Generation of bioactive peptides during food processing

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Running title: **Generation of bioactive peptides during food processing**

Abstract

Large amounts of peptides are naturally generated in foods through the proteolysis phenomena taking place during processing. Such proteolysis is carried out either by endogenous enzymes in ripened foods or by the combined action of endogenous and microbial enzymes when fermented. Food proteins can also be isolated and hydrolysed by peptidases to produce hydrolysates. Endo-peptidases act first followed by the successive action of exo-peptidases (mainly, tri- and di-peptidylpeptidases, aminopeptidases and carboxypeptidases). The generated peptides may be further hydrolysed through the gastrointestinal digestion resulting in a pool of peptides with different sequences and lengths, some of them with relevant bioactivity. However, these peptides should be absorbed intact through the intestinal barrier and reach the blood stream to exert their physiological action. This manuscript is reporting the enzymatic routes and strategies followed for the generation of bioactive peptides.

Keywords: proteolysis, peptides, bioactive peptides, proteomics, enzymes, peptidases, exo-peptidases

1 Introduction

Proteins are one of the main components in foods, especially in those of animal origin such as meat, fish, milk or eggs. Proteins exert nutritional, functional and biological properties which are frequently affected by the technology used in food processing. Main causes of alteration of proteins during processing are pH changes like acidification, chemical treatments such as acylation, glycosylation, and phosphorylation, heat treatments and fermentation (Pihlanto & Korhonen, 2003). These changes can be responsible for positive aspects such as the improvement in final textural/organoleptic characteristics, better stability of the product, or the generation of bioactive peptides, although some negative aspects such as the modification of one or several amino acids or the generation of allergenic compounds can affect the product. Changes occurred during specific food processes such as curing or fermentation in cheese, wine or dry-cured meats have been widely described as a source of bioactive peptides (Corrêa et al., 2014; Mohanty, Mohapatra, Misra & Sahu, 2016; Mora, Escudero, Arihara & Toldrá,2015). Other key mechanisms to obtain bioactive peptides are the hydrolyis using controlled and commercial peptidases or microorganisms mainly used to take advantage of food by-products (Ryder, Bekhit, McConnell & Carne, 2016). Finally, the gastrointestinal (GI) digestion due to the action of salivary, stomachal, intestinal and pancreatic enzymes constitute the final hydrolysis step in generating bioactive peptides (Capriotti, Caruso, Cavaliere, Samperi et al., 2015; Pepe et al., 2016). The role of food proteins as a source of bioactive peptides has been widely described in recent studies (Li-Chan, 2015; Oseguera-Toledo, González de Mejía, Reynoso-Camacho, Cardador-Martínez & Amaya-Llano, 2014; Lassoued et al., 2015). In this respect, the bioactive peptides are inactive when they are taking part of the parent protein, but turn active when released due to the action of enzymes during food

processing or GI digestion. Once released, the bioactive peptides may provide different functions that can be reproduced *in vitro* with biochemical assays or *in vivo* in cell or animal models and humans. Different open access databases report the bioactive peptides that are being discovered including data about their main chemical and structural characteristics, IC50, protein of origin, and references. Most studied biological functions to date according to the results reported by BIOPEP database (http://www.uwm.edu.pl/biochemia/index.php/pl/biopep) have been ACE-inhibitory activity, antioxidant activity, antimicrobial activity, opioid activity, inmunomodulating and antithrombotic activities.

Numerous studies in the literature have reported bioactivities derived from food protein sources. First discoveries of food derived peptides were from milk-based products which have been extensively studied in relation to their potential health promoting effects in humans (Nongonierma and FitzGerald, 2016). Also meat protein is considered a good source to obtain bioactive peptides due to the high quality of its proteins which has been widely described (Escudero, Sentandreu, Arihara & Toldrá, 2010; Udenigwe & Howard, 2013;) together with fish proteins (Ferraro, Carvalho, Piccirillo, Santos, Castro, Pintado, 2013). The use of by-products obtained from protein sources such as slaughterhouses, fisheries, olivemill wastewater, cheese whey, winery sludge, citrus peel, etc is done under controlled enzymatic conditions which permits the control of the hydrolisis and the generated peptides (Mora, Reig & Toldrá, 2014; Ryder, Bekhit, McConnell & Carne, 2016). Egg proteins, soybean proteins, or peanut proteins have also been extensively studied protein hydrolysates (Ji, Sun, Zhao, Xiong & Sun, 2014; Tanzadehpanah, Asoodeh & Chamani, 2012; De Oliveira, Corrêa, Coletto, Daroit, Cladera-Olivera & Brandelli, 2015).

The effect of natural bioactive peptides on health by preventing infection and diseases is of great interest nowadays due to the severe toxic side-effects that have been described to be caused by the use of synthetic peptides and drugs in the treatment and prevention of numerous diseases. Also the economic impact on health care in future years due to the effect of bad habits and ageing of population could be decreased by proportionally increasing the development and use of bioactive peptides.

