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

López-Pedrouso, M[1]., Pérez-Santaescolástica, C[2]., Franco, D[2]., Fulladosa, E.[3], Carballo, J.[4], Zapata, C[1]., and Lorenzo, J. M.[2*]

[1]Department of Zoology, Genetics and Physical Anthropology, University of Santiago de Compostela, Santiago de Compostela -15872, Spain

[2]Centro Tecnológico de la Carne de Galicia, Rúa Galicia Nº 4, Parque Tecnológico de Galicia, San Cibrán das Viñas, 32900 Ourense, Spain

[3]IRTA, XaRTA, Food Technology, Finca Camps i Armet, E-17121 Monells, Girona, Spain

[4]Área de Tecnología de los Alimentos, Facultad de Ciencias de Ourense, Universidad de Vigo, 32004 Ourense, Spain

* Corresponding author. Tel: +34 988 548 277; fax: +34 988 548 276

E-mail address: jmlorenzo@ceteca.net

Abstract

Excessive proteolysis during the 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 physico-chemical parameters, including the proteolytic index and instrumental adhesiveness. Proteins of samples with extreme proteolytic index were subsequently separated by two-dimensional electrophoresis and identified by tandem mass spectrometry (MALDI-TOF/TOF). We found that hams of higher proteolytic index had significantly ($P<0.05$) increased adhesiveness. Proteomic analysis revealed marked proteolytic index dependent qualitative and quantitative differences. Thus, protein fragments increased remarkably in samples with higher proteolytic index scores. In addition, a total of five non-redundant myofibrillar and sarcoplamic proteins showed increased degradation in hams of higher proteolytic index. However, myosin-1, α-actin and myosin-4 proteins seem to be the most reliable biomarkers for proteolysis and adhesiveness because they underwent the most intense response to proteolysis.

Keywords: Defective textures; Instrumental adhesiveness; Meat proteomics; Protein degradation; Relative change measure

1. Introduction

Dry-cured ham is a high-quality food product traditionally consumed in Europe. A wide variety of physico-chemical changes during the elaboration process influence the final product characteristics such as flavor and texture (Bermudez, Franco, Carballo, & Lorenzo, 2014a). 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, Sentandreu, Mozzarelli, Fadda, Hollung, & Almeida, 2013; Toldrá, Flores, & Sanz, 1997). The proteins undergo an intense proteolysis during the ripening process, which constitutes the most important enzymatic reactions regarding muscle proteins (Bermúdez, Franco, Carballo, Sentandreu, & Lorenzo, 2014b; Lorenzo, Cittadini, Bermúdez, Munekata, & Domínguez, 2015). Salt content together with other many factors, such as rearing conditions (e.g. feeding, sex and slaughter age), pig line, features of raw product (initial weigh, fat level and pH), type of muscle and the ripening process, have a recognized impact on protein denaturation of dry-cured hams (Škrlep e*t al.*, 2011; Théron, Sayd, Pinguet, Chambon, Robert, & Santé-Lhoutellier, 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 proteolytic 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, 2000; Harkouss *et al.*, 2015; Ruiz-Ramírez, Arnau, Serra, & Gou, 2006; Virgili, Parolari, Schivazappa, Bordini, & Borri, 1995). The

proteolytic index of good quality Spanish dry-cured ham is considered to be between 33 and 36%, whereas in Italian is between 22 and 30% (Careri, Mangia, Barbieri, Bouoni, Virgili, & Parolari, 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). But myofibrillar proteins are the major fraction of the total, accounting for around 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). 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. Therefore, the determination of instrumental adhesiveness could be 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, Almeida, & Mozzarelli, 2012; Paredi *et al.*, 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 its 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,

87 Mora, Toldrá, & Eikevik, 2016, Škrlep *et al.*, 2011; Théron *et al.*, 2011). To the best of
88 our knowledge, however, proteome changes linked to differential adhesiveness have not
89 been previously reported.

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

94 **2. Materials and methods**

95 *2.1. Dry-cured ham samples*

96 Two hundred raw hams (average weight of 11.72±1.06 kg) obtained from a
97 commercial slaughterhouse resulting from Large White × Landrace breed crosses were
98 elaborated according to the traditional system with some modifications regarding the
99 temperature at specific steps in order to ensure hams with high proteolysis. At the end
100 of process, hams were cut and boned and the cushion part containing *biceps femoris*
101 muscle was excised and sampled. Six biological replicates of low proteolysis and high
102 proteolysis dry-cured hams were selected for texture, chemical and proteomic analysis
103 according to their proteolytic index scores: low proteolysis, proteolytic index < 33%;
104 and high proteolysis, proteolytic index > 36%. Ten slices from each dry-cured ham were
105 vacuum packed and stored at room temperature for no longer than 4 weeks for analysis.

106 *2.2. Instrumental texture*

107 Textural analysis was performed using a texture analyzer (Stable Micro Systems,
108 TA-XT Plus, London, UK) by carrying out a separation test using different load cells
109 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 the 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 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 analysed by drying at 103 ± 2 °C until reaching a constant weight (AOAC, 1990); whereas the chloride content was analysed 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 (NT) was determined with Kjeldahl method (ISO R-937, 1978) using the Vapodest 50S analyser (Gerhardt, Königswinter, Germany). It concerns a semi-micro rapid routine method using block-digestion, copper catalyst and steam distillation into boric acid. A known quantity of the sample (1 ± 0.1 g) was taken in the Kjeldatherm digestion tube of the Vapodest. Added 20 mL of H_2SO_4 solution to the tube. Then, the tube was placed onto Vapodest and steam digestion was started for 4 minutes. 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, & Carballo (2008). 2.5 g of sample was homogenised in 25 mL of deionized water and centrifuged. Afterwards, 10 mL of 20% trichloroacetic acid (99.5% purity, Merck, Darmstadt, Germany) was added, stirred well and let to stabilize for 60 min at room temperature. After centrifugation, the supernatant was filtered and 15 mL of filtrate was used for determination of nitrogen as described above for total nitrogen (NT, ISO R-937, 1978). The proteolytic index was calculated as the ratio (non-protein nitrogen /nitrogen total) × 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 sonicating (Sonifier 250, Branson, Danbury, CC, USA) 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 indications. This method for selectively protein precipitating 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, USA) according to the manufacturer instructions in a Chromate® microplate reader (Awareness Technology, Palm City, FL, USA).

