peptides was tested on SHRs and resulted to be dose-dependent (Yang et al., 2003). Sesame peptide powder obtained from sesame proteins hydrolyzed with thermolysin exhibited ACE-inhibitory activity, and significantly and temporarily decreased the systolic blood pressure (SBP) in SHRs by a single administration (1 and 10 mg/kg, Nakano et al., 2006). Six ACE-inhibitory peptides were isolated and identified from this substrate: LSA, LQP, LKY, IVY, YIY, LVY, and MLPAY (Nakano et al., 2006). A novel ACE-inhibitory peptide, ACKEP was identified from pistachio kernel proteins after digestion with gastrointestinal enzymes pepsin and trypsin (Li et al., 2014). ACKEP has the same C-terminal construction as that of lisinopril and enalapril, which plays a key role in binding with ACE. That mechanism was explored at a molecular basis by docking experiments revealing that seven amino acids in the ACE active site and two atoms of ACKEP greatly contribute to the combinative stabilization (Li et al., 2014). Five antihypertensive peptides (WYT, WVYY, SVYT, PLSPA, and IPAGV) were isolated from hemp seed proteins (Girgih et al., 2014). Lopez-Barrios, Gutierrez-Uribe, & Serna-Saldıvar (2014) reviewed pulses as a rich source of proteins in the human diet and associated their consumption with the prevention of chronic diseases. Chickpea (*Cicer arietinum*), field pea (*Pisum sativum*), mung bean (*Vigna radiata*), and kidney bean (*Phaseolus vulgaris*) protein hydrolysates among others, have yielded ACE-inhibitory activity. As confirmed by Li, Wan, Le, & Shi (2006) mung bean proteins are good precursors of ACE-inhibitors such as KDYRL, VTPALR, KLPAGTLF, and alcalase hydrolysates of this plant could be used to produce functional foods with antihypertensive activity. Red bean proteins digested with alcalase, papain, pepsin, trypsin, and α-chymotrypsin produced an active peptide with sequence PVNNPQIH (Rui, Boye, Simpson &

Prasher, 2013), while chickpea proteins treated with alcalase and flavourzyme produced peptides MD, MDFLI, MFDL, MDL, and MDLA, (Yust et al., 2003). Three dipeptides (IR, KF, and EF), obtained by alcalase digestion of pea protein isolate, showed strong inhibition of ACE and renin, with higher potency against ACE than against renin (Li & Aluko, 2010). A hydrophobic residue at the N terminus and a bulky amino acid residue at C terminus was a preferred structural arrangement for renin inhibition by the three synthesized peptides (Li & Aluko, 2010). Two active peptides from yellow field pea proteins, WMP and ADMFPF were obtained using thermolysin (Aluko, Wu & Aukema, 2014).

Antihypertensive peptides were also isolated from marine algae protein hydrolysates, such as *Chlorella vulgaris* and *Spirulina platensis*, the most popular edible microalgae in Japan since ancient times. Peptide sequences were IVVE, AFL, FAL, AEL, and VVPPA from *Chlorella vulgaris*; IAE, FAL, AEL, IAPG, and VAF from *Spirulina platensis* protein hydrolysates (Suetsuna & Chen, 2001). The purified undecapeptide VECYGPNRPQF from *Chlorella vulgaris* (Sheih, Fang & Wu, 2009) and VEGY from *Chlorella ellipsoidea* protein hydrolysates (Ko et al., 2012) possess strong activity. The oral administration of the latter purified peptide can significantly decrease systolic blood pressure in SHRs. Recently, Samarakoon et al. (2013) purified two novel ACE-inhibitory from a protein hydrolysate obtained from the cultured marine microalga *Nannochloropsis oculata*, GMNNLTP and LEQ. Up to now, few renin inhibitory peptides from marine algae have been reported. The first example is IRLIIVLMPILMA isolated from the macroalga *Palmaria palmata* protein hydrolysate, which inhibited renin activities by 58.97% at 1 mg/ml (Fitzgerald et al., 2012). Recently, Furuta and co-workers (2016) synthesized two peptides previously obtained by hydrolysis of the red algae *Palmaria palmata* proteins using a combination of enzymes: thermolysin, pepsin, trypsin, and chymotrypsin. LRY had remarkably high activity, *in vitro*, followed by VYRT. The activity of the former is equivalent to that of sesame peptide LVY which is used as antihypertensive agent for the beverage of a Food for Specified Health Uses (FOSHU) in Japan. Therefore, it is suggested that the dulse may have a potential as functional foodstuff (Furuta, Miyabe, Yasui, Kinoshita, & Kishimura,

2016). Four active peptides (AIYK, YKYY, KFYG, and YNKL) were isolated from the peptic digest prepared from wakame (*Undaria pinnatifida*) proteins (Suetsuna & Nakano, 2000). Each tetrapeptide was synthesized and its antihypertensive activity was confirmed after oral administration (50 mg/kg) in SHRs (Suetsuna, & Nakano, 2000). The antihypertensive effect of wakame was shown in hypertensive patients where a significant decrease of the systolic blood pressure by 14 ± 3 mmHg, after daily oral administration of 3.3 g of dried wakame after 4 weeks, was observed (Nakano, Hidaka, Uchida, Nakajima, & Hata, 1998).

Seven ACE-inhibitory peptides were isolated from the hydrolysates of wakame by Protease S "Amano", from *Bacillus stearothermophilus*. These peptides, having sequences VY, IY, AW, FY, VW, IW, and LW, have resistance against gastrointestinal proteases *in vitro* and was determined to have an antihypertensive effect after a single oral administration in SHRs, which was particularly relevant for peptides VY, IY, FY, and IW when administered in a dose of 1 mg/kg of body weight (Sato et al., 2002).

3.2 Antioxidant peptides

Two different peptides showing strong antioxidant activities were isolated from a rice endosperm protein hydrolysate, obtained with neutrase, FRDEHKK and KHDRGDEF (Zhang, Zhang, Wang, Guo, Wang, & Yao, 2010) while highly antioxidant alcalase hydrolysate sequences seemed characterized by containing peptides with Tyr or Trp at the C-terminal fragment (Dei Più et al., 2014). Rice bran protein fractions were hydrolyzed with proteases M, N, P, S, and pepsin producing hydrolysates with antioxidative activity (Adebiyi, Adebiyi, Yamashita, Ogawa, & Muramoto, 2009). Pepsin hydrolysates gave the highest activity despite the low hydrolytic activity. Among the peptides isolated, YVAQGEGVVA and YLAGMN had the highest activity (Adebiyi et al., 2009). Hemp seeds can be a source of antioxidant peptides if digested with alcalase, with the formation of the peptides NHAV and HVRETALV (Lu et al., 2010) or with pepsin and pancreatin, originating WVYY and PSLPA that showed DPPH scavenging and metal chelation activity (Girgih et al., 2014). A strong

antioxidant peptide (VECYGPNRPQF) was obtained using pepsin from the algae *Chlorella vulgaris* protein waste which is normally discarded as animal feed (Sheih, Wu & Fang, 2009). The peptide could efficiently quench a variety of free radicals and had significant protective effects on DNA. In addition, the peptide had gastrointestinal enzyme-resistance and no cytotoxicity was observed in human lung fibroblasts cell lines *in vitro*. Enzymatic hydrolysates from the benthic diatom *Navicula incerta* proteins exhibited free radical scavenging effects and two antioxidative peptides from the papain hydrolysate (PGWNQWFL and VEVLPPAEL), were isolated (Kang et al., 2012). Using different proteases (pepsin, papain, trypsin, and α-chymotrypsin), protein hydrolysates were produced from the marine *Chlorella ellipsoidea*, and the antioxidant peptide LNGDVW was identified, which showed a strong free radical scavenging activities (Ko, Kim, & Jeon, 2012).