2. Main characteristics of peptides exerting biological activities

Data about the characteristics of bioactive peptides such as their length, amino acid composition or structural conformation results very useful in the identification and characterisation of novel active sequences, especially when empirical strategies are used in the detection of bioactive peptides. These characteristics are only well-known in the most studied activities as the lack of information about peptide sequences identified in minor bioactivities as well as the ideal conditions of proteolysis makes more difficult the standardisation of the peptides characteristics.

2.1. ACE-inhibitory activity

Angiotensin I-converting enzyme (ACE) is a key enzyme influencing the regulation of blood pressure. ACE is a central component of the renin-angiotensin system, and converts angiotensin I into the potent vasoconstrictor angiotensin II. It is also well-known to degrade the vasodilative bradykinin in the kinin–kallikrein system. For these reasons, the inhibition of ACE enzyme is of high interest in the search of antihypertensive peptides (Escudero, Mora & Toldrá, 2014).

ACE is an exopeptidase with an ability to cleave dipeptides from the C-terminal of peptides. It is a chloride-activated zinc metallopeptidase and it is assumed that the function of the anion activation in ACE provides high *in vitro* substrate specificity.

Studies with different peptide inhibitors showed that binding to ACE is strongly influenced by the C-terminal tripeptide sequence of the substrate. In fact, main inhibitors of ACE enzyme show hydrophobic amino acid residues at each of the three C-terminal positions with proline, lysine or arginine as C-terminal amino acids (Fernández, Benito, Martín, Casquete, Córdoba & Córdoba, 2016; Gu, Majumder & Wu, 2011).

2.2. Antioxidant activity

The antioxidant activity showed by peptides is classified into two groups depending on the basis of the chemical reactions involved: methods based on hydrogen atom transfer (HAT) and methods based on electron transfer (ET) (Huang, Ou, & Prior, 2005). The HAT-based assays evaluate the ability of a peptide to reduce free radicals by hydrogen donation in a competitive reaction. The *in vitro* assays used for its measurement are oxygen radical absorbance capacity assay (ORAC), total radical trapping antioxidant parameter (TRAP) and β-carotene bleaching assay. ET-based assays evaluate the ability of a potential antioxidant to transfer one electron to reduce an oxidant, so these reactions are pH dependent. The assays ABTS radical scavenging assay, ferric-reducing antioxidant power, and DPPH radical scavenging activity are used for its measurement (Huang, Ou & Prior, 2005; McDonald-Wicks, Wood, & Garg, 2006).

According to Liu,Xing, Fu, Zhou and Zhang (2016), most of the antioxidant peptides have between 4–16 amino acids, with molecular mass of about 400–2000 Da. The molecular weight affects the used routes to reach target sites and the capacity to suffer additional digestion by GI enzymes which could increase the antioxidant capacity *in vivo* (Li, Le, Shi & Shrestha, 2004). Peptides showing Pro residues have been described to be more resistant to further degradation by digestive enzymes (Fitzgerald & Meisel, 2000).

The type of amino acid plays an important role in determining the antioxidant activity of the peptides. In this respect, aromatic amino acids such as Tyr, His, Trp, and Phe can donate protons contributing to the radical-scavenging properties (Rajapakse, Mendis, Jung, Je & Kim, 2005). On the other hand, the hydrophobic amino acids have been described to be able to increase the presence of peptides at the water-lipid interface and then access to scavenge free radicals from the lipid phase (Ranathunga, Rajapakse & Kim, 2006). Finally, acidic amino acids utilise carbonyl and amino groups in the side chain which function as chelators of metal ions (Suetsuna, Ukeda & Ochi, 2000).

2.3. Antimicrobial activity

Antimicrobial peptides generated from dietary proteins show several characteristic properties. They are relatively small (20-46 amino acid residues), basic (lysine- or arginine-rich), and amphipathic. The mechanism of action of these antimicrobial peptides is still not well-known but it is believed that their effectivity depends on their capacity to form channels or pores within the microbial membrane impairing the possibility for anabolic processes (Castellano, Mora, Escudero, Vignolo, Aznar & Toldrá, 2016). Antibacterial peptides are usually described as long chains, which can adopt an α-helical linear or circular structure organized in a β-sheet which is essential against microorganisms.

2.4. Opioid activity

Exorphin is the name of those opioid peptides derived from exogenous proteins. The classic opioid peptides show the N-terminal tetra-peptide sequence Tyr-Gly-Gly-Phe, although many opioid active peptides have been described containing the N-terminal sequence Tyr-Pro. So, many opioid peptides isolated from mammalian and amphibian

sources share a common short sequence fragment with Tyr at the N-terminal (except a-casein opioids) separated from a Phe residue or the aromatic tyrosine by one or two amino acids (Stefanucci, Mollica, Macedonio, Zengin, Ahmed & Novellino, 2017). The C-terminal sequences of these peptides vary substantially both in sequence and length, but the described structural motif fits into the binding site of opioid receptors. The negative potential of the tyrosine amino acid is essential for opioid activity and the removal of Tyr residue from the active peptide results in the absence of activity (Guesdon, Pichon & Tomé, 2006).