2.6. Two-dimensional electrophoresis (2-DE)

154 The 2-DE was performed according to Franco *et al.* (2015a). Briefly, 250 µg of
155 protein in lysis buffer was mixed with rehydration buffer (7 M urea, 2 M thiourea, 4%
156 CHAPS, 0.002% bromophenol blue), reaching 450 µL of total volume. Finally, 0.6%
157 DTT and 1% IPG buffer (Bio-Rad Laboratories) were added. This protein extract was
158 loaded into immobilized pH gradient (IPG) strips (24 cm, pH 4-7 linear, Bio-Rad
159 Laboratories, Hercules, CA, USA). The isoelectric focusing (IEF) was carried out on a
160 PROTEAN IEF cell system (Bio-Rad Laboratories). Low voltage (50 V) was applied to
161 rehydrate the strips and then an increasing voltage ramp until to reach 70 kVh. After
162 IEF, strips were soaked in equilibration buffer (50 mM Tris pH 8.8, 6 M urea, 2% SDS,
163 30% glycerol) successively supplemented with 1% DTT and 2.5% iodoacetoamide for
164 15 min each. Second dimension was performed using an Ettan DALTsix vertical gel
165 system (GE Healthcare) with 12% SDS-PAGE gels at 18 mA/gel until the bromophenol
166 blue dye front reached the end of the gels. The 2-DE gels were stained with SYPRO
167 Ruby fluorescent stain (Lonza, Rockland, ME, USA).

168 *2.7. Image analysis of 2-DE gels*

169 Gels were visualized and digitalized using the Gel Doc XR+ system (Bio-Rad
170 Laboratories). The detection and quantification of spot volumes were performed with
171 PDQuest Advanced software v. 8.0.1 (Bio-Rad Laboratories) after background
172 subtraction. Relative volumes of spots were obtained considering the total intensity
173 value of image pixels. Observed values of molecular mass (M_r) were determined across
174 protein spots from standard molecular mass markers ranging from 15 to 200 kDa
175 (Fermentas, Ontario, Canada), whereas those of isoelectric point (p*I*) were established
176 according their position on the IEF-strips.

2.8. Protein identification by mass spectrometry (MS)

For MALDI TOF/TOF MS analysis, selected spots were excised from the gel and they were dehydrated with acetonitrile using a vacuum centrifuge. The gel piece was washed with Ambic buffer (50 mM ammonium bicarbonate in 50% methanol). The proteins were reduced with 10 mM DTT in 50 mM ammonium bicarbonate and alkylated with 55 mM acetoamide in 50 mM ammonium bicarbonate. Extracts were repeatedly rinsed with Ambic buffer, dehydrated by addition of acetonitrile and dried in a SpeedVac. Then the proteins were hydrolysed with 20 μg/μL of trypsin in 20 mM ammonium bicarbonate for a total volume of 30 μL overnight at 37 °C. The total digest was incubated three times in 40 μL of 60% acetonitrile with 5% formic acid, concentrated in a SpeedVac and stored at -20 °C until analysis. Dried samples were dissolved in 4 μL of 0.5 % acetic acid. Equal volumes (0.5 μL) of peptide and matrix solution, consisting of 3 mg of α-cyano-4-hydroxycinnamic acid dissolved in 1 mL of 50 % acetonitrile and 0.1 % trifluoroacetic acid, were deposited onto a 384 Opti-TOF MALDI plate (Applied Biosystems, Foster City, CA, USA) using the thin layer method. Mass spectrometric data were obtained in an automated analysis loop using 4800 MALDI-TOF/TOF analyzer (Applied Biosystems, Foster City, CA, USA). MS spectra were acquired in positive-ion reflector mode with a Nd:YAG, 355 nm wavelength laser, averaging 1000 laser shots, and at least three trypsin autolysis peaks were used as internal calibration. All MS/MS spectra were performed by selecting the precursors with a relative resolution of 300 (FWHM) and metastable suppression. Automated analysis of mass data were achieved using the 4000 Series Explorer Software v. 3.5 (Applied Biosystems, Foster City, CA, USA). Peptide mass fingerprint and peptide

fragmentation spectra data of each sample were combined using the GPS Explorer Software v. 3.6 and Mascot software v. 2.1 (Matrix Science, Boston, MA, USA) to search against UniProt/SwissProt database. A 50 ppm precursor tolerance, 0.6 Da MS/MS fragment tolerance, carbamidomethyl cysteine were used as fixed modification, oxidized methionine as variable modification and permitting one missed cleavage. Proteins with at least two matched peptides and statistically significant (*P*-value <0.05) MASCOT scores were selected as positively identified.

2.9. Statistical analysis

Statistical analysis of the results for physico-chemical parameters was performed by an analysis of variance (ANOVA) using IBM SPSS Statistics V21.0 (SPSS, Chicago, USA) software package.

Quantitative changes of 2d gel spot volumes in sample groups were assessed using the measures "fold change" (*FC*) and "relative change" (*RC*) (Franco et al., 2015a, b). The measure fold change is given by $FC = V_{high}/V_{low}$, where V_{high} and V_{low} are the mean volumes in samples with high and low proteolysis level, respectively. Fold change values less than one were represented as their negative reciprocal. The relative change is provided by the relationship $RC = DV/|DV_{max}|$, where $DV = V_{high}-V_{low}$ and DV_{max} is the maximum observed value of DV over spots.

Bootstrapping was used to obtain 95% confidence intervals for the means of spot volume across replicates as previously described (Franco *et al.*, 2015a, b). For each set of N (= 4) volume estimates, 20,000 bootstrap samples of size N were obtained following a Monte Carlo algorithm. The 95% bootstrap confidence intervals were obtained by the bias-corrected percentile method from distribution of bootstrap mean

223 replications (Efron, 1982). Confidence intervals were adjusted for multiple hypothesis
224 testing with the Bonferroni procedure.

