Bioactive peptides as natural antioxidants in food products – A review

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Abstract

Background: Over the last years, bioactive antioxidant peptides, extracted from food proteins, have been studied due to their potential as useful tools in the development of new natural drugs and food ingredients. These compounds can be used to decrease oxidative stress and food quality decay, thus being an interesting strategy to reduce economic losses in food production, as well as improve public health.

Scope and Approach: Antioxidant peptides are extracted from non-antioxidant precursor proteins from different origin by the activity of either proteolytic microorganisms or isolated enzymes. In the present review, the main sources of bioactive peptides will be discussed. Moreover, the current strategies to obtain these compounds as well as their health benefits and *in vivo* biological effects will be evaluated. Considerations for further research and development of strategies to increase the knowledge about this underexplored activity of peptides will be also considered.

Key Findings and Conclusions: Bioactive peptides´ content and profile differ according to the matrix studied and the method used. The utilization of fermentation processes and enzymes has been established to obtain antioxidant bioactive peptides from proteins, being isolated enzymes the most commonly used method due to their improved control over releasing and obtaining targeted peptides. Antioxidant peptides have the ability to reduce the formation of oxidative products along with the induction of antioxidant enzymes *in vivo*. However, at this stage of development more *in vivo* studies are needed in order to evaluate the specific effects on health of selected antioxidant peptides. In food technology, successful application in meat products strengthens the role of selected peptides as antioxidant additives, although there is

a need to observe the effects of the isolated bioactive peptides in other food matrices along with studies to scale-up its production.

Keywords: Proteolysis; active amino acid sequence; antioxidant defense; oxidative stress; food quality; food additives

1. Introduction

The oxidative balance is a critical and delicate status derived from the overcoming production of reactive species in living organisms due to endogenous reactions (such as phagocytosis and respiratory chain) and exposure to physical and chemical agents (*e.g.* UV radiation and air pollutants). Once reactive species are formed, several vital molecules (lipids, DNA, and proteins) and processes can be affected, which causes a disturbance on cell homeostasis and induce the development of severe diseases such as atherosclerosis and cancer (Lobo, Patil, Phatak, & Chandra, 2010).

Living organisms possess a complex protective system, which is activated to prevent the oxidative damage. In this line of defense, enzymatic and non-enzymatic antioxidant agents promote oxidative balance by reducing the concentration of reactive species and forming less reactive compounds. However, this line of defense can be overwhelmed by the constant generation of reactive species, thus being required additional protection to balance the oxidative status (Bouayed & Bohn, 2010).

The importance of antioxidants is recognized by the World Health Organization that has been arguing in favor of worldwide increasing consumption of dietary sources of antioxidants, being food intake the main form to acquire these compounds (WHO, 1990). The relevance of antioxidants in living organisms, along with increasing media divulgation, increased the efforts to characterize known sources of natural antioxidants (Brewer, 2011; Granato, Nunes, & Barba, 2017; Maiani et al., 2009; Pandey & Rizvi, 2009).

However, the ability to prevent oxidative reactions is not exclusive to well-known natural antioxidants. Peptides can exert antioxidant activity by the same mechanisms as those observed for other antioxidants. The exploration of antioxidant peptides requires additional technologies to release active amino acid sequences from proteins since the precursor proteins may not display the same antioxidant effect (Chi et al., 2014; Gallego, Mora, Hayes, Reig, & Toldrá, 2017; Homayouni-Tabrizi, Asoodeh, & Soltani, 2017; Jemil et al., 2016; Rizzello et al., 2017; Sachindra & Bhaskar, 2008).

The importance of such characteristics of peptides has been discussed in several reviews available in the scientific literature. For instance, Sarmadi and Ismail (2010), in an overview perspective, discussed several aspects related to antioxidant peptides. Authors highlighted the importance of bioactive peptides, particularly for allergic reactions, that may preserve part of its precursor protein allergenic activity. Liu et al. (2016) explored the peptide composition in meat and meat products, along with the biological and potential role in pharmacological applications. Halim, Yusof, and Sarbon (2016), evaluated the releasing, technological application such as water and fat holding capacity, and intended health benefits of antioxidant activity and other biological activities of peptides extracted from fish proteins. In a similar way, Mohanty et al. (2016) reviewed how digestion, fermentation and enzymatic activity affected the releasing of bioactive peptides from milk proteins. The authors also reported important findings related to biological activity, particularly as potential therapeutic agents against non-communicable diseases (*e.g.* hypertension and immunological diseases).

In all reviews, meat, fish, and milk proteins were shown as important sources of bioactive peptides. In a similar way, Sila and Bougatef (2016) supported the exploration of marine by-products as potential sources of antioxidant peptides and suggested the potential and significant application of these compounds in complex food systems. The production of antioxidant peptides of vegetable origin has been also discussed by Rizzello et al. (2016), who also agreed regarding the previous applications of bioactive peptides.

The biological importance of antioxidant peptides was the focus of Chakrabarti, Jahandideh, and Wu (2014). The authors stated that bioactive peptides can improve the actual frame of nutraceutical and functional foods by improving the biological defenses against oxidative stress inflammatory diseases. In a similar way, Cicero, Fogacci, and Colletti (2016) highlighted the multivariate activities that peptides can exert, particularly for heart-related diseases.

However, the biological activity of dietary antioxidants has some controversies. The first point of discussion is the relevance of pro-oxidant compounds in living cells that exerted a relevant role in some cell signaling pathways and a beneficial effect associated to oxidative stress. Another point in this context is the contrasting results reported in several studies

104 suggesting either no effect or potential negative effects in certain diseases. The influence of
105 experimentation level (*in vitro*, *in vivo*, and clinical trials) is believed to have a significant impact
106 on results. In addition, natural antioxidants can exert pro-oxidant activity, which induces
107 oxidative stress, and few is known about the interaction with medication and supplements
108 (Pham-Huy, He, & Pham-Huy, 2008; Carocho & Ferreira, 2013).

109 At this stage of development, an integrated approach to explore both medicinal/therapeutic
110 effects and successful integration to food matrix can shed some light on this new approach to
111 obtain and explore antioxidants from natural sources. **Figure 1** summarizes the beneficial
112 activities of bioactive peptides. Therefore, in the present review, the current and future
113 strategies to selectively release antioxidant peptides from several sources are explored.
114 Moreover, the therapeutic activity of antioxidant peptides along with their *in vivo* biological
115 effects are evaluated. Finally, the impact of their addition to foodstuff (as antioxidant additive)
116 on quality attributes are also discussed.

117 2. Strategies applied to promote the release of antioxidant peptides from protein
118 precursors

119 The release of antioxidant peptides can be promoted by the activity of endogenous and
120 exogenous microorganisms and proteolytic enzymes (**Figure 2**). The use of proteolytic
121 microorganisms, either autochthonous or exogenous, is one of the strategies to break proteins
122 and release antioxidant peptides. The other approach consists in the exploitation of
123 endogenous or exogenous proteolytic enzymes to break proteins into peptides. The traditional
124 processing of food use both microorganisms and enzymes to change food structure. Moreover,
125 both approaches have a deep impact on peptide profile and content as proteolysis
126 progressively evolves during processing.

