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.

Keywords: Defective ham texture, Instrumental adhesiveness, Meat proteolysis Meat proteomics, Porcine proteome, Processed meat

Introduction

Dry-cured ham is a traditional product with a great popularity among European consumers. The dry-cured ham process is a traditional method, which consists mainly of two steps: salting and ripening. During the whole process, there are many biochemical factors influencing the characteristics of the final product such as texture and flavour.

The curing processing of dry-cured ham requires salt as preserving agent, causing physicochemical and biochemical phenomena that are responsible of development of texture, flavor, final quality of the product and also influences the growth of microorganisms and enzymatic activities (Toldrá, Flores and Sanz, 1997).

In view of health worries, reducing salt intake has been identified as one of the most cost-effective measures countries can take to improve population health outcomes (WHO, 2007). The salting control is an important goal for industrial products and for traditional ones increasing demand for low-salt products (Martuscelli, Lupieri, Sacchetti, Mastrocola, and Pittia, 2017).

It is well known that a change in salt concentration affects protein denaturation depending on many factors such as rearing conditions (e.g. feeding, sex and slaughter age), pig line, features of raw hams (initial weigh, fat level and pH) and the ripening process (Mora, Calvo, Escudero, & Toldrá, 2016; Škrlep et al., 2011). In addition, during the ripening process proteins undergo an intense proteolysis, which constitutes the most important enzymatic reactions regarding muscle proteins (Pérez-Palacios, Ruiz, Barat, Aristoy, and Antequera, 2010). Raw material is pork muscle, which consists of 15-22% protein where there are three different groups of proteins based on their solubility: myofibrillar,

sarcoplasmic and connective tissue proteins (Tornberg, 2005). The most abundant proteins are the myofibrillar (50-55%) followed by sarcoplasmatic (30-34%) and both groups are intensely degraded contributing to dry-cured ham quality during the ripening process (Bermúdez, Franco, Carballo, Sentandreu, and Lorenzo, 2014).

Muscle proteins undergo an intense proteolysis during the curing process that contributes to singular dry-cured ham quality. However an excessive proteolysis may lead to higher pastiness, characterized by excessive softness, mushy texture and unpleasant flavours (Škrlep et al., 2011). The main myofibrillar proteins are myosin and actin, which builds up the myofibrillar structure together with other cytoskeletal proteins. In addition, they are important for the development of texture and sensorial characteristics the proteolitic changes of the myofibrillar proteins, especially responsible for changes of pastiness. On the other hand, sarcoplasmatic proteins include most of the enzymes such as enzymes related to energy metabolism (Petrova, Aasen, Rustad, and Eikevik, 2015).

Some studies have been developed to calculate proteolysis index (degree of proteolysis) and its relationship with meat tenderness (Bee, Anderson, Lonergan, and Huff-Lonergan, 2007; Harkouss et al., 2015; Fuentes, Estévez, Grèbol, Ventanas, and Ventanas, 2013). In recent years, omics techniques have used to understand the biological mechanisms of the muscle and the meat process ageing (Lana and Zolla, 2016). Recently, proteomics approach has been applied to study muscle proteins and further proteolysis in different types of meat (Mora Escudero, Fraser, Aristoy, and Toldrá, 2014; Petrova Tolstorebrov, Mora, Toldrá, and Eikevik, 2016; Degnes, Kvitvang, Haslene-Hox,

and Aasen, 2017). Accordingly, molecular markers have been identified to follow the conversion of muscle into meat as well as to control the quality requirements in meat products (Paredi et al., 2013).

The aim of the present study is to assess the degradation of myofibrillar and sarcoplasmatic proteins in dry-cured ham with different proteolysis level based on a combination of two dimensional electrophoresis (2-DE) and mass spectrometry, trying to find biomarkers as a first step in order to improve the final quality of the product.

