

1 **Recent progress in enzymatic release of food-derived peptides**  
2 **and assessment of bioactivity**

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19 Running title: Enzymatic release of food bioactive peptides

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22 **Abstract**

23 There is a wide variety of peptides released from food proteins which are able to exert a  
24 relevant benefit for human health like angiotensin-converting enzyme (ACE) inhibition,  
25 antioxidant, anti-inflammatory, hypoglycemic or antithrombotic activity, among others.  
26 This manuscript is reviewing the recent advances on enzymatic mechanisms for the  
27 hydrolysis of food proteins, including the types of enzymes and mechanisms of action  
28 involved, the strategies followed for the isolation and identification of bioactive  
29 peptides through advanced proteomic tools, the assessment of bioactivity and its  
30 beneficial effects. Specific applications in fermented and/or ripened foods where a  
31 significant number of bioactive peptides have been reported with relevant *in vivo*  
32 physiological effects on laboratory rats and humans, as well as the hydrolysis of food  
33 proteins for the production of bioactive peptides are also reviewed.

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35 **Keywords:** Proteolysis, Bioactive peptides, Proteomics, Mass spectrometry, Enzyme  
36 hydrolysis, Peptidases

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## 39 INTRODUCTION

40 The content of proteins in foods is very significant and has a great nutritional relevance  
41 because they constitute the source of essential amino acids in the diet. However,  
42 proteins are subject of changes during food processing and cooking. In this sense, it is  
43 outstanding the relevant enzymatic hydrolysis of proteins taking place during food  
44 processing, especially during fermentation and/or ripening like in fermented sausages,  
45 dry-cured meats, cheese, yogurt, wine, etc., that generates polypeptides, peptides and  
46 free amino acids<sup>1-3</sup>. Such peptides are considered bioactive because, once released from  
47 the protein, they can contribute to a positive effect on consumers' health. Numerous  
48 bioactive peptides have been reported in cheese<sup>4</sup>, meat products like fermented sausages  
49 and dry-cured ham<sup>5</sup> and fishes<sup>6</sup>.

50 The activity of the generated bioactive peptides has been extensively studied *in vitro*  
51 and its physiological effects confirmed through *in vivo* assays with laboratory animals  
52 and in recent trials with humans. The most reported bioactivities are ACE inhibitory,  
53 antioxidant, antimicrobial, opioid, immunomodulating and antithrombotic. Milk-based  
54 products were the first reported to generate peptides with relevant bioactivity  
55 contributing to health<sup>7</sup>. Further, no changes in blood pressure were reported with daily  
56 consumption of Camembert cheese<sup>8</sup> or even decreased diastolic and systolic blood  
57 pressure with Gouda-type cheese<sup>9</sup>. Similar trend in reduction of blood pressure was  
58 reported with daily consumption of dry-cured ham<sup>10</sup> that was also reported to impair  
59 platelet and monocyte activation, and the levels of plasmatic P-selectin and interleukin 6  
60 in healthy humans<sup>11</sup>. Peptides from salmon and sardine were also reported to have  
61 antihypertensive effects in humans<sup>6</sup>.

62 Bioactive peptides can also be obtained in large amounts through reactor-controlled  
63 hydrolysis of extracted food proteins with commercial proteolytic enzymes or  
64 microorganisms<sup>12,13</sup>. Food by-products from meat, fish, dairy, olive oil and wine  
65 constitute typical sources of proteins to be hydrolysed and produce bioactive peptides at  
66 industrial scale<sup>12,14-17</sup>. Other hydrolyzates have been reported from eggs, peanut, and  
67 soybean proteins<sup>18-20</sup>.

68 The possibility of diseases prevention through the ingesta of bioactive peptides is of  
69 high interest to health authorities because it would contribute to reduce the budget for  
70 health care treatments. However, bioavailability of bioactive peptides must be taken into  
71 account since some of them may be susceptible to partial or total loss of activity due to  
72 further hydrolysis by salivary, stomachal, intestinal and pancreatic enzymes, and even  
73 intestinal microbiota during gastrointestinal digestion<sup>21,22</sup>. Furthermore, peptides may be  
74 subject of reactivity (i.e. deamination, decarboxylation, oxidation, etc.) with the food  
75 matrix, being reduced its bioactivity and/or bioavailability<sup>23</sup>.

76 This manuscript is reviewing the recent advances on the generation of bioactive  
77 peptides in fermented and ripened foods, the enzymatic mechanisms involved in the  
78 hydrolysis of food proteins, including the types of enzymes and mechanisms of action  
79 involved, the strategies followed for the isolation and identification of such peptides  
80 through advanced proteomic tools, the assessment of bioactivity and physiological  
81 effects, and specific applications.

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### 83 **ROUTES FOR ENZYMATIC HYDROLYSIS OF FOOD PROTEINS**

84 Food proteins are subjected to hydrolysis during processing. Such proteolysis is more  
85 extensive when the food is fermented and/or ripened. The enzymes involved are either

86 intrinsically endogenous in the food (i.e. muscle enzymes in meat or fish), or microbial  
87 peptidases present in the microorganisms responsible for food fermentation (i.e. lactic  
88 acid bacteria peptidases in fermented sausages). The enzymes responsible for  
89 proteolysis are endopeptidases, also known as proteinases, and exopeptidases. The  
90 mechanism of action consists of proteins break down by endopeptidases into  
91 polypeptides that constitute the substrates for the action of exopeptidases. In this way,  
92 polypeptides are further hydrolysed generating smaller peptides and free amino acids<sup>13</sup>.  
93 Depending on the length and sequence of residues, some of the released peptides may  
94 be bioactive<sup>24</sup>. Peptidomics has become a very useful tool for obtaining the peptide  
95 profiles of hydrolyzed foods and helpful for their identification and quantification<sup>25</sup>.

96 There are many types of exopeptidases depending on the action on N- or C-terminal, its  
97 specificity and generated product of reaction. So, tripeptidylpeptidases (TPP) release  
98 tripeptides and dipeptidylpeptidases (DPP) release dipeptides from the N-terminal. DPP  
99 II and IV cleave preferently Gly-Pro and Arg-Pro, DPP III prefer dipeptides Ala-Arg  
100 and Arg-Arg while DPP I prefer Ala-Arg and Gly-Arg<sup>26</sup>. X-prolyl dipeptidyl peptidase  
101 (PepX) releases dipeptides X-proline in the N terminal. The generated tripeptides can be  
102 further hydrolysed by tripeptidases into a dipeptide and a single amino acid. Further,  
103 dipeptides can also be hydrolysed by dipeptidases into the two single constituent amino  
104 acids<sup>27</sup>.

105 Free amino acids are also released from the N-terminal by several types of  
106 aminopeptidases (Pep N, Pep A, Pep C, Pep P among others). Amino acids can also be  
107 released from the C-terminal by carboxypeptidases A and B, named so because its  
108 activity is optimal at acid or basic pH, respectively. The consequence for the action of  
109 these enzymes is the generation of peptides with reduced length. A scheme of mode of  
110 action for different types of peptidases on a fragment of myosin heavy chain is shown in

111 **Figure 1.** For such particular case, it can be observed that endopeptidases act on the  
112 internal linkage Phe-Pro. Aminopeptidases would release Thr from the N-terminal that  
113 would be followed by the release of the dipeptide Val-Lys by a dipeptidylpeptidase. On  
114 the C-terminal, carboxypeptidase would release Asp and then Glu, followed by  
115 dipeptides Lys-Ile and Phe-Asp released by peptidyl dipeptidase and Lys by  
116 carboxypeptidase<sup>28</sup>.

117 The generation of bioactive peptides depends on the proteolysis phenomena but there  
118 are many variables affecting the enzyme action such as the food ingredients used, the  
119 type of enzymes and their activity, the microorganisms used for fermentation, and the  
120 applied processing conditions<sup>29</sup>. Preliminary information on the profile of expected  
121 small peptides may be obtained by using model systems representing the food. For  
122 instance, proteolysis was studied by using model fermented sausages inoculated with  
123 *Lactobacillus curvatus* CRL705 and *Staphylococcus vitulinus* GV318<sup>30</sup>.