3.3 Anticancer/Antiproliferative peptides

Peptides with anticancer activity have been isolated from enzymatic hydrolysates obtained with vegetable proteins, in particular from rice, soy and marine algae. A peptide with anticancer properties has been isolated from rice bran proteins digested with alcalase (Kannan, Hettiarachchy, Lay, & Liyanage, 2010). The peptide EQRPR had antiproliferative activities on different cancer cell lines: at 600-700 µg/mL dose caused 84% inhibition to colon cancer cells (Caco-2, HCT-116) growth, 80% to breast cancer cells (MCF-7, MDA-MB-231) growth and 84% to liver cancer cells (HepG-2) growth (Kannan, Hettiarachchy, Lay, & Liyanage, 2010). An anticancer peptide from soy, with sequence XMLPSYSPY was purified and isolated from defatted protein hydrolyzed with thermoase. Anticancer activity was assayed by measuring in vitro cytotoxicity (determined by the viability of the cells measured by ^{3}H-thymidine uptake after 72 h incubation) on P388D1, a mouse monocyte macrophage cell line (Kim et al., 2000). The peptide showed high cytotoxicity (IC 50 if 0.16 mg/ml) affecting cell cycle progression by arresting P388D1 at G2/M phase.

Marine algae-derived peptides have been shown to possess cytotoxic effects on human cancer cells (Fan, Bai, Zhu, Yang, & Zhang, 2014; Kang & Kim, 2013). For example, the undecapeptide

VECYGPNRPQF, isolated from *Chlorella vulgaris* protein waste, exhibited strong dose-dependent antiproliferation and induced a post-G1 cell cycle arrest in gastric cancer AGS cells and no cytotoxicity in normal lung fibroblast WI-38 cells *in vitro* (Sheih, Fang, Wu, & Lin, 2010). The polypeptide CPAP from *Chlorella pyrenoidosa* proteins hydrolysate showed inhibitory activity on human liver cancer HepG2 cells potentially due to CPAP-induced apoptosis and necrotic death. In addition, the micro- and nanoencapsulation of CPAP demonstrated the resistance to gastrointestinal enzymatic degradation (Wang & Zhang, 2013).

3.4 Hypocholesterolemic peptides

Hypocholesterolemic effects of soybean protein hydrolysates obtained with different enzymes (trypsin, pepsin, neutrase, alcalase, etc.) are well known and different peptides responsible of this activity were isolated and characterized. The soybean glycinin fraction hydrolysed with trypsin enabled the isolation of the hypocholesterolemic peptide LPYP (Kwon et al. 2002) while the hydrolysis with pepsin produced the peptides IAVPGEVA and IAVPTGVA able to bind bile acids shielding them from reabsorption and stimulating the transformation of cholesterol in blood plasma (Pak, Koo, Kasymova, & Kwon, 2005). All the peptides modulate the cholesterol metabolism in HepG2 cells through activation of the low-density lipoprotein receptor (LDLR) sterol regulatory-element-binding protein 2 (SREBP2) and inhibition of 3-hydroxy-3-methylglutaryl CoA reductase (HMGCoAR), a key enzyme in the synthesis of endogenous cholesterol and the main target of statins, which interact with this enzyme as competitive inhibitors (Lammi, Zanoni, & Arnoldi, 2015). Two peptides from soybean β-conglycinin subjected to gastro-intestinal digestion, YVVNPDNDEN and YVVNPDNNEN (Lovati et al., 2000), can be absorbed by human enterocytes and behave as competitive inhibitors of 3-hydroxy-3-methylglutaryl CoA reductase (HMGCoAR) activity, with a statin-like mechanism (Lammi, Zanoni, Arnoldi, & Vistoli 2015).

An alcalase soy hydrolysate generating the peptide YGAPSL showed a strong cholesterol reducing capacity and was stable to gastrointestinal protease digestion (Zhong, Zhang, Ma, & Shoemaker,

2007). A protease from *Bacillus amyloliquefaciens* was used to hydrolyze soybean proteins, producing the peptide FVVNATSN which strongly stimulated transcription of low density lipoprotein receptors (LDL-R), which play an important role in the reduction of plasma LDL cholesterol (Cho, Juillerat, & Lee, 2008).

Recently, it was observed that the *in vitro* hydrolysis of amaranth (*Amaranthus cruentus*) proteins with pepsin and trypsin produced the peptides GGV, IVG, and VGVL, that strongly inhibited the activity of 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMG-CoA reductase), a key enzyme in cholesterol biosynthesis, suggesting a potential hypocholesterolemic effect (Soares, Mendonça, de Castro, Menezes, & Arêas, 2015).

3.5 Antinflammatory peptides

Inflammation is the body's response to nonlethal injury, which is characterized by increased endothelial permeability, leakage of protein-rich exudates, and infiltration of leukocytes into extravascular tissues. While inflammation is essential for resistance to microbial infections and wound healing, excessive and uncontrolled inflammatory changes often lead to chronic diseases such as asthma, cancer, cardiovascular disease, diabetes, obesity, inflammatory bowel disease, osteoporosis and neurological diseases like Parkinson's (Chakrabarti, Jahandideh, & Wu, 2014; Majumder, Mine, & Wu, 2016). Overall, bioactive peptides have a potential to modify intestinal barrier function (Martínez-Augustin, Rivero-Gutiérrez, Mascaraque, & Sánchez de Medina, 2014) whose alteration are related to many inflammatory and non-inflammatory disorders. Soybean, whey and corn enzymatic hydrolysates have shown anti-inflammatory effects both in *in vitro* and *in vivo* studies (Vernaza, Dia, Gonzalez de Mejia, & Chang, 2012; Hwang, Yoo, Songa, Kimb, Chunc, et al., 2011; Shahi, Rashidi, Mahboob, Haidari, Rashidi, et., 2012, Nagarajan, Burris, Stewart, Wilkerson, & Badger, 2008; Martinez-Villaluenga, Dia, Berhow, Bringe, & Gonzalez de Mejia, 2009; Mochizuki, Shigemura, & Hasegawa, 2010; Dia, Bringe, & de Mejia, 2014) but only few peptides have been identified, to date, as responsible of this activity. Recent studies demonstrated that the

soybean tripeptide VPY (Nakamori, 2010) is a PepT1 substrate that can inhibit the production of pro-inflammatory mediators in vitro in intestinal epithelial and immune cells, and reduce the severity of colitis in mice by down-regulating the expression of pro-inflammatory cytokines in the colon, suggesting that it may be promising for the treatment of inflammatory bowel disease (Kovacs-Nolan et al., 2012). The pyro-glutamyl leucine, obtained from wheat gluten hydrolysed with *Aspergillus oryzae* protease (Sato et al., 2013) was shown to protect against dextran sulphate-induced colitis in mice (Wada et al., 2013) and chemically induced hepatitis in rats (Sato et al., 2013).

