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

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4 *Carlo G. Rizzello¹, Davide Tagliazucchi², Elena Babini³, Giuseppina Sefora Rutella², Danielle L.*
5 *Taneyo Saa³, Andrea Gianotti^{3*}*

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8 *¹Department of Soil, Plant, and Food Sciences, University of Bari, 70126 Bari, Italy*

9 *²Department of Life Sciences, University of Modena and Reggio Emilia, 42122 Reggio Emilia, Italy*

10 *³Department of Agricultural and Food Science, Alma Mater Studiorum, Università di Bologna, 40127*

11 *Bologna, Italy*

12

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14

15 **Corresponding author: andrea.gianotti@unibo.it; tel +39 051 2096577*

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18

19 ***Abstract***

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

30

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

32

33

34 **1. Introduction**

35 Proteins are important macronutrients of foods, serving as source of energy and amino acids, that
36 contribute to growth and maintenance of the body (Shahidi & Zhong, 2008). Beside the nutritional
37 role, proteins are responsible for various physicochemical and sensory properties of foods, and may
38 act as functional and health-promoting ingredients (Shahidi & Zhong, 2008). Many of the
39 physiological and functional properties of proteins are attributed to biologically active peptides which
40 are often encrypted in the native sequence (Gobbetti, Minervini, & Rizzello, 2007; Shahidi & Zhong,
41 2008). Biogenic or bioactive peptides can be produced from the protein precursor by digestive
42 enzymes (gastrointestinal digestion), during food processing (ripening, fermentation, cooking),
43 storage, or by *in vitro* hydrolysis by proteolytic enzymes (Carrasco-Castilla, Hernandez-Alvarez,
44 Jimenez-Martinez, Gutierrez-Lopez, & Davila-Ortiz, 2012).

45 Bioactive peptides mainly contain 3-20 amino acid units, but in some cases the size is larger (Shahidi
46 & Zhong, 2008), and they can be considered as components of functional foods which may exert
47 regulatory activities in the human organism, irrespective of their nutritive functions (Gobbetti et al.,
48 2007). *In vitro* and some *in vivo* studies show a large spectrum of biological functions attributed to
49 bioactive peptides: opioid like, mineral-binding, immunomodulatory, antimicrobial, antioxidative,
50 antithrombotic, hypocholesterolemic, antihypertensive (Coda, Rizzello, Pinto, & Gobbetti, 2012),
51 and antitumoral (Rizzello, Nionelli, Coda, & Gobbetti, 2012; Rizzello et al., 2015a).

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

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

67 Overall, sequences showing the same bioactivities can be released from native proteins deriving from
68 vegetable or animal matrices. For example, the multi-functional dipeptide VY can be released through
69 the hydrolysis of vegetable (brewed sake) or milk proteins (Saito, Wanezaki, Kawato, & Imayasu,
70 1994; Tagliacruzchi, Shiamsia, & Conte, 2016). The ACE-inhibitory dipeptide AI was isolated from
71 a soy sauce and pinto bean proteins as well as from milk after gastro-intestinal digestion (Nakahara
72 et al., 2010; Tagliacruzchi, Martini, Bellesia, & Conte, 2015; Tagliacruzchi et al., 2016). The
73 antihypertensive peptide SY was identified in protein hydrolysates of soybean, garlic, cereals, milk,
74 pork sarcoplasmic and chicken proteins (García et al., 2013; Castellano, Aristoy, Sentandreu, Vignolo,
75 & Toldrá, 2013; Iwaniak & Dziuba, 2009; Weimann, Meisel, & Erhardt, 2009); the peptide YPFVV,
76 isolated from an hydrolysate of soy β -conglycinin showed high similarity with the human
77 casomorphin-5 (YPFVE) (Ohinata, Agui, & Yoshikawa, 2007); the bioactive peptide soymorphin-5
78 shares the same sequence (YPF), the same bioactivity (opioid agonist) and the same mechanism of
79 action (interaction with μ -receptors) of human and bovine casomorphins.

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

83

84 **2. Release of bioactive peptides by microbial fermentation**

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

91 Among microorganisms involved in food biotechnologies, lactic acid bacteria are recognized as the
92 most useful in bioactive peptides enrichment, thanks to the large adaptability to different
93 environments and animal or vegetable matrices, the safety, and, especially, the efficient proteolytic
94 system of which they are provided. Indeed, proteinase activity and a large portfolio of peptidases are
95 considered the pre-requisites to liberate bioactive peptides from proteins (De Angelis et al., 2006;
96 Gobbetti et al., 2007). Lactic acid bacteria belong to a microbial group having a large variability,
97 derived from the evolution in a wide range of environments. Their large variability is very suitable
98 for the selection of strains to be used for different biotechnology processes. An increasing number of
99 scientific articles (Coda et al., 2011; Coda et al., 2012; Rizzello, Cassone, Di Cagno, & Gobbetti,
100 2008; Rizzello et al., 2012) described the use of lactic acid bacteria isolated from sourdough for the
101 release of bioactive peptides in vegetable matrices. Sourdough is the natural starter traditionally used
102 for making baked goods. It was reported that fermentative and proteolytic activities of sourdough
103 lactic acid bacteria determined not only the sensory, technology and nutritional characteristics, but
104 also the functional features of the resulting baked goods (Gobbetti, De Angelis, Corsetti, & Di Cagno,
105 2005). Besides the microbial agents, also the fermentation conditions typical of the sourdough
106 technology (Coda et al., 2012; Rizzello et al., 2008; Rizzello et al., 2012) were adopted and optimized
107 to promote the peptides release in a wide range of food matrices. A description of the main
108 antihypertensive, antioxidant and anticancer peptides released by fermentation processes of vegetal
109 matrices is reported below. Moreover, in Table 1 a list of bioactive peptides identified from plant
110 matrices specifically fermented by lactic acid bacteria is also reported.

