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Proteomic analysis of *semimembranosus* and *biceps femoris* muscles from Bayonne dry-cured ham

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ABSTRACT

The aim of the study was to delineate and compare the proteomic maps of two muscles of dry-cured ham: the *biceps femoris* and the *semimembranosus*. For this purpose, we used two-dimensional electrophoresis on a subcellular muscle fraction: insoluble protein in low ionic strength buffer. After protein identification by MALDI–TOF mass spectrometry and bioinformatic analyses, we found differences in expression levels in the two muscles. Seventy-three proteins or fragments were differentially expressed: 43 were over-represented in *semimembranosus* and 30 in *biceps femoris*. Although the study was performed on the insoluble protein fraction in low strength ionic buffer, protein and fragment identifications by mass spectrometry showed that most of the proteins were involved in energy metabolism. The differences observed between the two muscles can be explained by the differences in salt and moisture content in the course of dry-cured ham processing.

1. Introduction

Bayonne ham enjoys EU Protected Geographical Indication (PGI) status. This certification requires professional processors to comply with specifications that provide the consumer with a finished product of optimal quality. The curing technology is based on the addition of sea salt on the surface of the ham, which acts as a preserving agent but is also responsible for causing physico-chemical and biochemical phenomena that contribute to development of the textural properties. Salt affects muscle proteins by inducing denaturation (Adamsen, Møller, Parolari, Gabba & Skibsted, 2006; Graiver, Pinotti, Califano & Zaritzky, 2006) in which the extent depends on salt concentration (Thorarinsdottir, Arason, Geirsdottir, Bogason & Kristbergsson, 2002) Proteolysis in dry-cured ham occurs throughout processing, but at different rates and to varying extents depending on salt penetration and water migration. In the first stage of processing i.e. salting phase, the outer and inner parts of the ham will clearly present differences in the values of these physical and chemical parameters. The *semimembranosus* (SM) muscle is an external muscle directly in contact with salt; it acquires a high NaCl content in the first stages of the process by the diffusion of salt, and its water content falls rapidly. By contrast, the *biceps femoris* (BF) is an internal muscle, and so will have a lower NaCl content and higher water content. This difference implies greater proteolytic activity in the *biceps femoris* muscle, which will affect its texture (Parolari, Virgili, & Schivazappa, 1994; Virgili, Parolari, Schivazappa, Soresi Bordini, & Borri, 1995; Rosell & Toldra, 1998; Virgili, Schivazappa, Parolari, Bordini, & Degni, 1998). Proteolytic activity on dry-cured ham proteins is essentially attributed to cathepsins, which act for a longer time (Toldrà & Etherington, 1988; Toldrà & Flores, 1998). However, in the first stage of processing, classical muscle ageing occurs, when calpains can also act. The time course of myofibrillar protein hydrolysis during the ripening process has been studied by one-dimensional gel electrophoresis (Toldrà, Flores, & Sanz, 1997; Toldrà, Rico, & Flores, 1993; Monin et al., 1997) and recently using protein labchip (Théron, Chevarin, Robert, Dutertre, & Santé-Lhoutellier, 2009) and by two-dimensional gel electrophoresis (Di Lucia et al., 2005). Myosin heavy chains (MHC), myosin light chains (MLC1 and MLC2) and troponin C and I are targets of proteolysis. In the soluble fraction, the presence of tropomyosin shows that solubility properties have changed, possibly due to environmental conditions such as salt. We aimed to evaluate both proteolysis and denaturation phenomena occurring in dry cured ham by a proteomic approach coupled with mass spectrometry. *Biceps femoris* and *semimembranosus* muscles were selected according to

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their salt diffusion rate, as shown above, and we focused our proteomic study on the insoluble protein fraction in low ionic strength medium.

2. Materials and methods

2.1. Origin of hams and sampling

The study was based on a total of 10 hams from pigs fed a cereal-based diet (60–80%), slaughtered at the Lahontan abattoir, and selected to meet the processing specifications of PGI Bayonne ham (a ham weight average of 10 kg, a fat cover average of 16 mm and a semimembranosus pH of 5.8). The processing of Bayonne hams, which lasts 9 months, was carried out at the Pyragena experimental station using the following sequence: salting for 12 days (temperature 3 °C, relative humidity RH 85%<RH<95%), curing for 10 weeks (1–4 °C, 70%<RH<80%), air drying for 10 weeks (14 °C, 68%< RH<76%), grease covering and ripening for 18 weeks (18 °C, RH = 70%) (Robert, Basly, & Dutertre, 2005). Sampling was carried out at 9 months, at the end of ripening. Proteomic analysis was performed on *biceps femoris* (internal muscle reference) and *semimembranosus* (external muscle reference).

2.2. Physical and chemical analysis

Moisture, protein, non-protein nitrogen (NPN) and salt content were determined on the whole slice of dry cured ham using the officially prescribed analysis methods of the French Ministry of Food, Agriculture and Fisheries (respectively NF V 04-401 of April 2001, NF V 04-407 and NF V 04-405).

