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Additional Information

**BIOACTIVE PEPTIDES GENERATED FROM MEAT INDUSTRY BY-
PRODUCTS**

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Running title: Bioactive peptides from meat by-products

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26 ABSTRACT

27 There is a large generation of meat by-products, not only from slaughtering but also in
28 the meat industry from trimming and deboning during further processing. This results in
29 extraordinary volumes of by-products that are primarily used as feeds with low returns
30 or, more recently, to biodiesel generation. The aim of this work was to review the state
31 of the art to generate bioactive peptides from meat industry by-products giving them an
32 added value. Hydrolysis with commercial proteases constitute the typical process and a
33 variety of peptides result from such extensive proteolysis. This review focuses on the
34 identification of a large number of peptides derived from the enzymatic hydrolysis of
35 specific meat by-products and its characterisation for bioactivity. The potential of some
36 of the identified peptides to be used as bioactive supplements in foods has also been
37 considered.

40 KEYWORDS

41 Peptides, bioactive peptides, protein hydrolysis, proteolysis, mass spectrometry,
42 bioactivity, antihypertensive peptides, antioxidant peptides, antimicrobial peptides

46 INTRODUCTION

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3 47 Meat industry annually produces tons of by-products that represent a cost for the meat
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6 48 processing sector as well as an important environmental problem. The generation of by-
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9 49 products depends on tradition, culture, and religion of the production countries, but
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11 50 usually includes trimming, bones, blood, and skin (Nollet & Toldrá, 2011). Nowadays,
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13 51 industries are making a strong effort converting by-products and wastes into useful
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16 52 sources of both, edible and non-edible products, producing valuable new products and
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18 53 functional ingredients with a significant added-value and/or a strong economic potential
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21 54 (Zhang, Xiao, Samaraweera, Lee & Ahn, 2010; Toldrá, Aristoy, Mora and Toldrá,
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23 55 2012). Fertilizers as well as biodiesel generation, pharmaceuticals, and plastic or energy,
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26 56 would be the main non-edible use of by-products (Pearl, 2004; Ockerman and Basu,
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28 57 2004a,b). However, due to its strong technologic and economic potential, the
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31 58 development and application of edible uses for meat by-products is a current concern in
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33 59 the research community. In this sense, one of the most studied and promising lines is the
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36 60 production of protein hydrolysates, that may be used as flavor enhancers, emulsifiers,
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38 61 enhancers of water bonding capacity or nutrients to be added to foods since they
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41 62 constitute an excellent source of nutrients like essential amino acids, minerals and
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43 63 vitamins (Aristoy and Toldrá, 2011, Honikel, 2011, Kim, 2011; García-Llatas, Alegría,
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45 64 Barberá and Farré, 2011), and functional ingredients like bioactive peptides (Toldrá &
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47 65 Reig, 2011; Zhang, Xiao, Samaraweera, Lee, & Ahn, 2010).

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51 66 Science and innovation is helping the meat industry to add value to its meat by-products
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53 67 reducing the environmental damage but most important, converting them into products
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56 68 capable of covering all the processing and disposal costs (Toldrá, Mora & Reig, 2012).

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58 69 A diagram showing the main routes for generation of bioactives from meat by-products
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70 is shown in Figure 1. This manuscript reviews the latest innovations to generate
71 bioactive peptides from meat by-products giving them a high added value.

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73 BY-PRODUCTS TREATMENT THROUGH ENZYMATIC HYDROLYSIS FOR
74 PEPTIDES GENERATION