111

112 ***2.1 Antihypertensive peptides***

113 Bioactive peptides having Angiotensin I-Converting Enzyme (ACE)-inhibitory properties, may be
114 used for preventing hypertension as well as for other therapeutic purposes (Rizzello et al, 2008).
115 Within the enzyme cascade of the renin-angiotensin system, ACE removes histidyl-leucine from
116 angiotensin I to form the physiologically active octapeptide angiotensin II, one of the most potent
117 known vasoconstrictors. Therefore, a rationale for treating hypertension would be to administer drugs
118 or natural compounds which selectively inhibit ACE. Although most of the studies on ACE-inhibitory
119 peptides referred to synthesis occurring in dairy products, some antihypertensive sequences have been
120 recently isolated from plant proteins. Although many experiments were carried out on enzymatic
121 hydrolysates (see below), the production of ACE-inhibitory peptides through microbial fermentation
122 has been also investigated (Rizzello et al., 2008). A pool of selected sourdough lactobacilli having
123 specific proteinase and peptidase activities towards cereal proteins, was successfully used for
124 releasing ACE-inhibitory peptides during a long-time sourdough fermentation (Rizzello et al., 2007;
125 Rizzello et al., 2008). In particular, high activity was found fermenting flours under semi-liquid
126 conditions and, especially, by using wholemeal wheat flour. The addition of commercial proteases to
127 the starters did not increase the ACE-inhibitory activity of the fermented matrices, since they caused
128 a too extensive proteolysis of the active sequences (Rizzello et al., 2008). Fourteen ACE-inhibitory
129 peptides (IC₅₀ ranging from 0.19 to 0.54 mg/mL) were identified from the fermented wholemeal
130 wheat. Almost all contained the well-known antihypertensive epitope VAP (Rizzello et al., 2008).
131 Previously, the epitope VAP was found either as antihypertensive peptide or as encrypted epitope of
132 ACE-inhibitory peptides from milk proteins (Gobbetti et al., 2007). Anti-ACE peptides deriving from
133 legume proteins were also isolated, as reported by Jakubczyk, Karaś, Baraniak, & Pietrzak (2013),
134 that used a *L. plantarum* strain to ferment and hydrolyze pea flour proteins. The active and purified
135 peptide fraction obtained from the pea proteins hydrolysates had an IC₅₀ of 64.04 µg/ml (Jakubczyk
136 et al., 2013). Antihypertensive peptides were also obtained in soybean products (soymilk, soy-yogurt,

137 soy sauce) through fermentation with lactic acid bacteria (*Lactobacillus casei*, *L. acidophilus*, *L.*
138 *bulgaricus*, *Streptococcus thermophilus*), but also using *Bacillus natto*, *B. subtilis*, *Bifidobacterium*
139 *longum* as starters (Singh, Vij, & Hati, 2014). Antihypertensive peptides also showing anti-
140 thrombotic, surface tension and antioxidant properties were isolated in the traditional soy-fermented
141 foods *natto* and *tempeh* (Gibbs, Zougman, Masse, & Mulligan, 2003). More recently, it was
142 demonstrated that lactic acid bacteria allowed the release of antihypertensive peptides during
143 *Phaseolus vulgaris* (navy bean) fermentation (Rui et al., 2015). Moreover, it was demonstrated that
144 fermented tropical legumes (Bambara groundnut, locust bean, soybean) are able to reduce the
145 hypertension state, together with the diabetes-induced dyslipidemia, of rats (Ademiluyi & Oboh,
146 2015).

147 Peptide fractions having ACE-inhibitory activity were purified also in fermented alcoholic beverages,
148 including red and white wines (Pozo-Bayòn, Alcaíde, Polo, & Pueyo, 2005) and sake (Saito et al.,
149 1994).