1. 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:(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 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 DALTsix vertical gel system (GE Healthcare) with 12% SDS-PAGE gels at 18 mA/gel until the bromophenol blue dye front reached the end of the gels. The 2-DE gels were stained with SYPRO Ruby fluorescent stain (Lonza, Rockland, ME).

2.7. Image analysis of 2-DE gels

Gels were visualized and digitalized using the Gel Doc XR+ system (Bio-Rad Laboratories). The detection and quantification of 2-DE spot volumes were performed with PDQuest Advanced software v. 8.0.1 (Bio-Rad Laboratories) after background subtraction. Spot volume normalization was performed using those validated across all replicate gels. Observed values of molecular mass (M_r) were determined across protein spots from standard molecular mass markers ranging from 15 to 200 kDa (Fermentas, Burlington, ON, Canada), whereas those of isoelectric point (p*I*) were established according to 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 acetamide 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 hydrolyzed with modified porcine trypsin (Promega, Madison, WI) at a final concentration of 20 ng/µL of trypsin in 20 mM ammonium bicarbonate 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) using the thin layer method (Vorm, Roepstorff, & Mann, 1994). Mass spectrometric data were obtained in an automated analysis loop using 4800 MALDI-TOF/TOF analyzer (Applied Biosystems). Mass spectra were acquired in positive-ion reflector mode with an Nd:YAG laser operating at 355 nm and an average accumulation of 1000 laser shots. A minimum of three trypsin autolysis peaks were used for internal calibration, in order to decrease peptide mass errors for protein identification. 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 was achieved using the 4000 Series Explorer Software v. 3.5 (Applied Biosystems). Peptide mass fingerprint and peptide fragmentation spectra data of each sample were combined using GPS Explorer Software v. 3.6 and Mascot software v. 2.1 (Matrix Science, Boston, MA) to search against UniProt/SwissProt database. Mascot search parameters were: precursor mass tolerance of 50 ppm, 0.6 Da MS/MS fragment tolerance, carbamidomethyl cysteine 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 for physicochemical parameters was performed using the IBM SPSS Statistics V21.0 (SPSS, Chicago, IL) software package.

Quantitative changes of 2-DE gel spot volumes in sample groups were assessed using the measures “fold change” (*FC*) and “relative change” (*RC*)

(Franco et al., 2015a, Franco et al., 2015b). 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 index, 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, Franco et al., 2015b). 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 replications (Efron, 1982). Confidence intervals were adjusted for multiple hypothesis testing with the Bonferroni procedure.

3. Results and discussion

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

Mean (±SE) values of instrumental adhesiveness, moisture, salt content, non-protein nitrogen and total nitrogen in dry-cured hams with extreme (<33% or >36%) proteolysis indices are shown in Table 1. Physicochemical parameter values both for the total of hams with extreme proteolysis indices and for those samples used in the proteomic analysis are presented separately. It is noteworthy that physicochemical values in samples used for proteomic analysis were representative of the entire set of selected samples. Therefore, we will

hereafter refer only to physicochemical data of the entire set of selected samples. Mean (±SE) proteolysis indices in samples with low and high proteolysis were 31.5 ± 0.2 and 38.5 ± 0.3, respectively ($p < 0.001$). Differences in proteolysis index can be attributed to a large number of factors, such as variable raw materials, salting procedures, ripening process, duration of the different steps involved in the elaboration, as well as variations of temperature and relative humidity in dry-cured ham processing (García-Garrido et al., 1999, Pugliese et al., 2015, Škrlep et al., 2011, Zhao et al., 2008). In the present study, however, hams were elaborated under uniform conditions. It suggests that proteolysis can undergo large variations even under similar processing systems.