225 **3. Results and discussion**

226 *3.1. Proteolysis index and instrumental adhesiveness of dry-cured hams*

227 A total of two hundred dry-cured hams were analyzed for the following physico-
228 chemical parameters: proteolysis index, instrumental adhesiveness, moisture, salt
229 content, non-protein nitrogen and total nitrogen. Four biological replicates exhibiting
230 extreme and statistically significant (*P*-value <0.05) differences in proteolytic index
231 were eventually selected for proteomic analysis. Mean (± SE, standard error) proteolytic
232 index values in the selected sample groups with low and high samples proteolytic index
233 were 30.3 ± 0.68 and 38.0 ± 0.88, respectively. Differences in proteolytic index can be
234 attributed to a large number of factors such as variable raw materials, salting
235 procedures, ripening process, duration of the different steps involved in the elaboration,
236 as well as variations of temperature and relative humidity in dry-cured ham processing
237 (García-Garrido *et al.,* 1999; Pugliese *et al.,* 2015; Škrlep *et al.,* 2011; Zhao, Tian, Liu,
238 Zhou, Xu, & Li, 2008). In the present study, however, hams were elaborated under
239 uniform conditions. It suggests that proteolysis can undergo large variations even under
240 similar processing systems.

241 Table 1 shows mean (± SE) values of instrumental adhesiveness, moisture, salt
242 content, total nitrogen and non-protein nitrogen in samples with different proteolytic
243 index (low and high samples) selected for proteomic analysis. It must be highlighted
244 that adhesiveness of sliced dry-cured ham was assessed, for the first time, by
245 mechanical procedures as alternative to sensory analysis panel. We found that the

instrumental adhesiveness was significantly (P<0.001) higher in high proteolysis batch (100.43 g) than in low proteolysis batch (66.75 g). Hams with a defective texture can exhibit high moisture/protein ratios as result of both increased moisture and decreased protein contents related to ham with a normal texture (García-Garrido *et al.*, 1999). In addition, several authors (Bermúdez *et al.*, 2014a; Ruiz-Ramírez *et al.*, 2006; Virgili, Parolari, Schivazappa, Bordini, & Borri, 1995) noticed that proteolytic activity in ham is governed by salt. However, García-Garrido *et al.* (1999) showed hams with normal and defective texture containing salt contents from 6.2% to 8.1% by wet weight. In this study, there were no significant differences between sample groups for moisture and salt content. In contrast, non-protein nitrogen showed significant (P<0.01) differences between treatments, since the lowest values were observed in low proteolysis batch (1.50 *vs*. 1.84%, for low and high proteolysis groups, respectively). This finding is in agreement with data reported by García-Garrido *et al.* (1999) who observed that non-protein nitrogen levels were 30% higher in hams of defective texture than in normal pieces.

3.2. Comparison of proteomic profiles by 2-DE

High-quality 2-DE gels were obtained despite dry-cured ham salt content. Representative 2-DE gel images of low and high proteolysis proteomes were shown in Fig. 1. The identification, matching and volume evaluation of 2-DE spots were obtained by PDQuest software. The total number of selected spots for proteomic analysis was 92 and 123 spots in low and high proteolysis groups, respectively; after the elimination of saturated or faint spots, as well as non-reproducible spots over replicates. We found that proteomic profiles of low and high proteolysis samples were remarkably differentiated

(Table 2). In total, 58 protein spots showed statistically significant differential abundance by the bootstrap re-sampling statistical method. Note that Bonferroni-corrected 95% bootstrap confidence intervals for means of spot volumes did not overlap in matched spots of different intensity or did not overlap zero in unique spots. It is important to highlight that only eight unique spots were observed in low proteolysis samples, whereas in high proteolysis were 37 spots (P<0.001, Fisher's exact test). This difference probably reflects an increased protein fragmentation in high proteolysis samples.

3.3. Evaluation of protein fragmentation

Protein fragmentation in low and high proteolysis hams was evaluated by the following procedure. First, protein identification of differentially abundant spots was performed by MALDI-TOF/TOF MS. Second, spots containing protein fragments were assessed by comparing the theoretical molecular mass of each protein with the molecular mass observed on 2-DE gel. Protein fragments were eventually validated when the ratio between theoretical and empirical masses was above 1.5 kDa. We found that most differentially abundant protein spots in low and high proteolysis ham samples (40 out of 58 spots) were successfully identified (P<0.05) by MALDI-TOF/TOF MS (Table 3). The comparison of theoretical and observed molecular masses revealed that an important number (55%) of identified spots contained protein fragments (Table 3). It is noteworthy, however, that most (86%) of these spots were actually unique spots present only in high proteolysis samples (Table 2). Accordingly, the proteomic profile in dry-cured ham samples of higher proteolysis index showed increased levels of protein fragmentation. It also shows that proteolysis index scores can be good indicators

292 of differential proteolysis over proteomes. The remaining spots, with theoretical and
293 empirical mass ratios below 1.5 kDa, were excluded for further analysis. It is not
294 possible to assess whether they actually contain either entire or slightly degraded
295 proteins at the level of resolution of 2-DE.

296 All fragments detected in our study corresponded to seven non-redundant
297 myofibrillar or sarcoplamic muscle proteins: myosin-1 (MYH1), myosin-4 (MYH4), α-
298 4 glucan phosphorylase (F1RQQ8), α-actin (ACTS or ACTA1), heat shock 70 kDa
299 protein 1-like (HS71L), myosin-7 (MYH7) and vinculin (VINC). However, most
300 fragments (86%) resulted from hydrolysis of myosin heavy chain and α-actin
301 myofibrylar proteins: nine MYH1 spots, four MYH4 spots, one MYH7 spots and five
302 ACTS spots (Table 3). It is noteworthy, however, that the amount of protein fragments
303 does not provide determinant information by itself to reliably evaluate the extent of
304 differential proteolysis over proteins and sample groups. A complete characterization of
305 differential proteolysis not only requires determining the number of protein fragments,
306 but also the quantification of their volumes.

307 *3.4. Candidate biomarkers for differential proteolysis and adhesiveness*

308 Quantitative differences in proteolysis intensity between low and high proteolysis
309 ham batches were assessed by fold and relative change statistics from protein fragment
310 volumes. Table 4 shows fold and relative change values for each protein found to be
311 differentially affected by proteolysis. There can be seen that fold and relative change
312 provide very discrepant information about the extent of proteolysis across proteins. It is
313 worth noting that fold change is a measure traditionally used to quantify differential
314 protein abundance between treatments. But it has the disadvantage that its range varies

315 from -∞ to +∞ and range boundaries are achieved with the presence of unique spots
316 independently of the existing differences in volume. In contrast, relative change always
317 ranges from -1.0 to +1.0. It provides; therefore, a more intuitive measure of the strength
318 of change and maximum values of its range are not necessarily achieved with the mere
319 occurrence of unique spots (see Table 4). Accordingly, relative change is particularly
320 appropriate measure for the analysis of degraded proteome profiles exhibiting large
321 number of unique spots. In the present study, we found that relative change values over
322 proteins ranged between -0.04 and +1.0 (Table 4). Only five proteins (i.e. MYH1,
323 ACTS, MYH4, HS71L and F1RQQ8) showed positive relative change values,
324 indicating that their fragments were over-represented in high proteolysis hams. In
325 contrast, MYH7 and VINC proteins underwent decreased proteolysis in high proteolysis
326 samples given that their relative change values were of negative sign. This result
327 suggests that MYH7 and VINC proteins are not useful biomarkers of proteolysis
328 intensity.