127 However, the activity of both agents has already been explored in the production of food
128 but with a traditional perspective: achieve expected sensorial and physico-chemical
129 characteristics. For instance, traditional processing of dry-cured hams can be briefly explained
130 by simple steps: salting with solid salt followed by dry ripening, both under controlled

temperature and relative humidity for several months until achieving the targeted characteristics (*e.g.* moisture content lower than 60%) (Bermúdez, Franco, Carballo, & Lorenzo, 2014). The exogenous and endogenous proteases, such as cathepsins, calpains, peptidases, and aminopeptidases, progressively break down sarcoplasmic and myofibrillar proteins and derived peptides for several months. The peptides generated may have molecular weight between 2700 and 4500 Da on early stages of ripening while peptides below 2700 Da and amino acids can be produced at the end of ripening period (Toldrá, 2006).

Likewise, cheese processing requires the addition of microorganisms and/or enzymes to achieve expected characteristics such as texture and flavour. At this stage of processing, cheese is held under controlled temperature, which favours proteolysis during long periods. The release of antioxidant peptides mainly occurs during ripening (Barac et al., 2016; Erkaya & Şengul, 2015; Gupta, Mann, Kumar, & Sangwan, 2009; Timón, Parra, Otte, Broncano, & Petrón, 2014). Several proteolytic agents can be involved in the formation of peptides such as the coagulant, milk enzymes, enzymes produced by starter, non-starter and secondary cultures, and exogenous enzymes. Although the main targets are the proteins known as caseins (α-, β-, and κ-casein), whey proteins (α- and β-lactoglobulin) can be also potential targets of microbial enzymes (Sousa, Ardö & McSweeney, 2001). In this sense, the final meat and dairy products contain a unique composition of peptides that is suggested to exert antioxidant activity *in loco*.

A biochemical approach in the release of antioxidant peptides has been explored by different researchers over the last decades. Selected microorganisms and enzymes have been used to break down proteins from many protein sources to obtain antioxidant peptides. The ultimate goal of such strategy is the use of the released peptides as food antioxidants. Proteases play a central role to optimize this process due to specific cleavage sites on proteins. For instance, trypsin (protease of natural occurrence in the digestive system of vertebrates) exclusively cleaves the peptide bonds of C-terminal in the presence of arginine or lysine (Olsen, Ong, & Mann, 2004). On the other hand, alcalase (produced by *Bacillus licheniformis*) displays wider specificity than other enzymes due to the production of peptides containing

159 glutamic acid, methionine, leucine, tyrosine, lysine, and glutamine (Adamson & Reynolds,
160 1996).

161 Moreover, pepsin (found in the stomach of humans and many animals) cleaves the peptide
162 bond after phenylalanine and leucine (Hamuro, Coales, Molnar, Tuske, & Morrow, 2008)].
163 Pancreatin (a combination of lipase, amylase and proteolytic enzymes) preferentially break
164 peptide bonds at the N-terminal phosphorylated region and the C-terminal hydrophobic regions
165 (Su et al., 2012). Finally, papain (naturally present in papaya) cleavage the peptide bonds of
166 hydrophobic regions that include the amino acids alanine, valine, leucine, isoleucine,
167 phenylalanine, tryptophan, and tyrosine (Schechter & Berger, 1968).

168 **3. Antioxidant hydrolysates and peptides**

169 Antioxidant compounds exert their activity by two main mechanisms: hydrogen transfer and
170 electron donation. However, the classification of methods is difficult due to the simultaneous
171 occurrence of both mechanisms in widely utilized antioxidant methods such as 2,2-diphenyl-
172 1-picrylhydrazyl (DPPH) and Trolox equivalent antioxidant capacity (TEAC) (Barba, Esteve,
173 Tedeschi, Brandolini, & Frígola, 2013). Although the use of *in vitro* (test-tube) antioxidant
174 capacity has generated a controversy over the last years due to the impossibility of
175 extrapolating the results to *in vivo* (human) effects (Anonymous, 2016), these methods are still
176 of great importance to reveal and facilitate the selection of potential antioxidant compounds in
177 complex solutions, which can be used for different food science and technology applications
178 (Touati, Barba, Louaileche, Frigola, & Esteve, 2016). The differences in the mechanism and
179 other crucial factors involved in the interaction of reactive molecules and antioxidants (*e.g.*
180 solubility and affinity) demand more than one methodology to characterize and interpret the
181 antioxidant activity of target compounds (Karadag, Ozcelik, & Saner, 2009).

182 Among the several methodologies applied to characterize the antioxidant capacity of
183 fermented foods, hydrolysates, fractions, and isolated peptides from several sources, the most
184 widely used methods are: i) 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS); ii)
185 2,2-diphenyl-1-picrylhydrazyl (DPPH); iii) hydroxyl and superoxide anion radical scavenging

activity; iv) Ferric Reducing Antioxidant Power (FRAP); v) metal chelating activity (MCA); and vi) Oxygen Radical Absorbance Capacity (ORAC) (**Tables 1-4**).

The hydrogen donating protocols (such as ORAC) have more relevance in the context of chain breaking reactions and its reaction is usually completed in up to few minutes. On the other hand, electron transfer protocols (*e.g.* ABTS and DPPH) involve a probe that changes its maximum absorbance due to antioxidant compound and are also relatively simple and fast methods (Karadag, Ozcelik & Saner, 2009; MacDonald-Wicks, Wood & Garg, 2006).

The relative simple protocol and reliable results obtained from most of these methodologies have increased the popularity among researchers on related areas. ABTS method can estimate the antioxidant potential of both hydrophilic and hydrophobic antioxidants, is simple and fast, and does not depend on pH of medium but the reaction time is compound-dependent and ABTS radical is not naturally found in biological systems (Barba, Esteve, Tedeschi, Brandolini, & Frígola, 2013). DPPH assay is fast, simple, require relatively simple equipment (*e.g.* spectrophotometer), and can be also used to measure multiple samples. However, this method requires an organic medium, has a maximum water content level before radical coagulation, and its maximum absorbance may overlap with that of tested antioxidant. In the same line, FRAP assay is considered as a simple, low-cost, and robust procedure. The main disadvantages of this method are: i) the sensibility to electron donating compound, ii) inaccurate results for samples contaminated with Fe(III) and iii) the lack of reactivity with hydrogen atom transfer antioxidants can be listed. Finally, ORAC assay can be applied to evaluate the capacity to break radical chain reactions for both hydrophilic and hydrophobic antioxidants but total estimation of ORAC value demands the evaluation of both hydrophilic and hydrophobic antioxidants and adaptations in the original method usually reduces the method sensibility (Karadag, Ozcelik & Saner, 2009). The use of several antioxidant assays may be seen as an important step in the characterization of the mechanism involved on the activity of hydrolysates and isolated peptides but at the same time the lack of standardization makes it difficult to compare between studies.

3.1. Endogenous enzymes and autochthonous microorganisms

The characterization and quantification of several antioxidant peptides in food, as a consequence of endogenous enzymes and autochthonous microorganisms, has been carried out in several studies. For instance, many studies can be found in the literature reporting the occurrence of antioxidant peptides in dry-cured hams (Escudero, Aristoy, Nishimura, Arihara, & Toldrá, 2012; Kęska, Libera, & Stadnik, 2017; Mora, Escudero, & Toldrá, 2016). The evaluation of antioxidant peptides extracted from Spanish dry-cured revealed that peptides SAGNPN and GLAGA displayed the highest antioxidant activity and reducing power, respectively, among the identified peptides (Escudero, Mora, Fraser, Aristoy, & Toldrá, 2013). Likewise, the peptide DLEE was identified as one of the main antioxidant compounds in dry-cured Xuanwei ham (Xing et al., 2016).

