BIOACTIVE PEPTIDES GENERATED FROM MEAT INDUSTRY BY-PRODUCTS

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ABSTRACT

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

KEYWORDS

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

Meat industry annually produces tons of by-products that represent a cost for the meat processing sector as well as an important environmental problem. The generation of by-products depends on tradition, culture, and religion of the production countries, but usually includes trimming, bones, blood, and skin (Nollet & Toldrá, 2011). Nowadays, industries are making a strong effort converting by-products and wastes into useful sources of both, edible and non-edible products, producing valuable new products and functional ingredients with a significant added-value and/or a strong economic potential (Zhang, Xiao, Samaraweera, Lee & Ahn, 2010; Toldrá, Aristoy, Mora and Toldrá, 2012). Fertilizers as well as biodiesel generation, pharmaceutics, and plastic or energy, would be the main non-edible use of by-products (Pearl, 2004; Ockerman and Basu, 2004a,b). However, due to its strong technologic and economic potential, the development and application of edible uses for meat by-products is a current concern in the research community. In this sense, one of the most studied and promising lines is the production of protein hydrolysates, that may be used as flavor enhancers, emulsifiers, enhancers of water bonding capacity or nutrients to be added to foods since they constitute an excellent source of nutrients like essential amino acids, minerals and vitamins (Aristoy and Toldrá, 2011, Honikel, 2011, Kim, 2011; García-Llatas, Alegría, Barberá and Farré, 2011), and functional ingredients like bioactive peptides (Toldrá & Reig, 2011; Zhang, Xiao, Samaraweera, Lee, & Ahn, 2010).

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

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

BY-PRODUCTS TREATMENT THROUGH ENZYMATIC HYDROLYSIS FOR PEPTIDES GENERATION

Meat by-products wastes (trimmings and mechanically recovered meat, collagen, blood) are, in general, very rich in proteins and thus, they constitute a good substrate for proteolysis. These proteins are subject to hydrolysis with specific commercial proteases like papain, bromelain, thermolysine, pronase or proteinase K (Vercruysse, Van Camp & Smagghe, 2005). Other commercial enzymes are Neutrase®, a metallo-protease from Bacillus amyloliquefaciens (4 hours at pH 7.0, 50°C), Alcalase®, a serine-protease from Bacillus licheniformis (4 hours at pH 8.0, 50°C) or crude enzyme extract from R. Clavata (4 hours at pH 8.0, 40°C). The hydrolysis reaction is usually carried out either in batch-fed reactors or in continuous reactors using ultrafiltration membrane. Once the desired degree of hydrolysis is reached, the product is then submitted to fractionation and partial purification through filtration and/or chromatographic techniques (Arihara, 2006). A typical industrial production is schematised in Figure 2. As the enzymatic hydrolysis is usually intense, a large number of peptides are generated.

Endogenous proteolytic activity may also contribute to the generation of peptides and free amino acids through proteolysis mechanisms (Toldrá, 2006). Meat by-products contain endogenous muscle enzymes like calpains and cathepsins that break proteins internally followed by the action of peptidylpeptidases that generate small peptides from the amino and carboxy termini (Arihara, 2006a; Sentandreu & Toldrá, 2007; Mora, Sentandreu, Koistinen, Fraser, Toldrá & Bramley, 2009).
Some of the generated peptides are denominated bioactive peptides because they may be able to exert a determined health benefit to the consumer like antihypertensive activity (Arihara & Ohata, 2010).

TYPES OF BIOACTIVITY IN THE GENERATED PEPTIDES

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

Bioactive peptides are able to inhibit the angiotensin I-converting enzyme (ACE), an enzyme that participates in the renin-angiotensin system where angiotensin I is converted into antiotensin II that constricts the arteries and, as a consequence, increases the blood pressure. So, the inhibition of ACE constitutes an efficient way to reduce blood pressure (Ahmed & Mugurama, 2010). This inhibitory activity can be measured...
in vitro and in vivo but its effects may not be similar because bioactive peptides must reach the cardiovascular system in an intact form. This is not always achieved because the proteases in the human gastrointestinal tract might hydrolyse some peptides and reduce them to smaller inactive peptides. Another drawback to exert its effect is the difficulty found in its absorption through the intestinal wall into the blood. In general, the bioactivity intensity is usually inversely correlated to the peptide length (Vermeissen, Van Camp & Verstraete, 2004). Therefore, it is necessary that peptides can inhibit ACE in vitro but also exert antihypertensive effect in vivo because then they can be object of the development of novel functional foods for preventing hypertension.

Other activities of interest are the antioxidant activity, antimicrobial or opioid activity among others. The antioxidant peptides can be detected through their DPPH radical-scavenging activity and reducing power. It is important because such antioxidant activity may reduce the reactive oxygen species (ROS) and other free radicals present in the food that might produce oxidative damage to DNA, proteins, and other macromolecules such as lipids (Escudero, Mora, Fraser, Aristoy and Toldrá, 2013). The opioid peptides received such name because they have an affinity for an opioid receptor that may exert an effect on the nerve system (Guesdon, Pichon & Tomé, 2005). The antimicrobial peptides are able to inhibit the growth of certain pathogen bacteria (Chan & Li-Chan, 2005).

