

## Proteomic Identification of Actin-Derived Oligopeptides in Dry-Cured Ham

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An intense proteolysis of muscle proteins, mainly due to the action of endogenous proteolytic enzymes, has been reported to occur during the processing of dry-cured ham. This gives rise to an important generation of free amino acids and peptides of small size that contribute directly or indirectly to flavor characteristics of the final product. The nature and properties of the free amino acids generated during postmortem proteolysis have been well established. However, little is known about the identity of peptides generated during the processing of dry-cured ham. In the present paper, we describe the isolation (by ethanol precipitation followed by size exclusion and reverse phase chromatographies) and identification (by matrix-assisted laser desorption/ionization time-of-flight MS and collision-induced dissociation MS/MS) in a Spanish dry-cured ham of the following four oligopeptides: (A) TKQEY-DEAGPSIVHR, (B) ITKQEYDEAGPSIVHRK, (C) DSGDGVTHNVPIYE, and (D) DSGDGVTHNVPIYEG. This is the first time that these peptide fragments have been reported in dry-cured ham at the end of processing. Sequence homology analysis revealed that the four peptides originated from muscle actin, indicating an extensive hydrolysis of this protein during dry-curing. Some of the cleavage sites corresponding to these fragments in actin were reproduced by other authors through the incubation of this myofibrillar protein in the presence of cathepsin D (EC 3.4.23.5), thus supporting a relevant action of this enzyme during the processing of dry-cured ham.

**KEYWORDS:** Dry-cured ham; actin; proteolysis; peptide sequencing; proteomics; cathepsin D

### INTRODUCTION

The sector of meat and its derived products represents an important part of the whole food and beverages area in terms of consumption and economy. Actually, the meat industry has to face important challenges due to an increasing demand for safe products with standardized and certified quality, subject to stable prices in the market. A proof of this is the increasing, but still insufficient, proliferation of meat products labeling guarantee of origin as a way to assess their quality, a fact that is especially remarkable in the case of dry-cured ham. There are several renowned dry-cured ham types in the world, most of them produced in the Mediterranean area such as Spanish “Ibérico” and “Serrano”, Italian “Parma” and “San Danielle”, or French “Bayonne” ham (*1*). The characteristics of raw material, together with the additives employed and the curing time required in each case, are different, though in general processing times as long as several months are required to reach the typical texture and flavor properties characteristic of each

ham type, a fact that considerably increases the price of these products with respect to fresh meat. Numerous biochemical reactions take place during dry-curing. In this respect, one of the most relevant features is the degradation of muscle proteins by endogenous enzymes. Despite the research carried out during the past few decades, this phenomenon is still not deeply understood, although it is determinant for the development of final dry-cured ham characteristics. During the first days after slaughter there is an initial breakdown of the myofibrillar structure by different proteolytic enzymes, resulting in a softening of this myofibrillar structure and giving rise to the generation of large polypeptides (*2, 3*). Subsequently, these large polypeptides serve as substrates for the action of other muscle enzymes such as different groups of exopeptidases. The consequence is an important accumulation of free amino acids and peptides of small size at the end of dry-curing (*4–6*). The final products of this proteolytic chain have great importance because they contribute directly, or indirectly as precursors of other compounds, to the unique dry-cured ham flavor characteristics, being hence responsible for the final quality of the product (*1*). Some of the difficulties that the meat industry has to face regarding the control and standardization of the final quality of dry-cured meat products are mainly due to the lack

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of knowledge still existing about the biochemistry of postmortem proteolysis. This includes better information about which are the main enzymes responsible for muscle protein degradation and their underlying specific mode of action, as well as the identification of the peptides present in the final product.

The aim of the present work was to contribute to the knowledge of postmortem muscle proteolysis as related to the quality of meat products by implementing proteomic technology, to overcome some of the difficulties found in the past by meat scientists in this field. Thus, we have accomplished the identification of peptides naturally generated during dry-cured ham processing by using a combination of chromatographic techniques along with matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) and tandem mass spectrometry. We investigated also the protein origin of the identified peptides, providing insights into the possible endogenous enzymes responsible for their generation.