150

151 **2.2 Antioxidant peptides**

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

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

163 antioxidant activity were obtained from many animal and plant proteins (Coda et al., 2012). They
164 were isolated from peanut kernels, rice bran, sunflower protein, alfalfa leaf protein, corn gluten meal,
165 frog skin, yam, egg-yolk protein, milk-kefir and soymilk kefir, mushrooms, mackerel, curry leaves,
166 cotton leaf worm, casein, algae protein waste, wheat, and buckwheat (Sarmadi & Ismail, 2010). It
167 was argued that antioxidant peptides act as inhibitors of lipid peroxidation, direct scavengers of free
168 radicals and/or as agents to chelate transition metal ions that catalyze the generation of radical species
169 (Shimada, Fujikawa, Yahara, & Nakamura, 1992). Usually, antioxidant peptides are constituted by
170 2-20 amino acidic residues, and have molecular masses less than 6.0 kDa (Coda et al., 2012). The
171 antioxidant activity seems to be strongly correlated to the amino acid composition, conformation and
172 hydrophobicity. A pool of selected lactic acid bacteria including ten strains, previously selected based
173 on proteinase and peptidase activities (Coda et al., 2012), was used to ferment various flours with the
174 aim to release antioxidant peptides. Fermentation by selected lactic acid bacteria was allowed for long
175 time and with semi-liquid conditions, which are indispensable to fully exploit microbial proteolysis
176 (Rizzello, Nionelli, Coda, De Angelis, & Gobbetti, 2010). It was found that the radical scavenging
177 activity of soluble extracts from fermented flours was significantly higher than that of unfermented
178 controls. Among different flours obtained from cereals, pseudo-cereals and legumes, the highest
179 activity was found for whole wheat, spelt, rye, and kamut sourdoughs. The purified active peptides,
180 having the size from 8 to 57 amino acid residues, were resistant to further hydrolysis by digestive
181 enzymes. Almost all their sequences shared compositional features which are typical of other
182 antioxidant peptides (Coda et al., 2012) and showed *ex vivo* antioxidant activity which was
183 comparable to that of α -tocopherol on mouse fibroblasts artificially subjected to oxidative stress.
184 Recently, it was reported that antioxidant peptides (together with anti-ACE peptides) were released
185 also by *Oenococcus oenis*, *B. subtilis*, *B. pumilus*, and *L. plantarum* during grape must (Apud,
186 Vaquero, Rollan, Stivala, & Fernández, 2013), rapeseed/flaxseed (He et al., 2012; Pihlanto,
187 Johansson, & Mäkinen, 2012), rice protein (Dei Più, Tassoni, Serrazanetti, Ferri, Babini, Tagliazucchi, &
188 Gianotti, 2014), and soybean (Singh et al., 2014) fermentation, respectively. *In vitro* and *in vivo*

189 evidences of the antioxidant activities of peptides isolated from *douchi* (a traditional Chinese salt-
190 fermented soybean food) were also reported (Wang et al., 2008), semi-fermented cacao seeds (Preza
191 et al., 2010), and commercial fermented mushrooms *Ganoderma lucidum* (Sun, He, & Xie, 2004) and
192 Abalone (Li et al., 2012). In particular, antioxidant peptides from cacao beans showed antitumor
193 activity against murine lymphoma L5178Y in BALB/c mice after a 15-days treatment with an oral
194 dose of 25 mg/kg/day, while the *G. lucidum* Peptide (GLP) showed scavenging activity toward
195 hydroxyl radicals produced in a deoxyribose system with an IC₅₀ value of 25 µg/mL, and effectively
196 quenched superoxide radical anion produced by pyrogallol autoxidation in a dose-dependent manner.

197

198 **2.3 Anticancer peptides**

199 It was emphasized that proteins, peptides and amino acids might be implicated at various levels in
200 the prevention of different types of cancer (Shahidi & Zhong, 2008). Lunasin is a 43-amino acid
201 peptide having molecular weight of 5 kDa. It corresponds to the small subunit peptide (Gm2S-1) of
202 2S soy albumin (Galvez, Chen, Macasieb, & de Lumen, 2001). Lunasin contains 9 aspartic acid
203 residues at the C-terminus, a tripeptide arginine-glycine-aspartic acid cell adhesion motif and a
204 predicted helix whose structure is similar to the conserved region of chromatin-binding proteins
205 (Galvez et al., 2001). The amino acid sequence of lunasin peptide from soybean is deposited at the
206 National Center for Biotechnology Information (NCBI) database with the accession number
207 AAP62458. During *in vitro* assays, lunasin showed an inhibitory effect against the core histone
208 acetylation of mammalian cells (Rizzello et al., 2012), suggesting an involvement in the chromatin
209 modification, the process implicated in cell-cycle control and suppression of carcinogenesis (de
210 Lumen, 2005). Recently, *in vivo* trials were performed with the aim to investigate the bioactivity and
211 the bioavailability of lunasin after oral administration (Gonzalez de Mejia & Dia, 2010) and during
212 food processing (Jeong, Jeong, Hsieh, Hernández-Ledesma, & de Lumen, 2010).

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

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

238

239 **2.4 Antifungal peptides**

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

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

262

263 **3. Synthesis of bioactive peptides by enzymatic hydrolysis**

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

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

272 The enzymatic process is based on the use of single or different combinations of proteinases like
273 pepsin, trypsin, chymotrypsin, bromelain, papain, alcalase, neutrase, flavourzyme and many others.
274 The critical hydrolysis parameters (temperature, pH, aqueous or buffered solution), must be optimized
275 for each protein substrate and each selected enzyme or combination of enzymes, and must be
276 maintained during proteolysis to ensure efficient release of peptides. The hydrolysis duration has a
277 substantial inverse relationship with peptide size, but in most cases a plateau is reached during which
278 further increases in hydrolysis time do not produce any effect on peptide size or activity (Aluko,
279 2015). Hydrolysis can be performed basically in three ways (but with many variations available in
280 the literature): i) under traditional batch conditions, ii) using immobilized enzymes or iii) by
281 ultrafiltration membranes. Batch enzymatic hydrolysis methods have certain disadvantages such as
282 the relatively high cost of enzymes, the inherent inefficiency (resulting in low yields and
283 productivity), and the generation of secondary metabolites due to enzymatic autolysis. These
284 limitations can be overcome separating the enzyme and product during the reaction using a two-phase
285 system, one phase containing the enzyme and the other containing the product. The enzyme is
286 imprisoned within its phase allowing its re-use or continuous use, but preventing it from
287 contaminating the product; other molecules, including the reactants, are able to move freely between
288 the two phases. As compared to soluble enzymes, immobilized proteases allow the process to be
289 performed in milder and more controlled conditions, prevent the generation of secondary metabolites
290 originating from autolysis of enzymes and do not need to be inactivated by heat or acidification,
291 which may be damaging for the product. Finally, immobilized enzymes can be easily recovered and