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76 Meat by-products wastes (trimmings and mechanically recovered meat, collagen, blood)
77 are, in general, very rich in proteins and thus, they constitute a good substrate for
78 proteolysis. These proteins are subject to hydrolysis with specific commercial proteases
79 like papain, bromelain, thermolysine, pronase or proteinase K (Vercruysse, Van Camp
80 & Smagghe, 2005). Other commercial enzymes are Neutrase[®], a metallo-protease from
81 *Bacillus amyloliquefaciens* (4 hours at pH 7.0, 50°C), Alcalase[®], a serine-protease from
82 *Bacillus licheniformis* (4 hours at pH 8.0, 50°C) or crude enzyme extract from *R.*
83 *Clavata* (4 hours at pH 8.0, 40°C). The hydrolysis reaction is usually carried out either
84 in batch-fed reactors or in continuous reactors using ultrafiltration membrane. Once the
85 desired degree of hydrolysis is reached, the product is then submitted to fractionation
86 and partial purification through filtration and/or chromatographic techniques (Arihara,
87 2006). A typical industrial production is schematised in Figure 2. As the enzymatic
88 hydrolysis is usually intense, a large number of peptides are generated.
89 Endogenous proteolytic activity may also contribute to the generation of peptides and
90 free amino acids through proteolysis mechanisms (Toldrá, 2006). Meat by-products
91 contain endogenous muscle enzymes like calpains and cathepsins that break proteins
92 internally followed by the action of peptidylpeptidases that generate small peptides from
93 the amino and carboxy termini (Arihara, 2006a; Sentandreu & Toldrá, 2007; Mora,
94 Sentandreu, Koistinen, Fraser, Toldrá & Bramley, 2009).

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95 Some of the generated peptides are denominated bioactive peptides because they may
96 be able to exert a determined health benefit to the consumer like antihypertensive
97 activity (Arihara & Ohata, 2010).

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99 TYPES OF BIOACTIVITY IN THE GENERATED PEPTIDES

100

101 Bioactive peptides usually contain between 3–20 amino acid residues and their
102 bioactivities are based on their amino acid composition and location within the
103 sequence of amino acids that form the peptide (Pihlanto-Leppala, 2001). They are
104 inactive in the sequences of their parent proteins, but may be released through
105 enzymatic hydrolysis (Kim et al., 1999; Lahl and Braun, 1994), by proteolytic enzymes
106 during gastrointestinal digestion (Escudero et al 2010), during fermentations with
107 generally recognised as safe (GRAS) bacteria such as Lactobacilli (Philanto et al., 2001)
108 or during food processing (Arihara and Ohata, 2010). In order to exert a positive health
109 effect, bioactive peptides must survive enzyme degradation in the gastrointestinal tract
110 following consumption. Once liberated in the human body, bioactive peptides can affect
111 numerous physiological functions. Depending on their amino acid sequence, they may
112 be involved in biological functions including prevention of hypertension (ACE-I-
113 inhibitory and antihypertensive peptides), opioid agonists or antagonists,
114 immunomodulatory, antithrombotic, antioxidant, anti-cancer, or antimicrobial activities.

115 Bioactive peptides are able to inhibit the angiotensin I-converting enzyme (ACE), an
116 enzyme that participates in the renin-angiotensin system where angiotensin I is
117 converted into antiotensin II that constricts the arteries and, as a consequence, increases
118 the blood pressure. So, the inhibition of ACE constitutes an efficient way to reduce
119 blood pressure (Ahmed & Mugurama, 2010). This inhibitory activity can be measured

120 *in vitro* and *in vivo* but its effects may not be similar because bioactive peptides must
121 reach the cardiovascular system in an intact form. This is not always achieved because
122 the proteases in the human gastrointestinal tract might hydrolyse some peptides and
123 reduce them to smaller inactive peptides. Another drawback to exert its effect is the
124 difficulty found in its absorption through the intestinal wall into the blood. In general, the
125 bioactivity intensity is usually inversely correlated to the peptide length (Vermeissen,
126 Van Camp & Verstraete, 2004). Therefore, it is necessary that peptides can inhibit ACE
127 *in vitro* but also exert antihypertensive effect *in vivo* because then they can be object of
128 the development of novel functional foods for preventing hypertension.
129 Other activities of interest are the antioxidant activity, antimicrobial or opioid activity
130 among others. The antioxidant peptides can be detected through their DPPH radical-
131 scavenging activity and reducing power. It is important because such antioxidant
132 activity may reduce the reactive oxygen species (ROS) and other free radicals present in
133 the food that might produce oxidative damage to DNA, proteins, and other
134 macromolecules such as lipids (Escudero, Mora, Fraser, Aristoy and Toldrá, 2013). The
135 opioid peptides received such name because they have an affinity for an opioid receptor
136 that may exert an effect on the nerve system (Guesdon, Pichon & Tomé, 2005). The
137 antimicrobial peptides are able to inhibit the growth of certain pathogen bacteria (Chan
138 & Li-Chan, 2005).