On the other hand, the peptide SNAAC, with a molecular weight of 464.17 Da, was reported from the degradation of myosin heavy chain protein and gave a large antioxidant activity, near the positive control BHT (butylated hydroxytoluene) (**Figure 3**). Evaluation of antioxidant activity for SNAAC revealed: IC_{50} value of 75.2 μM in DPPH radical scavenging assay and 205 μM in ferric-reducing antioxidant power capacity (Mora, Escudero, Fraser, Aristoy & Toldrá, 2014). Such peptide showed good heat stability after exposure to temperatures up to 90 °C, remained stable in the presence of NaCl, and was effective to inhibit almost half of linoleic acid oxidation (Gallego, Mora & Toldrá, 2018a). However, a sensible reduction of the antioxidant activity of the peptide SNAAC was reported after its simulated gastrointestinal digestion because it lost its terminal cysteine residue giving the tetrapeptide SNAA (Gallego, Mora & Toldrá, 2018a).

More recently, the antioxidant peptide AEEEYPDL was also isolated and identified in dry-cured ham (Gallego, Mora & Toldrá, 2018b). The concentration of this peptide was found to be 0.148 fg/g of dry-cured ham, was also resistant to different heat treatments and salt contents but lost its antioxidant activity after simulated gastrointestinal digestion because it was cleaved by pepsin into the smaller peptides AEEEY and PDL (Gallego, Mora & Toldrá, 2018b).

240 Recently, the generation of bioactive peptides was also described during the aging of beef
241 meat under chilled storage for up to 4 weeks. In addition, the effect of cooking and
242 gastrointestinal digestion on the ACE-inhibitory and antioxidant activity was studied (Mora,
243 Bolumar, Heres & Toldrá, 2017).

244 Mejri, Vásquez-Villanueva, Hassouna, Marina, & García (2017) observed a significant
245 increase in the production of antioxidant peptides during camel sausage ripening for 28 days,
246 particularly in the release of peptides of molecular weight above 3 kDa. The accumulation of
247 antioxidant peptides achieved the highest antioxidant values at the end of ripening, measured
248 by ABTS, DPPH, inhibition of hydroxyl radicals, and FRAP assays. In addition, the identification
249 of amino acid sequence revealed the production of 13-22 common peptides of molecular
250 weight <3 kDa among inoculated and non-inoculated batches. However, the presence of
251 autochthonous microorganisms in control batch had lower potential to produce antioxidant
252 peptides in comparison to inoculated batches.

253 In cheese, proteolytic enzymes alter the peptide profile and composition. Timón et al.
254 (2014), selected a fraction of peptides with molecular weight < 3 kDa for antioxidant evaluation
255 (DPPH and metal chelating activity) from Burgos cheese. This study revealed that animal
256 rennet (conventional processing; 95% chymosin and 5% bovine pepsin) used to produce
257 Burgos cheese was efficient to produce antioxidant peptides to scavenge DPPH radical and
258 chelate Fe^{2+}. However, the antioxidant potential of the same peptide fraction (<3 kDa) obtained
259 from inoculated Burgos cheese was higher than observed for control batch. In this sense, the
260 technological use of microorganisms and enzymes has an important role in the improvement
261 of antioxidant peptides release.

262 *3.2. Use of microorganisms*

263 Several studies in the scientific literature have evaluated the potential effect of bacterial strains
264 in the production of antioxidant peptides (**Table 1**). For instance, the growth of *Lactobacillus*
265 *plantarum* strains in whole bovine milk led to the production of crude extracts rich in
266 antioxidants (Aguilar-Toalá et al., 2017). After the isolation of two fractions (<3 kDa and 3-10

kDa), the authors observed a strong relationship between small peptides (molecular weight <3 kDa) with antioxidant activity measured by DPPH and ABTS assays. The same research group also observed that peptides of molecular weight in the range 3-10 kDa also contributed to antioxidant potential of fermented whole bovine milk. Moreover, other studies also support the use of different lactic acid bacteria such as *L. helveticus* (Elfahri, Vasiljevic, Yeager, & Donkor, 2016), *L. rhamnosus*, *L. paracasei*, and *L. casei* (Solieri, Rutella, & Tagliazucchi, 2015) to induce the release of antioxidant peptides. These studies strengthen the importance of selecting lactic acid bacteria species and strains.

The fermentation of sardinelle, zebra blenny, goby, and ray meat protein by *Bacillus subtilis* produced hydrolysates rich in peptides and amino acids with antioxidant activity. However, the authors also observed that in all antioxidant assays, BHA displayed significant higher values than protein hydrolysates at the same concentration (Jemil et al., 2014). In a recent study, the same research group identified 800 peptides, mainly from myosin, in sardinelle protein fermented by *B. subtilis* and *B. amyloliquefaciens*. They selected 15 short peptides (8 from *B. subtilis* and 7 from *B. amyloliquefaciens* fermentation) for synthesis and antioxidant assays since large and long peptides may be degraded or not absorbed by enterocytes. The highest antioxidant potential was obtained for synthesized peptide NVPVYEGY in DPPH, RP, ORAC and β-carotene bleaching assays (Jemil et al., 2016).

In another study, Sachindra & Bhaskar (2008) explored the production of antioxidant peptides from shrimp waste by *Pediococcus acidolactici* fermentation. The authors attributed the antioxidant activity observed in the fermented extracts to peptides and amino acids of molecular weight <19.4 kDa.

Moreover, foods of vegetable origin and residues from industrial processing are also interesting sources of antioxidant peptides (Barba, Esteve, & Frigola, 2014). The fermentation of quinoa flour by *L. plantarum* (22 strains), *L. rossiae* (1 strain) and *P. pentosaceus* (3 strains) produced fermented quinoa flour extracts of high antioxidant activity (Rizzello et al., 2017). The authors observed that antioxidant activity was strain-dependent wherein *L. plantarum* strains displayed the highest capacity to breakdown quinoa proteins to antioxidant. In addition, the

295 identification of 5 antioxidant peptides (IVLVQEG, TLFRPEN, VGFGI, FTLIIN, and
296 LENSGDKKY) on fermented quinoa flour had molecular weight < 1.2 kDa (Rizzello et al.,
297 2017).

298 Moayedi, Mora, Aristoy, Hashemi, Safari, and Toldrá (2017), prepared a tomato seed meal
299 from tomato wastes and used it to produce antioxidant peptides through fermentation with
300 *Bacillus subtilis*. The hydrolysate showed a 2-fold higher percentage of aromatic amino acids
301 which in addition to the increase in hydrophobic amino acids resulted in a higher antioxidant
302 activity. The peptidomic analysis revealed 10 antioxidant peptides of molecular mass below 1
303 kDa. The most antioxidant was the peptide GQVPP with very close activity to BHT used as
304 control, probably due to the presence of Gln, Val, and Pro.

305 In a similar way, mold growth can be explored to produce antioxidant peptides. The
306 fermentation of soybean flour by *Aspergillus oryzae* produced an extract of high antioxidant
307 activity. In this study, most of the peptides on antioxidant extracts, evaluated by DPPH and
308 inhibition of linoleic acid autoxidation assays, had molecular weight <3 kDa (Lee, Rho, Kim,
309 Lee, & Lee, 2013).