3.6 Immunomodulatory Peptides

The immune system is an interactive network of lymphoid organs, cells, humoral factors, and cytokines that represents the first line of defense in recognizing, repelling, and eradicating pathogens and other foreign molecules (Parkin & Cohen, 2001). Underactivity results in the severe infections and tumors of immunodeficiency; overactivity, in allergic and autoimmune disease. The immune system is a major target for development of treatment strategies by immunomodulation with cytokines or their antagonists, therapeutic vaccination with designer adjuvants to drive specified types of immune response, and regulation of cell function and survival by manipulation of coreceptor signalling molecules (Parkin & Cohen, 2001).

Some studies have evaluated the capacity of peptides to stimulate the immune system after their administration (Gobbetti, Stepaniak, De Angelis, Corsetti, & Di Cagno, 2002; Jang, Jo, Kang, & Lee, 2008; Kong, Guo, Hua, Cao, & Zhang, 2008). As recently reported (Santiago-López, Hernández-Mendoza, Vallejo-Cordoba, Mata-Haro, & González-Córdova, 2016), peptides may enhance or weaken immunity by up-regulating pro- and anti- inflammatory cytokines. These stimulate the synthesis of some antibodies, also may either contribute to the proliferation or elimination of T and B lymphocytes, in addition to influencing NK cells and the phagocytic activity of macrophages (Santiago-López et al. 2016). The immunomodulatory action of peptides is one of the least explored

among the many beneficial bioactivities exerted by these molecules and only two peptides from vegetable sources have been recognized to exert this activity (Table 2).

In particular, soymetide-13 (MITLAIPVNKPGR) is an immunostimulating tridecapeptide, released by trypsin digestion of soybean β-conglycinin, one of the major components of soybean proteins. This tridecapeptide exhibits affinity for the receptor of the chemotactic peptide fMLP (formyl-Met-Leu-Phe) contributing to a rapid response to bacterial infection, leading to bacterial death by phagocytosis and ROS-induced bactericidal effects (Tsuruki et al., 2003).

The tryptic digest of rice soluble protein originated a peptide named oryzatensin GYPMYPLPR with the ability to stimulate the immune system. In particular, oryzatensin showed phagocytosis-promoting activity for human polymorphonuclear leukocytes and augmented the production of superoxide anion by human peripheral leukocytes (Takahashi et al., 1996).

3.7 *Antifungal peptides*

Leguminous species naturally possess antifungal proteins, including a large number of thaumatin-like proteins, chitinases, glucanases, embryo-abundant proteins, miraculin-like proteins, cyclophilin-like proteins, allergen-like proteins, thionins, non-specific lipid transfer proteins, defensins, and vicilins. Moreover, antifungal peptides were also isolated in different leguminous seeds: a Bowman–Birk type trypsin–chymotrypsin inhibitor from faba bean; cicerin and arietin from chickpea; angularin from red beans; and other peptides were found in Yunnan bean *Gymnocladus chinensis* and *Arachis hypogea* (Ng, 2004). These proteins and peptides are components of the defense system of the plant. Recently, an hydrolysate of pea proteins, obtained by the use of a commercial protease (from *A. oryzae*), was characterized for its antifungal activity (Rizzello, Lavecchia, Gramaglia, & Gobbetti, 2015b). In particular, beyond the activity of native pea defensins 1 and 2, and a non-specific lipid transfer protein [nsLTP], different active peptides which were released during enzymatic hydrolysis, were purified from the hydrolysate and identified. The antifungal peptides corresponded to sequences encrypted in leginsulin A, vicilin, provicilin, and nsLTP. The pea flour hydrolysate was successfully used as

ingredient for bread making, to prolong the shelf-life by the inhibition of the spoilage molds (Rizzello et al., 2015b).

4. Peptidomic-based approach for the analysis of hydrolysed vegetable proteins

Peptidomics concerns the study of the entire panel of peptides related to a specific protein matrix or organism. The efficient screening of the peptidomic profile of hydrolysed proteins has been made possible by the recent advances in mass spectrometry (MS) and bioinformatics tools. In the typical procedure, the finding of novel peptides was achieved through extensive purification steps followed by Edman-degradation-based sequencing (Kuba, Tanaka, Sesoko, Inoue, & Yasuda, 2009; Marczak et al., 2003). The main bottlenecks in this process is represented by the purification process of the peptides, which is very time-consuming, and by the low sensitivity of the Edman degradation.

Recently, the mass spectrometry (MS)-based peptidomic approaches set a new standard in peptide research. The use of liquid chromatography (LC) coupled to sequencing techniques based on MS, allowed to quit the laborious Edman-degradation based approach. Highly sensitive and accurate mass spectrometers let single MS and tandem MS analysis, enabling simultaneous mass and sequence determination of peptides. The high acuteness as well as the need of much less sample for the analysis are the main advantages in the peptidomic-based approach. Besides, the possibility to select for fragmentation peptide ions from a complex mixture has dramatically reduced the sample preparation efforts. In this regard, the peptidomic-based approach can lead to the identification of the whole peptide pool present in the hydrolysed samples.

The typical experimental design in the peptidomic-based identification of bioactive peptides involves different steps as shown in Figure 1. Yang, Tao, Liu & Liu (2007) perform a representative example of this workflow studying the ACE-inhibitory peptides derived from corn gluten meal (a by-product of corn wet milling) hydrolysate. The hydrolysate was firstly subjected to ultrafiltration (UF) and later, the ACE-inhibitory peptides fractionated by size exclusion chromatography (SEC) followed by

two consecutive reverse phase-high performance liquid chromatography (RP-HPLC). Sequences of peptides in the fractions showing the highest ACE-inhibitory activity were identified by HPLC coupled online to electrospray ionization (ESI)-MS. Finally, the ACE-inhibitory activity was verified for selected synthesized peptides, besides the verification of their *in vivo* antihypertensive activity in rats.

RP-HPLC is the most applied method to separate peptides from vegetable matrices, due to its capability to successfully separate small peptides (Puchalska et al., 2012; Tagliazucchi et al., 2015). However, in some works, other HPLC techniques, such as SEC or ion exchange chromatography, have been employed (Coda et al., 2012; Dei Più et al., 2014). Many authors combined RP-HPLC and SEC for the prior separation before identification by MS. Nogata et al. (2009) identified ACE-inhibitory peptides from wheat milling by-products, combining SEC and two consecutive RP- HPLC with C4 and C18 columns. Anyway, when different separation techniques are used, the last dimension is usually C18 RP-HPLC (Sandra et al., 2009).