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140 PEPTIDES GENERATION FROM SPECIFIC BY-PRODUCTS IN THE MEAT

141 INDUSTRY

142

143 *Trimmings and cuttings*

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144 Trimmings are portions of meat remaining after the preparation of primal cuts from the
145 carcass and include fat, gristles, and meat. They can also include mechanically
146 recovered meat. Portions of the head meat, internal organs, major tendons, or ligaments,
147 are not considered as trimmings. They are mainly obtained by removing the last traces
148 of skeletal muscle meat from animal bones once the primal cuts that have been carved
149 off manually in the deboning process.

150 Despite the meat industry make a take care of trimmings and cuttings transforming them
151 into secondary quality meat products such as hot dogs, these by-products are as good
152 source for bioactive peptides as the primal cuts. In fact, a wide number of studies based
153 on the bioactive peptides generation resulting from meat protein hydrolysis have been
154 described. Antihypertensive activity is by far the most studied biological activity
155 although antioxidant or antimicrobial peptides derived from muscular proteins have also
156 been described. A wide variety of enzymes have been tested in these studies. As an
157 example, porcine skeletal muscle proteins were hydrolysed by using eight proteases and
158 ACE-inhibitory activity measured (Arihara et al., 2001). Among the digests,
159 thermolysin showed the best inhibitory activity, and peptides MNPPK and ITTNP were
160 isolated and identified as ACE-inhibitors with IC_{50} of 945.5 and 549 μ M, respectively.
161 These peptides were tested in spontaneously hypertensive rats administering single oral
162 doses, proving their *in vivo* antihypertensive activity (Nakashima et al., 2002). Another
163 peptide RMLGQTPTK (44–52 position of troponin C) was purified from porcine
164 skeletal troponin hydrolysed with pepsin and showed ACE-inhibitory activity Katayama
165 et al (2003). It showed an IC_{50} of 34 μ M. Same authors digested myosin light chain
166 extracted from Japanese domestic pork loin with pepsin enzyme, and measured the
167 ACE-inhibitory activity of the digest. This study resulted on the isolation and
168 identification of the octapeptide VKKVLGNP, with an IC_{50} of 28.5 μ M (Katayama et

169 al., 2007). On the other hand, antioxidant and free radical scavenging activities were
170 tested in a papain hydrolysate of pork myofibrillar proteins (Saiga et al., 2003). From
171 the isolated and identified peptides, DAQEKLE sequence showed the highest
172 antioxidant activity. In another study, peptides DLYA, SLYA, and VW were tested *in*
173 *vitro* and *in vivo* for their antioxidant activity showing anti-fatigue effect in
174 spontaneously hypertensive rats (Arihara et al 2006).

175 The industry of meat products is also an important producer of trimmings that would be
176 an interesting source of ACE-inhibitory and antioxidant peptides as indicated through
177 the studies carried out on dry-cured ham during the last decade. In this sense,
178 antihypertensive and antioxidant activities have been described in peptide fractions
179 extracted from Spanish dry-cured ham (Escudero et al., 2012). In this study, fractions
180 were tested for their antihypertensive activity *in vitro* and *in vivo* by measuring changes
181 in systolic blood pressure (SBP) of spontaneously hypertensive rats as shown in Figure
182 3, obtaining a decrease of 38.38 mmHg in one of the analysed fractions. Recent studies
183 focused on the purification and identification of specific peptide sequences extracted
184 from dry-cured ham pointed the potential of this product as a source of antihypertensive
185 and antioxidant peptides (Escudero et al., 2013b; Escudero, Mora, Fraser, Aristoy, &
186 Toldrá, 2013a).