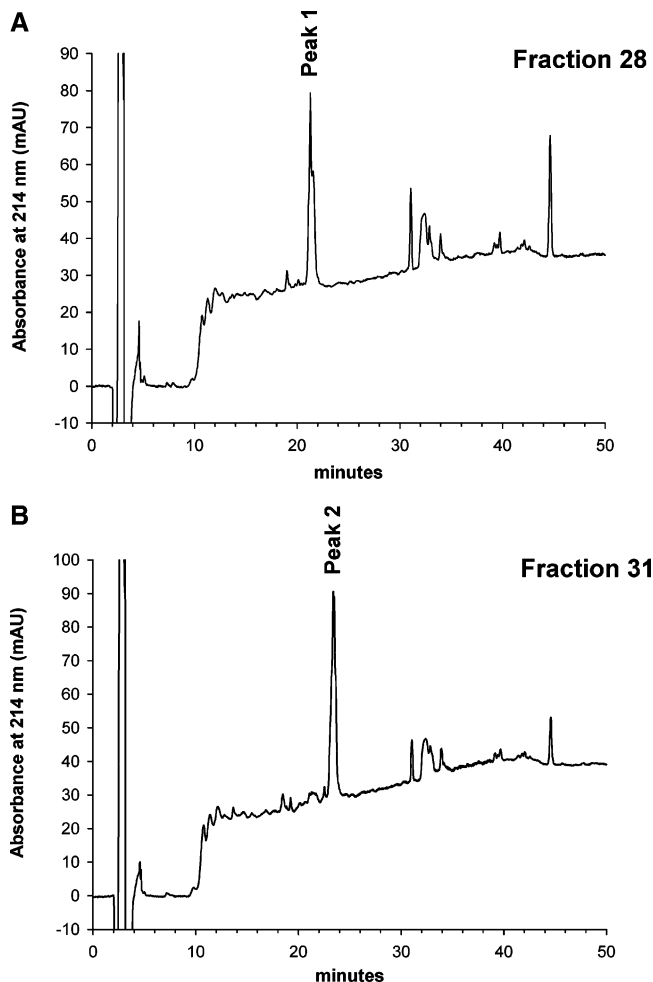
## MATERIALS AND METHODS

**Preparation of a Deproteinized Dry-Cured Ham Extract.** Spanish Serrano dry-cured ham (1 year of curing) was purchased from a local market. A 25 g sample of muscle semimembranosus, with no appreciable connective or adipose tissue, was minced and homogenized in 100 mL of 0.01 N HCl with a stomacher (Seward Laboratory). The homogenate was then centrifuged (10000g for 20 min at 4 °C) and the supernatant further deproteinized by adding 3 volumes of ethanol. After 20 h at 4 °C, the mixture was centrifuged (10000g for 20 min at 4 °C), the pellet containing the precipitated proteins was discarded, and the supernatant was dried in a rotatory evaporator. The dried extract was finally redissolved in 20 mL of 0.01 N HCl, filtered through a 0.45  $\mu$ m membrane filter (Millipore, Bedford, MA) and stored at -20 °C until use.

**Molecular Mass Fractionation.** A 5 mL sample of deproteinized ham extract was submitted to size exclusion chromatography on a Sephadex G-25 column (2.5  $\times$  65 cm, Amersham Biosciences, Uppsala, Sweden), equilibrated with 0.01 N HCl. Elution was performed at a flow rate of 15 mL/h with 0.01 N HCl at 4 °C and monitored by UV absorption at 214, 254, and 280 nm. Fractions of 5 mL were collected. Those fractions corresponding to an elution volume between 215 and 230 mL were pooled together, dried in a SpeedVac, and stored at -20 °C until use.

**Reversed-Phase HPLC.** Dried fractions were redissolved in 2 mL of 0.1% trifluoroacetic acid (TFA) in water (solution A) and filtered through a 0.22  $\mu$ m Millipore filter prior to injection on a SymmetryPrep C18 (7.8  $\times$  300 mm, 7  $\mu$ m particle size) reversed-phase column (Waters, Milford, MA). Peptide separation was performed on a Hewlett-Packard 1050 HPLC system (Palo Alto, CA) at a flow rate of 3 mL/min and using the following conditions: isocratic (solution A) for 15 min, followed by a linear gradient from 0% to 80% of 60% acetonitrile and 0.085% TFA in water (solution B) for 55 min. The separation was monitored at  $\lambda$  = 214 nm, and 1.5 mL fractions were collected. Selected reversed-phase fractions were rechromatographed on a Symmetry C18 (4.6  $\times$  250 mm, 5  $\mu$ m particle size) reversed-phase column using the same mobile phases as above, but eluting with a gradient from 0% to 60% buffer B in 20 min at a flow rate of 1 mL/min. Separation was monitored at  $\lambda$  = 214 nm, and peptide peaks were collected manually, dried in a speed vacuum, and stored at -20 °C.

