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Antihypertensive effect of peptides naturally generated during Iberian dry-cured ham processing

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ABSTRACT

Iberian dry-cured ham is considered a product of excellence in Spain and also highly valued by consumers overseas. Its processing conditions include a minimum time of ripening of 24 months which results on a very intense proteolysis of muscular proteins due to the action of endogenous enzymes and the generation of numerous peptides and free amino acids responsible for its characteristic flavour properties.
Previous studies in Spanish dry-cured ham of 10 months of ripening identified the amino acid sequences of many of the peptides generated during the proteolysis as well as described their potential as bioactive peptides. However, differences in genetics and processing conditions such as the time of ripening, could result on differences in the action of endogenous enzymes and the generated peptides.
In this study, differences in the generated peptides between Iberian dry-cured (24 months of ripening) and traditional Spanish dry-cured ham (14 months of ripening) have been detected. Iberian dry-cured ham extract was separated using size-exclusion chromatography and the ACE-inhibitory activity of the obtained fractions was evaluated in vitro showing up to 97.7% of inhibition in some of the fractions. The antihypertensive effect of the Iberian ham extract was also assayed in vivo using spontaneously hypertensive rats. A significant decrease of 12 mm Hg in SBP after 8 h of ingestion ($p < 0.05$) that returned to values similar to the control after 24 h of the treatment was observed. The analysis by mass spectrometry in tandem of the peptide extract revealed a total of 2632 peptides which contained the ACE inhibitory Pro–Pro–Lys, Pro–Ala–Pro, and Ala–Ala–Pro repeated in their sequences a total of 322, 302 and 119 times, respectively. This study reveals Iberian dry-cured ham as a source for natural antihypertensive peptides with very high potential in comparison with Spanish dry-cured ham, probably due to the differences in genetics and time of ripening which influence proteolysis and thus the generation of peptides.

1. Introduction

Iberian ham is the most valuable Spanish traditional meat product and it is obtained from at least 50% of Iberian bred pigs, compulsory conceived from 100% Iberian bred female and 100% Duroc bred male pigs (Boletín Oficial del Estado, BOE-A-2014-318, Spain). During its processing, Iberian dry-cured ham is subjected to a long (minimum of 24 months) and traditional ripening process responsible for the characteristic texture and flavour that differentiate this product in the market (Ventanas, Ventanas, Ruiz, & Estévez, 2005). For this reason, chemical and biochemical changes occurred during Iberian ham processing that are responsible for its flavour and texture characteristics have been widely investigated (Antequera et al., 1992; Ruiz et al., 1999).

Proteolysis is one of the most important biochemical processes occurred during the ripening of Iberian dry-cured ham. It influences texture and flavour development through the generation of small peptides and free amino acids (Kato, RaRhue, & Nishimura, 1989; Virigili et al., 1999). Recently, previous studies about Spanish dry-cured ham show that some of the generated peptides have been described to exert antioxidant and ACE inhibitory activities in vitro (Escudero, Aristoy, Nishimura, Arihara, & Toldrá, 2012, Escudero et al., 2013). In this respect, ACE inhibitory peptides have been identified in pork meat after hydrolysis (Arihara, Nakashima, Mukai, Ishikawa, & Itoh, 2001; Katayama et al., 2003) and also derived from in vitro digestion of pork meat (Escudero, Sentandreu, Arihara, & Toldrá, 2010; Escudero et al., 2012). Hypertension is recognized as a serious but controllable risk factor for cardiovascular disease (Collins & MacMahon, 1994), affecting 20–45% of the active population and 50–60% elderly people (Gokce, 2004). ACE enzyme is one of the major regulators of blood pressure through two different reactions in the renin–angiotensin–aldosterone system. Inhibition of ACE results in the decrease of blood pressure since ACE catalyses the conversion of the inactive angiotensin I into the powerful vasoconstrictor angiotensin II. Also, ACE acts as a catalyst in the inactivation of bradykinin which is a vasodilator that regulates vascular endothelial nitric oxide (NO) release. NO in turn has an important role

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in blood pressure regulation where it promotes vascular relaxation and angiogenesis (Fleming, 2007). Currently, the use of synthetic drugs in the treatment of hypertension is widely extended but has been described to produce serious side effects such as cough, skin rashes, and taste disturbances (Agostoni & Cicardi, 2001). For this reason, there is an increasing interest in identifying natural sources of ACE inhibitors.