310 Sun et al. (2015) studied the production of antioxidant peptides from cottonseed meal, a by-
311 product of cottonseed oil production, after fermentation by *B. subtilis*. Authors obtained
312 fermented extracts rich in peptides of molecular weight <1 kDa and concentration-dependent
313 antioxidant activity on DPPH, hydroxyl radical activity, metal-chelating ability, and reducing
314 power assays (0.5-8 mg/mL). This study also evaluated the protective potential of antioxidant
315 peptides against H_2O_2 in cultured cells. Decreasing of cell viability was partially inhibited by
316 antioxidant peptides in a concentration-dependent manner (0.01-2.5 mg/mL). The
317 microbiological activity on dietary and processing waste proteins can be effectively break-down
318 into peptides and yield hydrolysates of improved antioxidant activity. Due to several enzymes
319 produced during fermentation, a broader view of possible antioxidant peptides can be
320 obtained. Moreover, the fermentation of food also increases microbial stability, improves flavor
321 and aroma, and contributes to improving the value of the final product.

3.3. Use of isolated enzymes

The role of enzymes is directly associated with the effective breakdown of proteins into antioxidant peptides. Many studies in the scientific literature have explored the direct application of isolated microbial enzymes in the release of antioxidant peptides (**Table 2**). The enzyme thermolysin was used to generate peptides from bovine liver, a by-product of meat processing, that were separated according to molecular weight (<3 and 3-10 kDa). The authors observed similar antioxidant potential among peptide fractions and hydrolysates. The identification of antioxidant peptides revealed that main compounds were an amino acid sequence consisting of 2 peptides in the <3 kDa fraction and 42 peptides in the 3-10 kDa fraction (Di Bernardini et al., 2011). Furthermore, a recent study revealed that bones from Spanish dry-cured hams could be exploited as potential sources of antioxidant peptides. The effect of cooking and gastrointestinal digestion on the antioxidant activity of hydrolysates was studied (Gallego et al., 2017).

The hydrolysate produced by trypsin activity on Monkfish muscle proteins was studied by Chi et al. (2014). The authors isolated and identified 3 peptides (EWPAQ, FLHRP, and LMGQW) that displayed antioxidant activity in a concentration-dependent manner according to DPPH, hydroxyl and superoxide anion radical scavenging activity as well as lipid peroxidation inhibition assays.

Similarly, a study evaluated the release of antioxidant peptides from Nile tilapia scale gelatin by alcalase, pepsin, pronase E and trypsin (Ngo, Qian, Ryu, Park, & Kim, 2010). The assessment of antioxidant activity of hydrolysates indicated that alcalase released peptides of higher antioxidant potential than those produced by other enzymes. Authors also identified the antioxidant peptides in alcalase hydrolysate and argued that DPALATEPDPMPF peptide was the main active compound. In a study about antioxidant peptides from oyster proteins, Qian, Jung, Byun, & Kim (2008) isolated and identified 1 peptide after digestion with pepsin. Authors also observed that the identified peptide (LKQELEDLLEKQE) was able to scavenge radicals in both *in vitro* and in human embryonic lung fibroblasts cell line. The antioxidant potential of

349 the hydrolysates produced from the microalga *Palmaria palmate,* after using corolase PP, was
350 determined by FRAP and ORAC assays. Among the 15 peptides identified, SDITRPGGQM
351 displayed the highest antioxidant activity (Harnedy, O'Keeffe, & FitzGerald, 2017).
352 Memarpoor-Yazdi, Asoodeh, & Chamani (2012) explored the use of papain, trypsin and the
353 combination of both enzymes to hydrolysate hen egg white lysozyme. The hydrolysate
354 obtained from the association of papain and trypsin achieved the highest antioxidant potential
355 in ABTS, DPPH, and ion chelating assay. In this fraction, 10 peptides were identified and
356 NTDGSTDYGILQINSR was stated by the authors as the main antioxidant peptide.
357 Homayouni-Tabrizi et al. (2017) studied the antioxidant peptides released from camel milk by
358 the association of pepsin and pancreatin. The authors isolated and identified 3 peptides in the
359 hydrolysate wherein the highest antioxidant activity for peptide YLEELHRLNAGY in
360 comparison to peptides LEEQQQTEDEQQDQL and RGLHPVPQ.
361 A combination of two immobilized enzymes out of alcalase, pepsin, and trypsin were
362 explored to hydrolyse zein protein (a by-product from corn oil production) (Wang et al., 2015).
363 An isolated peptide, tentatively identified as M-I/L-P-P, was the main compound responsible
364 to scavenge DPPH radicals. Zhao & Song (2014) studied the effect of hydrolysis and plastein
365 reactions of soybean protein hydrolysates by alcalase in the release of antioxidant peptides.
366 The authors optimised the enzymatic reaction conditions by response surface methodology
367 (enzyme content 1037 U/g peptides, peptide content 29.7%, and reaction temperature 20.3
368 °C) and obtained a hydrolysate of improved antioxidant activity on ABTS, reducing power and
369 scavenging activity on hydroxyl radical assays. Interestingly, plastein reaction induced a slight
370 increase in the antioxidant activity.
371 A study explored the use of protease A Amano 2G on the production of antioxidant peptides
372 from hydrolysates obtained of sesame seed meal, which are usually discarded from sesame
373 oil production (Das, Ghosh, & Bhattacharjee, 2012). The fraction of peptides of molecular
374 weight <1 kDa displayed the highest antioxidant potential in comparison to other fractions and
375 the full hydrolysate. Similarly, Zhuang, Tang, Dong, Sun, & Liu (2013) isolated and identified 1
376 peptide from alkaline protease hydrolysis of corn gluten meal, a residue from corn oil

processing. The peptide identified as GHKPS displayed the highest antioxidant activity by the DPPH, metal ion-chelating activity, reducing power, and lipid peroxidation inhibition assays.

The utilization of isolated enzymes is a useful tool which can aid both food industry and academy to target the release of peptides from protein by performing experiments involving the use of one enzyme per experiment. It narrows the possibilities for protein cleavage and is particularly important to improve the control for further studies in pharmacology as well as in food science and technology.

4. Antioxidant activity

4.1. Biological effects

In biological systems, complex reactions and multiple factors influence the oxidative balance. Under normal conditions, living organisms (*e.g.* animals, human being) are able to produce and inactivate free radicals and reactive species due to catabolism of molecules essential for proper functioning of an organism (*e.g.* energy production in mitochondria). However, the accumulation of reactive species leads to oxidation, a condition which these species react with essential biomolecules and induces damage to tissues, impairs metabolic routes and genetic expression, and ultimately increases the risk and/or facilitates the evolution of diseases such as cancer, diabetes, atherosclerosis, and neurological disorders. In the face of such scenario, living organisms produce molecules to preserve this delicate oxidative balance (Bouayed & Bohn, 2010).

Among the different antioxidants, catalase (CAT), glutathione (GPH), glutathione peroxidase (GPx), glutathione-S-transferase (GST), superoxide dismutase (SOD), are considered to be at the first line of defense against oxidizing agents. Such enzymatic and non-enzymatic antioxidants act by dismutating, detoxifying and producing less and/or non-reactive species. Although the biological defense provided by CAT, GPx, SOD, and their related molecules against oxidative stress can keep oxidative balance in check, additional consumption of antioxidants, known as exogenous antioxidants, can prevent oxidative damage

403 when oxidants overcome this antioxidant defense (Baunthiyal, Singh, & Dwivedi, 2017;
404 Bouayed & Bohn, 2010).