329 MYH1, ACTS and MYH4 proteins showed the highest level of degradation in
330 high proteolysis samples (relative change values > 0.40). Previous proteomic studies
331 based on one-dimensional electrophoresis and 2-DE have systematically demonstrated
332 that myosin heavy chain and α-actin are main targets of proteolysis in the *biceps femoris*
333 muscle, particularly at the end of ripening (Larrea *et al.*, 2006; Tabilo, Flores, Fiszman,
334 & Toldrá, 1999; Théron *et al.*, 2011; Toldrá, Rico, & Flores, 1993). In 12-month old
335 Parma and S. Daniele dry-cured ham, most isoforms of myosin and actin were found to
336 be completely hydrolysed (Di Luccia *et al.*, 2005). We found that MYH1 (relative
337 change = +1) was a more sensitive biomarker for proteolysis than ACTS (relative

change = +0.60). This difference can be attributed to the fact that the myosin is more sensitive to denaturation by salt content (Graiver, Pinotti, Califano, & Zaritzky, 2006). However, we found that two specific isoforms of the myosin heavy chain (MYH1 and MYH4) were intensively degraded in response to proteolysis. It suggests that these two myosin heavy chain isoforms might exhibit differential susceptibility to degradation by proteolytic enzymes during dry-cured ham processing. In this regard, Théron *et al.* (2011) reported differential MYH1 or MYH4 fragmentation in *biceps femoris* and *semimembranosus* muscles with different proteolytic activity due to differences in salt and moisture content in the course of dry-cured ham processing. Specifically, fragments of these two myosin heavy chains isoforms were overrepresented in *biceps femoris* muscle that is an internal muscle with lower NaCl concentration, higher water content and increased proteolytic activity. Taken together, the available evidence suggests that MYH1 and MYH4 can be suitable biomarkers for proteolysis under different scenarios.

Of the five differentially fragmented proteins in the present study, two were sarcoplasmic proteins: HS71L and F1RQQ8. They are proteins with a considerably lower relative representation in the proteome of *biceps femoris* muscle, which explains their low relative change values (<0.10). The HS71L protein is a molecular chaperone that appears to play a critical role in multiple cellular functions, including protection of the proteome in response to stress, activation of proteolysis of misfolded proteins and controlling the targeting of proteins for subsequent degradation (Archivald *et al.*, 2010; Radons, 2016; The UniProt Consortium, 2017). On the other hand, the F1RQQ8 protein is a phosphorylase that catalyzes and regulates the breakdown of glycogen to glycose-1-phosphate for the generation of ATP during glycogenolysis (Archivald *et al.*, 2010;

Gautron, Daegelen, Mennecier, Dubocq, Kahn, & Dreyfus, 1987; The UniProt Consortium, 2017). Fragments of F1RQQ8 result from proteolytic activity were also detected in post-mortem *longissums dorsi* porcine muscle (Lametsch, Roepstorff, & Bendixen, 2002), as well as in dry-cured *biceps femoris* and *semimembranosus* muscles (Théron *et al.,* 2011). Specifically, the *biceps femoris* muscle showed more F1RQQ8 fragments than the *semimembranosus* muscle during the ripening of dry-cured ham due to its higher proteolytic activity (Théron *et al.,* 2011). It follows FIRQQ8 is a good biomarker of proteolysis in agreement with our observations.

In the present study, we found that the proteolytic activity correlated positively with the extent of sliced dry-cured ham instrumental adhesiveness. Therefore, the identified biomarkers also apply for the meat quality trait of adhesiveness. These biomarkers provide non-invasive tools alternative to sensory analysis panel or mechanical measures in order to assess variations in adhesiveness. The identified proteins can also be potential biomarkers for other proteolysis-related porcine quality traits. It is particularly true in the case of pastiness considering that pastiness variations are closely related with the extent of proteolysis and adhesiveness (Morales *et al.,* 2008; Škrlep *et al.,* 2011).

4. Conclusions

Comparison of dry-cured ham proteomic profiles with extreme proteolysis index scores, based on two-dimensional electrophoresis coupled to tandem mass spectrometry, allowed us to identify novel candidate biomarkers for differential proteolytic activity underlying meat quality traits. First of all, we found that the proteolytic index is a reliable indicator of the extent of protein hydrolysis at proteomic scale and instrumental

adhesiveness of sliced dry-cured ham. A total of five myofribrillar and sarcoplasmic proteins of *biceps femoris* muscle were identified as candidate markers for proteolysis and adhesiveness. However, two distinct isoforms of the myosin heavy chain (myosin-1 and myosin-4) and α-actin exhibited the strongest response to variable proteolysis as well as to adhesiveness according to the measure of relative change. These proteins could also be potential candidate biomarkers for quality traits closely linked to proteolysis such as pastiness. Further research is clearly needed to precisely assess the relationship of these markers with proteolysis-related quality traits under a wide range of dry-cured ham elaboration conditions.

Conflict of Interest statement

The authors declare no conflict of interest.

Acknowledgements

This research was supported by Grant RTA 2013-00030-CO3-03 from INIA (Spain). Acknowledgements to INIA for granting Cristina Pérez Santaescolástica with a predoctoral scholarship.

References

AOAC (1990). Official method 950.46, moisture in meat, B. Air drying. In K. Helrich (Ed.), *Official methods of analysis of the association of official analytical chemists*, Vol. II, (p. 931). Arlington: Association of Official Analytical Chemists.

Archibald, A. L., Bolund, L., Churcher, C., Fredholm, M., Groenen, M. A. M., Harlizius, B., Lee, K.-T., Milan, D., Rogers, J., Rothschild, M. F., Uenishi, H., Wang, J., & Schook, L.B. Pig genome sequence – analysis and publication strategy. *BMC Genomics, 11*, 438.