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## 125 **Hydrolysis in foods by endogenous and microbial peptidases and mode of action**

126 The hydrolysis of proteins in foods may be carried out by endogenous or microbial  
127 peptidases (see **Figure 2**). The released peptides may be bioactive but they must be  
128 resistant to gastrointestinal digestion and further hydrolysis by brush border peptidases  
129 in the intestine membrane in order to exert its physiological effect in humans<sup>31</sup>.

130 Endogenous peptidases like muscle peptidases are able to release small peptides during  
131 the ripening and/or drying of meat products. So, DPP I and II that are active at pH 5.5-  
132 6.5, near the pH found in most meat products, can release dipeptides Ala-Gln, Arg-Gly,  
133 Asn-Pro, Ile-Leu, Ala-Gly, Ser-Gly, Ser-Gln, Pro-Ala among other from the N-  
134 terminal<sup>32</sup>. TPP I, also active at pH 5.5-6.5, releases specific tripeptides like Ile-Ile-Pro,

135 Arg-Gly-Ala, Gly-Asn-Pro, Gly-Ala-Gly, Gly-Pro-Gly from the N-terminal<sup>33</sup>. Pro, Lys  
136 and Ala are also released by aminopeptidases<sup>34</sup>. Several antioxidant peptides were  
137 reported in different types of dry-cured ham (see **Table 1**). Some of them are Asp-Leu-  
138 Glu-Glu in Xuanwei ham<sup>36</sup>, Gly-Lys-Phe-Asn-Val, Phe-Leu-Lys-Met-Asn, Gly-Lys-  
139 Phe-Asn-Val and Leu-Pro-Gly-Gly-Gly-His-Gly-Asp-Leu in Jinhua ham<sup>35</sup> and Ala-Glu-  
140 Glu-Glu-Tyr-Pro-Asp-Leu<sup>38</sup>, Ser-Asn-Ala-Ala-Cys<sup>42</sup> in Spanish ham and Met-Trp-Thr-  
141 Asp and Phe-Trp-Ile-Ile-Glu in mutton ham<sup>39</sup>. ACE inhibitory peptides Leu-Gly-Leu,  
142 Gly-Val-Val-Pro-Leu and Ser-Phe-Val-Thr-Thr were isolated from Parma ham<sup>37</sup> and  
143 Ala-Ala-Ala-Thr-Pro<sup>43</sup> and Thr-Lys-Tyr-Arg-Val-Pro from Spanish ham<sup>42</sup> were also  
144 reported. Peptides Ala-Ala-Ala-Ala-Gly, Ala-Leu-Gly-Gly-Ala and Leu-Val-Ser-Gly-  
145 Met showed inhibitory activity against DPP IV and were also isolated from Spanish  
146 ham<sup>58</sup>.

147 Most fermented foods today use microbial starters that improve safety and allow for a  
148 better standard quality. Such microorganisms have complex enzyme system exerting  
149 different types of activities able to hydrolyse proteins, carbohydrates and lipids<sup>59</sup>.

150 Peptidases from lactic acid bacteria (LAB), yeasts or molds may be helpful in  
151 generating bioactive peptides, especially in short term processed foods<sup>60</sup>. LAB  
152 constitutes a large group of microorganisms typically used for food fermentation and  
153 characterized by having a high proteolytic activity as a consequence of their  
154 extracellular cell wall serine proteinase and the content of diverse intracellular  
155 peptidases with a wide range of specificity. This explains that different peptides patterns  
156 are obtained for a particular food depending on the LAB strain used for fermentation.  
157 Yeasts are also used in food fermentation and are able to hydrolyze proteins<sup>61</sup>.  
158 Proteinases A and D, and prolyl and arginyl aminopeptidases were reported in

159 *Debaryomyces hansenii*<sup>61</sup> and PepX, leucine aminopeptidase, and DPP IV and V in  
160 *Aspergillus oryzae* and DPP V in *Aspergillus fumigatus*<sup>62,63</sup>.

161 Aminopeptidase activity is usually present in LAB so that its activity is particularly high  
162 in *Leuconostoc mesenteroides* and *L. curvatus* and variable between strains of *L.*  
163 *plantarum*, *L. pentosus* and *Weissella cibaria*<sup>64</sup>. Ala, Lys, Pro and Leu have been  
164 reported to be released from the N-terminal by strains of *L. plantarum* and *L. brevis*<sup>65</sup>.  
165 *L. casei* subsp *casei* is also able to release Ala, Arg, Lys, Met and Leu<sup>66-68</sup>. *L. sakei*  
166 releases Ala and Leu and *L. plantarum* releases Leu<sup>67,68</sup>. In general, aminopeptidases are  
167 partially inhibited by salt and therefore, their activity modulated in such fermented and  
168 ripened foods where salt is typically used<sup>69</sup>. There is a wide variety of intracellular  
169 aminopeptidases present in LAB as reported in the literature. So, aminopeptidase N,  
170 PepN is present in many bacteria like *L. Helveticus*, *L. lactis* and *L. sakei*, to release  
171 preferently Arg and Pro from the N-terminal<sup>70</sup>. Glutamyl (aspartyl) specific  
172 aminopeptidase PepA, that releases Glu and Asp from the N-terminal, is found in  
173 *Streptococcus cremoris*, *Streptococcus thermophilus*, *L. lactis* sp. and *L. delbrueckii*  
174 ssp. *Lactis*<sup>71</sup>. Aminopeptidase PepC is a thiol peptidase that hydrolyzes Ala, Leu or Lys  
175 from the N-terminal and has been reported in *Streptococcus thermophilus* and *L. lactis*  
176 ssp. *Cremoris*<sup>72</sup>. Proline aminopeptidase PepP that releases Arg, Met, Lys and Tyr at the  
177 N terminal is found in *L. lactis* ssp. *Lactis*<sup>73</sup>.

178 The activity of carboxypeptidases to release amino acids from the C terminal has been  
179 reported to be very low or negligible in cell-free extracts of several LAB<sup>65,68</sup> and low for  
180 the release of Phe and Arg by *L. paracasei* subsp *paracasei*<sup>66,67</sup>. On the contrary,  
181 carboxypeptidase activity in muscle foods is higher and, in fact, several amino acids like  
182 Phe, Tyr, Trp, Met, Ile, Leu, Val and Pro have been reported to be released from the C-  
183 terminal by carboxypeptidases A and B during the processing of dry-cured ham<sup>33</sup>.



184 X-prolyl dipeptidyl peptidase PepX has been reported in *Leuconostoc mesenteroides*, *L.*  
185 *curvatus* and *L. sakei*<sup>64,70,74</sup>. Several tripeptides X-Pro-Pro were found in casein  
186 hydrolysates with *L. helveticus*<sup>70</sup>. Tripeptidase activity was reported for *L. sakei* and  
187 also by Pep N in LAB<sup>59,75</sup>. Generation of dipeptides through DPP action has been  
188 reported for *L. paracasei* where dipeptides like Ala-Phe, Pro-Leu, Lys-Leu, Leu-Gly  
189 and Lys-Phe were reported in fermented foods<sup>66</sup>. DPP activity has also been reported in  
190 *Leuconostoc mesenteroides*, releasing dipeptides Arg-Pro and Gly-Phe and *L. paracasei*  
191 subsp *casei* also releasing Gly-Pro<sup>67</sup>. It must be taken into account that the released  
192 dipeptides might be further hydrolysed into their individual amino acids by microbial  
193 dipeptidases activity. In fact, dipeptides can cross the membrane thanks to cellular  
194 transport systems and be further hydrolyzed by dipeptidases in *L. sakei*<sup>75</sup>. In such cases,  
195 the dipeptide is no longer bioactive and therefore no health benefits may be expected.  
196 Dipeptidase activity has been reported in microorganisms like *L. plantarum*, *L. brevis*,  
197 *L. helveticus*, *L. casei* sp *casei* and *L. paracasei*. Their dipeptidases are able to  
198 hydrolyse preferentially dipeptides Leu-Leu, Phe-Ala, but also dipeptides Ala-Phe, Tyr-  
199 Leu and Lys-Leu, at lower rate. However, some dipeptides like Ala-Ala or Leu-Gly are  
200 resistant to hydrolysis<sup>68</sup>. Dipeptidase activity is also present in *L. brevis* that hydrolyzes  
201 dipeptides Leu-Leu, Tyr-Leu, Ala-Ala, Leu-Gly, Ala-Phe, Lys-Leu and Phe-Ala. Lower  
202 dipeptidase activity is also present in *L. casei* sp *casei*<sup>63,64,68</sup>. Oligopeptidase PepO is a  
203 metallopeptidase in *Streptococcus thermophilus* that is specific for peptides with  
204 arginine and methionine<sup>76,77</sup>.