PEPTIDES GENERATION FROM SPECIFIC BY-PRODUCTS IN THE MEAT INDUSTRY

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

Despite the meat industry make a take care of trimmings and cuttings transforming them into secondary quality meat products such as hot dogs, these by-products are as good source for bioactive peptides as the primal cuts. In fact, a wide number of studies based on the bioactive peptides generation resulting from meat protein hydrolysis have been described. Antihypertensive activity is by far the most studied biological activity although antioxidant or antimicrobial peptides derived from muscular proteins have also been described. A wide variety of enzymes have been tested in these studies. As an example, porcine skeletal muscle proteins were hydrolysed by using eight proteases and ACE-inhibitory activity measured (Arihara et al., 2001). Among the digests, thermolysin showed the best inhibitory activity, and peptides MNPPK and ITTNP were isolated and identified as ACE-inhibitors with IC\(_{50}\) of 945.5 and 549 \(\mu\)M, respectively. These peptides were tested in spontaneously hypertensive rats administering single oral doses, proving their \textit{in vivo} antihypertensive activity (Nakashima et al., 2002). Another peptide RMLGQTPTK (44–52 position of troponin C) was purified from porcine skeletal troponin hydrolysed with pepsin and showed ACE-inhibitory activity Katayama et al (2003). It showed an IC\(_{50}\) of 34 \(\mu\)M. Same authors digested myosin light chain extracted from Japanase domestic pork loin with pepsin enzyme, and measured the ACE-inhibitory activity of the digest. This study resulted on the isolation and identification of the octapeptide VKKVLGNP, with an IC\(_{50}\) of 28.5 \(\mu\)M (Katayama et
al., 2007). On the other hand, antioxidant and free radical scavenging activities were tested in a papain hydrolysate of pork myofibrillar proteins (Saiga et al., 2003). From the isolated and identified peptides, DAQEKLE sequence showed the highest antioxidant activity. In another study, peptides DLYA, SLYA, and VW were tested in vitro and in vivo for their antioxidant activity showing anti-fatigue effect in spontaneously hypertensive rats (Arihara et al 2006).

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

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

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**Bones (Horn)**

Bones, horns, and hooves resulting from meat industry are mainly used as feed material,
organic fertilisers, or soil. Very few studies have described the purification and
identification of bioactive peptides from these by-products. In this respect, the
antioxidant peptides QYDQGV, YEDCTDCGN, and AADNANELFPPN, have been
identified from an aqueous extract of water buffalo horn, commonly used in Chinese
medicine. Results showed that these peptides could reduce the DPPH radical and protect
rat cerebral microvascular endothelial cells against H$_2$O$_2$-induced injury (Liu et al.,
2010).

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

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

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

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

Most of the studies about collagen peptides that are focused on their bioactive properties have dealt in their antioxidant and ACE-inhibitory activity. Thus, four antioxidant peptides were identified from hydrolysed porcine skin collagen obtained using different protease treatments. One of the antioxidative peptides, Gln-Gly-Ala-Arg, was synthesized and the antioxidant confirmed in vitro (Li et al., 2007). On the other hand, four peptides showing good in vitro and in vivo ACE-inhibitory activity against
spontaneous hypertensive rats were reported from chicken skin collagen hydrolysate obtained by treatment with an *Aspergillus* species derived enzyme (Saiga, et al, 2008).

More recently, two ACE-I inhibitory peptides with sequences AKGANGAGAPGFGPARGPSGPSGPP and PAGNPAGDQPGA, have been identified from bovine Achilles tendon collagen. Bacterial collagenase was used to hydrolyze the collagen and it was described that peptides retained 80% of ACE-I inhibitory activity after *in vitro* simulation of gastrointestinal tract (Banerjee & Shanti, 2012).

**Blood**

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

Blood represents up to 4% of animal weight and could become a problematic by-product in meat industry due to the tons of blood generated and its high pollutant characteristics for the environment. The interest in searching new blood uses exists since the beginning of slaughterhouses. In fact, its high content in proteins makes blood useful in food industry to increase the final nutritional value of some foods, enhance water binding, and because of its emulsifying capacity (Ofori and Hsieh, 2011).
Blood is mostly obtained from bovine and porcine sources and studies related with its value as generator of bioactive peptides used to be focused on the cellular fraction, specially hemoglobin cells, and the plasma fraction.