Bermúdez, R., Franco, D., Carballo, J., & Lorenzo, J. M. (2014a). Physicochemical changes during manufacture and final sensory characteristics of dry-cured Celta ham. Effect of muscle type. *Food Control, 43*, 263-269.

Bermúdez, R., Franco, D., Carballo, J., Sentandreu, M., & Lorenzo, J. (2014b). Influence of muscle type on the evolution of free amino acids and sarcoplasmic and myofibrillar proteins through the manufacturing process of Celta dry-cured ham. *Food Research International, 56*, 226-235.

Careri, M., Mangia, A., Barbieri, G., Bouoni, L., Virgili, R., & Parolari, G. (1993). Sensory property relationships to chemical data of Italian-type dry-cured ham. *Journal of Food Science, 58(5)*, 968-972.

Di Luccia, A., Picariello, G., Cacace, G., Scaloni, A., Faccia, M., Liuzzi, V., Alviti, G., & Spagna Musso, S. (2005). Proteomic analysis of water soluble and myofibrillar protein changes occurring in dry-cured hams. *Meat Science, 69(3)*, 479-491.

Efron, B. (1982). *The jackknife, the bootstrap and other resampling plans*. (1st ed.). Philadelphia; Society for Industrial and Applied Mathematics, (Chapter 10).

Franco, D., Mato, A., Salgado, F.J., López-Pedrouso, M., Carrera, M., Bravo, S., Parrado M., Gallardo J.M., & Zapata C. (2015a). Tackling proteome changes in the *longissimus thoracis* bovine muscle in response to pre-slaughter stress. *Journal Proteomics, 122*, 73-85.

Franco, D., Mato, A., Salgado, F., López-Pedrouso, M., Carrera, M., Bravo, S., Parrado, M., Gallardo, J. & Zapata, C. (2015b). Quantification of proteome changes in bovine muscle from two-dimensional electrophoresis data. *Data in Brief, 4*, 100-104.

García-Garrido, J. A., Quiles-Zafra, R., Tapiador, J., & Luque de Castro, M. (1999). Sensory and analytical properties of Spanish dry-cured ham of normal and defective texture. *Food Chemistry, 67(4)*, 423-427.

García-Garrido, J. A., Quiles-Zafra, R., Tapiador, J., & Luque de Castro, M. D. (2000). Activity of cathepsin B, D, H and L in Spanish dry-cured ham of normal and defective texture. *Meat Science*, *56*, 1-6.

Gautron, S., Daegelen, D., Mennecier, F., Dubocq, D., Kahn, A., & Dreyfus, J.-C. (1987). Molecular mechanisms of McArdle's disease (muscle glycogen phosphorylase deficiency). *Journal of Clinical Investigation*, *79*, 275-281.

Graiver, N., Pinotti, A., Califano, A., & Zaritzky N. (2006). Diffusion of sodium chloride in pork tissue. *Journal of Food Engineering*, *77*, 910-918.

Harkouss, R., Astruc, T., Lebert, A., Gatellier, P., Loison, O., Safa, H., Portanguen, S., Parafita, E., & Mirade, P.S. (2015). Quantitative study of the relationships among proteolysis, lipid oxidation, structure and texture throughout the dry-cured ham process. *Food Chemistry, 166*, 522-530.

ISO (1978). Determination of nitrogen content. ISO 937:1978 Standard. In: International standards meat and meat products. International Organization for Standardization. Ginebra. Suiza.

ISO 1841-2 (1996). Meat and meat products. Determination of chloride content —Part 2: Potentiometric method (reference method). Geneva: International Organization for Standardization.

Lametsch, R., Roepstorff, P., & Bendixen, E. (2002). Identification of protein degradation during post-mortem storage of pig meat. *Journal of Agricultural and Food Chemistry, 50(20),* 5508-5512.

Lana, A., & Zolla, L. (2016). Proteolysis in meat tenderization from the point of view of each single protein: A proteomic perspective. *Journal of Proteomics, 147,* 85-97.

Larrea, V., Hernando, I., Quiles, A., Lluch, M. A., & Pérez-Munuera, I. (2006). Changes in proteins during Teruel dry-cured ham processing. *Meat Science, 74,* 586-593.

Lorenzo, J. M., Cittadini, A., Bermúdez, R., Munekata, P. E., & Domínguez, R. (2015). Influence of partial replacement of NaCl with KCl, $CaCl_2$ and $MgCl_2$ on proteolysis, lipolysis and sensory properties during the manufacture of dry-cured lacón. *Food Control, 55,* 90-96.

Lorenzo, J. M., García Fontán, M.C., Franco, I., & Carballo, J. (2008). Proteolytic and lipolytic modifications during the manufacture of dry-cured lacón, a Spanish traditional meat product: Effect of some additives. *Food Chemistry, 110(1),* 137-149.

Mora, L., Escudero, E., Fraser, P., Aristoy, M., & Toldrá, F. (2014). Proteomic identification of antioxidant peptides from 400 to 2500Da generated in Spanish dry-cured ham contained in a size-exclusion chromatography fraction. *Food Research International, 56,* 68-76.

Mora, L., Sentandreu, M., & Toldrá, F. (2011). Intense degradation of myosin light chain isoforms in Spanish dry-cured ham. *Journal of Agricultural and Food Chemistry, 59(8),* 3884–3892.

Morales, R., Arnau, J., Serra, X., Guerrero, L., & Gou, P. (2008). Texture changes in dry-cured ham pieces by mild thermal treatments at the end of the drying process. *Meat Science, 80(2),* 231-238.

Paredi, G., Raboni, S., Bendixen, E., de Almeida, A. M., Mozzarelli, A. (2012). "Muscle to meat" molecular events and technological transformation: The proteomics insight. *Journal of Proteomics*, *75*, 4275-4289.

Paredi, G., Sentandreu, M., Mozzarelli, A., Fadda, S., Hollung, K., & de Almeida, A. (2013). Muscle and meat: New horizons and applications for proteomics on a farm to fork perspective. *Journal of Proteomics, 88,* 58-82.

Petrova, I., Tolstorebrov, I., Mora, L., Toldrá, F., & Eikevik, T. (2016). Evolution of proteolytic and physico-chemical characteristics of Norwegian dry-cured ham during its processing. *Meat Science, 121,* 243-249.