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

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

308

309 ***3.1 Antihypertensive peptides***

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

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

344 *Bacillus licheniformis*) was the source of the ACE-inhibitory peptide PSGQYY, composed of an
345 hydrophobic amino acid at the amino terminal, a basic amino acid residue at the center, and a carboxyl
346 terminal tyrosine. A dose of 30 mg per kg body weight antagonized the rat's pressor response to
347 angiotensin I, lowering the blood pressure (Suh et al., 1999). Corn gluten meal was hydrolyzed by
348 alcalase after starch removal and the ACE-inhibitory peptide AY was isolated. The activity of this
349 peptide was not affected by preincubation with ACE and persisted after oral administration to SHR.
350 A maximal reduction of systolic blood pressure of 9.5 mmHg was observed 2 h after oral
351 administration of AY at doses of 50 mg/kg (Yang, Tao, Liu & Liu, 2007). Enzymatic hydrolysates of
352 soybean glycinin were recognized as sources of ACE-inhibitors. Among them, the high activity of
353 the protease P glycinin hydrolysate resulted related to the peptide VLIVP which was a competitive
354 inhibitor of ACE and resistant to digestion by proteases of the gastrointestinal tract (Mallikarjun et
355 al., 2006). Its sequence corresponds to Val397-Pro401 of the glycinin subunit G2 (Swiss Prot:
356 P04405) of soybean (Mallikarjun et al., 2006). Hydrolysis of soybean glycinin by acid proteinase
357 from *Monascus purpureus* led to the release of the peptides SPYP and WL, while the hydrolysis of
358 soybean β -conglycinin with the same enzyme produced two other antihypertensive peptides,
359 LAIPVNKP and LPHF (Kuba, Tana, Tawata & Yasuda, 2005). The further hydrolysis of SPYP
360 peptide *in vitro* by pepsin, chymotrypsin and trypsin enhanced its ACE-inhibitory activity (Kuba et
361 al., 2005). Digestion of soybean with pepsin revealed activity against ACE due to the release of the
362 peptides IA, YLAGNQ, FFL, IYLL, and VMDKPQG (Chen, Okada, Muramoto, Suetsuna & Yang,
363 2002). Their antihypertensive activity was confirmed on SHR which were fed by saline solutions of
364 peptidic fraction powders (0.9% w/v; 2.0 g per kg body weight). A significant reduction of blood
365 pressure (17.5 mm Hg) was observed after 2 h, and continued for 6 h following oral administration
366 (Chen et al., 2002). The ACE-inhibitory peptides DLP and DG were identified in soy protein alkaline
367 hydrolysate, obtained with alcalase (Wu & Ding, 2002). The antihypertensive peptides YVVFQ,
368 PNNKPFQ, NWGPLV, and IPPGVYWT were obtained from soybean protein isolates using a
369 recombinant protease D3, a cathepsin L-like protease derived from germinating soybean cotyledons,

370 which was expressed and isolated from *Escherichia coli* (Kodera & Nio, 2006). Antihypertensive
371 activity was investigated in SHRs and the dose-dependent antihypertensive effect was confirmed,
372 with systolic blood pressure significantly reduced after the oral administration of doses exceeding
373 100 mg/kg. Yang, Marczak, Yokoo, Usui & Yoshikawa (2003) isolated four active peptides
374 (MRWRD, MRW, LRIPVA, and IAYKPAG) in pepsin-pancreatin digests of the spinach Rubisco
375 protein which inhibited ACE. The antihypertensive effect of these peptides was tested on SHRs and
376 resulted to be dose-dependent (Yang et al., 2003). Sesame peptide powder obtained from sesame
377 proteins hydrolyzed with thermolysin exhibited ACE-inhibitory activity, and significantly and
378 temporarily decreased the systolic blood pressure (SBP) in SHRs by a single administration (1 and
379 10 mg/kg, Nakano et al., 2006). Six ACE-inhibitory peptides were isolated and identified from this
380 substrate: LSA, LQP, LKY, IVY, YIY, LVY, and MLPAY (Nakano et al., 2006). A novel ACE-
381 inhibitory peptide, ACKEP was identified from pistachio kernel proteins after digestion with
382 gastrointestinal enzymes pepsin and trypsin (Li et al., 2014). ACKEP has the same C-terminal
383 construction as that of lisinopril and enalapril, which plays a key role in binding with ACE. That
384 mechanism was explored at a molecular basis by docking experiments revealing that seven amino
385 acids in the ACE active site and two atoms of ACKEP greatly contribute to the combinative
386 stabilization (Li et al., 2014). Five antihypertensive peptides (WYT, WVYY, SVYT, PLSPA, and
387 IPAGV) were isolated from hemp seed proteins (Girgih et al., 2014). Lopez-Barrios, Gutierrez-Urbe,
388 & Serna-Saldívar (2014) reviewed pulses as a rich source of proteins in the human diet and associated
389 their consumption with the prevention of chronic diseases. Chickpea (*Cicer arietinum*), field pea
390 (*Pisum sativum*), mung bean (*Vigna radiata*), and kidney bean (*Phaseolus vulgaris*) protein
391 hydrolysates among others, have yielded ACE-inhibitory activity. As confirmed by Li, Wan, Le, &
392 Shi (2006) mung bean proteins are good precursors of ACE-inhibitors such as KDYRL, VTPALR,
393 KLPAGTLF, and alcalase hydrolysates of this plant could be used to produce functional foods with
394 antihypertensive activity. Red bean proteins digested with alcalase, papain, pepsin, trypsin, and α -
395 chymotrypsin produced an active peptide with sequence PVNNPQIH (Rui, Boye, Simpson &