It has been described that in *in vitro* assays, exorphins resulted from one hundred to one thousand times more potent than endogenous opioid peptides. On the other hand, some exogenous opioid peptides are active after oral administration, in which none of the exorphins were active. One of the reasons could be that Tyr-Pro sequence is more resistant to enzymatic GI digestion than the characteristic sequences for endogenous opioid peptides.

2.5. Inmunomodulating activity

Inmunomodulating activity has been especially identified in peptides derived from milk and milk products. These studies show that their length can be very different comprising from 2 to 64 amino acids, although those smaller than 3000 Da are the most abundant. The most repeated amino acids in the active sequences are Pro and Glu, with Tyr and Lys in the N-terminal and C-terminal extremes, respectively, and Arg at both extremes. Also, their charges differ widely at physiological pH, between 7 and 8, being mainly of hydrophilic character (Reyes-Díaz, González-Córdova, Hernández-Mendoza & Vallejo-Córdoba, 2016).

3. Mechanisms for proteolysis phenomena

Proteins are hydrolysed step-wise by peptidases within the food, from initial proteins and polypeptides down to sequences with just a few amino acids. This proteolysis may take place within the food during processing by endogenous peptidases and/or by microbial peptidases in fermented foods as schematised in figure 1. Such microorganisms have a variety of enzymes which are able to hydrolyse proteins, carbohydrates and lipids (Flores & Toldrá, 2011).

The result of the combined action of endo and exo-peptidases is an accumulation of small peptides and free amino acids in foods. As it has been previously described, some of the released peptides may be bioactive if shoving the adequate length and sequence of residues. An scheme on how proteolysis proceeds in foods by endogenous or microbial peptidases generating small amounts of bioactive peptides is shown in figure 2. Proteins may be also isolated from foods and hydrolysed with commercial peptidases releasing large amounts of bioactive peptides (see figure 2). Of course, the generated peptides must be ingested, subject to gastrointestinal digestion and absorbed intact through the intestinal barrier and reach the blood stream to exert their physiological action (Gallego, Grootaert, Mora, Aristoy, Van Camp & Toldrá, 2015).

The application of peptidomics tools allow the obtention of peptide profiles resulting from an extensive protein hydrolysis. Furthermore, free amino acids are also released from the N- and C-terminals through the action of exopeptidases and, consequently, the remaining peptides are progressively reduced in size. Peptidases are commonly found in microorganisms. For instance, lactic acid bacteria contains an extracellular serin proteinase and several intracellular peptidases. In fact, many intracellular exopeptidases have been reported in the literature like the general aminopeptidase PepN in *L. Helveticus* and *L. sakei*, the glutamyl (aspartyl) specific aminopeptidase, PepA in

Streptococcus cremoris, Lactococcus lactis sp. and *Lb. delbrueckii* ssp. *lactis*, the proline specific peptidases, such as PepX and PepP in *Lactococcus lactis* ssp. *lactis*, X-prolyl di-peptidyl peptidase activity in *Leuconostoc mesenteroide, L. curvatus* and in *L. sakei*, di-peptidyl peptidases in *L. paracasei*, dipeptidase in *L. sakei, L. helveticus, L. plantarum, L.brevis, L. paracasei* and *L. casei* sp *casei*, arginyl aminopeptidase and tripeptidase in *L. sakei* (Bintsis et al., 2004; Macedo et al., 2010; Zotta et al., 2007; Streessler, González et al., 2010; Eisele, Schlayer, Lutz-Wahl & Fischer, 2013a; Stressler Eisele, Schlayer & Fischer, 2012; Stressler Eisele, Kranz & Fischer, 2014; Stressler et al., 2016; Flores & Toldrá, 2011). The yeast *Debaryomices hansenii*.also contains endopeptidases like protease A and D and intracellular exopeptidases like prolyl and arginyl aminopeptidases (Santos, Santos-Mendonça, Sanz, Bolumar, Aristoy & Toldrá, 2001), all of them reported in Endopeptidases like neutral and alkaline protease and exopeptidases like X-prolyl di-peptidyl peptidase, leucine aminopeptidase, and dipeptidyl peptidases (DPP) IV and V have been reported in molds like *Aspergillus oryzae* and DPP V in *Aspergillus fumigatus* (Matsushita-Morita et al., 2011; Stressler et al., 2016).

4. Endogenous protein hydrolysis in foods

Endogenous food peptidases may be responsible for the release of polypeptides and bioactive peptides. The first step of proteolysis is the breakdown of proteins by endo-peptidases into major fragments. Figure 3 shows how ubiquitin 60S ribosomal protein, a muscle protein, is degraded by endogenous muscle endo-peptidases into major fragments at cleaving sites Leu-Glu, Lys-Glu, Lei-Ile and Leu-Ser during the processing of dry-cured ham (Mora, Gallego, Aristoy, Fraser & Toldrá, 2015). In the case of short term processed foods, like fermented sausages, additional peptidases from different

sources such as certain lactic acid bacteria, yeasts or molds are needed for the generation of bioactive peptides. The extent of proteolysis can be confirmed after comparing the chromatographic profiles of the controls with those of the inoculated microorganism.