Pugliese, C., Sirtori, F., Škrlep, M., Piasentier, E., Calamai, L., Franci, O., & Čandek-Potokar, M. (2015). The effect of ripening time on the chemical, textural, volatile and sensorial traits of *Bicep femoris* and *Semimembranosus* muscles of the Slovenian dry-cured ham Kraški pršut. *Meat Science, 100,* 58-68.

Radons, J. (2016). The human HSP70 family of chaperones: where do we stand? Cell Stress and Chaperones, 21, 379-404.

Ruiz-Ramírez, J., Arnau, J., Serra, X., & Gou, P. (2006). Effect of pH 24, NaCl content and proteolysis index on the relationship between water content and texture parameters in *biceps femoris* and *semimembranosus* muscles in dry-cured ham. *Meat Science, 72(2),* 185-194.

Škrlep, M., Čandek-Potokar, M., Mandelc, S., Javornik, B., Gou, P., Chambon, C., & Santé-Lhoutellier, V. (2011). Proteomic profile of dry-cured ham relative to *PRKAG3* or *CAST* genotype, level of salt and pastiness. *Meat Science, 88(4),* 657-667.

Tabilo, G., Flores, M., Fiszman, S. M., & Toldrá, F. (1999). *Postmortem* meta quality and sex affect textural properties and protein breakdown of dry-cured ham. *Meat Science*, 60(1), 77-83.

The UniProt Consortium. (2017). UniProt: the universal protein knowledgebase. *Nucleic Acids Research, 45(D1),* D158-169.

Théron, L., Sayd, T., Pinguet, J., Chambon, C., Robert, N., & Santé-Lhoutellier, V. (2011). Proteomic analysis of *semimembranosus* and *biceps femoris* muscles from Bayonne dry-cured ham. *Meat Science, 88(1),* 82-90.

Toldrá, F., Flores, M., & Sanz, Y. (1997). Dry-cured ham flavour: enzymatic generation and process influence. *Food Chemistry, 59(4),* 523-530.

Toldrá, F., Rico, E., & Flores, J. (1993). Cathepsins L, D, H and L activities in the processing of dry-cured ham. *Journal of the Science of Food and Agriculture, 62(2),* 157-161.

Virgili, R., Parolari, G., Schivazappa, C., Bordini, C. S., & Borri, M. (1995). Sensory and texture quality of dry-cured ham as affected by endogenous cathepsin b activity and muscle composition. *Journal of Food Science, 60(6),* 1183-1186.

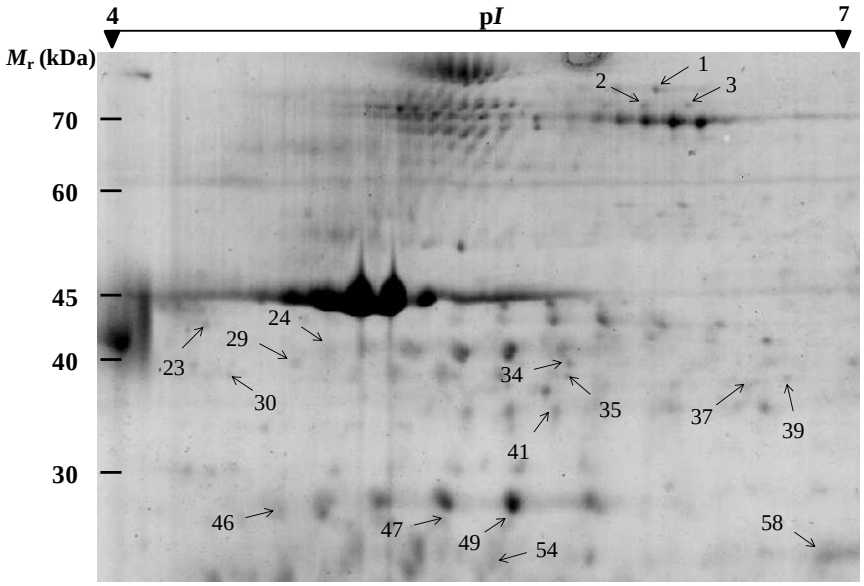
Zhao, G. M., Tian, W., Liu, Y. X., Zhou, G. H., Xu, X. L., & Li, M. Y. (2008). Proteolysis in *biceps femoris* during Jinhua ham processing. *Meat Science, 79(1),* 39-45.

518 **Figure captions**

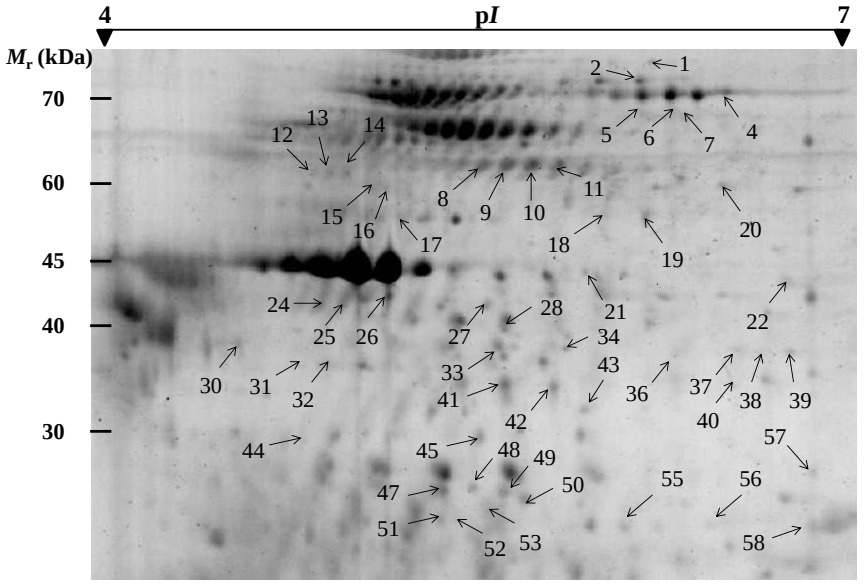
519 **Fig. 1.** 2-DE gel images showing the proteome profile of dry-cured ham with low
520 (A) and high (B) proteolysis index (LP and HP samples, respectively). Protein spots
521 with statistically significant qualitative (presence/absence) and quantitative (changes in
522 intensity) differences are marked and numbered. All these spots were excised for further
523 analysis by MALDI-TOF/TOF MS.