187 The *in vitro* simulation of pork meat proteins digestion with gastrointestinal enzymes
188 such as pepsin, chymotrypsin, and pancreatin, as well as the *in vivo* test of the identified
189 peptides, is necessary to know more data about their stability against digestive proteases
190 as well as their absorption through the intestinal wall. In this respect, the stability of
191 ACE-inhibitory activity of dry-cured ham peptides during processing and after *in vitro*
192 digestion has been recently investigated (Escudero et al., 2014). Results indicate that

193 peptides preserve almost the same ACE inhibitory activity before and after applying
194 diverse heating and time conditions, as well as simulated *in vitro* digestion with
195 gastrointestinal proteases.

196

197 ***Bones (Horn)***

198 Bones, horns, and hooves resulting from meat industry are mainly used as feed material,
199 organic fertilisers, or soil. Very few studies have described the purification and
200 identification of bioactive peptides from these by-products. In this respect, the
201 antioxidant peptides QYDQGV, YEDCTDCGN, and AADNANELFPPN, have been
202 identified from an aqueous extract of water buffalo horn, commonly used in Chinese
203 medicine. Results showed that these peptides could reduce the DPPH radical and protect
204 rat cerebral microvascular endothelial cells against H₂O₂-induced injury (Liu et al.,
205 2010).

206 However, in the sector of marine by-products, backbones hydrolysed using different
207 enzymes have been widely studied as a source of bioactive peptides, promoting human
208 health and preventing chronic disease (Šližytė et al, 2009; Ravallec et al, 2001; and Kim
209 et al., 2000). As an example, the antioxidant peptide VKAGFAWTANQQLS was
210 purified and identified in a study where tuna backbone was hydrolysed using various
211 proteases such as alcalase, α-chymotrypsin, neutrase, papain, pepsin, and trypsin (Je et
212 al., 2007).

213 Bones constitute one of the most important sources to obtain collagen and gelatin,
214 which have been described as proteins containing biologically active peptides on their
215 sequences, with promising health benefits for humans.

216

217

218 **Collagen**

219 Collagen is the most abundant protein in vertebrates as it is the main fibrous protein
220 constituent in bones, cartilages, and skin (Gómez-Guillén et al., 2011). Collagen is one
221 of the most useful proteins used in pharmaceutical companies as it has been proved that
222 orally administered collagen peptides have beneficial effects on bone metabolism.
223 Regarding this, ingested collagen hydrolysates obtained from chicken legs have been
224 described to improve bone mineral density in rat finding that exerts a beneficial effect
225 on osteoporosis by increasing the organic substance content of bone (Watanabe-
226 Kamiyana et al., 2010). On the other hand, chicken bone collagen hydrolysates
227 treatment might help to prevent atherosclerosis through their lipid-lowering effects as
228 well as inhibiting expression of inflammatory cytokines (Zhang et al., 2010).

229 Despite the nutritional value of collagen is very low because it is specially rich in non-
230 essential amino acids (Gly, Pro, and Hyp), it results a very important protein in food
231 industry as a source of bioactive peptides. During the last years, many studies have been
232 focused on the bioactive properties of collagen enzymatic hydrolysis prepared using
233 different by-product sources and different enzymes. Typically, collagen hydrolysate
234 peptides were produced from pig or bovine by-products, however, due to the incidence
235 of mad cow disease, an increase in results coming from marine processing waste
236 sources such as skin collagen has occurred (Alemán et al., 2013).

237 Most of the studies about collagen peptides that are focused on their bioactive properties
238 have dealt in their antioxidant and ACE-inhibitory activity. Thus, four antioxidant
239 peptides were identified from hydrolysed porcine skin collagen obtained using different
240 protease treatments. One of the antioxidative peptides, Gln-Gly-Ala-Arg, was
241 synthesized and the antioxidant confirmed *in vitro* (Li et al., 2007). On the other hand,
242 four peptides showing good *in vitro* and *in vivo* ACE- inhibitory activity against

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243 spontaneous hypertensive rats were reported from chicken skin collagen hydrolysate
244 obtained by treatment with an *Aspergillus* species derived enzyme (Saiga, et al, 2008).
245 More recently, two ACE-I inhibitory peptides with sequences
246 AKGANGAPGIAGAPGFPGARGPSGPQGPPSGPP and
247 PAGNPGADGQPGAKGANGAP, have been identified from bovine Achilles tendon
248 collagen. Bacterial collagenase was used to hydrolyze the collagen and it was described
249 that peptides retained 80% of ACE-I inhibitory activity after *in vitro* simulation of
250 gastrointestinal tract (Banerjee & Shanti, 2012).