396 Prasher, 2013), while chickpea proteins treated with alcalase and flavourzyme produced peptides MD,
397 MDFLI, MFDL, MDL, and MDLA, (Yust et al., 2003). Three dipeptides (IR, KF, and EF), obtained
398 by alcalase digestion of pea protein isolate, showed strong inhibition of ACE and renin, with higher
399 potency against ACE than against renin (Li & Aluko, 2010). A hydrophobic residue at the N terminus
400 and a bulky amino acid residue at C terminus was a preferred structural arrangement for renin
401 inhibition by the three synthesized peptides (Li & Aluko, 2010). Two active peptides from yellow
402 field pea proteins, WMP and ADMFPF were obtained using thermolysin (Aluko, Wu & Aukema,
403 2014).

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

422 2016). Four active peptides (AIYK, YKYY, KFYG, and YNKL) were isolated from the peptic digest
423 prepared from wakame (*Undaria pinnatifida*) proteins (Suetsuna & Nakano, 2000). Each tetrapeptide
424 was synthesized and its antihypertensive activity was confirmed after oral administration (50 mg/kg)
425 in SHR_s (Suetsuna, & Nakano, 2000). The antihypertensive effect of wakame was shown in
426 hypertensive patients where a significant decrease of the systolic blood pressure by 14 ± 3 mmHg,
427 after daily oral administration of 3.3 g of dried wakame after 4 weeks, was observed (Nakano, Hidaka,
428 Uchida, Nakajima, & Hata, 1998).

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

435

436 **3.2 Antioxidant peptides**

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

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

458

459 **3.3 Anticancer/Antiproliferative peptides**

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

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

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

481

482 **3.4 Hypocholesterolemic peptides**

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

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

500 2007). A protease from *Bacillus amyloliquefaciens* was used to hydrolyze soybean proteins,
501 producing the peptide FVVNATSN which strongly stimulated transcription of low density
502 lipoprotein receptors (LDL-R), which play an important role in the reduction of plasma LDL
503 cholesterol (Cho, Juillerat, & Lee, 2008).

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

509

510 ***3.5 Antinflammatory peptides***

511 Inflammation is the body's response to nonlethal injury, which is characterized by increased
512 endothelial permeability, leakage of protein-rich exudates, and infiltration of leukocytes into
513 extravascular tissues. While inflammation is essential for resistance to microbial infections and
514 wound healing, excessive and uncontrolled inflammatory changes often lead to chronic diseases such
515 as asthma, cancer, cardiovascular disease, diabetes, obesity, inflammatory bowel disease,
516 osteoporosis and neurological diseases like Parkinson's (Chakrabarti, Jahandideh, & Wu, 2014;
517 Majumder, Mine, & Wu, 2016). Overall, bioactive peptides have a potential to modify intestinal
518 barrier function (Martínez-Augustin, Rivero-Gutiérrez, Mascaraque, & Sánchez de Medina, 2014)
519 whose alteration are related to many inflammatory and non-inflammatory disorders. Soybean, whey
520 and corn enzymatic hydrolysates have shown anti-inflammatory effects both in *in vitro* and *in vivo*
521 studies (Vernaza, Dia, Gonzalez de Mejia, & Chang, 2012; Hwang, Yoo, Songa, Kimb, Chunc, et al.,
522 2011; Shahi, Rashidi, Mahboob, Haidari, Rashidi, et., 2012, Nagarajan, Burris, Stewart, Wilkerson,
523 & Badger, 2008; Martinez-Villaluenga, Dia, Berhow, Bringe, & Gonzalez de Mejia, 2009;
524 Mochizuki, Shigemura, & Hasegawa, 2010; Dia, Bringe, & de Mejia, 2014) but only few peptides
525 have been identified, to date, as responsible of this activity. Recent studies demonstrated that the

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

533

534 **3.6 Immunomodulatory Peptides**

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

543 Some studies have evaluated the capacity of peptides to stimulate the immune system after their
544 administration (Gobbetti, Stepaniak, De Angelis, Corsetti, & Di Cagno, 2002; Jang, Jo, Kang, & Lee,
545 2008; Kong, Guo, Hua, Cao, & Zhang, 2008). As recently reported (Santiago-López, Hernández-
546 Mendoza, Vallejo-Cordoba, Mata-Haro, & González-Córdova, 2016), peptides may enhance or
547 weaken immunity by up-regulating pro- and anti- inflammatory cytokines. These stimulate the
548 synthesis of some antibodies, also may either contribute to the proliferation or elimination of T and
549 B lymphocytes, in addition to influencing NK cells and the phagocytic activity of macrophages
550 (Santiago-López et al. 2016). The immunomodulatory action of peptides is one of the least explored

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

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

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

562

563 ***3.7 Antifungal peptides***

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

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

579

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

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

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

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

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

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

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

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

628 Indeed, ESI source can operate under variable solvent conditions and continuous sample infusion
629 (Emmett & Caprioli, 1994). Several examples regarding the use of LC-ESI-MS to identify bioactive
630 peptides from vegetables can be found in literature. Sato et al. (2002) and Nakahara et al. (2010)
631 identified, respectively, seven and nine new ACE-inhibitory and anti-hypertensive dipeptides from a
632 wakame hydrolysates and a soy sauce-like seasoning. Hybrids LC-ESI-Q-TOF and LC-ESI-Q-TRAP
633 were utilized by Puchalska et al. (2012) to identify anti-hypertensive peptides after thermolysin
634 digestion of maize and by Manolo Soares, Mendonça, Andrade de Castro, Carlos Menezes et al.
635 (2015) to identify the major 3-hydroxy-3-methyl-glutaryl-CoA reductase inhibitory peptides
636 generated by the *in vitro* hydrolysis of *Amaranthus cruentus* protein, respectively.

