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Identification of novel antioxidant peptides generated in Spanish dry-cured ham

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ABSTRACT

The objective of this study was to purify and identify antioxidant peptides present in a water soluble extract of Spanish dry-cured ham. The initial extract was loaded into a Sephadex G25 column and fractions showing antioxidant activity were collected, pooled together and subjected to reversed-phase chromatography for further purification. Using a nano-LC–MS/MS analysis, 27 peptides were identified in these fractions. Several key peptides were selected for synthesis and the determination of their antioxidant properties using the DPPH radical-scavenging assay and reducing power analysis. The strongest radical-scavenging activity was observed with peptide SAGNPN which showed 50% antioxidant activity at a concentration of 1.5 mg/ml. On the other hand, the peptide GLAGA showed the higher reducing power with 0.5 units of absorbance at 700 nm at a concentration of 1 mg/ml. Other synthesised sequences showed lower antioxidant activity. The results indicate the potential of Spanish dry-cured ham as a source of antioxidant peptides naturally generated during the dry-curing process.

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1. Introduction

Spanish dry-cured ham is a dry-cured product that has increased in popularity over the world due to its unique and typical quality characteristics. During ripening, the proteolysis of sarcoplasmic and myofibrillar proteins is one of the main biochemical phenomena (Díaz, Fernández, García de Fernando, De la Hoz, & Ordoñez, 1997; Fadda et al., 1999; Roseiro et al., 2008). Endogenous endopeptidases from skeletal muscle (calpains and specially cathepsins) have been considered as the main factors in the degradation of muscle proteins during dry-curing (Toldrá & Flores, 1998). The process involves a decrease of high molecular weight proteins, resulting in the formation of polypeptides that may be degraded by peptidyl peptidases and aminopeptidases into smaller peptides and free amino acids (Toldrá, Aristoy, & Flores, 2000). Some peptides have been identified through proteomic tools (Mora, Sentandreu, Fraser, Toldrá, & Bramley, 2009; Mora, Sentandreu, Koistinen, et al., 2009). The peptides resulting from the hydrolysis of sarcoplasmic and myofibrillar proteins are mainly related to the sensory aspects of dry-cured ham (Aristoy & Toldrá, 1995). Other properties and functionalities, such as bioactivity, await elucidation. Several examples exist where protein hydrolysates produced by enzymatic hydrolysis have been shown to have a wide range of biological activities, including antioxidant activity, which is one of the most common studied bioactivity in animal protein hydrolysates (Chang, Wu, & Chiang, 2007; Saiga, Tanabe, & Nishimura, 2003; Wang et al., 2008; Toldrá & Reig, 2011). Although a good deal of evidence has confirmed the *in vitro* antioxidant activity of bioactive peptides, it is important to consider the relationship between the *in vitro* antioxidant properties and *in vivo* antioxidant capacity of the peptides since they may be subject to further degradation and modification in the intestine, vascular system and liver. In this respect, it has been indicated that a portion of bioactive peptides of small size can pass the intestine barrier and exert the biological effects at the tissue level (Gardner, 1988, 1998). Intact absorption of peptides is regarded as a normal physiological process which is different from the regular peptide transporter route (Gardner, 1988).

Reactive oxygen species (ROS) and other free radicals produce oxidative damage to DNA, proteins, and other macromolecules such as lipids. ROS are implicated in the aetiology of many degenerative diseases such as diabetes, cancer, cardiovascular diseases, neurodegenerative disorders and, in general, ageing. So, the interest in research and use of antioxidants for the prevention and treatment of such diseases has increased (Pham-Huy, He, & Pham-Huy, 2008).

The antioxidant peptides posses metal-chelating or hydrogen/electron donating activity, which enables interaction with free radicals terminating or preventing the radical chain reactions proceeding (Wang, Zhao, Zhao, & Jiang, 2007).

Some studies concerned with the purification and characterisation of antioxidant peptides have been reported (Di Bernardini et al., 2011; Lee et al., 2010; Liu, Wang, Duan, Guo, & Tang, 2010;

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Ren, Zheng, Liu, & Liu, 2010). More recently, Spanish dry-cured ham has been studied as a natural source of peptides with antioxidant activity (Escudero, Aristoy, Nishimura, Arihara, & Toldrá, 2012). This study revealed that water soluble fractions of Spanish dry-cured ham exhibited antioxidant activity probably due to the content of some relevant peptides with antioxidant properties. To date, there are no studies about the purification and identification of these individual antioxidant peptides present in dry-cured ham. The purpose of the present study was to purify and identify the peptides present in the water soluble antioxidant fractions detected in our previous work (Escudero et al., 2012). Following purification and identification, some peptides were selected, synthesised and assayed to verify their individual antioxidant activity.

2. Materials and methods

2.1. Reagents

The chemicals trifluoroacetic acid (TFA), 1,1-diphenyl-2-picrylhydrazyl (DPPH), potassium ferricyanide and ferric chloride were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Acetonitrile HPLC grade and formic acid were from Scharlab (Barcelona, Spain). All other chemicals and reagents used were of analytical grade and water used was bidistilled milliQ quality (Millipore, Bedford, MA, USA).