405 The biological effect of antioxidant peptides extracted from several protein sources has
406 been evaluated by several *in vivo* studies (**Table 3**). For instance, Chou, Wang, Lin, & Chen
407 (2014) assessed the effects of antioxidant peptides released from chicken liver after enzyme-
408 assisted hydrolysis (pepsin) by using the inhibition of malondialdehyde (MDA) accumulation,
409 a well-known secondary lipid oxidation product, and the induction of CAT, GPx, and SOD
410 production in D-galactose-induced mice (chronic consumption of galactose can stimulate the
411 production of reactive species). The doses of chicken liver hydrolysate administered to mice
412 (0.05 and 0.25 g/kg) led to a similar or even improved antioxidant status in relation to control
413 and D-galactose-induced mice in brain, heart, liver, and kidney. The authors of the study
414 observed that doses of 0.25 and 0.5 g/kg prevented the oxidation of lipids in serum and liver
415 at the same level of control mice. In contrast to the reduction levels observed for D-galactose
416 treated mice, the serum and liver values of CAT, GPX, and SOD were significantly improved
417 or restored to similar levels reported for the control mice. A similar outcome was observed by
418 other authors in mice supplemented with chicken breast hydrolysates obtained after using
419 papain (Sun et al., 2012). The antioxidant effects of *in vivo* protein hydrolysates were also
420 supported by other authors who observed similar outcomes in loach meat hydrolyzed by
421 papain (You, Zhao, Regenstein, & Ren, 2011), tilapia collagen (Zhang, Chen, Jiang, Yin, &
422 Zhang, 2016) and rice proteins (Han, Park, Choi, & Suh, 2016) hydrolysed by alcalase.

423 It is worth noting that the *in vitro* antioxidant potential observed in protein sources subjected
424 to fermentation strategy also displayed an important *in vivo* activity. This outcome was reported
425 by Fazhi et al. (2014) who fermented sesame meal (a by-product from sesame oil extraction)
426 and isolated three peptides (tri-, tetra-, and hexapeptide). The supplementation with any
427 peptide at 0.1, 0.2, and 0.4 g/kg inhibited the accumulation of MDA in serum and liver.
428 Additionally, the levels of SOD and GPx were increased in all treated mice. In a similar way,
429 Kim et al. (2013) hydrolyzed Korean mussel proteins with papain and identified the main
430 antioxidant peptides. In this study, the peptide SLPIGLMIAM was used for supplementing the

diet of mice, observing a prevention in the increase of MDA level when it was used, while SOD concentration was increased. However, the GST level was not affected by peptide supplementation. This fact could be attributed to the activity of the antioxidants, which may consume reactive species before GST could be affected.

4.2. Effect in food

In contrast to living organisms, food displays an irreversible decay on its quality characteristics that can be delayed from few hours to several months and even years when appropriate strategies are applied. In this line, the use of antioxidant as food additives is a common trend in the food industry (Franco et al., 2018; Granato et al., 2017; Horita et al., 2018; Lorenzo et al., 2018). However, consumer´s growing awareness for foods without synthetic additives, due to their potentially harmful effects on health, has led both food industry and researchers to explore new ways to obtain natural antioxidants. Moreover, they are also showing an increased interest in the adequate ingestion of nutrients and bioactive compounds due to their preventive actions against the development of non-communicable diseases (Childs & Poryzees, 1997; Teratanavat & Hooker, 2006). This trend strengthens the need for obtaining and using food additives of natural origin that also exert bioactivity.

In this scenario, antioxidant peptides have been used, at laboratory level, as potential food additives. However, few studies have been performed to evaluate the effect of antioxidant peptides in real food matrices, which can support their potential use as additives (**Table 4**). Among the several food products available, meat products are susceptible to lipid oxidation and require additional protection against reactive species. However, controversial results were reported in the scientific literature regarding the use of antioxidant peptides.

For example, the strategy of using starter cultures to release antioxidant peptides from meat proteins and reduce the evolution of oxidation in dry-cured hams was evaluated (Okoń, Stadnik, & Dolatowski, 2017). The authors explored the *L. acidophilus* and *Bifidobacterium animalis* as starter cultures and obtained increasing antioxidant levels during ripening of hams

457 by the use of isolated or combined microorganisms. However, the authors reported similar
458 levels of antioxidant effect among all treatments.

459 A similar outcome was observed by Kęska & Stadnik (2017) that inoculated hams with one
460 of the following microorganisms: *L. acidophilus*, *L. acidophilus,* and *B. animalis*. The evaluation
461 of dry-cured hams revealed significant differences in the antioxidant status among treatments
462 but clustering analysis did not indicate remarkable differences among treatments. The authors
463 also argue that such differences in the antioxidant status seem to be associated with other
464 factors rather than the release of peptides.

465 On the other hand, the addition of starter cultures on dry-cured sausages caused a
466 significant decrease in lipid oxidation of the samples and a general increase of their antioxidant
467 status. Broncano, Timón, Parra, Andrés, & Petrón (2011) and Petrón, Broncano, Otte, Martín,
468 & Timón (2013) observed similar results when proteases extracted from *A. oryzae* and *B.*
469 *subtilis* were applied in meat samples. In both experiments, the radical scavenging activity and
470 reducing power were significantly increased in inoculated batches after the maturation period
471 while the lipid oxidation was significantly reduced.

472 A recent experiment with minced meat strengths the technological application of peptides
473 as food antioxidants (Przybylski, Firdaous, Châtaigné, Dhulster, & Nedjar, 2016). In this study,
474 the authors hydrolyzed bovine hemoglobin (obtained from a slaughterhouse) with pepsin and
475 isolated the peptide TSKYR. This peptide was further applied in minced meat (0.1 and 0.5%)
476 and stored up to 14 days at 4 °C. The lipid oxidation was inhibited in the same level as butylated
477 hydroxytoluene (BHT), a synthetic antioxidant commonly used in food industry, when BHT was
478 used at 0.1 and 0.5% during 14 days. Although promising results are reported in the literature
479 (particularly for minced meat and dry-cured sausages), further research is needed in order to
480 evaluate the effect of antioxidant bioactive peptides in sensory properties of final products and
481 consumer´s acceptance. To the best of our knowledge, only the presented studies were
482 published regarding the application of peptides as food antioxidants.

5. Conclusion and future perspectives

Antioxidant bioactive peptides have a huge potential to be used for both food and pharmacological applications. The use of fermentation process (microorganisms) and enzymes have been evaluated as potential tools to obtain antioxidant bioactive peptides from proteins, although most of the studies have been focused on the use of isolated enzymes instead of fermentation processes due to their superior control over releasing and obtaining targeted peptides.

Each step between the release and the application of antioxidant peptides have already been studied, particularly at laboratory scale. However, some improvements are necessary. The combination of protein and proteolytic agent is a crucial step since the final composition and content are dependent of this combination. Due to the immense number of combinations, advances in the elaboration and constant update of databases regarding the peptides formed in proteolytic reactions are necessary. Prediction of possible products and the consequent biological activity may improve the selection and production of new peptides. Separation of bioactive peptides is another critical step that currently has multiple alternatives: i) liquid chromatography, ii) gel filtration, iii) ultrafiltration, and iv) ion-exchange separation technologies, among others. The cost and time required to achieve the expected degree of separation are the primary targets to improve this step. The identification of peptides demands high-cost equipment to elucidate not only amino acid sequence but also secondary and even tertiary structure. The importance of elucidating all levels of peptide structure may lead to correlate the biological effect with its intrinsic characteristics, along with molecular weight and amino acid sequence (Wang et al., 2013; Mora et al., 2017).