Figure 1

A) Low proteolysis index (LP samples)



B) High proteolysis index (HP samples)



Highlights

- Instrumental adhesiveness was assessed for the first time in dry-cured ham
- The proteolysis index is indicator of differential adhesiveness
- Myosin-1, myosin-4 and actin underwent the strongest response to proteolysis
- Novel candidate biomarkers for proteolysis and adhesiveness

Table 1.- Mean (± SE) values of physico-chemical parameters in dry-cured hams with different proteolysis index selected for proteomic analysis.

Parameters	Batch		*p*-value
	LP	HP	
Instrumental adhesiveness (g)	66.75 ± 4.87	100.43 ± 2.86	0.001
Moisture (%)	59.10 ± 0.14	58.57 ± 0.16	0.052
Salt content (%)	4.67 ± 0.05	4.69 ± 0.10	0.884
Non-protein nitrogen (%)	1.50 ± 0.07	1.84 ± 0.04	0.010
Total nitrogen (%)	4.97 ± 0.19	4.84 ± 0.03	0.539

Batches: LP = low proteolysis (*PI* < 33%); HP = high proteolysis (*PI* > 36%).

Table 2.- Spot volumes with statistically significant (*p*-value < 0.05) differential abundance in dry-cured hams of low and high proteolysis level.

Spot No.	Low proteolysis (LP)			High proteolysis (HP)		
	Mean (± SE) Volume	$P(\hat{\theta}_B \leq \hat{\theta})$	95% bootstrap CI (CL, CU)	Mean (± SE) Volume	$P(\hat{\theta}_B \leq \hat{\theta})$	95% bootstrap CI (CL, CU)
1	684 ± 31	0.57	617, 746	280 ± 75	0.53	79, 409
2	741 ± 150	0.53	353, 962	1531 ± 128	0.52	1259, 1742
3	392 ± 81	0.55	247, 554	–	–	–
4	–	–	–	1360 ± 215	0.54	815, 1712
5	–	–	–	307 ± 18	0.75	281, 333
6	–	–	–	271 ± 25	0.73	236, 306
7	–	–	–	366 ± 113	0.58	121, 566
8	–	–	–	2010 ± 419	0.60	1241, 2904
9	–	–	–	2186 ± 473	0.56	1320, 3073
10	–	–	–	2360 ± 500	0.53	1348, 3212
11	–	–	–	1174 ± 342	0.56	647, 2156

12	–	–	–	688 ± 95	0.49	520, 881
13	–	–	–	667 ± 219	0.54	53, 1014
14	–	–	–	1302 ± 257	0.58	976, 1830
15	–	–	–	661± 58	0.55	509, 764
16	–	–	–	508± 43	0.56	422, 589
17	–	–	–	655 ± 185	0.64	377, 1074
18	–	–	–	619 ± 194	0.60	229, 1003
19	–	–	–	582 ± 193	0.56	237, 974
20	–	–	–	163 ± 13	0.75	145, 182
21	–	–	–	468 ± 116	0.53	259, 695
22	–	–	–	798 ± 176	0.49	437, 999
23	234 ± 16	0.75	211, 257	–	–	–
24	725 ± 183	0.49	341, 993	1801 ± 212	0.68	1419, 2259
25	–	–	–	1459 ± 56	0.76	1379, 1537
26	–	–	–	1980 ± 327	0.75	1518, 2443
27	–	–	–	477 ± 112	0.51	248, 602

28	–	–	–	3396 ± 855	0.62	2016, 5152
29	283 ± 122	0.52	67, 510	–	–	–
30	235 ± 65	0.67	84, 310	489 ± 65	0.67	409, 639
31	–	–	–	324 ± 95	0.51	99, 541
32	–	–	–	507 ± 160	0.61	185, 826
33	–	–	–	477± 112	0.51	248,602
34	1079 ± 177	0.75	829, 1329	443 ± 178	0.62	318, 652
35	524 ± 99	0.77	394, 674	–	–	–
36	–	–	–	387 ± 16	0.61	359, 422
37	255 ± 6	0.76	246, 263	333 ± 40	0.64	284, 426
38	–	–	–	142 ± 66	0.67	37, 289
39	252 ± 29	0.54	172, 302	455 ± 98	0.58	338, 658
40	–	–	–	266 ± 47	0.53	158, 358
41	1756 ± 408	0.56	957, 2485	3274 ± 249	0.56	2990, 3783
42	965 ± 267	0.55	649, 1511	2041 ± 254	0.56	1577, 2555
43	–	–	–	544 ± 82	0.52	372, 667

44	–	–	–	1103 ± 113	0.74	943, 1264
45	1145 ± 197	0.56	814, 1556	–	–	–
46	465 ± 43	0.76	405, 525	–	–	–
47	475 ± 86	0.73	354, 597	1469 ± 302	0.56	722, 1963
48	–	–	–	608 ± 31	0.63	567, 679
49	779 ± 34	0.62	706, 843	1517 ± 312	0.58	1112, 2441
50	–	–	–	1370 ± 46	0.59	1277, 1462
51	–	–	–	622 ± 33	0.71	0.569, 0.697
52	1089 ± 344	0.66	543, 1862	–	–	–
53	–	–	–	2544 ± 665	0.62	1485, 4037
54	1622 ± 462	0.55	654, 2496	–	–	–
55	–	–	–	313 ± 116	0.58	46, 537
56	–	–	–	661 ± 292	0.61	28, 1180
57	683 ± 67	0.74	589, 777	352 ± 62	0.75	264, 440
58	643 ± 90	0.63	634, 849	399 ± 121	0.56	156, 623

Gel position of spots is shown in Fig. 1.

Mean (± SE) volumes were obtained from four biological replicates.

Bootstrap confidence intervals (CIs) were obtained by the bias-corrected percentile method from 20,000 bootstrap mean replications; Bonferroni method was applied to obtain simultaneous CIs over comparisons; CL and CL are the lower and upper bounds, respectively.

The bootstrap distribution was median biased if $P(\hat{\theta}_B \leq \hat{\theta}) \neq 0.50$, where $\hat{\theta}_B$ and $\hat{\theta}$ are the bootstrap and sample mean estimates, respectively.

Table 3.- Protein identification by MALDI-TOF/TOF MS of differentially (p-value < 0.05) represented 2-DE spots in dry-cured hams with low and high proteolysis index.

