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Comparative proteomic profiling of myofibrillar proteins in dry-cured ham with different proteolysis indices and adhesiveness

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ABSTRACT

Excessive proteolysis during dry-cured ham processing may lead to high adhesiveness and consumer dissatisfaction. The aim of this research is to identify biomarkers for proteolysis and adhesiveness. Two hundred *biceps femoris* porcine muscle samples from Spanish dry-cured ham were firstly evaluated for various physicochemical parameters, including their proteolysis indices and instrumental adhesiveness. Proteins of samples with extreme proteolysis indices were separated by two-dimensional electrophoresis and identified by tandem mass spectrometry (MALDI-TOF/TOF). We found that hams of higher proteolysis index had statistically significant increased adhesiveness. Proteomic analysis revealed statistically significant qualitative and quantitative differences between sample groups. Thus, protein fragments increased remarkably in samples with higher proteolysis index scores. In addition, higher proteolysis index hams showed increased degradation for a total of five non-redundant myofibrillar and sarcoplasmic proteins. However, myosin-1, α-actin and myosin-4 proteins were the biomarkers that underwent the most intense response to proteolysis and adhesiveness.

1. Introduction

Dry-cured ham is a high-quality food product traditionally consumed in Europe. A wide variety of physicochemical changes during the elaboration process influence the final product characteristics, such as flavor and texture (Bermúdez, Franco, Carballo, & Lorenzo, 2014). Salting and ripening are the two main steps in the elaboration process of dry-cured ham. The curing processing requires salt as preserving agent. The amount and type of salt have a significant influence on flavor, texture, color and overall quality of the final product (Paredi et al., 2013; Toldrá, Flores, & Sanz, 1997). The proteins undergo an intense proteolysis during the ripening process, which constitutes the most important enzymatic reaction regarding muscle proteins (Bermúdez, Franco, Carballo, Sentandreu, & Lorenzo, 2014; Lorenzo, Cittadini, Bermúdez, Munekata, & Domínguez, 2015). Salt content together with many other factors, such as rearing conditions (e.g., feeding, sex and slaughter age), pig line, features of raw product (initial weight, fat level and pH), type of muscle and the ripening process, have a recognized impact on protein denaturation of dry-cured hams (Škrlep et al., 2011; Théron et al., 2011).

The intensity of proteolysis during dry-cured ham processing is often measured by the proteolysis index. It is defined as the percentage of non-protein nitrogen accounting for total nitrogen. The relationship between proteolysis index and texture throughout the dry-cured ham process has been previously studied under a variety of variables, including pH, water and NaCl content, and lipid oxidation (García-Garrido, Quiles-Zafra, Tapiador, & Luque de Castro, 1999; García-Garrido, Quiles-Zafra, Tapiador, & Luque de Castro, 2000; Harkouss et al., 2015; Ruiz-Ramírez, Arnau, Serra, & Gou, 2006; Virgili, Parolari, Schivazappa, Bordini, & Borri, 1995). The proteolysis index of good quality Spanish dry-cured ham is considered to be between 33 and 36%, whereas in Italian ham it is between 22 and 30% (Careri et al., 1993). Myofibrillar and sarcoplasmic proteins are intensively degraded during the ripening process contributing to dry-cured ham texture and ultimate quality (Bermúdez et al., 2014b). However, myofibrillar proteins are a major fraction of the total, accounting for 65–70% muscle proteins (Lana & Zolla, 2016). Accordingly, proteolytic changes in this protein fraction are important for the development of texture and sensorial characteristics. In particular, myosin and actin are two main targets of proteolysis (Mora, Sentandreu, & Toldrá, 2011; Théron et al., 2011).

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However, excessive proteolysis may generate the pastiness defect, characterized by excessive softness, mushy texture and unpleasant flavors (Škrlep et al., 2011). In this regard, Morales, Arnau, Serra, Guerrero, and Gou (2008) showed that there is a close relationship between pastiness and adhesiveness (degree to which the surface of the ham slice adheres to the palate when compressed by the tongue), as described by Guerrero, Gou, and Arnau (1999). Therefore, the determination of instrumental adhesiveness could be a good indicator of pastiness level in dry-cured ham.

Proteomics has great potential to enhance our knowledge on the biochemical processes underlying the conversion of muscle into meat and identify biomarkers for meat quality traits (Lana & Zolla, 2016; Paredi, Raboni, Bendixen, de Almeida, & Mozzarelli, 2012; Paredi, Sentandreu, Mozzarelli, Fadda, Hollung, & de Almeida, 2013). In dry-cured ham, proteomic studies, generally based on one- or two-dimensional electrophoresis coupled to mass spectrometry, have tackled a wide diversity of topics. For instance, variations in quality traits, evolution of proteolysis during processing, comparative proteomics profiling of *biceps femoris* and *semimembranosus* muscles and identification of antioxidant peptides (Di Luccia et al., 2005; Mora, Escudero, Fraser, Aristoy, & Toldrá, 2014; Petrova, Tolstorebrov, Mora, Toldrá, & Eikevik, 2016; Škrlep et al., 2011; Théron et al., 2011). To the best of our knowledge, however, proteome changes linked to differential adhesiveness have not been previously reported.

In this study, we undertook a comparative proteomic profiling in *biceps femoris* muscle from dry-cured hams with different proteolysis indices, to identify biomarkers for differential proteolytic activity and adhesiveness, using two-dimensional electrophoresis and tandem mass spectrometry (MALDI-TOF/TOF MS).