Dipeptides may be generated in foods through the action of di-peptidyl peptidases (DPP). So, such activity in *L. paracasei* is able to release dipeptides like Ala-Phe, Pro-Leu, Lys-Leu, Leu-Gly and Lys-Phe (Bintsis, Vafopoulou-Mastrojiannaki, Litopoulou-Tzanetaki, 2004), X-prolyl di-peptidyl peptidase activity releases particular proline-containing dipeptides in *Leuconostoc mesenteroides* and *L. curvatus* strains (Zotta, Ricciardi & Parente.2007), and DPP activity in *Leuconostoc mesenteroides,* releases Arg-Pro and Gly-Phe and additionally Gly-Pro in *L. paracasei* subsp *casei* (Macedo, Vieira, Poças & Malcata. 2010). Several dipeptides X.Pro and tripeptides X-Pro-Pro have been identified in casein hydrolysates with *Lb. helveticus* (Stressler, Eisele & Fischer, 2013).

Muscle foods contain endogenous muscle di-peptidyl peptidases, especially DPP I and DPP II, which are active at slightly acid pH (5.5-6.5) and are able to hydrolyse dipeptides like Ala-Gln, Arg-Gly, Asn-Pro, lle-Leu, Ala-Gly, Ser-Gly, Ser-Gln located in the N-terminal. An example for the action of such di-peptidyl-peptidases in shown in figure 4 where dipeptide Pro-Ala is sequentially released from the N-terminal of myosin light chain I (Mora, Sentandreu & Toldrá, 2011). Proline and alanine are also released by the action of aminopeptidase activity. Muscle tri-peptidyl peptidase I is also active at slightly acid pH (5.5-6.5) and is able to release certain tripeptides like Ile-Ile-Pro, Arg-Gly-Ala, Gly-Asn-Pro, Gly-Ala-Gly, Gly-Pro-Gly located at the N-terminal (Mora, Gallego, Escudero, Reig, Aristoy & Toldrá, 2015).

Some of the released di-peptides might be further hydrolysed by di-peptidase activity into their individual amino acids. This is especially relevant when those bioactive di-peptides because the bioactivity would be lost when broken down and no beneficial health effects would be observed. So, di-peptidase activity has been reported several microoorganisms like *L. plantarum* and *L. paracasei* that can hydrolyse Leu-Leu, Phe-Ala, and also Ala-Phe, Tyr-Leu and Lys-Leu, at lower rate while other dipeptides like Ala-Ala or Leu-Gly remain unaffected (González, Sacristán, ,Arenas, ,Fresno & Tornadijo, 2010). *L. brevis* has higher di-peptidase activity on Leu-Leu, Tyr-Leu, Ala-Ala, Leu-Gly, Ala-Phe, Lys-Leu and Phe-Ala and also *L. casei* sp *casei* but at much lower rate (González et al 2010). Di-peptides are reported to be more efficiently taken up by cellular transport systems and peptidases in *L. sakei* (Sinz & Schwab, 2012).

The released tri-peptides may be also hydrolysed into a single amino acid and a di-peptide. As mentioned for di-peptides, this would be also damaging if those tri-peptides are bioactive. A tri-peptidase from *L. sakei* was reported although tripeptides are also readily cleaved by Pep N of a variety of lactic acid bacteria (Flores & Toldrá, 2011). High aminopeptidase activity has been reported for *Leuconostoc mesenteroides* and *L. curvatus* while *L. plantarum, L. pentosus* and *Weissella cibaria* showed a variable enzymatic activity between strains (Zotta et al., 2007). In general, lactic acid bacteria show aminopeptidase activity being able to release different amino acids from the N-terminal. So, *L. plantarum, L. brevis* and *L. casei subsp casei* have been reported to release alanine, lysine, proline and leucine (Herreros et al., 2003), *L. paracasei subesp casei* releases alanine, arginine, lysine, methionine and leucine (Bintsis et al., 2003; Macedo et al., 2010), *L. sakei* releases alanine and leucine, *L. plantarum* releases leucine and *L. paracasei subsp paracasei* releases alanine, lysine, proline and leucine (González et al., 2010; Macedo et al., 2010). The yeast *Debaryomyces hansenii* was