Mass spectrometry is the pivotal analytical technique on which the peptidomic-based approaches are established. In recent years, new MS technologies and techniques have been developed. At the same time, the updating and development of bioinformatics tools allow a better and more accurate identification of the peptides released after hydrolysis of food proteins. Electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI) are the most important ionization sources used in peptidomic. It is possible to operate combining them with different mass analyzers, expanding the possible configurations of mass spectrometers. Typically, ESI source is coupled with a triple quadrupole (Q) mass analyzer whereas MALDI source is paired with time-of-flight (TOF) analyzer. The introduction of hybrid mass spectrometers, such as Q-TOF or TOF-TOF instruments, allowed enhancing mass accuracy (up to low ppm) and sensitivity (low attomole level).

Connection of liquid chromatography (LC) systems and MS analyzer benefit greatly analysis of complex mixtures. The peptides are separated and gradually eluted from the column before the ionization run by ESI source, which represent the interface of choice for coupling LC to the MS.

Indeed, ESI source can operate under variable solvent conditions and continuous sample infusion (Emmett & Caprioli, 1994). Several examples regarding the use of LC-ESI-MS to identify bioactive peptides from vegetables can be found in literature. Sato et al. (2002) and Nakahara et al. (2010) identified, respectively, seven and nine new ACE-inhibitory and anti-hypertensive dipeptides from a wakame hydrolysates and a soy sauce-like seasoning. Hybrids LC-ESI-Q-TOF and LC-ESI-Q-TRAP were utilized by Puchalska et al. (2012) to identify anti-hypertensive peptides after thermolysin digestion of maize and by Manolo Soares, Mendonça, Andrade de Castro, Carlos Menezes et al. (2015) to identify the major 3-hydroxy-3-methyl-glutaryl-CoA reductase inhibitory peptides generated by the *in vitro* hydrolysis of *Amaranthus cruentus* protein, respectively.

Nowadays, ESI technology is in continuous expansion thanks to implementation of miniaturized instrumentation. Indeed, nano-ESI allows working with small samples and buffer volumes. Nano flow LC-ESI has been coupled with single (Coda et al., 2011; Esteve, Marina, & García, 2015), or hybrid (Q-TOF) mass analyzers (Dei Più et al., 2014; Chang, Ismail, Yanagita, Esa, & Baharuldin, 2015).

MALDI ionization is an alternative source used in peptidomic and proteomic investigations. The use of MALDI-TOF spectrometer has some advantages and disadvantage compared to ESI. Lower susceptibility to impurities and salts and higher sensitivity are the most important advantages. LC is not combinable with MALDI spectrometer, therefore it is suggested using of ESI ionization source for peptide analysis and sequencing (Clynen, De Loof, & Schoofs, 2003). MALDI-TOF can generate exact peptide mass values, nevertheless peptide fragment not occurs. This disadvantage precludes its use for *de novo* sequencing of peptides. In peptide identification and sequencing, MALDI-TOF is used in combination with Edman degradation like in the identification of ACE-inhibitory peptides in hydrolyzed wheat milling byproducts (Nogata et al., 2009).

The recently developed MALDI-TOF-TOF spectrometer allows obtaining complex mass spectra of peptide fragment ions from a specific peptide precursor ion to develop complete *de novo* peptides sequence. Zhang et al. (2009) used MALDI-TOF-TOF MS/MS analysis to reveal the sequence of an

antioxidant peptide released from glutelin protein after hydrolysis of rice endosperm proteins. Data processing requires a tandem mass spectra database to identify bioactive peptides. Since cleavage sites are often unknown, database searching is not always simple, leading to an increase in database searching time, the number of false positive and the score needed to certainly identify the peptides.

5. Recovery stages and conventional technologies

Although some processes have been applied for peptide separation at industrial scale, a large number of laboratory scale procedures are too complex and expensive to be industrialized (Agyei, Potumarthi, & Danquah, 2015). Industrial-scale limitations include problems in making a reproducible product; lack of clinical trials to confirm bioactivity, efficacy, and safety; and food sensory changes such as bitterness and undesirable color). (Carrasco-Castilla et al., 2012, Chaves-López, Tofalo, Serio, Paparella, & Suzzi, 2012; Chaves-López et al., 2014) The extraction, fractionation and isolation of high added-value compounds from food matrices usually follow the rules of analytical chemistry (Galanakis, 2012). Afterwards, modifications are therefore introduced into the applied methodology with an ultimate objective of:

(a) maximizing the yield of the target compounds,

(b) accommodating the needs of industrial processing,

(c) purified the high added-value ingredients and remove toxic compounds,

(d) preventing deterioration and loss of functionality during processing,

(e) assuring the quality the final product.

One of the big challenge frequently faced in food protein-derived peptide research is to maximize the yield of the peptide products with high bioactivity. This constraint leads to carrying out supplementary processing on the enzymatic protein hydrolysates, often based on physicochemical and structural properties of the bioactive peptides. Based on the target of the industrial application, the peptide properties that can be considered in the purification processes are molecular mass, net charge, and hydrophobicity. Aiming at concentrating peptides having specific molecular mass, on a

large production scale, pressure-driven membrane technologies, electrically-driven membrane technologies, and chromatography techniques can be used (Langevin, 2012).

The membrane technologies processes has some advantages, including operation at low temperature, absence of phase transition and low energy expenditure (Conde et al., 2013; Murakami et al., 2011). Membranes are semipermeable barriers that split up two distinct phases and limit the movement of definite components in a selective way (Bazinet & Firdaous, 2013). Membranes allow the diffusion of some elements while retaining others, enabling the enrichment of the permeate and the retentate in specific components (de Morais Coutinho et al., 2009). The diffusion of the different compounds through the membrane can be assisted by applying a driving force (concentration gradient, pressure, temperature or electric potential). Membranes technologies include microfiltration (MF), ultrafiltration (UF), nanofiltration (NF) and reverse osmosis (RO) which use hydraulic pressure as a driving force. Nevertheless, the major part of the industrial applications for the isolation of specific bioactive peptides or proteins is based on UF and NF (Herrero et al., 2012; Szydłowska-Czerniak, Trokowski, & Szłyk, 2011). UF operates at 2-10 bar, even if in some cases up to 30 bar have been employed (Nawaz, Shi, Mittal, & Kakuda, 2006) and it is used to retain macromolecules and colloids with molar masses between 1 and 300 kDa, while allowing water and small molecules to permeate. NF is commonly carried out at 10-40 bar and it can separate particles with molar masses between 0.35 and 1 kDa. RO, performed at 40-100 bar, can hold on essentially all solutes in concentrate, as well as small ions such as sodium (de Morais Coutinho et al., 2009; Murakami et al., 2011). The hydraulic pressure used in these technologies depends on the pore size of the filtration membrane and can be set up depending on the concentration rate needed. The performance and efficiency of the separation are influenced by the solution properties, charges from which originate the repulsions and attractions of ions and the type of membrane used (Langevin et al., 2012; Martin-Orue, Bouhallab, & Garem, 1998). To bypass the formation of a polarization layer that usually affect the membrane selectivity, it could be suitable to work at low concentration which could affect the behavior and the efficiency of the separation method. Even though membrane separations are efficient and flexible,

they own high sensitivity in feed content, i.e. potential variations of the latest generate fouling problems that restrict membrane applications.