The use of antioxidant peptides still demands more attention and more studies are necessary to recommend their potential applications. Moreover, the antioxidant effects observed in the *in vivo* studies highlighted the importance of peptides on the defense against reactive species. However, further studies are necessary to evaluate the effects in clinical trials with both health subjects and patients with diseases related to oxidative unbalance (*e.g.*

atherosclerosis and cancer) and to either confirm and evolve preventive and therapeutic treatments or refute the consumption of antioxidant peptides under well-defined biological conditions. Nevertheless, the potential application of antioxidant peptides as prophylactic and therapeutic agents should be investigated in further studies as means of improving quality of life.

Regarding food processing, future studies should also explore the protective strategies of antioxidant peptides in other food matrices. Since a long period can occur between the processing and consumption of food, antioxidant peptides can interact with food components and their antioxidant potential is suggested to decay over time. Another relevant aspect of this approach is the increase in the number of protein sources by the reuse of food wastes and by-products generated by agro-industry to reduce the cost of production. It is crucial that further studies explore the scale-up of antioxidant peptides production for food and pharmacological purposes. The large body of evidence about the technological use of microorganisms and enzymes to hydrolyse several protein sources (in laboratory scale) support the need of further studies to produce/release such compounds in medium and large scale.

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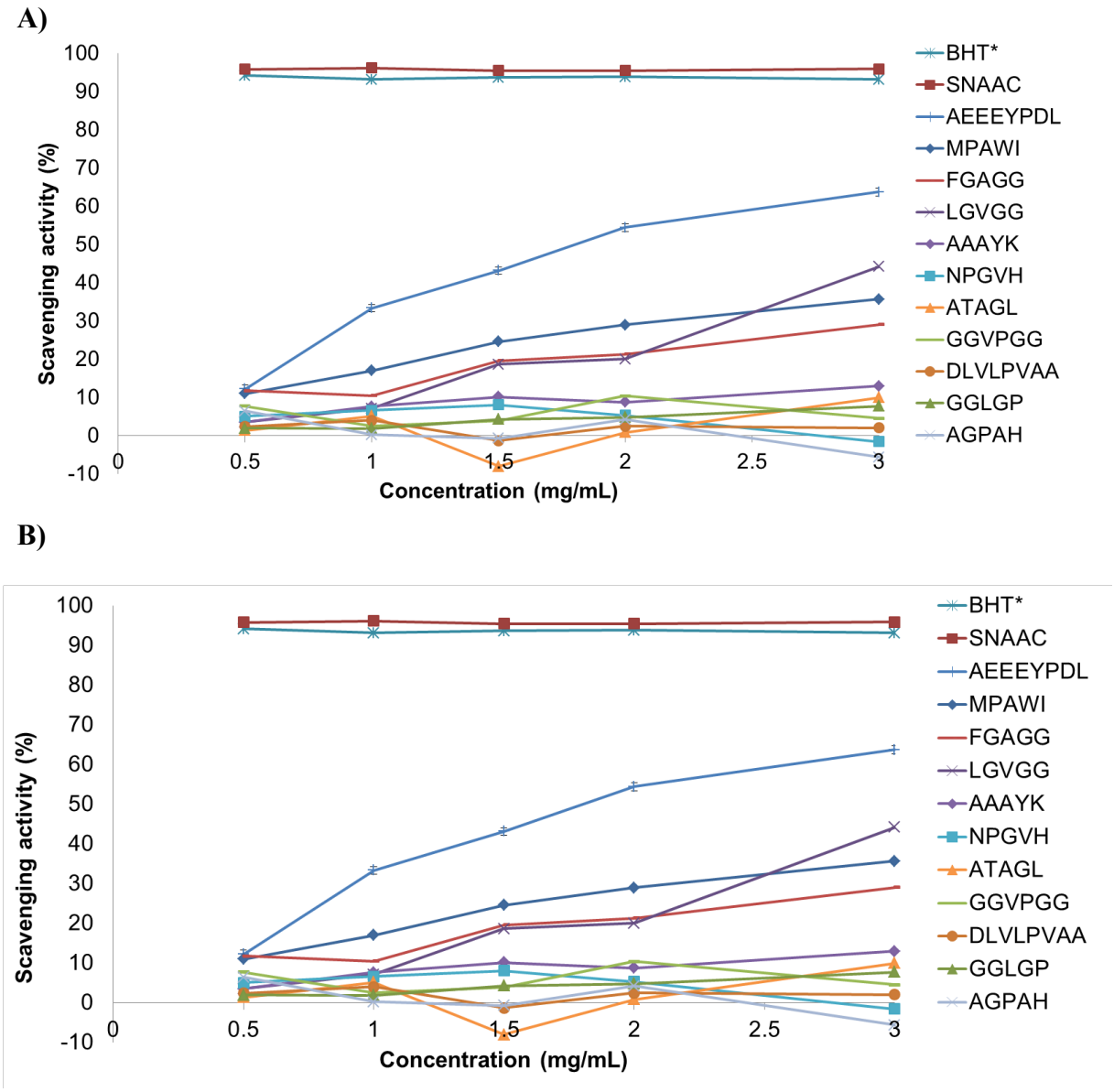

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789 **Figure 1.** A) DPPH radical-scavenging activity at different concentrations of 10 synthesised
790 antioxidant peptides. B) Reducing power of different concentrations of synthesised peptides.
791 Values represent means of three independent replicates (n=3). *The synthetic compound 2,6-
792 di-tert-butyl-4-methylphenol (BHT) was used as positive control. Reprinted from Mora et al
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796 **Table 1.** Microorganisms applied on food and sources of proteins to obtain antioxidant peptides