303 reported to hydrolyse sarcoplasmic proteins and generate large amounts of most amino
304 acids (Santos et al., 2001).
305 On the other hand, very low or negligible carboxypeptidase activity has been reported in
306 cell-free extracts of several lactic acid bacteria (González et al., 2010; Herreros, et al.,
307 2003), and a low activity for *L. paracasei subsp paracasei* to release phenylalanine and
308 arginine (Bintsis et al., 2003; Macedo et al., 2010). However, endogenous
309 carboxypeptidase activity is more evident in muscle-based foods where the presence of
310 hydrophobic amino acids like phenylalanine, tyrosine, tryptophan, methionine,
311 isoleucine, leucine, valine and proline residues in the C-terminal promotes its hydrolysis
312 by endogenous muscle carboxypeptidase A. The rest of amino acids are preferentially
313 hydrolysed by muscle carboxypeptidase B (Mora et al., 2015a).
314 The extent of proteolysis and the amount of generated bioactive peptides depends on
315 multiple variables including the raw materials, the type of enzyme activity, the
316 microbial population, and processing conditions. A first insight on small peptides
317 generated in a model fermented sausage inoculated with *Lactobacillus curvatus*
318 CRL705 and *Staphylococcus vitulinus* GV318 gave some information on potential
319 routes for proteolysis during fermentation and ripening (López et al., 2015). Bioactive
320 peptides were generated in dry-sausages containing added sodium caseinate and
321 fermented with *Lactobacillus pentosus* and *Staphylococcus carnosus* (Mora et al.,
322 2015b). In both cases, Staphilococci peptidases might be involved in peptide generation
323 because CNS have been reported to exert an important proteolytic activity against meat
324 proteins (Mauriello, Casaburi, Blaiotta & Villani, 2004). In addition, *L. pentosus* and *S.*
325 *carnosus* have a proteinase attached to the cell wall that supports the extracellular casein
326 degradation into oligopeptides that can be further eluted into the cytoplasm and be
327 degraded by intracellular peptidases into smaller peptides and free amino acids (Chaves-

López et al., 2014). β-casein has been reported to be more hydrolysed that other types of caseins probably due to its abundance in proline, leucine and valine residues which are preferred by aminopeptidases and carboxypeptidases (Mora et al., 2015b).

Two hexapeptides with relevant antioxidant activity were isolated and identified after the simulated gastrointestinal digestion of Stracchino which is a soft cheese produced in the Northern Italy (Pepe et al., 2016).

The peptide profiles of nine months dry-cured ham after fractionation by gel filtration are shown in Figure 5 for ACE inhibitory activity and antioxidant activity measured through the DPPH and ferric reducing power. It can be observed that all 3 activities are concentrated in similar fractions corresponding to small peptides, with size <2500 Da.

5. Hydrolysis of food proteins by peptidases

Alternatively, proteins may be isolated and hydrolysed by specific commercial peptidases which can be obtained from different origins as listed in table 1. The hydrolysis is carried out in a reactor followed by separation/purification operations.

The cleavage site of food proteins is very relevant and changes for each enzyme. For instance, trypsin may cleave proteins at the carboxy side of arginine and lysine residues, chimotrypsin cleaves on the carboxy side of aromatic or hydrophobic amino acids. Pepsin A prefers phenylalanine, leucine or glutamic acid at the C-terminal. Alcalase prefers the carboxy side of hydrophobic residues.

The progress of protein hydrolysis is usually followed with the degree of hydrolysis (DH). Figure 5 is showing the progress of hydrolysis of thornback ray muscle hydrolysate treated with Alcalase, Neutrase, an enzyme preparation from *Bacillus subtilis* A26 and an extract of crude alkaline proteases from *Raja clavata* (Lassoued et al., 2015a). So, the hydrolysis of a food protein with different peptidases will result in

different peptides patterns. Furthermore, the number or amount of released peptides as such is not the final target which must be focused on the number and amount of bioactive peptides. This was clearly reported for muscle hydrolysates treated with an extract of crude alkaline proteases and Neutrase, although not showing the highest DH (see figure 5) were reported as the most powerful to prevent DNA oxidation (Lassoued et al., 2015a). However, a similar study with Thornback ray gelatin showed that the hydrolysate treated with Alcalase was the most protective against DNA oxidation (Lassoued et al., 2015b). Similarly, lentil protein concentrates that were hydrolysed with Alcalase gave the highest yield of peptides even though the hydrolysis with Savinase gave more bioactive peptides (García-Mora, Peñas, Frías & Martínez-Villaluenga, 2014). So, the choice of the most adequate peptidase for each type of protein and target peptide bioactivity must be carefully studied and considered.

Commercial peptidases are sometimes not clearly defined in the manufacturers specifications and this may affect the degree of hydrolysis and its content in small peptides and free amino acids. In addition to the main enzyme activity, some side activities may be found (see Table 1). A good example is Flavourzyme, a peptidase extracted from *Aspergillus oryzae*, that was recently subjected to a nine step purification and characterization. The results showed the activity of 3 endopeptidases but also other enzymes like 2 aminopeptidases, 2 dipeptidylpeptidases and one amylase (Merz et al., 2015). Further, a characterisation of 10 commercial peptidases was performed through a three-step metholodogy (Merz, Claaβen, Appel, Berends, Rabe, Blank et al., 2016). Exopeptidase activity, based on the release of free amino acids, was found in Alcalase 2.4L (Novozymes), Maxazyme NNP DS (DSM), Flavourzyme 1000L (Novozymes) and Protease AN (Amano Enzyme Inc.). Such exopeptidase activity can be attributed to aminopeptidase and carboxypeptidase activity. The rest of assayed commercial