205 Furthermore, the net amount of bioactive peptides is a balance that does not only  
206 depend on peptides generation through hydrolysis but also on cells consumption. In  
207 LAB, the transportation of oligopeptides through the cell membrane consists of 5  
208 proteins (OppA, B, C, D and F). This system, typical of lactobacilli and lactococci,

209 allows the transport of peptide chains of up to 12 amino acids<sup>78</sup>. *Streptococcus*  
210 *thermophilus* has lower activity of peptidases but this is compensated by more efficient  
211 transport of peptide chains of up to 23 amino acids integrated in the Ami system<sup>77</sup>.

212 *Lactobacillus helveticus* has been reported to hydrolyze K-casein and releases short  
213 peptides with a variety of bioactivities<sup>79</sup>. However,  $\beta$ -casein and  $\alpha_{s1}$ -casein found to be  
214 more resistant to hydrolysis probably due to the presence of phosphoserine in their  
215 respective structures<sup>80,81</sup> even though several peptides were reported to be generated  
216 from such  $\alpha_{s1}$ -casein in Brazilian Canastra artisanal cheese<sup>45</sup> and hard cow milk  
217 cheese<sup>46</sup>. Resistance to proteolysis by *L. acidophilus* LA-5 was reported for  $\alpha_{s2}$ -casein<sup>82</sup>.

218 Other authors reported that the abundance of Pro, Leu and Val in  $\beta$ -casein, that are  
219 preferred by aminopeptidases and carboxypeptidases, was the probable reason for better  
220 hydrolysis than other types of caseins<sup>27</sup>. In fact, hexapeptides Ala-Val-Pro-Tyr-Pro-Gln  
221 and Glu-Ala-Met-Ala-Pro-Lys with antioxidant activity were released from  $\beta$ -casein  
222 after simulated gastrointestinal digestion of Stracchino cheese that is produced in  
223 Northern Italy<sup>22</sup> and longer ACE inhibitory peptides in Brazilian Prato cheese<sup>47</sup>. A  
224 significant correlation between the release of ACE inhibitory peptides Val-Leu-Ser-  
225 Arg-Tyr-Pro and Leu-Arg-Phe-Phe and aminopeptidase and carboxypeptidase activity  
226 was reported in milk fermented with the yeast *Kluyveromyces marxianus* Z17<sup>53</sup>.

227 A recent research with *L. helveticus* LH-2 and *L. acidophilus* La-5 growing in whey  
228 protein isolate medium generated peptides with antivirulence effect against *Salmonella*  
229 *enterica* subsp. *enterica* serovar Typhimurium after growth. A large number of  
230 bioactive peptides, especially with ACE inhibitory activity were also reported to be  
231 generated for both strains<sup>83</sup>. The released peptides remained and accumulated in the  
232 media because they were not transported into the cells and thus were not further

233 hydrolysed due to their composition and low affinity to the oligopeptide-binding protein  
234 (OppA) of both strains<sup>83</sup>.

235 When using staphilococci for meat fermentation, they have been reported to exert  
236 proteolytic activity preferently on myofibrillar meat proteins and peptidases action  
237 might also be expected<sup>84</sup>. Dry-fermented sausages with *Lactobacillus pentosus* and  
238 *Staphylococcus carnosus* containing added sodium caseinate as ingredient were  
239 reported to generate large amounts of bioactive peptides<sup>27</sup>. Both microorganisms, *L.*  
240 *pentosus* and *S. carnosus* are able to hydrolyze casein extracellularly thanks to the  
241 proteinase attached to the cell wall. The generated oligopeptides can be transported into  
242 the cell for further hydrolysis by intracellular peptidases into smaller peptides and free  
243 amino acids<sup>85</sup>.

#### 244 **Hydrolysis of food proteins with commercial peptidases.**

245 Bioactive peptides are generally produced through the enzymatic hydrolysis of food  
246 proteins. Depending on the type of bioactivity searched, the protein source and degree  
247 of hydrolysis will be fixed<sup>86</sup>. Food proteins may be isolated and hydrolyzed in reactors  
248 using commercial peptidases or microorganisms with proteolytic activity in order to  
249 produce large amounts of bioactive peptides at industrial scale (see **Figure 2**). Typical  
250 commercial enzymes used for proteins hydrolysis are derived from cheap sources like  
251 microorganisms. This is the case of Alcalase from *Bacillus licheniformis*, Protamex  
252 from *Bacillus* sp., Flavorzyme from *Aspergillus oryzae*, Neutrased from *Bacillus subtilis*  
253 or *Bacillus amyloliquefaciens*, Bioprased from *Bacillus* sp., Thermolysin from *Bacillus*  
254 *stearothermophilus*, Prolidase from *Lactobacillus casei*, and Corolase 7089 from  
255 *Bacillus subtilis*, among other. Other enzymes may be obtained from animal and plant  
256 but the costs tend to be much higher. This is the case of trypsin from bovine or pig

257 pancreas, and bromelain from pineapple stem<sup>13</sup>. These enzymes have, in general, a  
258 broad specificity because they usually contain endopeptidases that can be combined  
259 with one or more exopeptidases<sup>87</sup>. An exception is Prolidase which is a dipeptidase<sup>88</sup>.  
260 Examples of food protein hydrolyzates, the enzymes and hydrolysis conditions used, the  
261 main obtained peptides and major assayed bioactivity are reported in **Table 2**. As can be  
262 observed in the table, most of the sequences of bioactive peptides contain less 10 amino  
263 acids residues. Longer peptides like those generated from the hydrolysis of spent hens<sup>99</sup>,  
264 duck<sup>100</sup>, goat milk<sup>103</sup> or *Spirulina platensis* algae<sup>92</sup> may be subject of further hydrolysis  
265 during gastrointestinal digestion. In general, the most usual recovered bioactivities are  
266 ACE inhibitory and antioxidant activity. In some cases, peptides with anti-inflammatory  
267 and antidiabetic activities are also obtained. Peptide Asp-Gly-Val-Val-Tyr-Tyr with  
268 outstanding ACE inhibitory activity, IC<sub>50</sub>=2 μM, was obtained through the fermentation  
269 of tomato seeds with *Bacillus subtilis*<sup>106</sup>.

270 Defatted salmon backbones were hydrolysed with commercial enzymes obtaining  
271 protein hydrolysates with bioactivity. Hydrolysis with trypsin gave the highest ACE  
272 inhibitory, bromelain and papain gave the best cellular glucose transporter  
273 (GLUT/SGLT) inhibitory activity and the highest antioxidant activity was obtained  
274 hydrolyzing with protamex<sup>107</sup>.

275

## 276 **IDENTIFICATION OF BIOACTIVE PEPTIDES**

277 Traditionally, proteomics are used for the identification of proteins through the previous  
278 analysis of the peptides generated from their controlled hydrolysis using trypsin  
279 enzyme. This experimental methodology is called “bottom-up” approach and uses  
280 peptide mass fingerprint (PMF) for the final identification of the protein of origin.

281 However, the generation of bioactive peptides frequently occurs during the processing  
282 of foods or during gastrointestinal digestion, where the action of endogenous, microbial,  
283 or gastrointestinal enzymes results on unspecific peptide sequences that cannot be  
284 trypsin-digested due to their small size. Thus, the classic PMF approach, oftenly used in  
285 proteomics, is not useful, and it is thus necessary to adapt strategies used for the  
286 identification of proteins such as tandem mass spectrometry (MS) and modern  
287 bioinformatics tools<sup>108,109</sup>.

288 In this sense, peptidomics would permit the identification of the peptides generated  
289 during different food processes or controlled hydrolysis although the identification of  
290 naturally generated peptides is very difficult because: (i) the analysis of small bioactive  
291 peptides is near the limits of standard MS techniques, and (ii) longer peptides face up  
292 the difficulty to control hydrolysis. The major challenge is the complexity of the  
293 numerous peptides released and furthermore the associated difficulty due to the  
294 unspecific cleavage sites in proteins<sup>25</sup>.