Food or source of proteins	Microorganism	Antioxidant assays	Antioxidant activity	Peptides (N; mf)	Ref.
Whole milk	*Lactobacillus plantarum* strains	ABTS and ORAC	Bacterial strain dependency	n.d.; <3 kDa	(Aguilar-Toalá et al., 2017)
Skim milk	*L. helveticus*	DPPH	Influenced by time of fermentation and bacterial strain	n.d.; n.d.	(Elfahri, Vasiljevic, Yeager, & Donkor, 2016)
Skim milk	*L. rhamnosus*, *L. paracasei*, and *L. casei*	ABTS	The peptides of highest AA were produced *L. casei*	n.d.; n.d.	(Solieri, Rutella, & Tagliazucchi, 2015)
Camel sausage	*L. pentosus*; *L. plantarum*; *L. sakei*, *Staphylococcus xylosus* and *S. carnosus*	ABTS, DPPH, inhibition of hydroxyl radicals, and FRAP	Increasing activity was associated with ripening time and lactic acid bacteria strains	13-22 peptides; <3 kDa	(Vaštag, Popović, Popović, Petrović, & Peričinn, 2010)
Petrovac Sausage	n.d.	DPPH and RP	Partially responsible for AA	n.d.; n.d.	(Vaštag et al., 2010)
Fish meat flours	*Bacillus subtilis*	DPPH, βCBM, RP, and DNA nicking assay	Concentration-dependent effect	n.d.; n.d.	(Jemil et al., 2014)
Sardinelle protein hydrolysates	*B. subtilis* and *B. amyloliquefaciens*	DPPH, βCBM, RP, MCA, and ORAC	Highest AA: NVPVYEGY and GTEDELDKY	800 peptides; <1.2 kDa	(Jemil et al., 2016)
Shrimp waste	*Pediococcus acidolactici*	DPPH, ABTS, Hydroxyl RSA, Peroxyl RSA, and Singlet oxygen quenching activity	Release of peptides and amino acids of elevated AA	n.d.; <19.4 kDa	(Sachindra & Bhaskar, 2008)
Soybean flour	*Aspergillus oryzae*	DPPH and ILAAO	Concentration-dependent activity	n.d.; 1-3 kDa	(Lee, Rho, Kim, Lee, & Lee, 2013)
Defatted wheat germ	*B. subtilis*	DPPH, RP, and Iron chelating capacity	Peptides were the main fraction associated with AA in early stages of fermentation	n.d.; n.d.	(Liu, Chen, Shao, Wang, & Zhan, 2017)
Okara	*B. subtilis*	DPPH, ABTS, RP, βCBM	Increased after 24 h of fermentation	n.d.; n.d.	(Zhu, Fan, Cheng, & Li, 2008)
Quinoa flour	*L. plantarum*, *L. rossiae* and *P. pentosaceus*	DPPH, ABTS, and ILAAO	Highest AA was obtained from *L. plantarum* fermentation	5 peptides; <1.1 kDa	(Rizzello et al., 2017)
Cottonseed meal	*B. subtilis*	DPPH, Hydroxyl RAS, MCA, and RP	Concentration-dependent AA; protection against oxidative damage in cells	n.d.; <1 kDa	(Sun et al., 2015)
Tomato seed meal	*B. subtillis*	DPPH, RP	Concentration-dependent AA. Highest AA: GQVPP	10 peptides; <1 kDa	(Moayedi et al., 2017, 2018)

797 N: number of identified peptides. mf: main fraction of peptides (kDa). n.d.: dot determined. AA: antioxidant activity. ABTS: 2,2'-azino-
798 bis(3ethylbenzothiazoline-6-sulphonic acid). βCBM: β-carotene bleaching method. DPPH: 2,2-diphenyl-1-picrylhydrazyl. FRAP: Ferric
799 Reducing Antioxidant Power. ILAAO: Inhibition of linoleic acid autoxidation. ORAC: Oxygen Radical Absorbance Capacity. RSA: radical
800 scavenging activity. RP: Reducing Power. MCA: Metal chelating activity.

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802 **Table 2.** Enzymes applied on food and sources of proteins to obtain antioxidant peptides

Source of proteins	Enzyme/Source	Antioxidant assays	Antioxidant activity	Peptides (N; mf)	Reference
Bovine liver sarcoplasmic protein	Thermolysin	DPPH, FRAP, FICA	Similar AA between <3 kDa and 3-10 kDa	44; <10 kDa	(Di Bernardini et al., 2011)
Silkie fowl blood protein	ALC	DPPH, FICA, ILAAO and RP	AA was dependent on hydrolysis time	n.d.; 1-3 kDa	(Cheng, Lai, Lin, & Sakata, 2016)
Yak skin	ALC, FLA, Protamex, Proteinase K, PEP + TRYP	DPPH, Hydroxyl and Superoxide RSA	The hydrolysates produced by ALC and Proteinase K had the highest AA	n.d.; <3 kDa	(Tian et al., 2017)
Monkfish muscle proteins	TRYP	DPPH, Hydroxyl and Superoxide anion RSA and LPI	Isolated antioxidant peptides EWPAQ, FLHRP, and LMGQW displayed AA in concentration-dependent manner	3; 630-670 kDa	(Chi et al., 2014)
Bones of dry-cured hams	PEP, TRYP, CTRYP	ABTS,DPPH,FRAP, ORAC, βCBM	Preservation of AA after simulated digestion	459; <700 Da	(Gallego et al., 2017)
Camel milk	PEP, Pancreatin	ABTS, DPPH, Hydroxyl and Superoxide anion RSA	Peptide YLEELHRLNAGY with highest AA compared to LEEQQQTEDEQQDQL RGLHPVPQ	3; <2 kDa	(Homayouni-Tabrizi et al., 2017)
Goat milk proteins	PEP	DPPH and Superoxide RSA	Peptides from whey proteins displayed the highest AA	33; <2 kDa	(Ahmed, El-Bassiony, Elmalt, & Ibrahim, 2015)
Hen egg white lysozyme	Papain and TRYP	ABTS, DPPH, MICA, LPI	The highest AA with 2 enzymes; NTDGSTDYGILQINSR peptide was the main antioxidant compound	10; <3 kDa	(Memarpoor-Yazdi, Asoodeh, & Chamani, 2012)
Nile tilapia scale gelatin	ALC, PEP, Pronase E and TRYP	DPPH, Hydroxyl radical and Superoxide anion RSA	ALC hydrolysate had the highest AA; DPALATEPDPMPF was the main antioxidant peptide	1; 1382.57 Da	(Qian, Jung, Byun, & Kim, 2008)
Oyster	PEP, TRYP, CTRYP	ILAAO, Hydroxyl and Superoxide anion RSA	Isolated peptide LKQELEDLLEKQE displayed AA and prevented oxidative damage on DNA	1; 1.60 kDa	(Ngo, Qian, Ryu, Park, & Kim, 2010)
Thornback ray skin	ALC, *BSIE*, Neutrase, and Thornback ALKPROT	DPPH, DNA nicking assay, RP, TAC, and βCBM	Thornback ALKPROT hydrolysates displayed the highest AA except for DNA nicking assay; ALC hydrolysate was the most effective to prevent DNA oxidation	46; <2 kDa	(Lassoued et al., 2015a)
Thornback ray muscle	ALC, *BSIE*, Neutrase, and Thornback ALKPROT	DPPH, DNA nicking assay, RP, TAC, and βCBM	Neutrase displayed the highest AA	<3 kDa	(Lassoued et al., 2015b)
Smooth hound viscera	Neutrase, esperase, purafect and endogenous viscera proteases from smooth hound	DPPH, DNA breakage assay, MICA, RP, LPI, and βCBM	Enhanced AA after simulated gastrointestinal digestion	<3kDa	(Abdelhedi et al., 2016)
Microalga *Palmaria palmata*	Corolase PP	FRAP and ORAC	The peptide SDITRPGGQM displayed the highest AA	15; <1 kDa	(Harnedy, O'Keeffe, & FitzGerald, 2017)
Zein (Corn gluten meal protein)	ALC, PEP, TRYP	DPPH	Isolated peptide M-I/L-P-P displayed high AA	1, 452.3 Da	(Wang et al., 2015)
Bambara groundnut flour	ALC, PEP, TRYP	ABTS and ILAAO	Higher AA for ALC produced peptides; lower AA for isolated fractions than hydrolysates	n.d.; <1-3 kDa	(Arise et al., 2017)
Defatted almond flour	ALC, CTRYP FLA, PEP, TRYP	ABTS and RP	ALC hydrolysate had the highest AA	n.d.; 6.5-14.3 kDa	(Mirzapour, Rezaei, Sentandreu, & Moosavi-Movahedi, 2016)
Quinoa	Papain,Microbial papain-like enzyme	ORAC	The highest AA was observed in both papain and papain-like hydrolysates	n.d.; <1 kDa	(Nongonierma, Le Maux, Dubrulle, Barre, & FitzGerald, 2015)