Hemoglobin and plasma hydrolysates have been described to mainly exert antihypertensive, antioxidant, antimicrobial, and opioid activity. Some peptidic sequences showing these activities have been isolated and characterized using modern proteomic techniques. In this sense, peptides GFPTTKTYFPFH and VVYPWT, corresponding to the 34–46 fragment of the α-chain and the 34–39 fragment of the β-chain of porcine hemoglobin, and obtained from a hydrolysate with pepsin enzyme, resulted to be ACE-inhibitory peptides, showing IC₅₀ values of 4.92 and 6.02 μM, respectively (Yu et al., 2006). Antimicrobial activity of peptides derived from hemoglobin chain is, by far, the most studied. Peptides TKAVEHLDDLPGALSELSDLHAHKLRVDPVNFKLLSHSLL,

LLDLPGALSELSDLHAHKLRVDPVNFKLLSHSL, KLLSHSL, and LLSHSL, obtained from the hydrolysis of bovine α-chain hemoglobin with pepsin, presented antibacterial activity against Kocuria luteus, Listeria innocua, Escherichia coli, and Staphylococcus aureus, as well as showed ACE inhibitory activity in an IC₅₀ range from 42.55 to 1,095 μM (Adje et al., 2011). Catiau et al. (2011a) studied the minimal peptide sequence necessary to show antimicrobial activity when a digestion of bovine α-chain hemoglobin with pepsin was done. Results showed that KYR, which was studied against five bacterial strains including Escherichia coli and Salmonella enteritidis as Gram-negative bacteria and Listeria innocua, Micrococcus luteus and Staphylococcus aureus as Gram-positive bacteria, was contained in all active peptides showing antimicrobial activity. Same authors did a similar work but studying bovine β-chain
hemoglobin and concluded with the sequence RYH as the minimal antimicrobial
sequence of this protein (Catiau et al., 2011b). In previous studies, Daoud et al. (2005)
isolated and purified an antimicrobial peptide with sequence
VTLASHLPSDLTPAVHASLDKFLANVSTVL from α-chain bovine hemoglobin by
hydrolysis with pepsin. The peptide displayed antimicrobial activity against M. luteus
A270, Listeria innocua, Enterococcus faecalis, Bacillus cereus, Staphylococcus
saprophyticus and Staphylococcus simulans. In fact, a MIC of 38 μM was reported
against L. innocua and 76 μM for the other bacterial species (Daoud et al., 2005).
Nedjar-Aroume et al. (2006) identified three peptides corresponding to positions 107–
141, 137–141, and 133–141 fragments of α-chain bovine hemoglobin, and 126–145
from β-chain, all of them showing antibacterial activity against Micrococcus luteus
A270, Listeria innocua, Escherichia coli, and Salmonella enteritidis (Nedjar-Aroume
et al., 2006). Same authors identified in another study with pepsin enzyme a total of
thirty antibacterial peptides, and twenty-four and six of them derived from α- and β-
chains of hemoglobin, respectively (Nedjar-Aroume et al., 2008). More recently, Hu et
al. (2011) identified a novel antimicrobial peptide derived from α-chain bovine
hemoglobin sequenced as VNFKLHSLHLLVTLASHL. The peptide showed
antimicrobial activity against Escherichia coli, Staphylococcus aureus, and Candida
albicans when assessed using the radial-diffusion plate assay (Hu et al., 2011).
Many of the studies related to opioid peptides from meat-derived sources are based on
blood hydrolysates. In fact, originally, hemorphins were isolated from enzymatically
treated bovine blood. Brantl et al. (1986) isolated and determined the sequence of an
opioid active tetrapeptide (YPWT) from bovine blood hydrolysed with gastrointestinal
enzymes. During the past decades, a number of opioid active peptides containing this
sequence have been reported such as LVVYPWT, LVVYPWTQR, and
LVYPWTQRF, which were found to be relatively stable and are believed to interact
with opioid receptors in the brain and cardiovascular system (Nyberg, Carlsson, &
Hallberg, 2013; Collinder, Nyberg, Sanderson-Nydhall, Gottlieb-Vedi, & Lindholm,
2005).

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

FUTURE TRENDS

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

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REFERENCES


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**Legends for the figures**

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

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

**Figure 3.** Fractionation of dry-cured ham extract on a Sephadex G-25 gel filtration column. Fractions were collected and assayed for in vitro ACE-inhibitory activity. For antihypertensive in vivo assay in the present study, fractions corresponding to an elution volume from 200 mL to 320 mL were pooled and named sample 1 (S1). The same procedure was followed for fractions corresponding to elution volumes from 325 mL to 450 mL (S2) and those from 505 mL to 625 mL (S3). Reprinted from Escudero et al Meat Science, 2012, 91. 306-311 with permission from Elsevier.
Meat industry

Blood
- Plasma
  - Functional agents
  - Hydrolysis
    - Protein hydrolysate
    - Antimicrobial peptides
- Blood cells
  - Globin
  - Thrombin Fibrinogen
- Trimmings (incl MRM) Bones
  - Hydrolisis
    - Bioactive peptides
    - Protein hydrolysate
  - Rendering
    - Collagen
      - Feed
    - Pharmaceuticals Chemicals
    - Lard Tallow
    - Biodiesel
- Bones
  - Heme Iron peptide