295 The identification of bioactive peptides from complex food matrices has been  
296 traditionally done using empirical approaches including (i) the release of bioactive  
297 sequences from the parent protein; (ii) a preliminary separation to screen the bioactivity  
298 using *in vitro* assays; (iii) a secondary purification and separation of the fractions  
299 showing the best bioactivity using high-resolution techniques; (iv) additional *in vitro*  
300 assays to determine the most active fractions; (v) identification of peptides included in  
301 those active fractions using MS in tandem; and (vi) the synthesis of the identified  
302 sequences in order to confirm their *in vitro* and *in vivo* bioactivity<sup>108</sup>. A scheme of the  
303 traditional empirical procedure followed for bioactive peptides is shown in **Figure 3**.

304 The development of this approach is very challenging as there are multiple factors to  
305 consider that could finally affect the generation of the bioactive peptides and it results  
306 very complicated when the objective is the generation of controlled sequences showing  
307 certain activity of interest. In this case, the use of in silico approaches considering  
308 different bioinformatics tools for computer simulation results very useful and permits to  
309 choose/discard between different experimental procedures in a reasonable amount of  
310 time and low economical cost. In silico procedures will permit to select best protein of  
311 origin and proteolytic enzymes to obtain certain peptide sequences as well as predict  
312 their bioactivity, structure, and physical-chemical properties. After the simulation  
313 studies, the confirmation of in silico results is done through a traditional empirical  
314 approach<sup>110</sup>. **Figure 4** shows the main steps followed for the identification of bioactive  
315 peptides through computational prediction.

316

## 317 **MAJOR BIOACTIVITIES OF RELEASED PEPTIDES**

318 The health benefits of fermented foods like antioxidant, antiinflammatory,  
319 antihypertensive, antidiabetic, antimicrobial, etc., are most times associated to the  
320 generated bioactive peptides as reported in the literature<sup>111</sup>. Tripeptides Val-Pro-Pro and  
321 Ile-Pro-Pro generated in fermented milk are well known for their high ACE inhibitory  
322 activity. A meta-analysis of the relevant literature on the effect of both tripeptides on  
323 blood pressure in humans was recently performed<sup>50</sup> revealing that there was a  
324 significant but low hypotensive effect on blood pressure when those tripeptides were  
325 included in the diet. In fact, the observed effect was much lower than many  
326 antihypertensive drugs<sup>50</sup>.

327 The bioactivity of released peptides is always tested *in vitro* using different assays  
328 depending on the expected activity. However, in order to prove and confirm the  
329 bioactivity of the peptides, subsequent *in vivo* tests are done using cellular models, rat  
330 models, or even clinical trials with humans. In this regard, *in vitro* results do not  
331 guarantee a real physiological effect. Quite often, peptides with a high bioactivity *in*  
332 *vitro* are inactive after oral administration<sup>112</sup>. The reason is that, once ingested, peptides  
333 can be hydrolyzed by salivary, gastric and intestinal enzymes so that those peptides with  
334 longer sequences may be further hydrolyzed into smaller size peptides and therefore,  
335 lose their bioactivity. Small bioactive peptides can be hydrolyzed in the intestine by  
336 peptidases of the microbial flora or by brush border peptidases in the epithelium of the  
337 intestinal membrane. Finally, the released peptides have to cross the intestinal  
338 membrane and reach the bloodstream in order to exert its physiological benefit (see  
339 **Figure 2**).

340 The bioactivity of the generated peptides also depends on the amino acid composition of  
341 the sequence and its size, but peptide structure and hydrophobicity also play an  
342 important role influencing the accessibility of the peptides to the active sites of the  
343 enzymes<sup>113</sup>.

#### 344 **4.1 ACE-inhibitory peptides**

345 The ACE-inhibitory activity is the most extensively studied bioactivity in relation to  
346 food-derived peptides. Main interest is due to the ability of ACE-inhibitory peptides to  
347 prevent hypertension by decreasing the blood pressure. Its mechanism of action is based  
348 on the inhibition of ACE enzyme that converts the inactive decapeptide angiotensin-I  
349 into the potent vasoconstricting octapeptide angiotensin-II, whereas also inactivates the  
350 vasodilator bradykinin, resulting in an increase in blood pressure. Thus, by inhibiting

351 the catalytic action of ACE, the hypertension can be regulated by reducing the blood  
352 pressure in the body.

353 Currently, thousands of potential ACE-inhibitory peptides have been isolated and  
354 identified from food products after fermentation or curing processes such as dry-cured  
355 ham, cheese, yogurt, and other fermented products, as well as from the controlled  
356 digestion using commercial enzymes such as trypsin, Corolase, Thermolysin, Alcalase,  
357 as well as controlled microbial fermentation, in food products such as fish, algae or  
358 meat. In this respect, **Tables 1 and 2** show examples of bioactive peptides that have  
359 been described in the literature with respective calculated IC<sub>50</sub> values. However, the  
360 identification of ACE -inhibitory peptides is of high interest and other interesting  
361 sequences have been described in mushroom<sup>114</sup>, and cereals such as wheat, quinoa and  
362 corn<sup>115-117</sup>.

#### 363 **4.2 Antioxidant peptides**

364 Antioxidant peptides are the second most studied group of food-derived peptides with  
365 biological activity. These peptides can act as antioxidants in foods, naturally protecting  
366 against oxidation, avoiding sensory and nutritional defects that are frequently associated  
367 with oxidative patterns. On the other hand, antioxidant peptides can also exert their  
368 function after ingestion in the human body, decreasing the negative effects of reactive  
369 oxygen species (ROS) and the risk for development of some degenerative diseases such  
370 as cardiovascular diseases or certain types of cancer<sup>118</sup>.

371 The mechanism of action for antioxidant peptides can be very variable, depending on  
372 the transference mechanism. Certain mechanisms like ORAC and TRAP are based on  
373 hydrogen atom transfer mechanism while other such as DPPH and ABTS are based on  
374 electron transfer<sup>119</sup>. The antioxidant activity strongly depends on their composition in



375 amino acids. So, peptides containing His, Tyr, Met, Lys and Trp are more able to exert  
376 antioxidant activity.

377 Carnosine and anserine are the two most abundant natural antioxidant peptides in foods,  
378 as they are very common in fish and meat products. However, many different peptides  
379 showing antioxidant activity have been described to be generated during the processing  
380 of some products such as dry-cured ham<sup>38,42,120</sup>, mutton ham<sup>49</sup>, cheese<sup>46</sup>, yogurt<sup>48</sup>, or  
381 fermented fish<sup>56</sup>. Also the use of commercial enzymes alone or in combination has  
382 resulted in extensive hydrolysis generating antioxidant peptides in algae, fish, legumes  
383 or meat as shown in **Tables 1 and 2**.

#### 384 **4.3 Anti-obesity and antidiabetic peptides**

385 Obesity is the most important risk factor for type-2 diabetes, involves the accumulation  
386 of fat in the body, and it is associated to numerous health problems also related to  
387 cardiovascular diseases. Synthetic drugs are frequently used as anti-obesity substances  
388 with the disadvantage of showing multiple negative side effects. For this reason, the  
389 search for natural peptides derived from food sources is of high interest. Apolipoprotein  
390 A-I, melanocortin-4 receptor-specific agonist, GLP-1 dual and triple agonists,  
391 neuropeptides and prolactin-releasing peptide mimetics are the most studied for anti-  
392 obesity properties<sup>121</sup>.

393 On the other hand, diabetes mellitus is characterised by insufficient insulin production  
394 or insulin resistance, and the potential peptides that participate in the control of glucose  
395 level in carbohydrates pathway are  $\alpha$ -amylase and  $\alpha$ -glucosidase inhibitors, and  
396 dipeptidyl peptidase-IV inhibitors<sup>122</sup>.

397 The most studied food-related peptides showing anti-obesity properties have been  
398 soybean peptides due to their body fat-decreasing characteristics<sup>123,124</sup>. On the other

399 hand, milk has been described to suppress appetite due to its content in satiating  
400 peptides, preventing weight gain and obesity. In this sense, camel milk peptides  
401 displayed novel antidiabetic and anti-obesity activity<sup>125,126</sup>. Also peptides derived from  
402 the controlled digestion of algae have been described as antidiabetic ( $\alpha$ -amylase  
403 inhibitory) and anti-obesity<sup>91,92,127</sup>.