Defatted rapeseed protein meal	ALC, FLA, PEP+ Pancreatin, Proteinase, Thermolysin	ORAC	Peptide fraction of molecular weight <3 kDa had the highest antioxidant potential	n.d.; <3 kDa	(He et al., 2013)
Isolated soybean protein	ALC	ABTS, DPPH, RP, Hydroxyl RSA	AA increased as hydrolysis time increased; plastein reaction induced a slight increase on hydrolysates AA	n.d.; n.d.	(Zhao & Song, 2014)
Flaxseed cake	ALC, Cellulase, Pancreatin, Papain, TRYP	ABTS, FRAP, PCL-ACL, and FICA	ALC and pancreatin produced the highest AA hydrolysates; higher AA in hydrolysates than protein isolates	n.d.; n.d.	(Karamać, Kosińska-Cagnazzo, & Kulczyk, 2016)
Brown sesame seed meal	Protease A Amano 2G	DPPH and ILAAO	AA increased as molecular weight reduced	n.d.; <1 kDa	(Das, Ghosh, & Bhattacharjee, 2012)
Corn gluten meal	ALKPROT, FLA, Papain, TRYP	DPPH, MICA, RP, LPI	The hydrolysate produced from ALKPROT had the highest AA; the main antioxidant peptide had the amino acid sequence: GHKPS	1; 507.2 Da	(Zhuang, Tang, Dong, Sun, & Liu, 2013)

N: number of identified peptides. mf: main fraction of peptides (kDa). n.d.: dot determined. AA: Antioxidant activity. ALC: alcalase. ABTS: 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid). ALKPROT: Alkaline protease. βCBM: β-carotene bleaching method. BSIE: *B. subtilis* isolated enzymes DPPH: 2,2-diphenyl-1-picrylhydrazyl. CTRYP: Chymotrypsin. FICA: Ferrous ion chelating ability. FLA: Flavourzyme. FRAP: Ferric Reducing Antioxidant Power. ILAAO: Inhibition of linoleic acid autoxidation. LPI: Lipid peroxidation inhibition. MICA: Metal ion-chelating activity. ORAC: Oxygen Radical Absorbance Capacity. PCL-ACL: Photochemiluminescence Antioxidant Capacity of lipid-soluble compounds. PEP: Pepsin. RP: Reducing Power. RSA: radical scavenging activity. TAC: Total Antioxidant Capacity. TRYP: Trypsin.

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804 **Table 3**. Biological effects of antioxidant peptides *in vivo*

Source of proteins	Enzyme/ Microorganisms	Peptides/ Hydrolysate	Experimental conditions*	Effect in animals	Ref.
Chicken liver	Pepsin	Hydrolysate	24 male C57BL/6 mice; 6 weeks; oral gavage; 0.05 and 0.25 g/kg	Inhibition of MDA level in brain and liver (0.25 g/kg); effect on endogenous antioxidants (CAT, GPx, and SOD) was organ-dependent	(Chou, Wang, Lin, & Chen, 2014)
Chicken breast	Papain	Hydrolysate	60 ICR male mice; 42 days; oral gavage; 0.125, 0.25, and 0.5 g/kg	Doses , 0.25, and 0.5 g/kg reduced MDA levels in both serum and liver; these doses also induced the production of serum CAT, GPx, and SOD	(Sun, Pan, Guo, & Li, 2012)
Loach (*Misgurnus anguillicaudatus*)	Papain	Hydrolysate	54 male NIH mice; 4 weeks; intraperitoneal injection; 1 and 5 g/kg	The dose 5 g/kg indiced a significant increase in CAT, GPx and SOD serum levels	(You, Zhao, Regenstein, & Ren, 2011)
Tilapia collagen	Alcalase	Hydrolysate	25 female and 25 male KM mice; 20 days; gastric gavage; 0.85 and 1.7 g/kg	Decreased the increasing of MDA level in a dose-dependent manner; Induced the production of CAT and SOD	(Zhang, Chen, Jiang, Yin, & Zhang, 2016)
Rice protein	Alcalase	Hydrolysate	30 male Imprinting Control Region mice; 5 days; Intraperitoneal injection; 0.5, 1, and 2 g/kg	Reduction of serum MDA level; Induced the production of CAT, GPH, and GPx; downregulated NADPH oxidade 4	(Han, Park, Choi, & Suh, 2016)
Tilapia skin gelatin	Properase E	Hydrolysate (LSGYGP)	40 ICR male mice; n.i.; gastric intubation; 0.05, 0.1, and 0.2 g/kg	MDA content was dose-dependent reduced in skin; reduction of CAT, GPH, GPx and SOD levels were inhibited in a dose-dependent manner	(Sun, Zhang, & Zhuang, 2013)
Sesame meal	*Lactobacillus plantarum* and *Bacillus subtilis*	Tri-, tetra-, and hexapeptide	120 male Kunming strain mice; 30 days; oral gavage; 0.1, 0.2, and 0.4 g/kg	All isolated peptides at all concentration reduce MDA content in serum and liver; SOD and GPx levels were increased by all isolated peptides	(Fazhi et al., 2014)
Korean mussel (*Mytilus coruscus*)	Papain	SLPIGLMIAM	40 adult male mice; n.i.; oral gavage; 0.005 g/kg	Inhibited the increase in MDA level, induced SOD level but did not influence GST level	(Kim et al., 2013)

*Number of animals/time of supplementation/administration routes/doses g/kg of body weight. n.d.: not determined; n.i.: not informed; MDA: Malondialdehyde; CAT: Catalase; GPH: Glutathione; GPx: Glutathione peroxidase; GST: Glutathione-S-Transferase; NADPH: reduced Nicotinamide Adenine Dinucleotide Phosphate; SOD: Superoxide dismutase.

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807 **Table 4** – Effect of antioxidant peptides addition in food

Source of proteins	Enzyme/ Microorganisms	Peptides/ Hydrolysate	Product	Effect in food	Ref.
Pork meat	*L. acidophilus* and *B. animalis*	-*	Dry-cured loin	Antioxidant potential increase over time for all batches without differences among batches; no effect on pH	(Okoń, Stadnik, & Dolatowski, 2017)
Pork meat	*L. rhamnosus*, *L. acidophilus*, and *Bifidobacterium animali*	-*	Dry-cured loin	No relation between peptide release and antioxidant activity; slight reduction on pH and water activity	(Kęska & Stadnik, 2017)
Pork meat	Proteases from *B. subtilis* and *A. oryzae*	-*	Dry-cured sausage	*B. subtilis* protease caused the highest increase on radical scavenging activity and *A. oryzae* protease the highest reducing power, respectively; both proteases inhibited the accumulation of MDA; negative association between MDA and antioxidant potential	(Broncano, Timón, Parra, Andrés, & Petrón, 2011)
Pork meat	*B. subtilis.* and *A. oryzae*	-*	Dry-cured sausage	*A. oryzae* concentrated protease batch displayed the highest antioxidant capacity, inhibition of lipid oxidation and loss of redness	(Petrón, Broncano, Otte, Martín, & Timón, 2013)
Bovine hemoglobin	Pepsin	TSKYR (0.1 and 0.5%)	Ground beef	0.5% TSKYR peptide treatment displayed similar capacity to prevent lipid oxidation as 0.1 and 0.5% BHT treatments	(Przybylski, Firdaous, Châtaigné, Dhulster, & Nedjar, 2016)

808 *Direct addition of protease or microorganism in the product.

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