peptidases were Bioprase SP-20FG (Nagase), Collupulin 200 L (DSM), Corolase2TS (AB Enzymes), Promod 439 L (Biocatalysts Ltd.), Proteinase T (DuPont) and Protin SD-AY10 (Amano Enzyme Inc.) and they were reported to exert majorly endopeptidase activity so that low degree of hydrolysis and poor generation of free amino acids may be expected (Merz et al., 2016). Other enzymes have shown also different peptide patterns. For instance, Neutrase was reported to give shorter peptide fragments than papain when hydrolysing rawhide collagen (Damrongsakkul, Ratanathammapan, Komolpis & Tanthapanichakoon, 2008). Whey protein concentrates hydrolysed with Neutrase also gave better iron absorption than those with papain or Alcalase (Ou et al., 2010). Pepsin, trypsin, protease M and flavourzyme have been successfully tested to produce calcium chelating peptides from different food protein sources (Sun, Wu, Du, Tang, Liu & Fu, 2016).

Sequential hydrolysis with different peptidase preparations may be used to produce bioactive peptides of interest. For instance, the hydrolysis of hen egg white lysozyme combining trypsin and papain gave a better yield of antioxidant and antimicrobial peptides than the use of trypsin or papain alone (Memarpoor-Yazdi, Asoodeh & Chamani, 2012). *Brassica carinata* proteins were sequentially hydrolysed with immobilised trypsin, chymotrypsin, and carboxypeptidase A and resulted in an enriched fraction with antioxidant peptides (Pedroche et al., 2007). Smooth hound viscera from *M. mustelus* was hydrolysed using commercial proteases (Purafeet, Neutrase and Esperase) and combinations of such commercial enzymes with endogenous enzymes, being the last one the best option for the higher recovery of antioxidant, ACE-inhibitory and antibacterial peptides (Abdelhedi et al., 2016). Eight commercial enzyme preparations were combined and used to obtain bioactive peptides from protein hydrolysates of defatted salmon backbone. The highest antioxidant and ACE inhibitory

activity was obtained with trypsin, bromelain, papain and protamex treatment (Slizyte, Rommi, Mozuraityte, Eck, Five & Rustad, 2016). Other authors reported an original way to improve the peptide profile and its bioactivity in a protein hydrolysate (Xu, Kong & Zhao, 2014). This was achieved with plastein that has the ability to reverse the hydrolytic action by peptidases, forming polypeptides. So, casein was first hydrolysed with Neutrase to generate ACE inhibitory peptides and this hydrolysate was then used as substrate for further plastein reaction that once optimised could increase the ACE inhibitory activity of the hydrolysate (Xu et al., 2014).

Some caution must be taken when using commercial enzymes especially in the efficacy and reproducibility of protein hydrolysis and also the enzymes stability. Some batch to batch variability may be observed due to variations in the activity of certain enzymes. For instance, flavorzyme was reported to have some variability in casein hydrolysis that was attributed to loss of endopeptidase activity along the storage time (Merz, Appel, Berends, Rabe & Blank, 2016). In the case of endogenous hydrolysis, the peptide profile may also change for similar types of foods due to different raw materials that may have different endogenous enzymes profiles but also to changes in processing.

6. Conclusions

The final result of protein hydrolysates consists of a pool of peptides with different sequences and lengths, some of them with a relevant bioactivity depending on the particular food and type and conditions of hydrolysis. Thus, the generated small peptides may exhibit a wide range of bioactivities such as angiotensin converting enzyme (ACE) inhibitory activity, antioxidant, antithrombotic, hypoglucemic, hypocholesterolemic, and antimicrobial activity among others. However, it must be considered that the generated bioactive peptides, either endogenously in food or a

protein hydrolysate, may be further hydrolysed when ingested through the gastrointestinal digestion. Further, those peptides should be absorbed intact through the intestinal barrier and reach the blood stream to exert their physiological action, overcoming the potential sequence modifications by brush border peptidases during transepithelial transport.

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Conflicts of interest

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

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724 LEGENDS FOR THE FIGURES
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726 Figure 1.- Scheme of protein hydrolysis and enzymes involved.
727 Figure 2.- Scheme of the generation of bioactive peptides from protein hydrolysis in
728 foods and/or the hydrolysis of isolated food proteins.
729 Figure 3.- Peptides identified by nanoESI-LC-MS/MS derived from ubiquitin 60S
730 ribosomal protein (UniProtKB/TrEMBL protein database accession number P63053).
731 Endopeptidase activity is showed in black arrows. Adapted from Mora, Gallego,
732 Aristoy, Fraser, Toldrá. 2015. Peptides naturally generated from ubiquitin-60S
733 ribosomal protein as potential biomarkers of dry-cured ham processing time. Food
734 Control, 48, 102-107.
735 Figure 4-. Intense degradation of Myosin Light Chain 1 (accession number
736 A1XQT6_PIG in UniProtKB/TrEMBL database), evidencing the action of amino
737 peptidases (in dark black) and dipeptidyl peptidases (in light black). Adapted from
738 Mora, Sentandreu and Toldrá. 2011. Intense degradation of myosin light chain isoforms
739 after dry-cured ham processing. Journal of Agricultural & Food Chemistry, 59, 3884-
740 3892.