2. Materials and methods

2.1. Dry-cured ham samples

Two hundred raw hams from Large White × Landrace crosses (average weight of 11.72 ± 1.06 kg), obtained from a commercial slaughterhouse, were elaborated according to the traditional system with some modifications regarding the temperature at specific steps, in order to ensure hams with high proteolysis. At the end of the process, hams were cut and boned and the cushion part containing *biceps femoris* muscle was excised and sampled. Ten slices from each dry-cured ham were vacuum packed and stored at room temperature for no longer than 4 weeks, for texture and chemical analysis. Dry-cured hams of low proteolysis and high proteolysis were selected according to extreme proteolysis index scores: low proteolysis samples with proteolysis index lower than 33%, and high proteolysis samples with proteolysis index higher than 36%. Four biological replicates per treatment (i.e. low and high proteolysis hams) were used for proteomic analysis. Samples for proteomic analysis were lyophilised separately and subsequently frozen at −80 °C until the time of protein extraction.

2.2. Instrumental texture

Textural analysis was performed using a texture analyzer (TA-XT Plus; Stable Micro Systems, Godalming, UK) by carrying out a separation test using different load cells with a specific probe. Instrumental adhesiveness was measured in sliced ham samples (1 mm) by applying probe tests and calculating the negative area of a force-time curve in tension tests with a single-cycle. The texturometer was equipped with a probe connected to a special device that enables horizontal probe displacement. After separation of the slices, the probe returned to the initial position. The conditions for the measurement of adhesiveness of dry cured ham slices were: load cell = 5 N; speed = 0.5 mm/s and distance = 100 mm. From the obtained graph of force *vs*. distance, the adhesiveness was calculated. All the measurements were made in triplicate, at room temperature.

2.3. Chemical analysis

After instrumental adhesiveness determination, *biceps femoris* samples were minced and subjected to chemical analysis in triplicate. Water content was analyzed by drying at 103 ± 2 °C until reaching a constant weight (AOAC, 1990); whereas the chloride content was analyzed according to ISO 1841-2 (1996) using a potentiometric titrator 785 DMP Titrino (Metrohm, Herisau, Switzerland) and results were expressed as percentage of NaCl.

2.4. Proteolysis index

Total nitrogen content was determined with Kjeldahl method (ISO R-937, 1978) using the Vapodest 50S analyzer (Gerhardt, Königswinter, Germany). It involves a semi-micro rapid routine method using block-digestion, copper catalyst and steam distillation into boric acid. A known quantity of the sample (1.0 ± 0.1 g) was taken in the Kjeldatherm digestion tube of the Vapodest and 20 mL of H_2SO_4 solution were added to the tube. Then, the tube was placed onto the Vapodest and steam digestion was started for 4 min. The steam vapor was collected and titrated in a 250-mL volumetric flask.

For non-protein nitrogen, preparation of sample was performed as described by Lorenzo, García Fontán, Franco, and Carballo (2008) Sample (2.5 g) was homogenized in 25 mL of deionized water and centrifuged. Afterwards, 10 mL of 20% trichloroacetic acid (99.5% purity, Merck, Darmstadt, Germany) were added, stirred well and left to stabilize for 60 min at room temperature. After centrifugation, the supernatant was filtered and 15 mL of filtrate were used for determination of nitrogen, as described above for total nitrogen (NT, ISO R-937, 1978). The proteolysis index was calculated as the ratio:

$$(\text{non-protein nitrogen}/\text{nitrogen total}) \times 100,$$

according to Ruiz-Ramírez et al. (2006).

2.5. Protein extraction for proteomic analysis

Total protein from *biceps femoris* muscle was extracted from 50 mg of lyophilized dry-cured ham. Samples were homogenized with 1 mL of lysis buffer (7 M urea; 2 M thiourea; 4% CHAPS; 10 mM DTT, and 2% Pharmalyte™ pH 3–10; GE Healthcare, Uppsala, Sweden) and sonicated (Sonifier 250; Branson Ultrasonics Corporation, Danbury, CT) in short pulses at 0 °C. Excess salts and other interfering substances were removed twice using the 2-D Clean-Up Kit (GE Healthcare) following manufacturer's instructions. This method for selectively precipitating protein was carried out using 200 μL of sonicated sample and the resulting pellet was dissolved in 210 μL of lysis buffer. The protein concentration was assessed using a commercial CB-X protein assay kit (G-Biosciences, St. Louis, MO) according to the manufacturer's instructions in a Chromate® microplate reader (Awareness Technology, Palm City, FL).

2.6. Two-dimensional electrophoresis (2-DE)

The 2-DE was performed according to Franco et al. (2015a). Briefly, 250 μg of protein in lysis buffer were mixed with rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 0.002% bromophenol blue), reaching 450 μL of total volume. Finally, 0.6% DTT and 1% IPG buffer (Bio-Rad Laboratories, Hercules, CA) were added. This protein extract was loaded into immobilized pH gradient (IPG) strips (24 cm, pH 4–7 linear, Bio-Rad Laboratories). The isoelectric focusing (IEF) was carried out on a PROTEAN IEF cell system (Bio-Rad Laboratories). Low voltage (50 V) was applied to rehydrate the strips and then an increasing voltage ramp at 70 kVh. After IEF, strips were soaked in equilibration buffer (50 mM Tris pH 8.8, 6 M urea, 2% SDS, 30% glycerol) successively supplemented with 1% DTT and 2.5% iodoacetamide for 15 min each. The second dimension separation was performed using an Ettan