2.2. Spanish dry-cured ham preparation

Three Spanish dry-cured hams were produced using raw hams from 6 months old pig (Landrace × Large White). Hams were bled and prepared according to traditional procedures consisting of the pre-salting stage using a mixture of curing ingredients (salt, 300 mg nitrate/kg ham and 100 mg nitrite/kg ham) for 30 min in order to avoid microbiological spoilage, the salting stage, where hams were completely covered with solid salt, piled up without touching each other and placed in a cold room (2–4 °C and 90–95% relative humidity for 12 days), post-salting (4–5 °C, 75–85% relative humidity for 60 days) and finally, the ripening period (14–20 °C, relative humidity progressively reduced to 70% for 10 months). The muscle *Biceps femoris* in the final dry-cured hams had pH values within the range 6.1–6.2, the water content 55.0–55.5% and the salt content 5.8–6.1 g/100 g.

2.3. Sample extraction and deproteinisation

Fifty grams of *B. femoris* muscle from the processed Spanish dry-cured hams, previously removed from their extramuscular fat, were minced and homogenised with 200 ml of 0.01 N HCl in a stomacher (IUL Instrument, Barcelona, Spain) for 8 min in cold. The homogenate was centrifuged (12,000×g for 20 min at 4 °C) and, after filtering through glass wool, the supernatant was deproteinised by adding three volumes of ethanol and maintaining the sample for 20 h at 4 °C. Then, the sample was centrifuged again (12,000×g for 20 min at 4 °C) and the supernatant was dried in a rotatory evaporator. Finally, the dried deproteinised extract was dissolved in 25 ml of 0.01 N HCl, filtered through a 0.45 μm nylon membrane filter (Millipore, Bedford, MA, USA) and stored at −20 °C until use.

2.4. Size-exclusion chromatography

An aliquot (5 ml) of the deproteinised extract from the Spanish dry-cured ham was subjected to size-exclusion chromatography in order to fractionate the peptides according to their molecular mass. For this purpose, a Sephadex G25 gel filtration column (2.5 cm × 65 cm, Amersham Biosciences, Uppsala, Sweden), previously equilibrated with 0.01 N HCl filtered through a 0.45 μm nylon membrane filter (Millipore), was employed. The separation was performed at 4 °C using 0.01 N HCl as eluent, at a flow rate of 15 ml/h. The first 195 ml was discarded, and then 5 ml fractions were collected using an automatic fraction collector and monitored by ultraviolet (UV) absorption at 214 nm (Agilent 8453 UV spectrophotometer, Agilent Technologies, Palo Alto, CA, USA). Fractions corresponding to elution volumes from 280 to 325 ml, that showed antioxidant activity in a previous work (Escudero et al., 2012) were pooled together, dried under vacuum and redissolved in 3 ml of 0.1% trifluoroacetic acid (TFA) in bidistilled water.

2.5. Reversed-phase high performance liquid chromatography

An aliquot (500 μl) of the redissolved pool of peptide fractions was injected into an Agilent 1100 HPLC system (Agilent Tech.). The column used in this experiment was a Symmetry C18 (4.6 mm × 250 mm, 5 μm) from Waters (Milford, MA, USA). Mobile phases consisted of solvent A, containing 0.1% TFA in water and solvent B containing 0.085% TFA in acetonitrile:water (60:40, v/v). Both mobile phases A and B were filtered through a 0.45 μm nylon membrane filter and degassed prior to any analytical run. Peptides were first eluted with an isocratic gradient of 99% solvent A for 5 min, followed by a linear gradient from 1% to 100% of solvent B in 80 min at a flow rate of 0.8 ml/min. The separation was monitored using a diode array detector at a wavelength of 214 nm and 0.8 ml fractions were collected and dried in a vacuum centrifuge. Then, dried collected fractions were redissolved in bidistilled water and assayed for antioxidant activity. Those fractions showing remarkable antioxidant activity were further analysed by Nano-LC/MS/MS mass spectrometry in order to identify the peptides.

2.6. Antioxidant activity

2.6.1. DPPH radical-scavenging assay

The DPPH radical-scavenging activity was determined as described by Bersuder, Hole, and Smith (1998). One hundred microlitres of each peptide fraction or a 100 μl aliquot of synthesised peptides at different concentrations (0.5–3 mg/ml) was mixed with 500 μl of ethanol and 25 μl of a DPPH solution (0.02% in ethanol). The mixtures were incubated for 60 min in the dark at room temperature, and the reduction of DPPH radicals was measured at 517 nm. In its radical form, DPPH has an absorption band at 517 nm which disappeared upon reduction by an antiradical compound. Lower absorbance of the reaction mixture indicated higher free radical-scavenging activity. DPPH radical-scavenging activity was calculated as: DPPH radical-scavenging activity (%) = (absorbance of control − absorbance of sample)/absorbance control × 100. The control was conducted in the same manner than sample, except that bidistilled water was used instead of peptide sample. The test was carried out in triplicate.

2.6.2. Ferric-reducing antioxidant power (reducing power)

The reducing power was determined by measuring the ability to reduce ferric iron to ferrous iron (Huang, Tsai, & Mau, 2006). Briefly, 500 μl of each peptide fraction or synthesised peptide at different concentrations (0.16–1.00 mg/ml) was mixed with sodium phosphate buffer (500 μl, 200 mM, pH 6.6) and potassium ferricyanide (500 μl, 10 mg/ml), after which the mixture was incubated at 50 °C for 20 min. Then, trichloroacetic acid (500 μl, 100 mg/ml) was added and the mixture was centrifuged at 200×g for 10 min. The upper layer (1 ml) was then mixed with distilled deionised water (1 ml) and ferric chloride (200 μl, 1 mg/ml), after which the absorbance at 700 nm was measured against a

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