Another technology recently employed for the recovery of bioactive peptides includes the combined use of ion-exchange membranes and filtration membranes, for example applying electrodialysis with ultrafiltration membrane (EDUF), in which the electric field is the main driving force of process. The movement of the molecule is due to their charges and the flux is affected by the strength of the electric field (Doyen, Beaulieu, Saucier, Pouliot, & Bazinet, 2011; Doyen, Saucier, Beaulieu, Pouliot, & Bazinet, 2012; Poulin, Amiot, & Bazinet, 2007). The application of EDUF for the separation of bioactive peptides already gave good results (Doyen et al., 2011; Doyen et al., 2012). This modern technology allows the separation of molecules according to their charges and molecular masses (Langevin et al., 2012).

Nowadays, the recovery of peptides has been applied to some protein hydrolysates showing antioxidant activity against the peroxidation of lipids and/or fatty acids (Wang, Zhao, Zhao, Bao, & Jiang, 2007). Different processes for proteins and peptides recovery from plant matrices have been set-up. The use of membrane technologies has been successfully applied for producing protein isolates from defatted soy flour hydrolysates. Rapeseed protein isolates, relevant in the food industry because of their functional properties and health benefits, were obtained by precipitation at controlled pH and by ultrafiltration. The ultrafiltered protein isolate showed good functional properties, while the precipitated protein isolate demonstrated to have ACE-inhibitory capacity, bile acid-binding capacity and DPPH radical-scavenging capacity (Yoshie-Stark, Wada, & Wäsche, 2008). Protein from alfalfa leaf were hydrolyzed with proteases, and the resulting products were separated by UF and purified by adsorption, showing high nutritive importance, chelating ability, reducing power, and radical scavenging activity (Yoshie-Stark et al., 2008). Protein hydrolysates from potato tubers and by-products from the potato industry were fractionated by ultrafiltration with 3-10 kDa membranes to separate the ACE-inhibitory compounds in permeate (Pihlanto, Akkanen, & Korhonen, 2008). Protein hydrolysates of wheat gluten showing strong antioxidant properties were purified by

molecular weight using a 5 kDa membrane. It was found that the antioxidant activity of the filtrate was similar to the vitamin E at pH 7.0 (Yoshie-Stark et al., 2008). UF was used to recover antioxidant peptide fractions from enzymatic hydrolysates of wheat gluten (Kong, Zhou, & Hua, 2008). Hydrolysates from barley glutelin were separated by UF and reverse-phase chromatography to obtain large-size peptides (MW > 10 kDa) with enhanced DPPH scavenging activity and reducing power, and small-size peptides (MW < 1 kDa) valuable for chelating Fe^{2+} and for scavenging OH• (Xia, Bamdad, Gänzle, & Chen, 2012).

As mentioned before, bioactive peptides can also be fractioned using chromatography techniques. HPLC is commonly used for separation, identification, and purification of bioactive peptides at laboratory and pilot-plant scales. Size-exclusion chromatography can be used to purify peptides based on molecular mass. RP-HPLC can be employed to separate peptides according to their hydrophobic properties and, in particular, it was largely applied in the study of the structural and functional properties of bioactive sequences (Pownall, Udenigwe, & Aluko, 2010). Gibbs et al. (2004) purified and identified bioactive peptides from fermented soy by LC-MS. A soy milk hydrolysate was purified to recover ACE-inhibitory peptides using size exclusion chromatography, that allowed to recover one fraction with a very high ACE inhibitory effect (Chiang, Tsou, Tsai, & Tsai, 2006). An adipogenesis inhibitory tripeptide IQN, having an IC_{50} value of 0.014 mg protein/mL, was purified and identified from black soybean hydrolysate using chromatographic and protein sequencer, (Kim, Bae, Ahn, Lee, & Lee, 2007). The ACE-inhibitory tripeptide GPP, having IC_{50} value of 6.25 mg protein/mL, was purified and isolated from a buckwheat protein hydrolysate by protein sequencing system and electrospray-LC-mass spectrometry (Mallikarjun Gouda, Gowda, Rao, & Prakash, 2006). Seber et al. (2012) set-up a scalable method using a sequential application of anion-exchange chromatography combined with UF, and RP chromatography to produces lunasin preparations of 99% purity with a yield of 442 mg/kg defatted soy flour (Singh et al., 2014). A peptide preparation having active metal chelators was recovered from bean proteins and phaseolin treated with pepsin and pancreatin applying

a filtration through a 1 kDa MWCO membrane coupled with size exclusion chromatography (Carrasco-Castilla et al., 2012; Langevin et al., 2012).

Although many processes will be optimized before the large application in food and pharmaceutic industry, only two patented technologies are available in literature (https://patents.google.com/): the patent N°CN103052717, describing a method for preparing antihypertensive peptides from corn germ proteins using membrane technologies, and the patent N°CN102876765, dealing with a method for obtaining antioxidant peptides from waste tea leaves proteins hydrolysate using UF.

6. Future trends

Only few commercial products including bioactive peptides from vegetable sources are currently available on the market (Carrasco-Castilla et al., 2012). The industrial-scale production of bioactive peptides is similar to the laboratory-scale, nevertheless, hydrolysis, separation and purification steps are still limited by a lack of suitable technologies although few advances have been made in the last decade. Downstream processing involves several energy-expensive procedures including drying, cellular disruption and extraction of bioactive molecules, and production/cost relation studies of the different technologies are needed (Hayes & Tiwari, 2015). It was recently reported that novel processing technology utilization is limited by a number of factors including: (i) affordable technologies for the optimized processing of protein/peptide-rich biomass; (ii) lack of sustainability and high costs associated with pre-treatment and processing and extraction of proteins; (iii) costs associated with the isolation and downstream processing of valuable protein/peptide isolates, hydrolysates and co-products; (iv) health and safety legislation (Hayes & Tiwari, 2015).

Nevertheless, bioactive peptides are suitable candidates for a new era of pharmaceutical products, in particular with the sensitive concerns of side effects of small molecule drugs and the increased care to fresher and ‘greener’ foods and nutraceuticals possessing health-preventing or health-promoting properties (Danquah & Agyei, 2012; Lemes et al., 2016; McClean, Beggs, & Welch, 2014).

Until the last decade, the production of bioactive peptides was focused almost exclusively on animal protein sources, and in particular from milk. The latest researches, however, opened up new possibilities for obtaining peptides from plant sources and also from alternative and cheap matrices, such as agricultural surplus and waste, by-products and non-conventional vegetables. Indeed, the production of bioactive peptides from vegetable sources can be cheaper than that from animal origin: in many case the substrates for production are food by-products, and their production has a lower environmental impact. The variety of plants that can be used is larger than the animal protein sources. Nevertheless, vegetable-derived peptides present some limits, mainly related to the more difficult hydrolysis and the necessity for a thermal treatment (legumes).