637 Nowadays, ESI technology is in continuous expansion thanks to implementation of miniaturized
638 instrumentation. Indeed, nano-ESI allows working with small samples and buffer volumes. Nano
639 flow LC-ESI has been coupled with single (Coda et al., 2011; Esteve, Marina, & García, 2015), or
640 hybrid (Q-TOF) mass analyzers (Dei Più et al., 2014; Chang, Ismail, Yanagita, Esa, & Baharuldin,
641 2015).

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

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

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

658

659 **5. Recovery stages and conventional technologies**

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

- 669 (a) maximizing the yield of the target compounds,
- 670 (b) accommodating the needs of industrial processing,
- 671 (c) purified the high added-value ingredients and remove toxic compounds,
- 672 (d) preventing deterioration and loss of functionality during processing,
- 673 (e) assuring the quality the final product.

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

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

682 The membrane technologies processes has some advantages, including operation at low temperature,
683 absence of phase transition and low energy expenditure (Conde et al., 2013; Murakami et al., 2011).

684 Membranes are semipermeable barriers that split up two distinct phases and limit the movement of
685 definite components in a selective way (Bazinet & Firdaous, 2013). Membranes allow the diffusion
686 of some elements while retaining others, enabling the enrichment of the permeate and the retentate in
687 specific components (de Morais Coutinho et al., 2009). The diffusion of the different compounds
688 through the membrane can be assisted by applying a driving force (concentration gradient, pressure,
689 temperature or electric potential). Membranes technologies include microfiltration (MF),
690 ultrafiltration (UF), nanofiltration (NF) and reverse osmosis (RO) which use hydraulic pressure as a
691 driving force. Nevertheless, the major part of the industrial applications for the isolation of specific
692 bioactive peptides or proteins is based on UF and NF (Herrero et al., 2012; Szydłowska-Czerniak,
693 Trokowski, & Szlyk, 2011). UF operates at 2-10 bar, even if in some cases up to 30 bar have been
694 employed (Nawaz, Shi, Mittal, & Kakuda, 2006) and it is used to retain macromolecules and colloids
695 with molar masses between 1 and 300 kDa, while allowing water and small molecules to permeate.

696 NF is commonly carried out at 10-40 bar and it can separate particles with molar masses between
697 0.35 and 1 kDa. RO, performed at 40-100 bar, can hold on essentially all solutes in concentrate, as
698 well as small ions such as sodium (de Morais Coutinho et al., 2009; Murakami et al., 2011). The
699 hydraulic pressure used in these technologies depends on the pore size of the filtration membrane and
700 can be set up depending on the concentration rate needed. The performance and efficiency of the
701 separation are influenced by the solution properties, charges from which originate the repulsions and
702 attractions of ions and the type of membrane used (Langevin et al., 2012; Martin-Orue, Bouhallab, &
703 Garem, 1998). To bypass the formation of a polarization layer that usually affect the membrane
704 selectivity, it could be suitable to work at low concentration which could affect the behavior and the
705 efficiency of the separation method. Even though membrane separations are efficient and flexible,

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

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

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

732 molecular weight using a 5 kDa membrane. It was found that the antioxidant activity of the filtrate
733 was similar to the vitamin E at pH 7.0 (Yoshie-Stark et al., 2008). UF was used to recover antioxidant
734 peptide fractions from enzymatic hydrolysates of wheat gluten (Kong, Zhou, & Hua, 2008).
735 Hydrolysates from barley glutelin were separated by UF and reverse-phase chromatography to obtain
736 large-size peptides (MW > 10 kDa) with enhanced DPPH scavenging activity and reducing power,
737 and small-size peptides (MW < 1 kDa) valuable for chelating Fe²⁺ and for scavenging OH• (Xia,
738 Bamdad, Gänzle, & Chen, 2012).

739 As mentioned before, bioactive peptides can also be fractioned using chromatography techniques.
740 HPLC is commonly used for separation, identification, and purification of bioactive peptides at
741 laboratory and pilot-plant scales. Size-exclusion chromatography can be used to purify peptides based
742 on molecular mass. RP-HPLC can be employed to separate peptides according to their hydrophobic
743 properties and, in particular, it was largely applied in the study of the structural and functional
744 properties of bioactive sequences (Pownall, Udenigwe, & Aluko, 2010). Gibbs et al. (2004) purified
745 and identified bioactive peptides from fermented soy by LC-MS. A soy milk hydrolysate was purified
746 to recover ACE-inhibitory peptides using size exclusion chromatography, that allowed to recover one
747 fraction with a very high ACE inhibitory effect (Chiang, Tsou, Tsai, & Tsai, 2006). An adipogenesis
748 inhibitory tripeptide IQN, having an IC₅₀ value of 0.014 mg protein/mL, was purified and identified
749 from black soybean hydrolysate using chromatographic and protein sequencer, (Kim, Bae, Ahn, Lee,
750 & Lee, 2007). The ACE-inhibitory tripeptide GPP, having IC₅₀ value of 6.25 mg protein/mL, was
751 purified and isolated from a buckwheat protein hydrolysate by protein sequencing system and
752 electrospray-LC-mass spectrometry (Mallikarjun Gouda, Gowda, Rao, & Prakash, 2006). Seber et al.
753 (2012) set-up a scalable method using a sequential application of anion-exchange chromatography
754 combined with UF, and RP chromatography to produces lunasin preparations of 99% purity with a
755 yield of 442 mg/kg defatted soy flour (Singh et al., 2014). A peptide preparation having active metal
756 chelators was recovered from bean proteins and phaseolin treated with pepsin and pancreatin applying