Spot No.	Protein	Abbrev.	Accesion number (Uniprot)	Mascot score	Sequence coverage (%)	Number of matched peptides	p*I* Th/IObs	M_r Th/Obs (kDa)	
1	Vinculin	VINC	P26234	60	19	17	5.6/6.2	124.4/76.1	Fragment
2	Serum albumin	ALBU	P08835	144	21	13	6.1/6.1	71.6/72.9	
3	Serum albumin	ALBU	P08835	125	21	14	6.1/6.3	71.6/73.2	
4	Serum albumin	ALBU	P08835	601	42	19	6.1/6.5	71.6/70.7	
5	Serum albumin	ALBU	P08835	56	10	7	6.1/6.1	71.6/66.3	
9	Myosin-1	MYH1	Q9TV61	503	17	36	5.6/5.6	224.4/59.6	Fragment
10	Myosin-1	MYH1	Q9TV61	373	15	31	5.6/5.6	224.4/62.6	Fragment
11	Myosin-1	MYH1	Q9TV61	493	16	35	5.6/5.7	224.4/62.8	Fragment
12	Myosin-1	MYH1	Q9TV61	582	16	30	5.6/4.7	224.4/53.3	Fragment
13	Myosin-1	MYH1	Q9TV61	331	8	15	5.6/4.8	224.4/52.9	Fragment
14	Myosin-1	MYH1	Q9TV61	467	15	28	5.6/4.9	224.4/52.8	Fragment

15	Myosin-1	F1SS62	Q9TV61	287	24	34	5.5/5.1	171.0/61.2	Fragment
16	Myosin-4	MYH4	Q9TV62	249	11	19	5.6/5.1	224.0/60.8	Fragment
17	Myosin-1	MYH1	Q9TV61	249	15	25	5.6/5.2	224.4/59.4	Fragment
20	α-1,4 glucan phosphorylase	F1RQQ8	F1RQQ8	102	13	10	6.7/6.5	97.7/55.4	Fragment
21	α-actin, skeletal muscle	ACTS	P68137	180	28	9	5.2/5.9	42.4/45.5	
22	Heat shock 70 kDa protein 1-like	HS71L	A5A8V7	66	6	4	5.6/6.7	70.7/45.1	Fragment
23	Myosin-7	MYH7	P79293	380	13	21	5.6/4.4	223.0/44.2	Fragment
24	α-actin, skeletal muscle	ACTS	P68137	96	14	4	5.2/4.9	42.4/40.1	
25	Myosin-4	MYH4	Q9TV62	241	12	21	5.6/4.9	224.0/43.4	Fragment
26	Myosin-4	MYH4	Q9TV62	701	15	30	5.6/5.1	224.0/43.8	Fragment
28	α-actin, skeletal muscle	ACTS	P68137	255	34	10	5.2/5.6	42.4/40.1	
29	α-actin, skeletal muscle	ACTS	P68137	69	19	5	5.2/4.7	42.4/39.1	
30	Desmin	DESM	P02540	87	10	4	5.2/4.4	53.6/38.0	
31	α-actin, skeletal muscle	ACTS	P68137	94	13	4	5.2/4.8	42.4/42.6	
32	Myosin-4	MYH4	Q9TV62	424	11	22	5.6/4.9	224.0/39.3	Fragment

34	F-actin-capping protein subunit alpha-2	CAZA2	Q29221	269	47	11	5.6/5.8	33.1/39.1	
36	F-actin-capping protein subunit alpha-2	CAZA2	Q29221	67	9	2	5.6/6.1	33.1/35.7	
40	β-enolase	ENOB	Q1KYT0	92	23	7	8.1/6.5	47.4/35.1	
44	F-actin-capping protein subunit beta	CAPZB	A0PFK7	395	46	13	5.5/4.9	31.6/31.0	
45	α-actin, skeletal muscle	ACTS	P68137	149	17	5	5.2/5.3	42.4/32.6	
46	α-actin, skeletal muscle	ACTS	P68137	159	30	8	5.2/4.5	42.4/25.5	Fragment
47	α-actin, skeletal muscle	ACTS	P68137	117	12	4	5.2/5.3	42.4/25.4	Fragment
48	Myosin-1	MYH1	Q9TV61	415	15	32	5.6/5.5	224.4/62.5	Fragment
49	α-actin, skeletal muscle	ACTS	P68137	180	17	5	5.2/5.6	42.4/25.4	Fragment
50	Peroxiredoxin-6	PRDX6	Q9TSX9	665	58	15	5.7/5.7	25.0/25.5	
51	α-actin, skeletal muscle	ACTS	P68137	126	14	4	5.2/5.3	42.4/24.0	Fragment
53	α-actin, skeletal muscle	ACTS	P68137	180	14	4	5.2/5.5	42.4/24.2	Fragment
55	Multiprotein bridging factor 1	A6N8P5	A6N8P5	70	49	10	10.0/6.1	16.4/24.0	
56	Triosephosphate isomerase	TPIS	Q29371	85	33	8	7.0/6.6	26.9/24.0	

All identified proteins were matched to *Sus scrofa*.(pig) proteins.

The Mascot baseline statistically significant (p-value < 0.05) score was 56.

Sequence coverage (%): percentage of coverage of the entire amino acid sequence by matched peptides.

Number of matched peptides: total number of identified spectra matched for the protein.

Theoretical (Th) isoelectric point (pI) and molecular mass (M_r). were obtained from UniProtKB/Swiss-Prot databases.

Observed (Ob) pI and M_r were obtained from the spot position on the gel.

Protein fragments: M_r (Th)/M_r (Obs) ratio higher than 1.5.

Table 4.- Fold change (*FC*) and relative change (*RC*) of differentially ($P < 0.05$) represented protein fragments in dry-cured ham with different proteolysis index.

Spot No.	Protein (abbrev.) fragment	Fold change (*FC*)	Relative change (*RC*)
9-17, 48	Myosin-1 (MYH1)	+∞	+1.00
46, 47, 49, 51, 53	α-actin, skeletal muscle (ACTS)	13.23	+0.60
16, 25, 26, 32	Myosin-4 (MYH4)	+∞	+0.43
22	Heat shock 70 kDa protein 1-like (HS71L)	+∞	+0.08
20	α-1,4 glucan phosphorylase (F1RQQ8)	+∞	+0.02
23	Myosin-7 (MYH7)	-∞	-0.02
1	Vinculin (VINC)	-2.44	-0.04