Numerous progresses have been obtained in biotechnology, thanks to the specific applications of spontaneous fermentations, selected microbial starters and enzymes. The development of bioreactors and immobilized cells systems at experimental or industrial levels, are expected. Moreover, protein engineering strategies and techniques will continue to expand the commercial protease markets. The modern analytical techniques and the possibility of using *ex vivo* screening assays, allow to quickly collect data on the structure and functionality of the active sequences and to target more effectively the *in vivo* trials.

In particular, peptidomic is currently the uncontested tool to detect and quantify peptides, allowing more specific and sensitive identification. However, there are still some limitations in the use of MS techniques such as instrumental cost, technology, and software appropriateness. Indeed, development of targeted bioinformatic tools is highly required. In this regard, it was recently demonstrated, using a predictive informatic tool based on the BIOPEP database, that wheat, barley, oat, and rice storage proteins showed high occurrence frequencies of antihypertensive, antithrombotic, antioxidant, and opioid peptide sequences (Cavazos & Gonzalos de Mejia, 2013). The growing demand of functional formulations involve a more thoroughly knowledge of bioactive peptides so the employment of MS-based approaches is expected to increase during the next years.

Despite the need to implement the screening on plant matrices and to set-up of efficient bio-technological processes for large-scale production and recovery, the great variability of plant proteins already allowed to discover new potential compared to animal proteins, such as the isolation of antioxidant and anti-tumoral peptides. Nevertheless, a comparison of the bioactivities of peptides derived from vegetable to that of animal (especially dairy) proteins has not been adequately investigated, and further research is warranted (Roy, Boye, & Simpson, 2010). Some *in silico* studies revealed the existence of complementarity between vegetables and animals proteins as precursors of bioactive peptides (Cavazos & Gonzalos de Mejia, 2013). For example, pea and whey proteins were evaluated as precursors of ACE inhibitory peptides through the use of a sequences database and *in silico* gastro-intestinal digestion (Vermeirssen, van der Bent, Van Camp, van Amerongen, & Verstraete, 2004). Despite the higher potential of whey proteins compared to pea proteins, *in silico* digestion revealed that gastro-intestinal digestion of both the matrices may result in the release of complementary and identical sequences. Through *in silico* studies, Iwaniak & Dziuba (2009) confirmed that bovine caseins are the elective sources of ACE inhibitors; nevertheless, among the other matrices analyzed, soy globulins showed higher potential compared to β-lactoglobulins, and wheat gliadins higher than chicken meat proteins.

Few works were addressed to the quantification of the potential release of specific bioactive peptides, and most of them are focused on milk-derived peptides or based on the total peptide content: for these reasons, at the moment, the quantitative comparison between the potential production of bioactive peptides in vegetable sources is hard to fulfil. Also the necessity of a more accurate comparison between the *in vivo* effects related to bioactive peptides from vegetables and pharmaceutical preparations has been reported (Roy et al., 2010). Overall, drugs may have a higher potency and more readily metabolized, since pharmaceutical compounds are typically encapsulated to protect the active ingredient from adverse conditions such as microbial activity, protease enzymes and low stomach pH. Although specific *in vivo* test should be carried out in order to evaluate the effect on human health, novel applications as functional food dietary supplements or pharmaceutical preparations

could be expected. The recovery of bioactive peptides represents one of the most crucial challenges for the future. In fact, beside their widely application, the aforementioned technologies have some disadvantages. For example, membrane processes like nanofiltration need high energy consumption, whereas others, such as chromatography, are operationally expensive. Nowadays, to overcome the disadvantages of conventional technologies new emerging technologies are explored at laboratory scale and some of them are applied in the food industry. Given that the production of nutraceuticals from by-products is still a concern in food science due to elevated initial investment and safety considerations, the most popular emerging technologies applied in food science are: radio-frequency drying, electro-osmotic dewatering, low temperature plasma treatment, high-hydrostatic pressure, ultrasound-assisted extraction, laser ablation, high voltage electrical discharge, pulsed electric field, pulsed fluid bed agglomeration, nanotechnology. Further studies on the safety considerations and sensory impact as well as consumer acceptance should be done in order to take emerging technologies closer to a commercial breakthrough in the field.

Legends to figures

Figure 1. Analytical workflow for the identification of bioactive peptides from vegetable foods and by-products. The release of bioactive sequences is the first step. Released peptides are checked for their biological activity. The large number of peptides contained in samples usually leads their prior separation before identification by mass spectrometry (MS). Ultrafiltration with different molecular cut-off membranes is often used for fractionation step. High performance liquid chromatography (HPLC), in various modes, is the preferred technique to the separation of peptides because its versatility, efficiency, and automation capabilities. Recently, different membrane technologies have been proposed for separation and purification of bioactive peptides. The different fractions, obtained after separation, are assayed for the biological activity and the most active injected in the MS. At the end, the biological activity of identified peptides is verified *in vitro* and/or *in vivo* after chemical synthesis.

Table 1. List of bioactive peptides identified in vegetable matrices fermented by lactic acid bacteria.