741 Figure 5.- Hydrolysis curves of thornback ray muscle hydrolysates (TRMHs) treated
742 with Alcalase (TRMH-Alcalase), Neutrase (TRMH-Neutrase), enzyme preparation from
743 Bacillus subtilis A26 (TRMH-A26) and crude alkaline protease extract from R. clavata
744 (TRMH-Crude). Reproduced from Lassoued, Mora, Nssri, Aydi, Toldrá, Aristoy,
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746 of hydrolysates obtained from Thornback Ray (*Raja clavata*) muscle. Journal of
747 Proteomics, 128, 458-468, with permission from Elsevier.

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749

750 Table 1.- Commercial enzyme preparations with specific characteristics and some relevant
751 applications.

752

Commercial preparation	*Origin*	Manufacturers	Activity	Cleavage sites	Application	Literature
Flavourcyme 1000M	*Aspergillus oryzae*		3 endopeptidases 2 aminopeptidases 2 dipeptidylpeptidases 1 α-amylase		Cereals Calcium chelating peptides, Soy	Merz et al., 2015 Huang et al., 2015 Meinlschmidt et al, 2016
Valkerase	*Bacillus licheniformis*	Bri Enzymes	Keratinase, Serin Endopeptidase	Non especific	Feather meal	-
Prolidase	*L-lactis cremoris* *Other many sources*		Dipeptidase	Bonds including proline or hydroxiproline	Cheese making	Kitchener and Grunden, 2012
Bioprase SP-20FG	*Bacillus sp*		Subtilisin Endo metalloprotease Aminopeptidase			Merz et al., 2015
Neutrase	*Bacillus subtilis* *B. amyloliquefaciens*	Novozymes	Metalloprotease		Collagen Calcium- and iron-chelating peptides Soy	Ou et al., 2010 Meinlschmidt et al, 2016
Alcalase 2.4 L	*Bacillus licheniformis*	Novozymes	Subtilisin Alkaline serin endopeptidase Extracellular neutral metallo protease Aminopeptidase	Non especific	Calcium-chelating peptides	Choi et al., 2012 Charoenphun et al., 2013

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755 Figure 1

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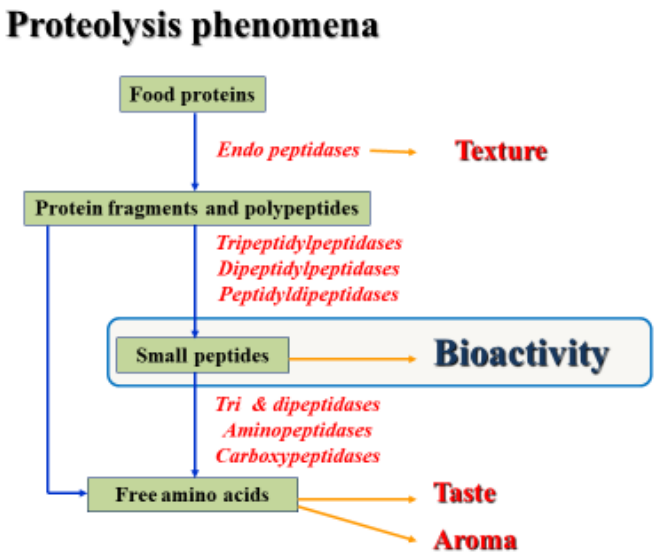

Figure 1.- Scheme of protein hydrolysis and enzymes involved.

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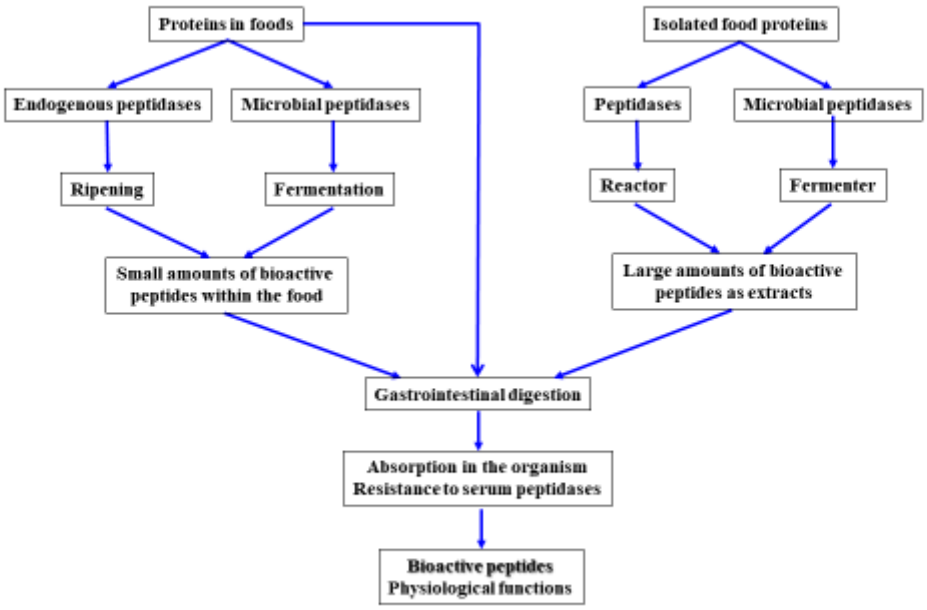
759 Figure 2