251

252 ***Blood***

253 Blood is a body fluid that constitutes a rich protein by-product. It is composed of blood
254 cells suspended in blood plasma, being the cellular elements red blood cells (also called
255 erythrocytes) and white blood cells, including leukocytes and platelets. Plasma contains
256 proteins such as fibrinogen, globulins and albumins (Bah et al., 2013). Albumin is the
257 main protein in plasma, and is a key element in the regulation of fluid distribution,
258 colloidal osmotic pressure and the transport of small metabolites in blood (Rondeau &
259 Bourdon, 2011). Red blood cells are the most abundant cells in vertebrate blood and
260 contain hemoglobin, an iron-containing protein. This protein facilitates the reversibly
261 binding of oxygen increasing its solubility and transportation in blood.

262 Blood represents up to 4% of animal weight and could become a problematic by-
263 product in meat industry due to the tons of blood generated and its high pollutant
264 characteristics for the environment. The interest in searching new blood uses exists
265 since the beginning of slaughterhouses. In fact, its high content in proteins makes blood
266 useful in food industry to increase the final nutritional value of some foods, enhance
267 water binding, and because of its emulsifying capacity (Ofori and Hsieh, 2011).

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1 269 Blood is mostly obtained from bovine and porcine sources and studies related with its
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3 270 value as generator of bioactive peptides used to be focused on the cellular fraction,
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6 271 specially hemoglobin cells, and the plasma fraction.
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8 272 Hemoglobin and plasma hydrolysates have been described to mainly exert
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10 273 antihypertensive, antioxidant, antimicrobial, and opioid activity. Some peptidic
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12 274 sequences showing these activities have been isolated and characterized using modern
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14 275 proteomic techniques. In this sense, peptides GFPTTKTYFPHF and VVYPWT,
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16 276 corresponding to the 34–46 fragment of the α -chain and the 34–39 fragment of the
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18 277 β -chain of porcine hemoglobin, and obtained from a hydrolysate with pepsin enzyme,
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21 278 resulted to be ACE-inhibitory peptides, showing IC_{50} values of 4.92 and 6.02 μ M,
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23 279 respectively (Yu et al., 2006). Antimicrobial activity of peptides derived from
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26 280 hemoglobin chain is, by far, the most studied. Peptides
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28 281 TKAVEHLDDLPGALSELSDLHAHKLRVDPVNFKLLSHSL,
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31 282 LDDLPGALSELSDLHAHKLRVDPVNFKLLSHSL, KLLSHSL, and LLSHSL,
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34 283 obtained from the hydrolysis of bovine α -chain hemoglobin with pepsin, presented
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36 284 antibacterial activity against *Kocuria luteus*, *Listeria innocua*, *Escherichia coli*, and
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38 285 *Staphylococcus aureus*, as well as showed ACE inhibitory activity in an IC_{50} range from
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41 286 42.55 to 1,095 μ M (Adje et al, 2011). Catiau et al. (2011a) studied the minimal peptide
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44 287 sequence necessary to show antimicrobial activity when a digestion of bovine α -chain
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46 288 hemoglobin with pepsin was done. Results showed that KYR, which was studied
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49 289 against five bacterial strains including *Escherichia coli* and *Salmonella enteritidis* as