Bioactivity	Peptide sequences [a]	Matrix	Lactic acid bacteria strains	Reference
antifungal	DPVAPLQRSGPEIP - PRSGNVGESGLID - ESVSLVA - PHAVAAVPPVLR – LLGWGHKGSSIID - HCNDPEKKNL - PILQSLIRFDGGACSSF - RSQIKREQYTPQDVEMLFSSF	soft wheat	*L. brevis* AM7	Coda *et al*., 2008
anti-ACE	DPVAPLQRSGPEI – PVAPQLSRGLL - ELEIVMASPP - QILLPRPGQAA – PVAPLQRSGPE - PRSGNVGESGL – VAPSRPTPR - DIIIPD – PRSGNVGESGLID – DPVAPLQRSGPEI – DPVAPLQRSGPEIP - PVAPLPRKGS – DPVAPLQRSGPE -SFTAGARTFNFDENPCDYFQGGKIKAT	wholemeal wheat	*L. alimentarius* 15M; *L. brevis* 14G; *L. hilgardii* 51B *L. sanfranciscensis* 7A, LS3, LS10, LS19, LS23, LS38 and LS47	Rizzello *et al*., 2008
antifungal	VLHEPLF - YNNPIIYVTENGIAEGNNKSLPITEAL - ALKAAPSPA - AILIIVMLFGR - AAAAVFLSLLAVGHCAAADFNATDADADFAGNGVDFNSSDAAVYWGPWTKAR	wheat germ	*L. plantarum* LB1; *L. rossiae* LB5	Rizzello *et al*., 2011
antifungal	PPDVLTKLTAVPAAQQLDEADGHPR - SAFEFADEHKGAYS - AAIIFGSIFWNVGMKR - ALGFEMTPEQIHQMI - IGRDVQNQSLFSPQVDSSSLLYNMVPNLTSNVSDGNLSTIPSGSTYLQNAMYG - SAFEFADEHKGAYS - AEGEVILEDVQPSSVQS - EGTYLDYIQNNGKTLGAEDSTNEFGWDNK - DEEIMLDITTCAMAFRLLR - WFVELTGIPVTTTLMGLGNFPSDDPLSLRMLGMHGTVYANYAVDK	soft wheat	*L. plantarum* 1A7	Coda *et al*., 2011
antioxidant	MAPAAVAAAEAGSK - DNIPIVIR	whole wheat	*L. alimentarius* 15M; *L. brevis* 14G; *L. hilgardii* 51B *L. sanfranciscensis* 7A, LS3, LS10, LS19, LS23, LS38 and LS47	Coda *et al*., 2012
antioxidant	AIAGAGVLSGYDQLQILFFGK - GNQEKVLELVQR - PAGSAAGAAP - EALEAMFL - AAGAAAAARSAGQCGR - ITFAAYRR - HPVPPKKK	spelt		
antioxidant	VFVDEGLEVLGWRPVPFNVSVVGRNAK - RLSLPAGAPVTVAVSP - NANGELCPNNMCCSQWGYCGLGSEFCGNGCQSGACCPEK - LCPVHRAADL - PAEMVAAALDR - KVALMSAGSMH - DLADIPQQQRLMAGLALVVATVIFLK - KNGSIFNSPSATAATIIHGHNYSGLAYLDFVTSK - GTIFFSQEGDGPTSVTGSVSGLKPGLHGFHVHALGDTTNGCMSTGPHFNPTGK	rye		
antioxidant	YEWEPTVPNFDVAKDVTDM - GVSNAAVVAGGH - DAQEFKR - PPGPGPGPPPPPGAAGRGGGG - HKEMQAIFDVYIMFIN - TGGGSTSSSSSSSSSLGGGASRGSVVEAAPPATQGAAAANAPAVPVVVVDTQEAGIR - DTAAGYVAPPDPAVSTGDYGLAGAEAPHPHESAVMSGAAAAAVAPGGEAYTR	kamut		
anti-ACE	KEDDEEEEQGEEE	pea	*L. plantarum* 299v	Jakubczyk *et al.* 2013
antitumoral	SKWQHQQDSCRKQKQGVNLTPCEKHIMEKIQGRGDDDDDDDDD (and relative fragments f4-43; f12-43; f20-43	soybean	*L. curvatus SAL33, L. rossiae CD76, L. brevis AM7, L. pentosus 12H6 , L. plantarum 3DM*	Rizzello *et al*., 2012

[a] The single-letter amino acid code is used.

862 **Table 2.** Bioactive peptides obtained by enzymatic hydrolysis of vegetable food matrices.

Bioactivity	**Peptide sequence**[a] (IC50 μM)[b]	**Matrix**	**Enzymes**	**Reference**
Antihypertensive	IAP (2,7)[c]	wheat gliadin	acid protease	Motoi & Kodama, 2003
	IVY (0,48)[c]	wheat germ	alcaline protease	Matsui, Li & Osajima, 1999
	FVNPQAGS (6,9)[c]	sunflower	pepsin, pancreatin	Megías et al. 2004
	TQVY (18,2)[c]	rice	alcalase	Li, Qu, Wan, & You, 2007
	RF	rice	chymotrypsin	Kagebayashi et al., 2012
	IHRF	rice	chymotrypsin	Kontani et al., 2014
	VNP (6.4)[c] – VWP (4.5)[c]	rice	alcalase, trypsin	Chen et al., 2013
	IY (3,7)[c] – RIY, *rapakinin* (28)[c] - VW (1,6)[c] – VWIS (30)[c]	rapeseed	alcalase	Marczak et al., 2003
	LY (110)[c] (1870)[d] – RALP (650)[c] (970)[d] - TF (810)[c] (3100)[d]	rapeseed	alcalase	He et al., 2013 (a)
	GHS (1740)[c] (1090)[d]	rapeseed	pepsin, pancreatin	He et al., 2013 (b)
	LAY (3,9)[c] - LQP (1,9)[c] – LRP (0,27)[c] – LSP (1,7)[c]	maize	thermolysin	Miyoshi et al., 1991
	PSGQYY (100)[c]	maize	pescalase	Suh et al., 1999
	AY (14.2)[c]	maize	alcalase	Yang, Tao, Liu & Liu, 2007
	VLIVP (1,69)[c]	soybean	protease P	Mallikarjun Gouda et al., 2006
	LAIPVNKP (70)[c] - LPHF (670)[c] – SPYP (850)[c] – WL (65)[c]	soybean	acid proteinase from *Monascus purpureus*	Kuba, Tana, Tawata & Yasuda, 2005

	FFL (37)[c] – IA (153)[c] – IYLL (42)[c] – YLAGNQ (14)[c] – VMDKPQG (39)[c]	soybean	pepsin	Chen, Okada, Muramoto, Suetsuna & Yang, 2002
	DLP (4,8)[c] - DG (12,3)[c]	soybean	alcalase	Wu & Ding, 2002
	NWGPLV (21)[c] - YVVFK (44)[c] – PNNKPFQ (33)[c] – IPPGVPYWT (64)[c]	soybean	recombinant protease D3	Kodera & Nio, 2006
	MRW (0,6)[c] – MRWRD (2,1)[c] - LRIPVA (0,38)[c] – IAYKPAG (4,2)[c]	spinach Rubisco	pepsin, pancreatin	Yang, Marczak, Yokoo, Usui & Yoshikawa, 2003
	LSA (7,81)[c] – LQP (1,04)[c] – LKY (0,78)[c] – IVY (14,74)[c] – VIY (4,50)[c] - LVY (1,80)[c] - MLPAY (1,58)[c]	sesame	thermolysin	Nakano et al., 2006
	ACKEP (126)[c]	pistachio	pepsin, trypsin	Li et al., 2014
	WYT - WVYY - SVYT - PLSPA - IPAGV	hemp seed	pepsin, pancreatin	Girgih et al., 2014
	KDYRL (26,5)[c] - VTPALR (82,4)[c] – KLPAGTLF (13,4)[c]	mung-bean	alcalase	Li, Wan, Le, & Shi, 2006
	PVNNPQIH (206,7)[c]	red bean	alcalse, papain, pepsin, trypsin, α chimtrypsin	Rui, Boye, Simpson & Prasher, 2013
	MD (0.021)[e] - MDFLI (0.011)[e] - MFDL (0.013)[e] – MDL (0.021)[e] – MDLA (0.013)[e]	chickpea	alcalase, flavourzyme	Yust et al, 2003
	IR (2250)[c] (9200)[d] – KF (7230)[c] (17840)[d] - EF (2980)[c] (22660)[d]	chickpea	alcalase	Li & Aluko, 2010
	ADMFPF - WMP	yellow field pea	thermolysin	Aluko, Wu & Aukema, 2014
	LTFPG – IFENLQN - FEGTVFENG	yellow field pea	thermolysin	Aluko et al., 2015