#### 404 **4.4 Anti-inflammatory activity**

405 The inflammation is the response of the body to local injury or infection, where it is  
406 necessary to fight infection and repair the tissue. However, excessive and uncontrolled  
407 inflammation is often associated with chronic diseases<sup>99,128,129</sup>.

408 Anti-inflammatory peptides might participate in multiple physiological systems by  
409 modulating or regulating the inflammatory response. However, as food-derived  
410 bioactive peptides are ingested, the regulation of gastrointestinal system has been the  
411 most studied<sup>130</sup>. The oxidative stress is often associated with inflammatory processes.  
412 However, there are other complex mechanisms related to the renin-angiotensin-  
413 aldosterone system (RAAS), proinflammatory cytokines, proinflammatory signalling  
414 kinases, and integrin-dependent signalling<sup>51</sup>. Anti-inflammatory peptides from milk,  
415 egg, fish and soy have been reported<sup>129,131,132</sup>.

#### 416 **4.5 Antimicrobial activity**

417 Certain peptides are effective against certain bacteria like *Staphylococcus aureus* and  
418 *Escherichia coli* and yeasts. They can exert such antimicrobial activity by defending the  
419 organism against pathogens as well as in food by preventing its contamination. They  
420 can interact with the bacterial cells by nonreceptor-mediated or receptor-mediated  
421 mechanisms and invader cells by disturbing the membrane integrity<sup>133,134</sup>.

422 Antimicrobial peptides are generated during the processing of foods such as  
423 fermentation, and during controlled hydrolysis using commercial enzymes. They have  
424 been isolated from fish and marine products<sup>135,136</sup>, milk and milk products<sup>137,138</sup>, meat  
425 products<sup>40</sup>, legumes<sup>139</sup> and eggs<sup>140</sup>.

426 In summary, bioactive peptides can be generated either endogenously in food or through  
427 enzymatic hydrolysis of extracted food proteins. Depending on the particular food  
428 protein, a pool of peptides may be obtained. Those peptides with smaller size may be  
429 more bioaccessible and exhibit bioactivity that, depending on the sequence, can be  
430 either ACE inhibitory, antioxidant, antithrombotic, hypoglycemic, hypocholesterolemic,  
431 or antimicrobial among others. In any case, bioactive peptides must be bioavailable to  
432 exert its physiological action in the way that they must be resistant to gastrointestinal  
433 digestion and be able to be absorbed through the intestinal barrier and reach the  
434 bloodstream.

435

#### 436 **ABBREVIATIONS USED**

437 ABTS: 2, 2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) radical scavenging  
438 assay; ACE: Angiotensin converting enzyme;  $\beta$ -CBA:  $\beta$ -carotene bleaching activity;  
439 Ch: Fe<sup>2+</sup>-chelating activity; DPP: dipeptidylpeptidases; DPPH: 2, 2-diphenyl-1-picryl-  
440 hydrazyl radical scavenging activity; LAB: Lactic acid bacteria; MS: mass  
441 spectrometry; ORAC: oxygen radical absorbance capacity; PMF: peptide mass  
442 fingerprint; RAAS: renin-angiotensin-aldosterone system; ROS: Radical oxygen  
443 species; RP: reducing power; TRAP: total radical trapping antioxidant parameter;  
444 DPPH: radical scavenging activity; TPP: tripeptidylpeptidases.

445 Three letter abbreviations for amino acids are used. Ala: Alanine; Arg: Arginine; Asn:  
446 Asparagine; Asp: Aspartic Acid; Cys: Cysteine; Gln: Glutamine; Glu: Glutamic acid;  
447 Gly: Glycine; His: Histidine; Ile: Isoleucine; Leu: Leucine; Lys: Lysine; Met:  
448 Methionine; Phe: Phenyl alanine; Pro: Proline; Ser: Serine; Thr: Threonine; Trp:  
449 Tryptophan; Tyr: Tyrosine; Val: Valine.

450

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455

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463 All authors of this manuscript declare that they do not have any conflict of interest. All  
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465

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923

924 **LEGENDS FOR THE FIGURES**

925

926 Figure 1.- Scheme of food protein hydrolysis and enzymes involved. The amino acids  
927 sequence is a fragment belonging to myosin heavy chain. Aminopeptidase (A),  
928 Dipeptidylpeptidase (D), Endopptidase (E), Carboxypeptidase (C) and  
929 Peptidylpeptidase (P). Adapted from (29).

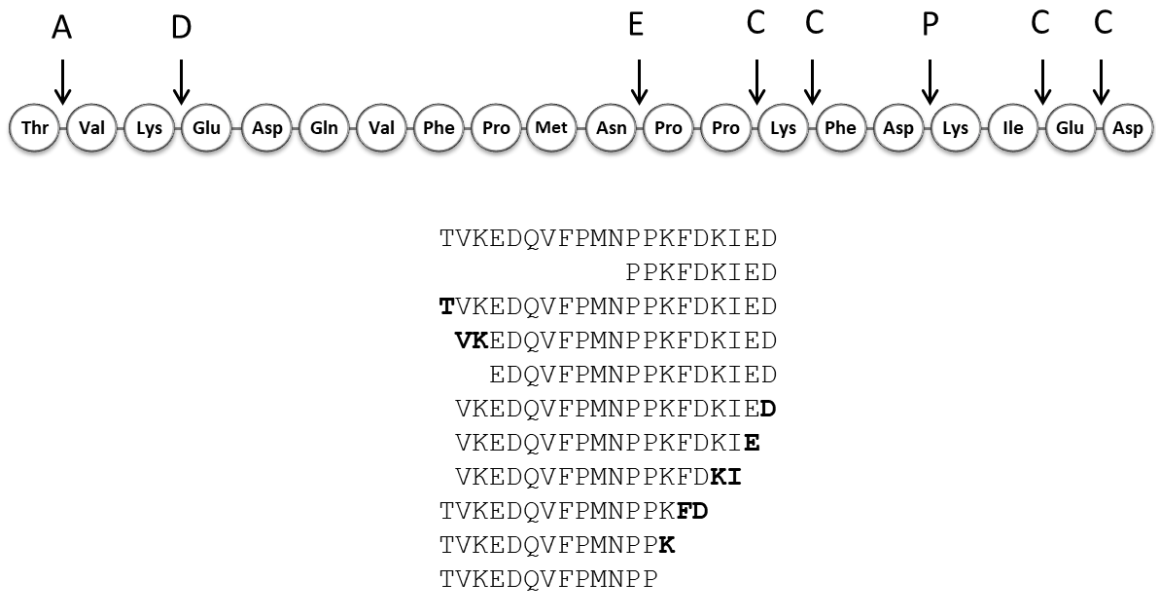
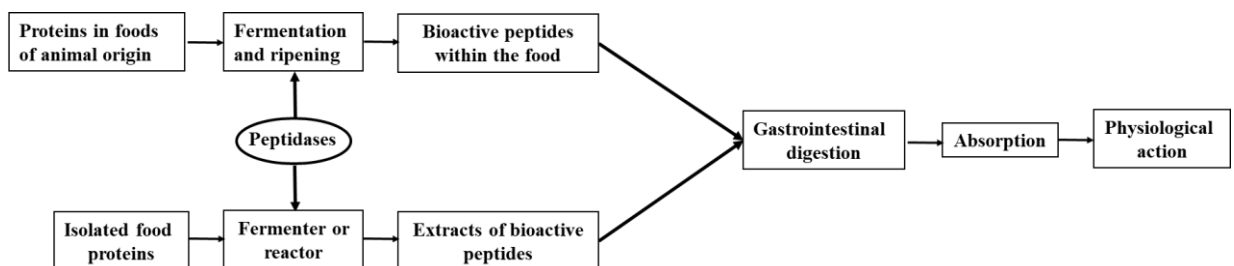


Figure 1

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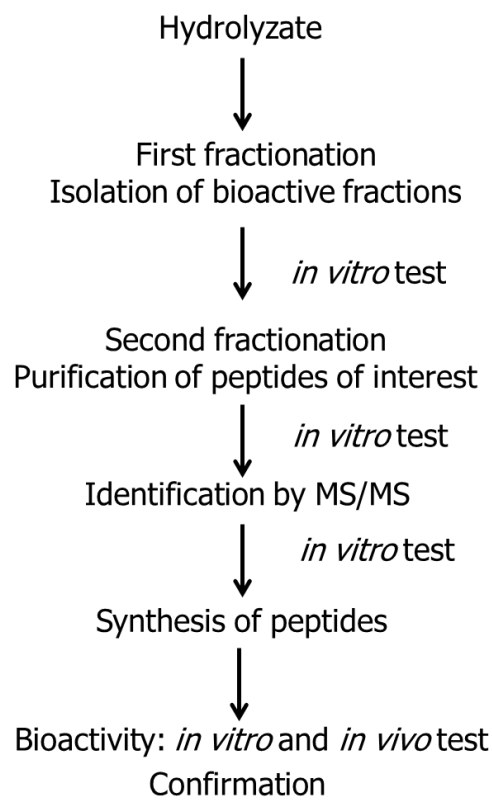
932 Figure 2.- Scheme of the generation of bioactive peptides from protein hydrolysis in  
933 foods and/or the hydrolysis of isolated food proteins.