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52 290 Gram-negative bacteria and *Listeria innocua*, *Micrococcus luteus* and *Staphylococcus*
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55 291 *aureus* as Gram-positive bacteria, was contained in all active peptides showing
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58 292 antimicrobial activity. Same authors did a similar work but studying bovine β -chain
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293 hemoglobin and concluded with the sequence RYH as the minimal antimicrobial
294 sequence of this protein (Catiau et al., 2011b). In previous studies, Daoud et al. (2005)
295 isolated and purified an antimicrobial peptide with sequence
296 VTLASHLPSDFTPAVHASLDKFLANVSTVL from α -chain bovine hemoglobin by
297 hydrolysis with pepsin. The peptide displayed antimicrobial activity against *M. luteus*
298 A270, *Listeria innocua*, *Enterococcus faecalis*, *Bacillus cereus*, *Staphylococcus*
299 *saprophyticus* and *Staphylococcus simulans*. In fact, a MIC of 38 μ M was reported
300 against *L. innocua* and 76 μ M for the other bacterial species (Daoud et al., 2005).
301 Nedjar-Arroume et al. (2006) identified three peptides corresponding to positions 107–
302 141, 137–141, and 133–141 fragments of α -chain bovine hemoglobin, and 126–145
303 from β -chain, all of them showing antibacterial activity against *Micrococcus luteus*
304 A270, *Listeria innocua*, *Escherichia coli*, and *Salmonella enteritidis* (Nedjar-Arroume
305 et al., 2006). Same authors identified in another study with pepsin enzyme a total of
306 thirty antibacterial peptides, and twenty-four and six of them derived from α - and β -
307 chains of hemoglobin, respectively (Nedjar-Arroume et al., 2008). More recently, Hu et
308 al. (2011) identified a novel antimicrobial peptide derived from α -chain bovine
309 hemoglobin sequenced as VNFKLLSHSLLVTLASHL. The peptide showed
310 antimicrobial activity against *Escherichia coli*, *Staphylococcus aureus*, and *Candida*
311 *albicans* when assessed using the radial-diffusion plate assay (Hu et al., 2011).
312 Many of the studies related to opioid peptides from meat-derived sources are based on
313 blood hydrolysates. In fact, originally, hemorphins were isolated from enzymatically
314 treated bovine blood. Brantl et al. (1986) isolated and determined the sequence of an
315 opioid active tetrapeptide (YPWT) from bovine blood hydrolysed with gastrointestinal
316 enzymes. During the past decades, a number of opioid active peptides containing this
317 sequence have been reported such as LVVYPWT, LVVYPWTQR, and

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318 LVVYPWTQRF, which were found to be relatively stable and are believed to interact
319 with opioid receptors in the brain and cardiovascular system (Nyberg, Carlsson, &
320 Hallberg, 2013; Collinder, Nyberg, Sanderson-Nydahl, Gottlieb-Vedi, & Lindholm,
321 2005).

322 Some studies about the antioxidant properties of porcine plasma protein hydrolysate
323 have been published during the last decade, but no sequences of the responsible
324 peptides have been described (Liu et al., 2009 and 2010; Xu et al., 2009; and Wang et
325 al., 2008).

326

327 FUTURE TRENDS

328 There is a large variety of applications of meat by-products. Traditional applications are
329 primarily human and animal foods. Other applications consist of rendered fat for
330 cosmetics and chemicals and hides for leather. More recent innovations are related to
331 the use of proteins taking profit of its technological properties or for improved
332 nutritional properties, and the hydrolysis of proteins for the generation of peptides with
333 biological activity. So, it is still necessary to analyze by-products for nutritional
334 properties, in order to search for key active molecules in food and nutrition. This is
335 basic when considering innovative value-addition for such meat by-products.

336

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669 **Legends for the figures**

670

671 Figure 1.- Flow diagram of main routes for value-addition to meat by-products

672

673 Figure 2.- Flow diagram for the generation of bioactive peptides through the enzymatic
674 hydrolysis of edible meat by-products.