2.3. Insoluble protein extraction and electrophoresis

The method was adapted from Sayd et al. (2006). Twenty grams of Semimembranosus and Biceps femoris were frozen in liquid nitrogen and stored at −80 °C. The samples muscles were grinded in liquid nitrogen to obtain a fine powder. They were then homogenized using a glass bead agitator MM2 (Retsch, Haan, Germany), in 40 mM Tris HCl (pH 8) at 4 °C in a ratio of 1:8 (w/v). The homogenate was centrifuged at 4 °C for 10 min at 10 000 *g*. The supernatant was removed. The pellet was washed five times with this buffer to obtain only insoluble protein in low ionic strength buffer. After the last centrifugation, the supernatant was removed and the pellet was homogenized in 7 M urea, 2 M thiourea, 4% CHAPS (w/v), 1% DTT (w/v), at 4 °C in the same ratio as the first step. The homogenate was centrifuged at 4 °C for 10 min at 10 000 *g*. The supernatant, forming the protein fraction soluble at high ionic strength, was stored at −80 °C. The protein concentration was determined by the RC-DC assay (Bio-Rad). First 1 mg of proteins was incorporated in a buffer containing 7 M urea, 2 M thiourea, 2% CHAPS (w/v), 0.4% carrier ampholyte (v/v), 1% DTT (w/v), and bromophenol blue. Samples were loaded onto immobilized pH gradient strips (pH 3–10 NL, 17 cm, Bio-Rad), and isoelectric focusing was performed using a Protean IEF cell system (Bio-Rad). Gels were passively rehydrated for 16 h. Rapid voltage ramping was subsequently applied to reach a total of 86 kVh. After strip equilibration, proteins were resolved on 12% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) gels using a Protean II XL system (Bio-Rad) for the second dimension. Gels were stained with Coomassie Blue (colloidal blue). Three gels were produced per sample, giving 60 gels in all.

2.4. Image analysis and statistical treatment of data

Gels were visualized and analysed using the two-dimensional electrophoresis (2DE) image analysis software Samespots (non-linear dynamics). Aligned spots were normalized by expressing the relative quantity of each spot as the ratio of individual spot quantity to the total quantity of valid spots. Relative quantities were expressed in ppm. For one sample and one spot, the mean of three values (corresponding to the gels in triplicate) was calculated. The resulting set of average spot quantities and the physical/chemical data underwent a one-way analysis of variance (ANOVA) using XLSTAT with the muscle as a factor. A difference was considered significant when associated with $p<0.05$ in ANOVA.

2.5. Protein identification by mass spectrometry

Coomassie stained spots of interest were manually excised using pipette tips. The spots where then destained with 100 µL of 25 mM NH4HCO3 with acetonitrile 95/5 (v/v) for 30 min, followed by two washes in 100 µL of 25 mM NH4HCO3 with acetonitrile 50/50 (v/v) and then dehydrated in 100% acetonitrile. Gel spots were completely dried using a Speed Vac before trypsin digestion at 37 °C over 5 h with 15 µL of trypsin (10 ng/µL; V5111, Promega) in 25 mM NH4HCO3. Peptide extraction was optimized by adding 8 µL of acetronitile, followed by 10 min of sonication.

For matrix-assisted laser desorption/ionization–time of flight (MALDI–TOF) mass spectrometry analysis, 1 µL of supernatant was loaded directly onto the MALDI target. The matrix solution (5 mg/mL of -cyano-4-hydroxycinnamic acid in 50% acetonitrile, 0.1% trifluoro-acetic acid) was immediately added and allowed to dry at room temperature.

Peptide Mass Fingerprint (PMF) of trypsin digested spots was determined in positive-ion reflector mode using a Voyager DE Pro MALDI–TOF–MS (*Applied Biosystems*, Courtaboeuf, France). External calibration was performed with a standard peptide solution (Peptide Mix 4, Proteomix, LaserBio Labs, Sophia-Antipolis, France). PMFs were compared to SwissProt (01/2008, 290 484 seq) protein sequence databases (ftp://ftp.ebi.ac.uk/pub/databases/uniprot/knowledgebase/uniprot_sprot.fasta.gz) using MASCOT 2.2 software [http://www.matrixscience.com]. The initial search parameters allowed a single trypsin missed cleavage, partial carbamidomethylation of cystein, partial oxidation of methionine and mass deviation lower or equal to 25 ppm. The validations are based on the significant score given by Mascot software which takes into account the number of matched peptides per protein (at least five peptides) and the decoy score. When identification by MALDI–TOF proved unsuccessful, identification was also attempted using nano LC-ion trap MS/MS analysis. HPLC was performed with an ULTIMATE LC SYSTEM combined with Famos autosample and Switchos II microcolumn switching for preconcentration (LC Packings, Amsterdam, The Netherlands). Six µL of the supernatant containing peptides were loaded on the column PEPMAP C18, 5 µm, 75 µm ID, 15 cm (Dionex, Labège, France) using a pre-concentration step in a micro pre-column cartridge (300 µm ID, 1 mm) at 30 µL/min. After 3 min, the pre-column was connected with the separating column and the gradient was started at 200 nL/min. The buffers were 5% ACN, 0.5% HCOOH in water (A) and 5% H_2O, 0.5% HCOOH in ACN (B). A linear gradient from 10 to 90% B for 45 min was applied. For ion trap MS, a LCQ DECA with a nano electrospray interface (Thermo Fisher Scientific, Les Ulis, France) was used. Ionisation (2.2 kV ionisation potential) was performed with a liquid junction and a non-coated capillary probe (New Objective, Cambridge, USA). Peptide ions were analysed by the data-dependent "triple play" method: (i) full MS scan (m/z 400–1400), (ii) zoomscan (scan of the major ion with bigger resolution), and (iii) MS/MS of this ion. Identification of peptides was performed with Mascot 2.2, restricting the taxonomy to *mammalia* (20080417, 1177111 sequences) in the protein NCBInr database. Mass deviation tolerance was set at 1.5 Da for parent ion and 0.8 for fragments ions. Protein identification was validated when at least two peptides originating from one protein showed significant identification scores.