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936 Figure 3.- Scheme of the traditional empirical procedure for the identification and  
937 confirmation of bioactive peptides from food matrices. SEC: size-exclusion  
938 chromatography; CE: capillary electrophoresis; LC: liquid chromatography; IEF:  
939 isoelectric focusing; HPLC: high performance liquid chromatography; MS/MS: mass  
940 spectrometry in tandem. Adapted from (84).



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943 Figure 4.- Main steps of *in silico* approaches and open access databases for the selection  
944 of the protein, hydrolysis simulation and bioactivity prediction. Adapted from (84).

***In silico* approach**

Selection of the protein



Hydrolysis simulation



Bioactivity prediction



**Confirmation**



Synthesis of peptides



Bioactivity assays *in vitro* and *in vivo*

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Table 1.- Examples of bioactive peptides recently identified in fermented and dry-cured products.

Food	Type / Fermentation	Peptide sequence	Parent protein	Potential activity	Activity values*	Reference
Dry-cured ham	Chinese Jinhua	FLKMN	—	Antioxidant	DPPH: 70% at 1 mg/mL	35
		GKFNV	—	Antioxidant	DPPH: 92.7% at 1 mg/mL	35
		LPGGGHGDL	—	Antioxidant	OH: 85% at 1 mg/mL	35
	Chinese Xuanwei	DLEE	—	Antioxidant	DPPH: 74.4% at 0.5 mg/mL	36
Italian Parma		GVVPL	—	Antihypertensive	ACE inhibition: IC <sub>50</sub> = 956 μM	37
		LGL	—	Antihypertensive	ACE inhibition: IC <sub>50</sub> = 145 μM	37
		SFVTT	—	Antihypertensive	ACE inhibition: IC <sub>50</sub> = 395 μM	37
Spanish		AEEEYPDL	Creatine kinase	Antioxidant	ABTS: 1474.08 nmol TEAC/mg, ORAC: 960.04 nmol TE/mg	38
		FNMPLTIRITPGSKA	LIM domain-binding 3	Anti-inflammatory	PAF-AH: 26.06 % at 1mM	39
				Antihypertensive	68.34% at 1mM	39
		HCNKKYRSEM	Dynein heavy chain	Antimicrobial	MIC ( <i>L. monocytogenes</i> )= 50 mM	40
			Anti-inflammatory	LOX: 23.33% at 1mM	41	

			Antioxidant	ORAC: 1767.56 nmol TE/mg	41
			Antihypertensive	ACE inhibition: 99.34% at 1 mM	41
	MDPKYR	Titin	Antimicrobial	MIC ( <i>L. monocytogenes</i> )= 50 mM	40
			Anti-inflammatory	PAF-AH: 13.48% at 1mM, ATX: 14.51% at 1mM	41
			Antioxidant	ABTS: 5444.3 nmol TEAC/mg, ORAC: 3087.5 nmol TE/mg	41
			Antihypertensive	ACE inhibition: 60.64% at 1mM	41
	SNAAC	Myosin heavy chain	Antioxidant	ABTS: 3097.04 nmol TEAC/mg, ORAC: 2737.4 nmol TE/mg	42
	TKYRVP	Titin	Anti-inflammatory	PAF-AH: 11.04% at 1mM, ATX: 22.47% at 1mM	41
			Antioxidant	ABTS: 6987.8 nmol TEAC/mg, ORAC: 2886.8 nmol TE/mg	41
			Antihypertensive	ACE inhibition: 80.85% at 1mM	41
	AAATP	Allantoicase	Antihypertensive	ACE inhibition: IC <sub>50</sub> = 100,00 μM	43
	TSNRYHSYPWG	Ser/Thr-protein kinase	Anti-inflammatory	PAF-AH: 16.30 % at 1mM, ATX:18.93% at 1mM	41
			Antioxidant	ABTS: 3036.03 nmol TEAC/mg	41
			Antihypertensive	ACE inhibition: 71.62% at 1mM	41
Mutton ham	MWTD	—	Antioxidant	ABTS: IC <sub>50</sub> = 0.4 mg/mL	44
	APYMM	—	Antioxidant	ABTS: IC <sub>50</sub> = 0.12 mg/mL	44
	FWIIE	—	Antioxidant	ABTS: IC <sub>50</sub> = 0.23 mg/mL	44

Cheese	Italian <i>Stracchino</i>	AVPYPQ	$\beta$ -Casein	Antioxidant	ABTS: 19.5 $\mu$ mol TE/mg	22
		EAMAPK	$\beta$ -Casein	Antioxidant	ABTS: 22.9 $\mu$ mol TE/mg	22
Brazilian Canastra artisanal Minas	RPKHPIKHQ	$\alpha_{S1}$ -Casein	Antimicrobial	MIC ( <i>E. coli</i> )= 15 $\mu$ g/mL	45	
		$\alpha_{S1}$ -Casein	Antimicrobial	MIC ( <i>E. coli</i> )= = 17 $\mu$ g/mL	45	
Hard cow milk cheese	EIVPN	$\alpha_{S1}$ -Casein	Antioxidant	DPPH inhibition, Metal chelating activity	46	
	DKIHPF	$\beta$ -Casein	Antioxidant	DPPH inhibition, Metal chelating activity	46	
	VAPFPQ	$\alpha_{S1}$ -Casein	Antioxidant	Metal chelating activity	46	
Brazilian Prato / <i>Lactobacillus helveticus</i> (10%, 40°C, 18h)	QEPVLGPVRGPFPIV	$\beta$ -Casein	Antihypertensive	ACE inhibition	47	
	YQEPVLGPVRGPFPP	$\beta$ -Casein	Antihypertensive	ACE inhibition	47	
Yoghurt	Chinese Feng Wei Suan Ru / <i>Streptococcus thermophellolus</i> + <i>Lactobacilus bulgaricus</i>	FVAPFPEVF	$\alpha_{S1}$ -Casein	Antidiabetic	DPP-IV inhibition: IC <sub>50</sub> = 2.52 $\mu$ M	48
				Antihypertensive	ACE inhibition: IC <sub>50</sub> = 35.76 $\mu$ M	48
		PPFLQPEVM	$\beta$ -Casein	Antidiabetic	DPP-IV inhibition: IC <sub>50</sub> = 0.44 $\mu$ M	48
				Antihypertensive	ACE inhibition: IC <sub>50</sub> = 34.63 $\mu$ M	48
		QEPVLGPVRGPFPIV	$\beta$ -Casein	Antihypertensive	ACE inhibition: IC <sub>50</sub> = 160.76 $\mu$ M	48
Probiotic yoghurt with pineapple peel / <i>S. thermophilus</i> + <i>L.</i>	SLPQNIPPLTQTPVVVPPF	$\beta$ -Casein	Antioxidant	ABTS: IC <sub>50</sub> = 1.44 mg/mL, OH <sup>-</sup> : 34.97% at 1 mg/mL	49	