Adhesiveness of sliced dry-cured ham was assessed, for the first time, by mechanical procedures as an alternative to sensory analysis. Our observations indicate that the instrumental adhesiveness was significantly ($p < 0.05$) higher in the high proteolysis batch than in the low proteolysis batch. In addition, we found a significant positive relationship between proteolysis index and adhesiveness using the Pearson product-moment correlation coefficient (r) and Spearman's nonparametric coefficient (r_s) of rank correlation ($r = +236$, $p = 0.026$, $n = 89$; $r_s = +0.242$, $p = 0.023$). These results support the conclusion that adhesiveness is dependent on the proteolysis index. Hams with defective texture can exhibit high moisture/protein ratios as a result of both increased moisture and decreased protein contents relative to ham with normal texture (García-Garrido et al., 1999). In addition, several authors (Bermúdez et al., 2014a, Ruiz-Ramírez et al., 2006, Virgili et al., 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 ($p < 0.001$) differences between treatments, since the lowest mean values were observed in the low proteolysis batch (1.55 *vs*. 1.83%, 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. Tyrosine crystals could be considered markers of advanced and intense proteolysis. However, in our study, we did not observe tyrosine crystals in any dry-cured ham samples (our hams were ripened for 14 months, not more).

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 are shown in Fig. 1. The identification, matching and volume evaluation of 2-DE spots were obtained using PDQuest software. Saturated, faint and non-reproducible spots over replicates were excluded from further analysis. The total numbers of selected spots for proteomic analysis were 92 and 123 spots in low and high proteolysis groups, respectively. 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. It should be noted 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 samples there were 37 spots ($p < 0.001$, Fisher's exact test). This difference probably reflects an increased protein fragmentation in samples with high proteolysis.

3.3. Evaluation of protein fragmentation

Protein fragmentation was evaluated by the following procedure. First, protein identification of differentially abundant spots between treatments was performed by MALDI-TOF/TOF MS. Second, 2-DE gel 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. 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 of differential proteolysis over proteomes. The remaining spots, with theoretical and empirical mass ratios below 1.5, were excluded from further analysis. It is not possible to assess whether they actually contain either entire

or slightly degraded proteins at the level of resolution of 2-DE. The use of an internal standard in multiplexing methods such as two-dimensional difference gel electrophoresis (2-D DIGE) could reduce inter-gel variation, resulting in an increase of statistical power (Chevalier, 2010). However, 2-DE is able to identify the strongest protein changes between sample groups, and therefore the most useful biomarkers for proteolysis and adhesiveness.

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

3.4. Candidate biomarkers for differential proteolysis and adhesiveness

Quantitative differences in proteolysis intensity between low and high proteolysis ham batches were assessed by *FC* and *RC* statistics from protein fragment volumes. Table 4 shows *FC* and *RC* values for each protein found to be differentially affected by proteolysis. There can be seen that both statistics provide very different information about the extent of proteolysis across

proteins. It is worth noting that *FC* is a measure traditionally used to quantify differential protein abundance between treatments. But it has the disadvantage that its range varies from -∞ to +∞ and range boundaries are achieved with the presence of unique spots independently of the existing differences in volume. In contrast, *RC* always ranges from −1.0 to +1.0. It provides; therefore, a more intuitive measure of the strength of change and maximum values of its range are not necessarily achieved with the mere occurrence of unique spots (see Table 4). Accordingly, *RC* is a particularly appropriate measure for the analysis of degraded proteome profiles exhibiting large number of unique spots. In the present study, we found that *RC*-values of proteins ranged between −0.04 and +1.0 (Table 4). Only five proteins (i.e. MYH1, ACTS, MYH4, HS71L and F1RQQ8) showed positive *RC*-values, indicating that their fragments were over-represented in high-proteolysis hams. In contrast, MYH7 and VINC proteins underwent decreased proteolysis in high-proteolysis samples given that their *RC*-values were of negative sign. This result suggests that MYH7 and VINC proteins are not useful biomarkers of proteolysis intensity.