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

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762

763 Figure 3

P_0	Sequence ↓ ↓ ↓ ↓	P_f
3'	**FVKTLTGKTITLEVEPSDTIENVKAKIQDKEGIPPDQQRLIFAGKQLEDGRTLSDYNIQKESTL**	67'
I	FVKTLTGKTITL	E
L	EVEPSDTIENVKAKIQDK	E
K	EGIPPDQQRL	I
L	IFAGKQLEDGRTL	S
L	SDYNIQKESTL	H

Figure 3. Peptides identified by nanoESI-LC-MS/MS derived from ubiquitin 60S ribosomal protein (UniProtKB/TrEMBL protein database accession number P63053). Endopeptidase activity is showed in black arrows. Adapted from Mora, L., Gallego, M., Aristoy, MC., Fraser, PD., Toldrá, F. (2015) Peptides naturally generated from ubiquitin-60S ribosomal protein as potential biomarkers of dry-cured ham processing time. Food Control, 48, 102-107.

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767 Figure 4

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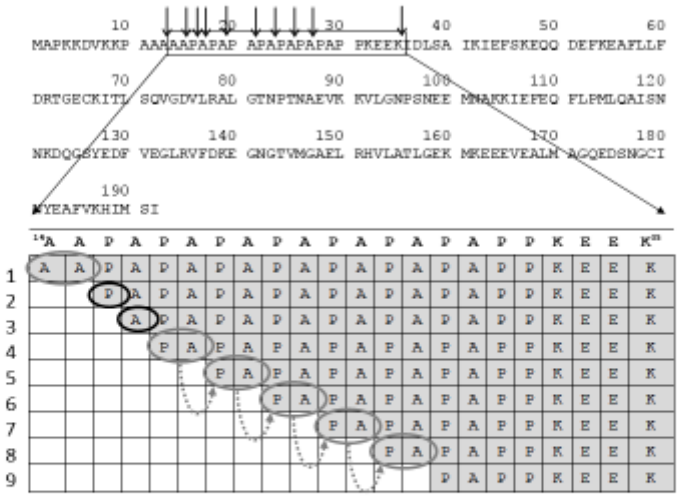

Figure 4. Intense degradation of Myosin Light Chain 1 (accession number A1XQT6_PIG in UniProtKB/TrEMBL database), evidencing the action of amino peptidases (in dark black) and dipeptidyl peptidases (in light black).

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772 Figure 5

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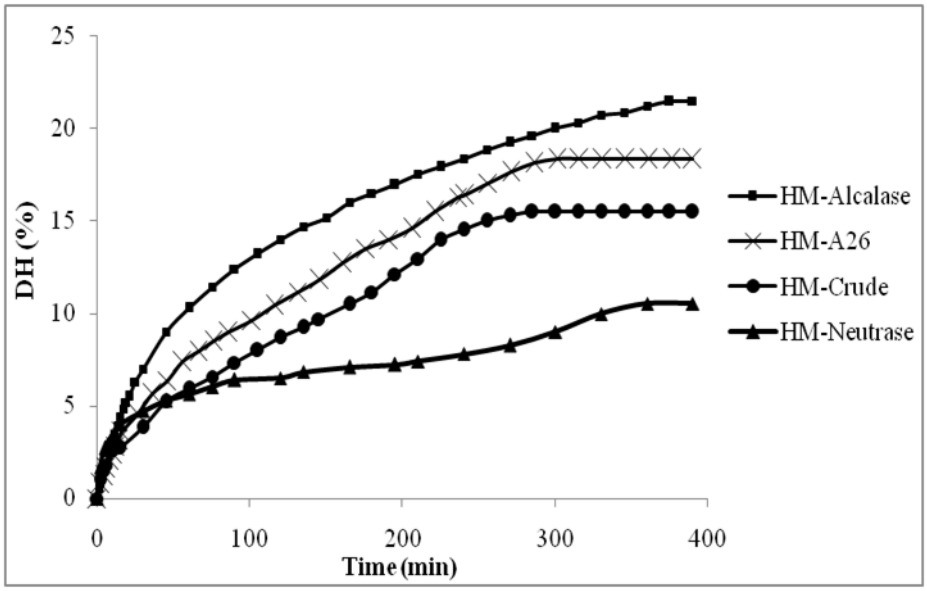

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