	<i>bulgaricus</i> + <i>L. acidophilus</i> + <i>L. casei</i> + <i>L. paracasei</i> (1%, 42°C, pH 4.5)	YQEPVLGPPVRGPFPIIV	$\beta$ -Casein	Anticancer	Antiproliferation colon cancer cells: 38.55% at 3 mg/mL	49
				Antioxidant	ABTS: IC <sub>50</sub> = 29.88 $\mu$ g/mL	49
				Anticancer	Antiproliferation colon cancer cells: 41.49% at 3 mg/mL	49
Fermented milk	<i>Lactobacillus, Saccharomyces</i>	IPP, VPP	$\beta$ -Casein	Antihypertensive	SBP: -2.95 mmHg	50
				Anti-inflammatory	Suppression of cytokine mediated inflammatory responses	51
				Adipogenic	Insulin-mimetic adipogenic effects	51
				Antidiabetic	Insulin sensitizing actions in adipocytes	52
	<i>Kluyveromyces marxianus</i> (6%, 32°C, pH 6.5, 48h)	LRFF	$\kappa$ -Casein	Antihypertensive	ACE inhibition: IC <sub>50</sub> = 116.9 $\mu$ M	53
		VLSRYP	$\alpha$ <sub>S1</sub> -Casein	Antihypertensive	ACE inhibition: IC <sub>50</sub> = 36.7 $\mu$ M	53
Kombucha culture (1%, 37°C, 72h)		FVAPEPFVFGKEK	$\alpha$ <sub>S1</sub> -Casein	Antihypertensive	ACE inhibition: IC <sub>50</sub> = 0.75 $\mu$ M	54
		LVYFPFGPLH	$\beta$ -Casein	Antihypertensive	ACE inhibition: IC <sub>50</sub> = 0.03 $\mu$ M	54
		VAPFPEVFGK	$\alpha$ <sub>S2</sub> -Casein	Antihypertensive	ACE inhibition: IC <sub>50</sub> = 0.03 $\mu$ M	54
<i>Lactobacillus casei</i> (1%, 37°C, 72h)		LVESPELNTVQ	$\kappa$ -Casein	Antihypertensive	ACE inhibition: IC <sub>50</sub> = 0.11 $\mu$ M	54
		VLESPELN	$\kappa$ -Casein	Antihypertensive	ACE inhibition: IC <sub>50</sub> = 0.23 $\mu$ M	54
		WGYLAYGLD	—	Antihypertensive	ACE inhibition: IC <sub>50</sub> = 0.10 $\mu$ M	54

Fermented cucumber pickles	<i>Lactobacillus pentosus</i> (28°C, 43d)	IPP	—	Antihypertensive	ACE inhibition: IC <sub>50</sub> = 5 μM	55
		KP	—	Antihypertensive	ACE inhibition: IC <sub>50</sub> = 22 μM	55
		LPP	—	Antihypertensive	ACE inhibition: IC <sub>50</sub> = 9.6 μM	55
		VPP	—	Antihypertensive	ACE inhibition: IC <sub>50</sub> = 9 μM	55
Fermented fish	Malaysian pekasam / <i>Lactobacillus plantarum</i> (27°C, 15d)	AIPHPYYP	—	Antioxidant	IC <sub>50</sub> (mg/mL): DPPH = 1.38, ABTS = 0.87, RP = 0.45	56
		IAEVFLITDPK	—	Antioxidant	IC <sub>50</sub> (mg/mL): DPPH = 0.89, ABTS = 0.594, RP = 0.69	56
Fermented shrimp pastes	Thai Kapi Ta Dam	IF	—	Antihypertensive	ACE inhibition: IC <sub>50</sub> = 70.03 μM	57
		SV	—	Antihypertensive	ACE inhibition: IC <sub>50</sub> = 60.68 μM	57
		WP	—	Antioxidant	ABTS: EC <sub>50</sub> = 17.52 μM	57

\* Activity values: IC<sub>50</sub> value is the peptide concentration that inhibits 50% of activity. SBP is the maximum decrease in systolic blood pressure after administration of the peptides to human subjects. Antioxidant activity: ABTS

radical-scavenging activity (ABTS), DPPH radical scavenging assay (DPPH), hydroxyl radical scavenging activity (OH<sup>-</sup>), and oxygen radical absorbance capacity assay (ORAC). MIC is the minimum concentration of peptide that inhibits the visible growth of bacteria. Anti-inflammatory activity: platelet-activating factor-acetylhydrolase inhibition (PAF-AH), lipoxygenase inhibition (LOX), and autotaxin inhibition (ATX).

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951 Table 2.- Examples of bioactive peptides recently identified in hydrolyzates of different types of foods.

Food	Type	Treatment hydrolysis	Peptide sequence	Parent protein	Potential activity	Activity values*	Reference
Algae	<i>Gracilariopsis lemaneiformis</i> (Rhodophyta)	Trypsin (2%, 2h)	FQIN[M(O)]CILR	—	Antihypertensive	ACE inhibition: IC <sub>50</sub> = 9.64 μM, SBP: -34 mmHg (2h)	89
			TGAPCR	—	Antihypertensive	ACE inhibition: IC <sub>50</sub> = 23.94 μM, SBP: -28 mmHg (2h)	89
	<i>Palmaria palmata</i>	Corolase PP (2%, 50°C, pH 7, 4h)	SDITRPGGQM	Allophycocyanin β-chain	Antioxidant	ORAC: 152.43 nmol TE/μmol, RP: 21.23 nmol TE/μmol	90
Red seaweed ( <i>Porphyra spp</i> )		Pepsin (1%, 37°C, pH 2, 3h)	GGSK	—	Antidiabetic	α-Amylase inhibition: IC <sub>50</sub> = 2.58 mM	91
			ELS	—	Antidiabetic	α-Amylase inhibition: IC <sub>50</sub> = 2.62 mM	91
<i>Spirulina platensis</i>		Pepsin (6%, 37°C, pH 2, 10h)	CANPHELPNK	—	Anti-obesity	Antiproliferation adipocytes: 60.08% at 2 mg/mL Triglyceride accumulation: -19.5% at 600 μg/mL	92
			LNNPVCDCDCMMKAAR	—	Anti-obesity	Antiproliferation adipocytes: 32.29% at 2 mg/mL	92
			NALKCCHSCPA	—	Anti-obesity	Antiproliferation adipocytes: 37.86% at 2 mg/mL	92
			NPVWKRK	Hydrolase protein	Anti-obesity	Antiproliferation adipocytes: 46.89% at 2 mg/mL Triglyceride accumulation: -23.7% at 600 μg/mL	92
Fish	Atlantic salmon ( <i>Salmo salar</i> )	Corolase PP (1%, 50°C, pH 7, 1h)	GPAV	—	Antihypertensive	ACE inhibition: IC <sub>50</sub> = 415.91 μM	93
					Antidiabetic	DPP-IV inhibition: IC <sub>50</sub> = 245.58 μM	93
					Antioxidant	ORAC: 9.51 μmol TE/μmol	93

		FF	—	Antihypertensive	ACE inhibition: IC <sub>50</sub> = 59.151 μM	93
				Antidiabetic	DPP-IV inhibition: IC <sub>50</sub> = 546.84 μM	93
				Antioxidant	ORAC: 8.47 μmol TE/μmol	93
Cuttlefish ( <i>Sepia officinalis</i> )	<i>Bacillus mojavensis</i> (3U/mg, 50°C, pH 10)  Cuttlefish hepatopancreas enzymes (3U/mg, 50°C, pH 8)	AFVGYVLP	—	Antihypertensive	ACE inhibition: IC <sub>50</sub> = 18.02 μM	94
		EKSYELP	—	Antihypertensive	ACE inhibition: IC <sub>50</sub> = 14.41 μM	94
		VELYP	—	Antihypertensive	ACE inhibition: IC <sub>50</sub> = 5.22 μM, SBP: -20 mmHg (6h)	94
Leatherjacket ( <i>Meuschenia</i> sp.)	Insoluble bromelain (0.5%, 50°C, 2h)	AER	—	Antihypertensive	ACE inhibition: IC <sub>50</sub> = 0.11 g/L	95
		EQIDNLQ	—	Antihypertensive	ACE inhibition: IC <sub>50</sub> = 0.24 g/L	95
	Insoluble papain (0.5%, 50°C, 6h)	DPHI	—	Antihypertensive	ACE inhibition: IC <sub>50</sub> = 0.02 g/L	95
		EPLYV	—	Antihypertensive	ACE inhibition: IC <sub>50</sub> = 0.05 g/L	95
	Insoluble flavourzyme (1.25%, 50°C, 2h)	WDDME	—	Antihypertensive	ACE inhibition: IC <sub>50</sub> = 0.01 g/L	95
Sardinelle ( <i>Sardinella aurita</i> )	<i>Bacillus amyloliquefaciens</i> (4%, 37°C, 24h)	ITALAPSTM	Actin	Antihypertensive	ACE inhibition: IC <sub>50</sub> = 0.23 mM	96
				Antioxidant	β-CBA: IC <sub>50</sub> = 0.64 mM	96
		SLEAQAEKY	Tropomyosin	Antihypertensive	ACE inhibition: IC <sub>50</sub> = 0.41 mM	96
			Antioxidant	RP, ORAC	96	
		GTEDELDKY	Tropomyosin	Antioxidant	DPPH: IC <sub>50</sub> = 1.32 mM, RP, ORAC	96
	<i>Bacillus subtilis</i> (4%, 37°C, 24h)	NVPVYEGY	Actin	Antihypertensive	ACE inhibition: IC <sub>50</sub> = 0.21 mM	96