	IVVE (315,3)[c] – AFL (63,8)[c] – FAL (26,3)[c] - VVPPA (79,5)[c] - AEL (57,1)[c]	*Chlorella vulgaris*	pepsin	Suetsuna, K., & Chen, J. R., 2001
	IAE (34,7)[c] - FAL (26,3)[c] - AEL (57,1)[c] – IAPG (11,4)[c] – VAF (35,8)[c]	*Spirulina platensis*	pepsin	Suetsuna, K., & Chen, J. R., 2001
	VECYGPNRPQF (29,6)[c]	*Chlorella vulgaris*	pepsin	Sheih, I. C., Fang, T. J., & Wu, T. K., 2009
	VEGY (128,4)[c]	*Chlorella ellipsoidea*	protamex, kojizyme, neutrase, flavourzyme, alcalase, trypsin, α-chymotrypsin, pepsin, papain	Ko et al., 2012
	GMNNLTP (123)[c] - LEQ (173)[c]	*Nannochloropsis oculata*	pepsin, trypsin, α-chymotrypsin, papain	Samarakoon et al., 2013
	IRLIIVLMPILMA (3344)[d]	*Palmaria palmata*	papain	Fitzgerald et al., 2012
	LRY - VYRT	*Palmaria palmata*	thermolysin, pepsin, trypsin, chymotrypsin	Furuta, Miyabe, Yasui, Kinoshita, & Kishimura, 2016
	AIYK (213)[c] – YKYY (64,2)[c] – KFYG (90,5)[c] – YNKL (21)[c]	*Undaria pinnatifida (wakame)*	pepsin	Suetsuna & Nakano, 2000
	VY (35,2)[c] – IY (6,1)[c] - AW (18,8)[c] - FY (42,3)[c] - VW (3,3)[c] – IW (1,5)[c] – LW (23,6)[c]	*Undaria pinnatifida (wakame)*	protease S "Amano", from *Bacillus stearothermophilus*	Sato et al., 2002
Antinflammatory	VPY	soybean	*not available*	Nakamori, 2010; Kovacs-Nolan et al., 2012
	pyro-EL	wheat	protease from	Sato et al., 2013

			Aspergillus oryzae	
Antioxidant	FRDEHKK	rice	neutrase	Zhang, Zhang, Wang, Guo, Wang, & Yao, 2010
	YVAQGEGVVA - YLAGMN	rice bran	pepsin	Adebiyi, Adebiyi,Yamashita, Ogawa, & Muramoto, 2009
	NHAV - HVRETALV	hempseed	alcalase	Lu et al., 2010
	WVYY - PSLPA	hempseed	pepsin, pancreatin	Girgih et al., 2014
	VECYGPNRPQF	algae protein waste	pepsin	Sheih, I. C., Wu, T. K., & Fang, T. J., 2009
	PGWNQWFL - VEVLPPAEL	*Navicula incerta*	papain	Khang et al., 2012
	LNGDVW	*Clorella ellipsoidea*	papain, pepsin, trypsin, α chimtrypsin	Ko, Kim, & Jeon, 2012
Immunomodulatory	MITLAIPVNKPGR (Soymetide-13)	soybean	trypsin	Tsuruki et al., 2003
	GYPMYPLPR (oryzatensin)	rice	trypsin	Takahashi et al., 1996
Anticancer	EQRPR	rice bran	alcalase	Kannan, Hettiarachchy, Lay, & Liyanage, 2010
	XMLPSYSPY	soybean	thermoase	Kim et al., 2000
	VECYGPNRPQF	*Chlorella vulgaris*	pepsin	Sheih, Fang, Wu, & Lin, 2010
	CPAP	*Chlorella pyrenoidosa*	papain, trypsin, alcalase	Wang & Zhang, 2013
Hypocholesterolemic	LPYP	soybean	trypsin	Kwon et al., 2002
	IAVPGEVA - IAVPTGVA	soybean	pepsin	Pak, Koo, Kasymova, & Kwon, 2005

	YVVNPDNDEN - YVVNPDNNEN	soybean	pepsin and trypsin	Lovati et al., 2000
	YGAPSL	soybean	alcalase	Zhong, Zhang, Ma, & Shoemaker, 2007
	FVVNATSN	soybean	protease from *Bacillus amyloliquefaciens*	Cho, Juillerat, & Lee, 2008
	GGV - IVG - VGVL	amaranth	pepsin and trypsin	Soares, Mendonça, de Castro, Menezes, & Arêas, 2015

[a] The single-letter amino acid code is used
[b] IC50 values are reported only for *in vitro* antihypertensive activity when available
[c] Antihypertensive activity against ACE activity
[d] Antihypertensive activity against renin
[e] (mg/mL)

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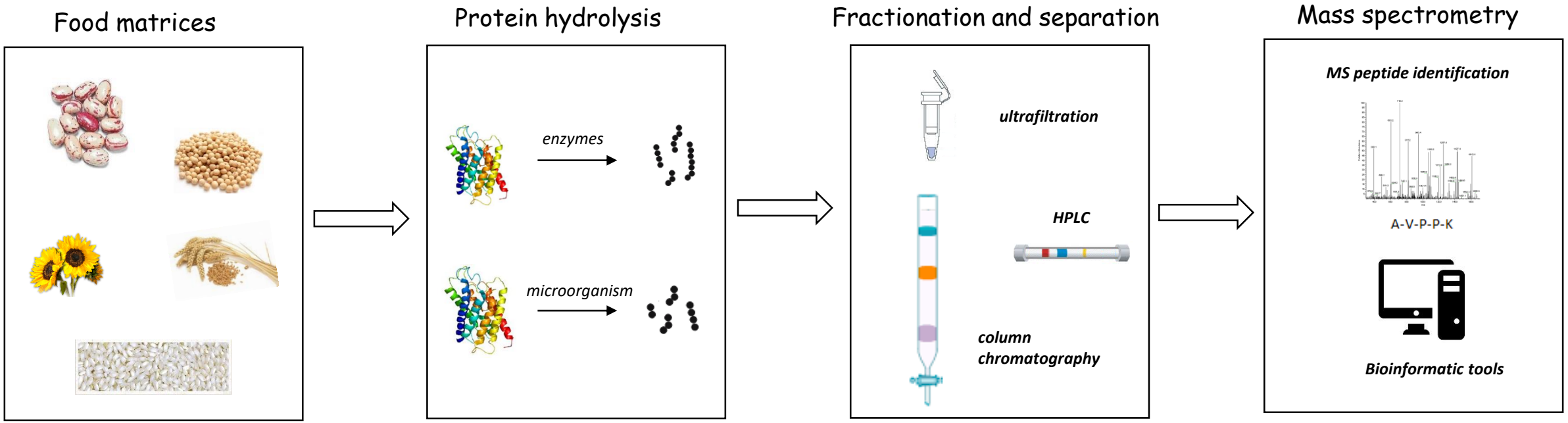
Food matrices
Protein hydrolysis
enzymes
microorganism
Fractionation and separation
ultrafiltration
HPLC
column chromatography
Mass spectrometry
MS peptide identification
A-V-P-P-K
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