The purposes of this work were to relatively quantify differences between Iberian dry-cured ham and traditional Spanish dry-cured ham generated peptides and study the soluble fraction of Iberian ham to assess its in vitro ACE-inhibitory activity and in vivo antihypertensive effect. The occurrence of these bioactive peptides in the Iberian ham would increase even more its value, not only as a high-quality meat product but as a healthy source of bioactive peptides.

2. Material and methods

2.1. Material and reagents

Iberian dry-cured ham was obtained from cross-breed pigs with at least 50% Iberian pork breed. Pigs were fed with cereals and pulses in an intensive farming system, and slathering was done at a minimum of 10 months age. Iberian hams were cured for a minimum of 24 months. Traditional Spanish dry-cured hams with a time of ripening of 14 months were also used. Angiotensin–converting enzyme (from rabbit lung) was purchased from Sigma Chemical Co. (St. Louis, Mo., USA). Abz-Gly-*p*-nitro-phe-pro-OH trifluoroacetate salt was obtained from Bachen AG. (Bubendorf, Switzerland). Other chemicals and reagents used were of analytical grade.

2.2. Peptides extraction

A total of 50 g obtained from six Iberian dry-cured hams and 6 Traditional Spanish dry-cured hams were minced and homogenized with 200 mL of 0.01 N HCl in a stomacher (IUL Instrument, Barcelona, Spain) for 8 min. The homogenate was centrifuged in the cold (12,000 $\times g$ for 20 min at 4 °C) and, after filtering through glass wool, proteins were precipitated by adding 3 volumes of ethanol and keeping the sample at 4 °C overnight. After that, the sample was centrifuged again (12,000 $\times g$ for 20 min at 4 °C) and the supernatant was dried in a rotary evaporator and lately a lyophilisation system. All twelve samples of Iberian (six) and traditional Spanish dry-cured ham (six) were resuspended at 625 mg/mL of TFA 0.1% and analysed using nLC–MS/MS for the statistical analysis. Remaining dry deproteinised extracts were dissolved in 25 mL of 0.01 N HCl, filtered through a 0.45 μm nylon membrane filter (Millipore, Bedford, MA) and stored at −20 °C until use.

2.3. Size-exclusion chromatography

A 5 mL aliquot of the deproteinised extract from Iberian ham was subjected to size-exclusion chromatography in order to fractionate the peptides according to their molecular mass. For this purpose, a Sephadex G25 column (2.5 × 65 cm, Amersham Biosciences, Uppsala, Sweden), previously equilibrated with 0.01 N HCl, was employed. The separation was performed at 4 °C using 0.01 N HCl as eluent, at a flow rate of 15 mL/h and 5 mL fractions were collected using an automatic fraction collector and further monitored by ultraviolet (UV) absorption at 214 nm (Agilent 8453 UV spectrophotometer, Agilent Technologies, Palo Alto, CA). Each fraction was lyophilised and dissolved in 2 mL of distilled water for subsequent bioactivity analysis.

2.4. Measurement of angiotensin converting enzyme (ACE) inhibition activity

The ACE inhibitory activity of the collected fractions from Iberian ham was measured according to the method developed by Sentandreu and Toldrá (2006). This assay is based on the ability of ACE to hydrolyze the internally quenched fluorescent substrate *o*-aminobenzoylglycyl-*p*-nitro-*L*-phenylalanyl-*L*-proline (Abz-Gly-Phe-(NO_2)-Pro). A sample solution (50 μL each collected fraction) was mixed with 50 μL of 150 mM Tris-base buffer (pH 8.3) containing 3 mU/mL of ACE. The reaction was initiated by the addition of 200 μL of 150 mM Tris-base buffer (pH 8.3) containing 1.125 M NaCl and 10 mM Abz-Gly-Phe-(NO_2)-Pro. The reaction mixture was then incubated for 45 min at 37 °C. The generation of fluorescence due to the release of *o*-aminobenzoylglycine (Abz-gly) by the action of ACE was measured at initial time and after 45 min of incubation using excitation and emission wavelengths of 355 and 405 nm, respectively.