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

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

396 Of the five fragmented proteins over-represented in high proteolysis hams, two
397 were sarcoplasmic proteins: HS71L and F1RQQ8. They are proteins with a
398 considerably lower relative representation in the proteome of *biceps*
399 *femoris* muscle, which explains their low *RC* values (<0.10). The HS71L protein
400 is a molecular chaperone that appears to play a critical role in multiple cellular
401 functions, including activation of proteolysis of misfolded proteins, controlling
402 the targeting of proteins for subsequent degradation and protection of the
403 proteome in response to stress (Archibald et al., 2010; Radons, 2016, The
404 UniProt Consortium, 2017). On the other hand, the F1RQQ8 protein is a
405 phosphorylase that catalyzes and regulates the breakdown of glycogen to

406 glucose-1-phosphate for the generation of ATP during glycogenolysis (Archibald
407 et al., 2010; Gautron et al., 1987, The UniProt Consortium, 2017). Fragments of
408 F1RQQ8 resulting from proteolytic activity were also detected in post-
409 mortem *longissimus dorsi* porcine muscle (Lametsch, Roepstorff, & Bendixen,
410 2002), as well as in dry-cured *biceps femoris* and *semimembranosus* muscles
411 (Théron et al., 2011). Specifically, the *biceps femoris* muscle showed more
412 F1RQQ8 fragments than the *semimembranosus* muscle during the ripening of
413 dry-cured ham, due to its higher proteolytic activity (Théron et al., 2011). It
414 follows FIRQQ8 is a good biomarker of proteolysis in agreement with our
415 observations.

416 In the present study, we found that the instrumental adhesiveness is dependent
417 on the proteolytic activity in dry-cured ham. Therefore, the identified biomarkers
418 also apply for the meat quality trait of adhesiveness. These biomarkers provide
419 non-invasive tools alternative to sensory analysis or mechanical measures, to
420 assess variations in adhesiveness. The identified proteins can also be potential
421 biomarkers for other proteolysis-related ham quality traits. It is particularly true
422 in the case of pastiness, considering that pastiness variations are closely
423 related with the extent of proteolysis and adhesiveness (Morales et al.,
424 2008, Škrlep et al., 2011).

425 **4. Conclusions**

426 Comparison of dry-cured ham proteomic profiles with extreme proteolysis index
427 scores allowed us to identify novel candidate biomarkers for differential
428 proteolytic activity underlying quality traits. First of all, we found that the
429 proteolysis index is a reliable indicator of the extent of protein hydrolysis at

proteomic scale. In addition, hams with higher proteolysis indices showed increased instrumental adhesiveness. A total of five myofibrillar 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.

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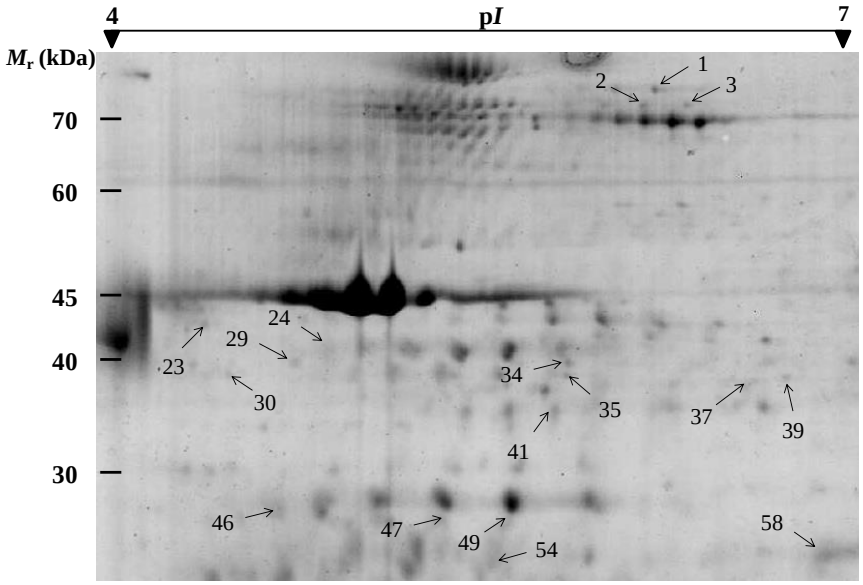
CAPTION TO FIGURE