675

676 Figure 3. - Fractionation of dry-cured ham extract on a Sephadex G-25 gel filtration
677 column. Fractions were collected and assayed for in vitro ACE-inhibitory activity. For
678 antihypertensive in vivo assay in the present study, fractions corresponding to an elution
679 volume from 200 mL to 320 mL were pooled and named sample 1 (S1). The same
680 procedure was followed for fractions corresponding to elution volumes from 325 mL to
681 450 mL (S2) and those from 505 mL to 625 mL (S3). Reprinted from Escudero et al
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683

Figure 1

Figure 1

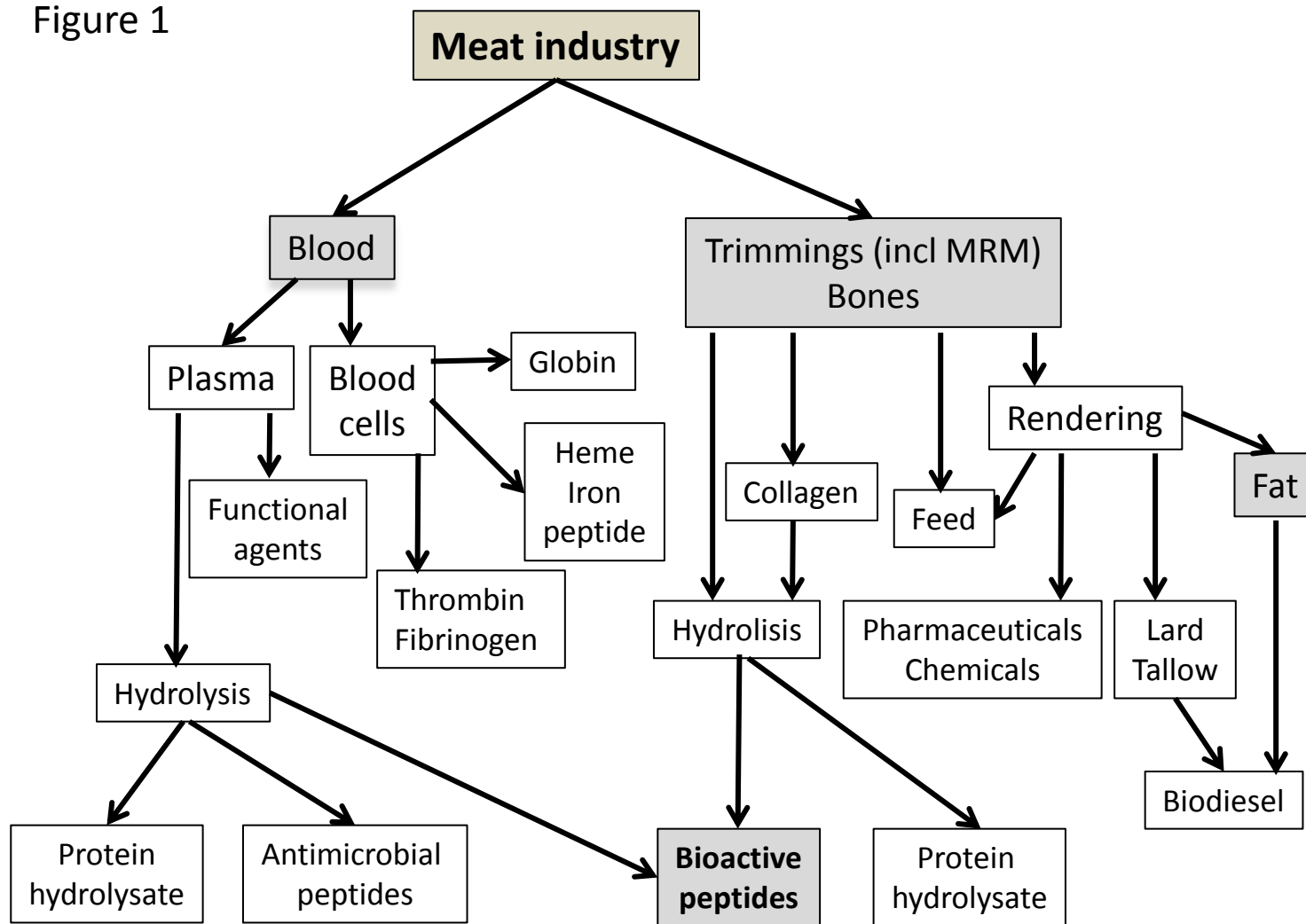


Figure 2

Figure 2

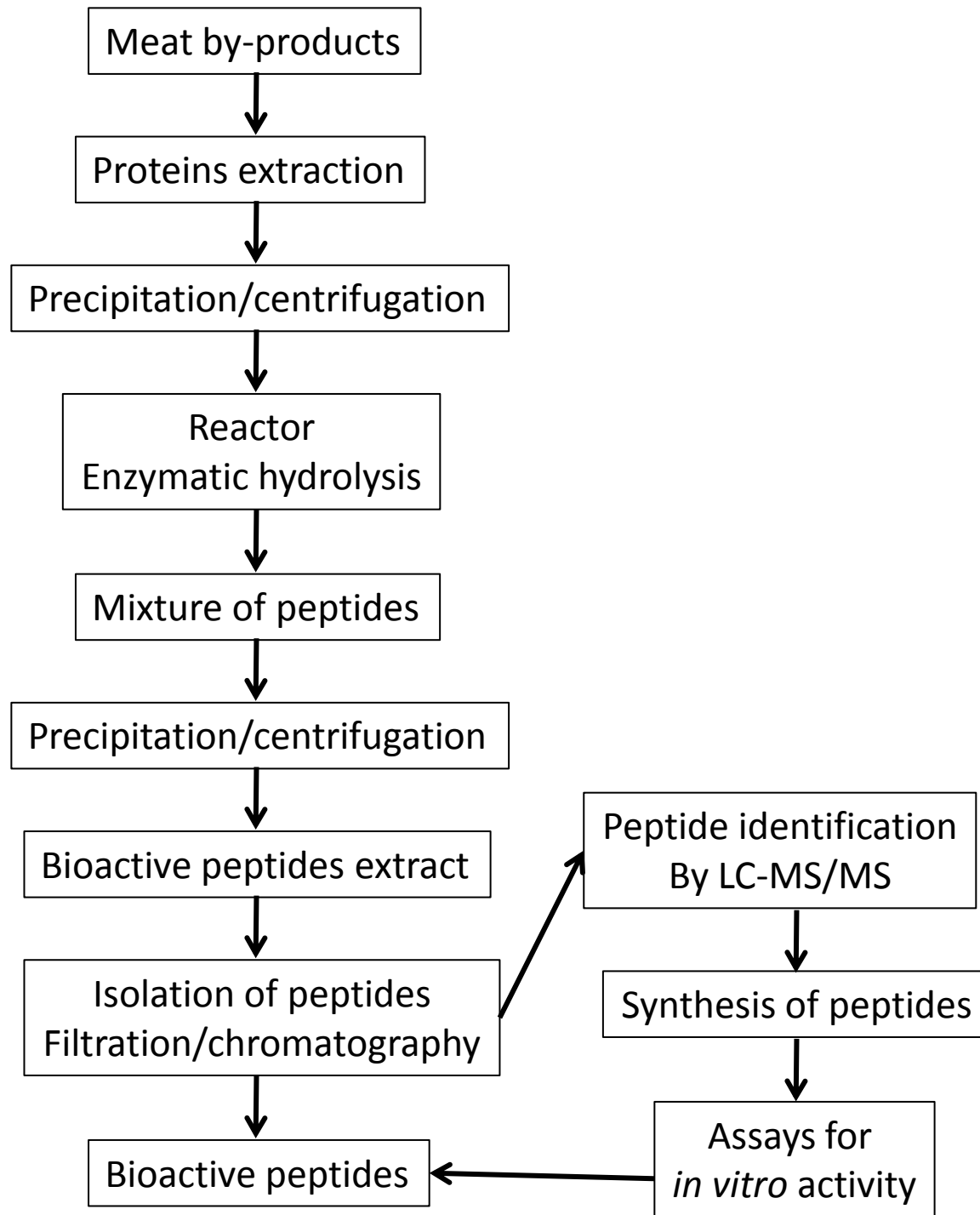


Figure 3