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

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

764

765 **6. Future trends**

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

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

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

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

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

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

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

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

846

847 **Legends to figures**

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

859

860 **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	DPVAPLQRSQPEIP - PRSGNVGESGLID - ESVSLVA - PHAVAAPPPVLR - LLGWGHKGSIIID - HCNDPEKKNL - PILQSLIRFDGGACSSF - RSQIKREQYTPQDVEMLFSSF	soft wheat	<i>L. brevis</i> AM7	Coda <i>et al.</i> , 2008
anti-ACE	DPVAPLQRSQPEI - PVAPQLSRGLL - ELEIVMASPP - QILLPRPGQAA - PVAPLQRSQPEI - PRSGNVGESGL - VAPSRPTPR - DIIIPD - PRSGNVGESGLID - DPVAPLQRSQPEI - DPVAPLQRSQPEIP - PVAPLPRKGS - DPVAPLQRSQPEI - SFTAGARTFNFDENPCDYFQGGKIKAT	wholemeal wheat	<i>L. alimentarius</i> 15M; <i>L. brevis</i> 14G; <i>L. hilgardii</i> 51B <i>L. sanfranciscensis</i> 7A, LS3, LS10, LS19, LS23, LS38 and LS47	Rizzello <i>et al.</i> , 2008
antifungal	VLHEPLF - YNNPIIYVTENGIAEGNNKSLPITEAL - ALKAAPSPA - AILIIVMLFGR - AAAAVFLSLLAVGHCAAADFNATDADADFAGNGVDFNSSDAAVYWGPPWTKAR	wheat germ	<i>L. plantarum</i> LB1; <i>L. rossiae</i> LB5	Rizzello <i>et al.</i> , 2011
antifungal	PPDVLTKLTAVPAAQQLDEADGHPR - SAFEFADDEHKGAYS - AAIFGSIWVNVGMKR - ALGFEMTPEQIHQMI - IGRDVQNSQLFSPQVDSSLLYNMVPNLTSNVSDGNLSTIPSGSTYLQNAMYG - SAFEFADDEHKGAYS - AEGEVILEDVQPSSVQS - EGTYLDYIQNNGKTLGAEDSTNEFGWDNK - DEEIMLDITTCAMAFRLLR - WFVELTGIPVTTTLMGLGNFSPDDPLSLRMLGMHGTVYANYAVDK	soft wheat	<i>L. plantarum</i> 1A7	Coda <i>et al.</i> , 2011
antioxidant	MAPAAVAAAEEAGSK - DNIPIVIR	whole wheat	<i>L. alimentarius</i> 15M; <i>L. brevis</i> 14G; <i>L. hilgardii</i> 51B <i>L. sanfranciscensis</i> 7A, LS3, LS10, LS19, LS23, LS38 and LS47	Coda <i>et al.</i> , 2012
antioxidant	AIAGAGVLSGYDQLQILFFGK - GNQEKVLELVQR - PAGSAAGAAP - EALEAMFL - AAGAAAAARSAGQCGR - ITFAAYRR - HPVPPKKK	spelt		
antioxidant	VFVDEGLEVLGWRPVPFNVSVVGRNAK - RLSLPAGAPVTVAVSP - NANGELCPNNMCCSQWGYCGLGSEFCGNGCQSGACCPEK - LCPVHRAADL - PAEMVAAALDR - KVALMSAGSMH - DLADIPQQRLMAGLALVVATVIFLK - KNGSIFNSPSATAATIIHGHNYSGLAYLDFVTSK - GTIFFSQEGDGPTSVTGSVSLKPLGHLGHFVHALGDTTNGCMSTGPHFNPTGK	rye		
antioxidant	YEWPTVPNFDVAKDVTDM - GVSNAAVVAGGH - DAQEFKR - PPGPGPGPPPPGAAGRGGGG - HKEMQAIQFDVYIMFIN - TGGGSTSSSSSSSSSLGGGASRGSVVEAAPPATQGAAAANAPAVPVVVVDVTQEAGIR - DTAAGYVAPPDPAVSTGDYGLAGAEAPHPHESAVMSGAAAAAVAPGGEAYTR	kamut		
anti-ACE	KEDDEEEEQGEEE	pea	<i>L. plantarum</i> 299v	Jakubczyk <i>et al.</i> 2013
antitumoral	SKWQHQQDSCRKQKQGVNLTPEKHIMEKIQGRGDDDDDDDDDD (and relative fragments f4-43; f12-43; f20-43)	soybean	<i>L. curvatus</i> SAL33, <i>L. rossiae</i> CD76, <i>L. brevis</i> AM7, <i>L. pentosus</i> 12H6, <i>L. plantarum</i> 3DM	Rizzello <i>et al.</i> , 2012