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

554

a) Low proteolysis



b) High proteolysis

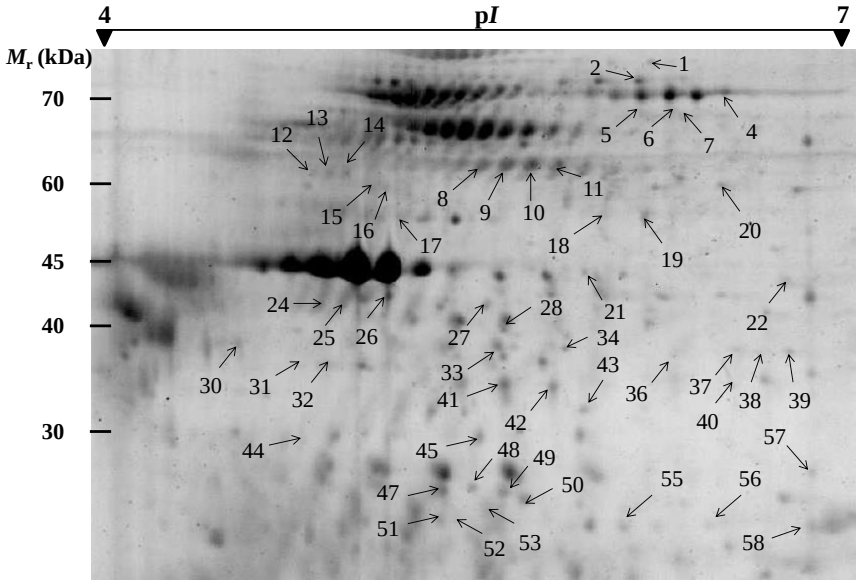


Figure 1.

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556

557 **Table 1.** Mean (±SE) values of physicochemical parameters in dry-cured hams with
558 different proteolysis indices.

	LP	HP	SEM	p-value
Initial weight (kg)	11.14 ±0.43	11.44 ±0.14	0.11	0.236
Loss weight (kg)	36.65 ±0.86	36.11 ±0.08	0.22	0.278
pH	5.48 ±0.008	5.49 ±0.03	0.007	0.645
Salt (%)	4.67 ±0.11	4.69 ±0.20	0.05	0.884
Moisture (%)	59.10 ±0.29	58.57 ±0.32	0.14	0.052
Sensorial traits				
Pastiness	0.61 ±1.03	1.52 ±1.19	0.40	0.296
Adhesiveness	1.10 ±0.61	1.78 ±1.31	0.35	0.385
Viscosity saliva	1.38 ±0.83	3.18 ±1.93	0.59	0.137
Instrumental Adhesiveness (N)	66.75 ±9.74	100.43±5.73	6.88	0.001
Total nitrogen (%)	4.97 ±0.39	4.84 ±0.06	0.09	0.539
Non-protein nitrogen (%)	1.50 ±0.15	1.84 ±0.09	0.07	0.010
Basic volatile nitrogen (mg/100g)	164.87 ±13.49	168.95 ±5.22	3.43	0.593
Proteolysis index	30.25 ±1.37	37.96 ±1.76	1.54	<0.001