					Antioxidant	DPPH: IC <sub>50</sub> = 1.41 mM, RP, ORAC	96
	Pacific herring ( <i>Clupea pallasii</i> )	Trypsin (1.39U/Kg, 32.06°C, pH 6.78, 7h)	KEEKFE	—	Antioxidant	IC <sub>50</sub> (mg/mL): OH= 3.78, DPPH = 4.37, Cell = 1.04	97
			LHDELT	—	Antioxidant	IC <sub>50</sub> (mg/mL): OH= 4.57, DPPH = 5.14, Cell = 1.19	97
Legumes	Soy	Alkaline proteinase (6U/Kg, 50°C, pH 9)	LLPLPVLK	—	Antidiabetic	α-Glucosidase inhibition: IC <sub>50</sub> = 237.43 μM	97
			SWLRL	—	Antidiabetic	α-Glucosidase inhibition: IC <sub>50</sub> = 182.05 μM	97
			WLRL	—	Antidiabetic	α-Glucosidase inhibition: IC <sub>50</sub> = 162.29 μM	97
	<i>Erythrina edulis</i> (pajuro)	Alcalase (0.5%, 50°C, pH 8.3, 2h)	CCGDYY	—	Antioxidant	ABTS: 1.18 μmol TE/μmol, ORAC: 3.61 μmol TE/μmol	98
			DGLGYY	—	Antioxidant	ABTS: 0.63 μmol TE/μmol, ORAC: 3.83 μmol TE/μmol	98
			GESWCR	—	Antioxidant	ABTS: 1.12 μmol TE/μmol, ORAC: 2.43 μmol TE/μmol	98
			SQLPGW	—	Antioxidant	ABTS: 0.53 μmol TE/μmol, ORAC: 2.95 μmol TE/μmol	98
			WAL	—	Antioxidant	ABTS: 0.58 μmol TE/μmol, ORAC: 3.38 μmol TE/μmol	98
			YDLHGY	—	Antioxidant	ABTS: 0.64 μmol TE/μmol, ORAC: 3.59 μmol TE/μmol	98
Mea	Spent hens	Protex 50FP (4%, 50°C, pH 3, 3h)	AFMNVKHWPW	Myosin	Anti-inflammatory	IL-6 inhibition: 59% at 100 μg/mL	99
			FLWGKSY	Myomesin	Anti-inflammatory	IL-6 inhibition: 79% at 100 μg/mL	99
			SFMNVKHWPW	Myosin	Anti-inflammatory	IL-6 inhibition: 68% at 100 μg/mL	99
			WPW	Myosin	Anti-inflammatory	IL-6 inhibition: 63% at 100 μg/mL	99
	Duck ( <i>Anas platyrhynchos</i> )	Protamex (0.75%, 50°C, pH 6, 4h)	AGRDLTDYLMKIL	—	Antioxidant	DPPH: 85.45%, OH= 30.75%, Fe-Ch=74.74% at 1mg/mL	100

			GYDLGEAEFARIM	—	Antioxidant	DPPH: 91.81%, OH= 31.30%, Fe-Ch=58.94% at 1mg/mL	100
			IEDPFDQDDWGAWKK	—	Antioxidant	DPPH: 90.39%, OH= 46.51% at 1mg/mL	100
			LQAEVEELRAALE	—	Antioxidant	DPPH: 93.36%, OH= 20.52%, Fe-Ch=87.13% at 1mg/mL	100
			NWDDMEK	—	Antioxidant	DPPH: 16.36%, OH= 43.34%, Fe-Ch=37.20% at 1mg/mL	100
Kacang goat ( <i>Capra aegagrus hircus</i> )	Protamex + Flavourzyme (0.5%, 50°C, pH 7, 4h)	FQPS	Actin	Antihypertensive	ACE inhibition: IC <sub>50</sub> = 27.0 µM, SBP: - 10.6 mmHg (8h)	101	
Pork loin	Thermolysin (0.008%, 5°C, 24h)	LVGRPRHGQ	—	Antihypertensive	ACE inhibition: IC <sub>50</sub> = 15.69 µM	102	
		VFPS	—	Antihypertensive	ACE inhibition: IC <sub>50</sub> = 3.60 µM	102	
Milk	Goat ( <i>Capra hircus</i> ) milk	Trypsin (3%, 37°C, pH 8, 3h)	INNQFLPYPY	κ-Casein	Antidiabetic	DPP-IV inhibition: IC <sub>50</sub> = 40.08 µM	103
			MHQPPQPL	β-Casein	Antidiabetic	DPP-IV inhibition: IC <sub>50</sub> = 350.41 µM	103
			SPTVMFPPQSVL	β-Casein	Antidiabetic	DPP-IV inhibition: IC <sub>50</sub> = 376.31 µM	103
By-products	Chicken combs and wattles	Alcalase (5%, 4h)	APGLPGPR	Collagen and elastin	Antihypertensive	ACE inhibition: IC <sub>50</sub> = 53 µM	104
			FPGPPGP	Collagen and elastin	Antihypertensive	ACE inhibition: IC <sub>50</sub> = 38 µM	104
			Piro-GPPGPT	Collagen and elastin	Antihypertensive	ACE inhibition: IC <sub>50</sub> = 88 µM	104
	Oil palm ( <i>Elaeis guineensis Jacq</i> ) kernel expeller	Alcalase (0.5%, 45°C, pH 8.5, 2h) + flavourzyme (0.5%, 50°C, pH 7, 2h) + pepsin (0.3%, 37°C, pH 2, 1h)+ trypsin (0.3%, 37°C, pH 7, 1h).	ADVFNPR	Glutelin-2	Antihypertensive	ACE inhibition: IC <sub>50</sub> = 485.7 µM	105
			LPILR	Glutelin-2	Antihypertensive	ACE inhibition: IC <sub>50</sub> = 779.8 µM	105

		VIEPR	Glutelin-2	Antihypertensive	ACE inhibition: IC <sub>50</sub> = 632.0 μM	105
		VVLYK	Glutelin-2	Antihypertensive	ACE inhibition: IC <sub>50</sub> = 533.9 μM	105
Tomato seeds	<i>Bacillus subtilis</i> (2%, 37°C, 24h)	DGVVYY	—	Antihypertensive	ACE inhibition: IC <sub>50</sub> = 2 μM	106
		GQVPP	—	Antioxidant	DPPH: 97% at 0.4mM, RP: 0.95 UA at 0.5 mM	106

\* Activity values: IC<sub>50</sub> value is the peptide concentration that inhibits 50% of activity. SBP is the maximum decrease in systolic blood pressure after administration of the peptide to spontaneously hypertensive rats. Antioxidant activity: ABTS radical-scavenging activity (ABTS),

Radical scavenging assay (DPPH), hydroxyl radical scavenging activity (OH<sup>-</sup>), β-carotene bleaching activity (β-CBA), reducing power (RP), oxygen radical absorbance capacity assay (ORAC), cytotoxic effects on HepG2 cells (Cell), and Fe<sup>2+</sup>-chelating activity (Ch).