861 ^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 (IC ₅₀ 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 <i>Monascus purpureus</i>	Kuba, Tana, Tawata & Yasuda, 2005	

FFL (37) ^c – IA (153) ^c – IYLL (42) ^c – YLAGNQ (14) ^c – VMDKPDG (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 - YVDFK (44) ^c – PNNKPFQ (33) ^c – IPPGVYWT (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
ACEP (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	alcalase, 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	<i>Chlorella vulgaris</i>	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	<i>Spirulina platensis</i>	pepsin	Suetsuna, K., & Chen, J. R., 2001
	VECYGPNRPQF (29,6) ^c	<i>Chlorella vulgaris</i>	pepsin	Sheih, I. C., Fang, T. J., & Wu, T. K., 2009
	VEGY (128,4) ^c	<i>Chlorella ellipsoidea</i>	protamex, kojizyme, neutrase, flavourzyme, alcalase, trypsin, α -chymotrypsin, pepsin, papain	Ko et al., 2012
	GMNNLTP (123) ^c - LEQ (173) ^c	<i>Nannochloropsis oculata</i>	pepsin, trypsin, α -chymotrypsin, papain	Samarakoon et al., 2013
	IRLIIVLMPILMA (3344) ^d	<i>Palmaria palmata</i>	papain	Fitzgerald et al., 2012
	LRY - VYRT	<i>Palmaria palmata</i>	thermolysin, pepsin, trypsin, chymotrypsin	Furuta, Miyabe, Yasui, Kinoshita, & Kishimura, 2016
	AIYK (213) ^c – YKYY (64,2) ^c – KFYG (90,5) ^c – YNKL (21) ^c	<i>Undaria pinnatifida</i> (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	<i>Undaria pinnatifida</i> (wakame)	protease S “Amano”, from <i>Bacillus stearothermophilus</i>	Sato et al., 2002
Antinflammatory	VPY	soybean	<i>not available</i>	Nakamori, 2010; Kovacs-Nolan et al., 2012
	pyro-EL	wheat	protease from	Sato et al., 2013

			<i>Aspergillus oryzae</i>	
Antioxidant	FRDEHKK	rice	neutrased	Zhang, Zhang, Wang, Guo, Wang, & Yao, 2010
	YVAQGEVVA - 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 - VEVLPPEL	<i>Navicula incerta</i>	papain	Khang et al., 2012
	LNGDVW	<i>Clorrella ellipsoidea</i>	papain, trypsin, α chimtrypsin	Ko, Kim, & Jeon, 2012
Immunomodulatory	MITLAIPVKNPGR (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	<i>Chlorella vulgaris</i>	pepsin	Sheih, Fang, Wu, & Lin, 2010
	CPAP	<i>Chlorella pyrenoidosa</i>	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 <i>Bacillus amyloliquefaciens</i>	Cho, Juillerat, & Lee, 2008
	GGV - IVG - VGVL	amaranth	pepsin and trypsin	Soares, Mendonça, de Castro, Menezes, & Arêas, 2015

863

864 ^a The single-letter amino acid code is used

865 ^b IC₅₀ values are reported only for *in vitro* antihypertensive activity when available

866 ^c Antihypertensive activity against ACE activity

867 ^d Antihypertensive activity against renin

868 ^e (mg/mL)

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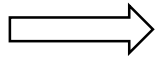
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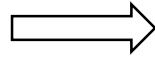
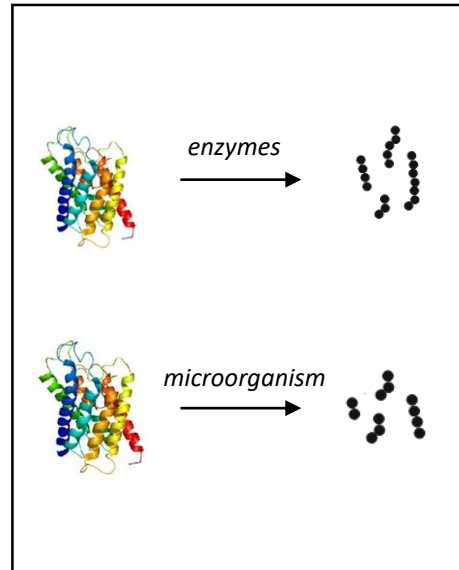
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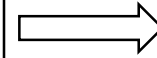
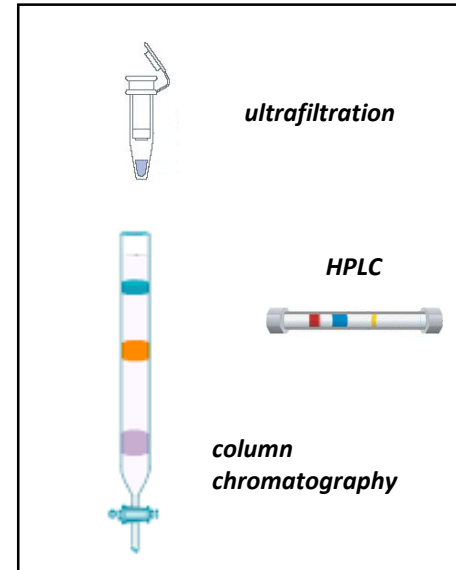
Food matrices



Protein hydrolysis



Fractionation and separation



Mass spectrometry