559

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

	Low proteolysis (LP)			High proteolysis (HP)			
Spot No.[a]	Mean (± SE) Volume	$P(\hat{\theta}_B \leq \hat{\theta})$[b]	95% bootstrap CI (CL, CU)[c]	Mean (± SE) Volume	$P(\hat{\theta}_B \leq \hat{\theta})$[b]	99% bootstrap CI (CL, CU)[c]	*P*-value
1	684 ± 31	0.57	617, 746	280 ± 75	0.53	79, 409	< 0.05
2	741 ± 150	0.53	353, 962	1531 ± 128	0.52	1259, 1742	< 0.05
3	392 ± 81	0.55	247, 554	–	–	–	< 0.05
4	–	–	–	1360 ± 215	0.54	815, 1712	< 0.05
5	–	–	–	307 ± 18	0.75	281, 333	< 0.05
6	–	–	–	271 ± 25	0.73	236, 306	< 0.05
7	–	–	–	366 ± 113	0.58	121, 566	< 0.05
8	–	–	–	2010 ± 419	0.60	1241, 2904	< 0.05

9	–	–	–	2186 ± 473	0.56	1320, 3073	< 0.05
10	–	–	–	2360 ± 500	0.53	1348, 3212	< 0.05
11	–	–	–	1174 ± 342	0.56	647, 2156	< 0.05
12	–	–	–	688 ± 95	0.49	520, 881	< 0.05
13	–	–	–	667 ± 219	0.54	53, 1014	< 0.05
14	–	–	–	1302 ± 257	0.58	976, 1830	< 0.05
15	–	–	–	661± 58	0.55	509, 764	< 0.05
16	–	–	–	508± 43	0.56	422, 589	< 0.05
17	–	–	–	655 ± 185	0.64	377, 1074	< 0.05
18	–	–	–	619 ± 194	0.60	229, 1003	< 0.05
19	–	–	–	582 ± 193	0.56	237, 974	< 0.05
20	–	–	–	163 ± 13	0.75	145, 182	< 0.05
21	–	–	–	468 ± 116	0.53	259, 695	< 0.05

22	–	–	–	798 ± 176	0.49	437, 999	< 0.05
23	234 ± 16	0.75	211, 257	–	–	–	< 0.05
24	725 ± 183	0.49	341, 993	1801 ± 212	0.68	1419, 2259	< 0.05
25	–	–	–	1459 ± 56	0.76	1379, 1537	< 0.05
26	–	–	–	1980 ± 327	0.75	1518, 2443	< 0.05
27	–	–	–	477 ± 112	0.51	248, 602	< 0.05
28	–	–	–	3396 ± 855	0.62	2016, 5152	< 0.05
29	283 ± 122	0.52	67, 510	–	–	–	< 0.05
30	235 ± 65	0.67	84, 310	489 ± 65	0.67	409, 639	< 0.05
31	–	–	–	324 ± 95	0.51	99, 541	< 0.05
32	–	–	–	507 ± 160	0.61	185, 826	< 0.05
33	–	–	–	477± 112	0.51	248,602	< 0.05
34	1079 ± 177	0.75	829, 1329	443 ± 178	0.62	318, 652	< 0.05

35	524 ± 99	0.77	394, 674	–	–	–	< 0.05
36	–	–	–	387 ± 16	0.61	359, 422	< 0.05
37	255 ± 6	0.76	246, 263	333 ± 40	0.64	284, 426	< 0.05
38	–	–	–	142 ± 66	0.67	37, 289	< 0.05
39	252 ± 29	0.54	172, 302	455 ± 98	0.58	338, 658	< 0.05
40	–	–	–	266 ± 47	0.53	158, 358	< 0.05
41	1756 ± 408	0.56	957, 2485	3274 ± 249	0.56	2990, 3783	< 0.05
42	965 ± 267	0.55	649, 1511	2041 ± 254	0.56	1577, 2555	< 0.05
43	–	–	–	544 ± 82	0.52	372, 667	< 0.05
44	–	–	–	1103 ± 113	0.74	943, 1264	< 0.05
45	1145 ± 197	0.56	814, 1556	–	–	–	< 0.05
46	465 ± 43	0.76	405, 525	–	–	–	< 0.05
47	475 ± 86	0.73	354, 597	1469 ± 302	0.56	722, 1963	< 0.05