The soluble extract of peptides was also analysed for its ACE-inhibitory activity at different concentrations: 5, and 0.25 mg/mL.

2.5. Peptide profile characterization and identification by mass spectrometry

2.5.1. MALDI–ToF analysis

Matrix-Assisted Laser Desorption/Ionization (MALDI) coupled to a Time-of-Flight (ToF) detector was used to characterize the profile of peptides contained in the extract. The analysis was done in a 5800 MALDI ToF/ToF instrument (ABSciex) in positive reflectron mode (3000 shots every position) in two different ranges: from 150 to 800 Da and from 800 to 1800 Da. Laser intensity was manually adjusted to maximize the signal to noise ratio. Plate model and acquisition method were calibrated by ABSCIEX calibration mixture (des-Arg1-Bradykinin at 1 fmol/μL; Angiotensin I at 2 fmol/μL; Glu1-Fibrinopeptide B at 1.3 fmol/μL; ACTH (1–17 clip) at 2 fmol/μL; ACTH (18–39 clip) at 5 fmol/μL; and ACTH (7–38 clip) at 3 fmol/μL).

Dried hydrolysates were dissolved in H_2O:acetonitrile (ACN), (95:5, *v*/*v*) with 0.1% of TFA, and 1 μL of every sample was directly spotted on the MALDI plate and allowed to air dry. Once dried, 0.5 μL of matrix solution (5 mg/mL of α-Cyano-4-hydroxycinnamic acid (CHCA) in H_2O:ACN (70:30, *v*/*v*) with 0.1% of TFA was spotted. The analysis of data was done by using mMass software (http://www.mmass.org/).

2.5.2. LC–MS/MS analysis

The nanoLC–MS/MS analysis was performed using an Eksigent Nano-LC Ultra 1D Plus system (Eksigent of AB Sciex, CA, USA) coupled to a quadrupole/Time-of-Flight (Q/ToF) TripleTOF® 5600+ system from AB Sciex Instruments (Framingham, MA, USA) that is equipped with a nanoelectrospray ionization source.

Iberian ham peptide extract was resuspended in H_2O with 0.1% of trifluoroacetic acid (TFA) to be analysed by mass spectrometry in tandem. After centrifuge in cold for 3 min at 200 $\times g$, 15 μL of a sample was cleaned and concentrated using Zip-Tip C18 cartridges with standard bed format (Millipore Corporation, Bedford, MA) according to manufacturer's instructions. Finally, 5 μL of the eluate was injected into the LC–MS/MS system through the autosampler.

Samples were then preconcentrated on an Eksigent C18 trap column (3 μ, 350 μm × 0.5 mm) (Eksigent of AB Sciex, CA, USA) at a flow rate of 3 μL/min and using 0.1% *v*/*v* TFA as mobile phase. After 5 min of preconcentration, the trap column was automatically switched inline onto a nano-HPLC capillary column (3 μm, 75 μm × 12.3 cm, C18) (Nikkyo Technos Co., Ltd., Japan). The mobile phases consisted of solvent A, containing 0.1% *v*/*v* formic acid in water, and solvent B, containing 0.1% *v*/*v* FA in 100% acetonitrile. Chromatographic conditions were a linear gradient from 5% to 35% of solvent B over 90 min, and 10 min from 35% to 65% of solvent B, at a flow rate of 0.3 μL/min and running temperature of 30 °C.

The outlet of the capillary column was directly coupled to a nanoelectrospray ionization system (nano-ESI). The Q/ToF was operated in positive polarity and data-dependent acquisition mode, in which a 250 ms ToF MS scan from *m*/*z* of 100 to 1200 was performed, followed by 50 ms product ion scans from *m*/*z* of 100 to 1500 on the 50 most intense 2 to 4 charged ions. Samples were injected in a randomized way.