48	–	–	–	608 ± 31	0.63	567, 679	< 0.05
49	779 ± 34	0.62	706, 843	1517 ± 312	0.58	1112, 2441	< 0.05
50	–	–	–	1370 ± 46	0.59	1277, 1462	< 0.05
51	–	–	–	622 ± 33	0.71	0.569, 0.697	< 0.05
52	1089 ± 344	0.66	543, 1862	–	–	–	< 0.05
53	–	–	–	2544 ± 665	0.62	1485, 4037	< 0.05
54	1622 ± 462	0.55	654, 2496	–	–	–	< 0.05
55	–	–	–	313 ± 116	0.58	46, 537	< 0.05
56	–	–	–	661 ± 292	0.61	28, 1180	< 0.05
57	683 ± 67	0.74	589, 777	352 ± 62	0.75	264, 440	< 0.05
58	643 ± 90	0.63	634, 849	399 ± 121	0.56	156, 623	< 0.05

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562 Spot volume: means ± standard error

563 CI, Confident interval; CL lower bound; CU, upper bound

564 **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
565 and high proteolysis index.

Spot	Protein	Abbrev.	Accesion No. (Uniprot)	Mascot score[a]	Sequence coverage (%)[b]	Number of matched peptides[c]	p*I* Th/Obs[d]	M_r Th/Obs (kDa)[e]	
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	Alpha-1,4 glucan phosphorylase	F1RQQ8	F1RQQ8	102	13	10	6.7/6.5	97.7/55.4	Fragment
21	Actin, alpha 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	Actin, alpha 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	Actin, alpha skeletal muscle	ACTS	P68137	255	34	10	5.2/5.6	42.4/40.1	

29	Actin, alpha 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	Actin, alpha 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	Beta-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	Actin, alpha skeletal muscle	ACTS	P68137	149	17	5	5.2/5.3	42.4/32.6	
46	Actin, alpha skeletal muscle	ACTS	P68137	159	30	8	5.2/4.5	42.4/25.5	Fragment
47	Actin, alpha 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	Actin, alpha 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	Actin, alpha skeletal muscle	ACTS	P68137	126	14	4	5.2/5.3	42.4/24.0	Fragment
53	Actin, alpha 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	

[a]Mascot score greater than 56 is significant ($p<0.05$)

[b]Sequence coverage (%): percentage of the protein sequence covered by identified peptides.

[c]Number of matched peptides: total number of distinct peptides identified in the identified protein.

[d]p*I* Th/Obs: isoelectric point values were based on Uniprot database (Theoretical) and gel position (Observed)

[e]M_r Th/Obs (kDa): molecular weight values were based on Uniprot database (Theoretical) and gel position (Observed)

Protein fragments were considered when ratio between theoretical and observed molecular weight was higher than 1.5

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

Spot	Protein	Fold change (FC)	Relative change (RC)
2	Serum albumin	+2.02	+0.482
3	Serum albumin	- ∞	- 0.244
4	Serum albumin	+∞	+0.848
5	Serum albumin	+∞	+0.192
21	Actin, alpha skeletal muscle	+∞	+0.292
24	Actin, alpha skeletal muscle	+2.49	+0.671
28	Actin, alpha skeletal muscle	- 1.47	- 1.000
29	Actin, alpha skeletal muscle	- ∞	- 0.176
30	Desmin	+2.08	+0.158
31	Actin, alpha skeletal muscle	+∞	+0.202
34	F-actin-capping protein subunit alpha-2	- 1,99	- 0.335
36	F-actin-capping protein subunit alpha-2	+∞	+0.241
40	Beta-enolase	+∞	+0.166
44	F-actin-capping protein subunit beta	+∞	+0.688
45	Actin, alpha skeletal muscle	- ∞	- 0.714
50	Peroxiredoxin-6	+∞	+0.854
55	Multiprotein bridging factor 1	+∞	+0.195
56	Triosephosphate isomerase	+∞	+0.412

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