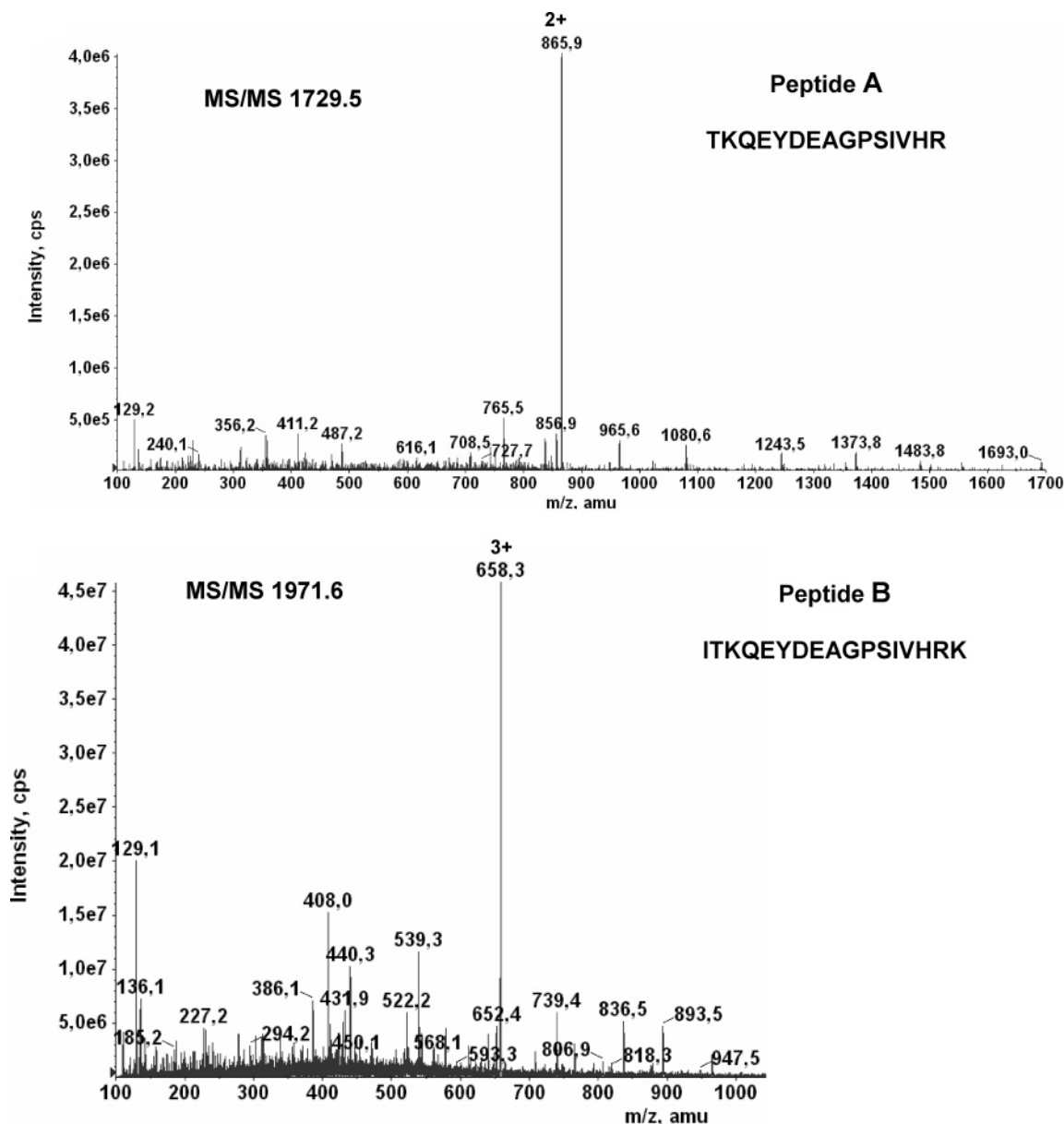
**Mass Spectrometry.** Dried fractions were redissolved in 6  $\mu$ L of 50% acetonitrile (ACN) and 0.1% TFA. A 0.85  $\mu$ L sample of each fraction was spotted onto a MALDI-TOF sample holder, mixed with an equal volume of a saturated solution of  $\alpha$ -cyano-4-hydroxycinnamic acid (Sigma, St. Louis, MO) in 50% ACN containing 0.1% TFA, air-dried, and analyzed with an Applied Biosystems Voyager-DE Pro MALDI-TOF mass spectrometer, operated in delayed extraction and reflector modes using the following parameters: 20 kV accelerating voltage, 74% grid voltage, 0.002 V guide wire voltage, 200 ns delay, and low mass gate of 500. Mass calibration was done by using a tryptic peptide mixture of *Cratylia floribunda* seed lectin (Swiss Prot accession



**Figure 1.** Analytical C18 reversed-phase HPLC chromatogram of fractions 28 and 31 obtained after size exclusion chromatography and preparative reversed-phase chromatography of a dry-cured ham extract. Peaks designated as 1 (fraction 28) and 2 (fraction 31) were further characterized by MS/MS analysis (Figures 2 and 3).

code P81517), which was previously prepared and characterized in the laboratory (7).

For peptide sequencing, the peptide mixture fraction was loaded in a nanospray capillary and subjected to electrospray ionization mass spectrometric analysis using a QTrap mass spectrometer (Applied Biosystems) equipped with a nanospray source (Protana, Denmark). Doubly or triply charged ions selected after enhanced resolution MS analysis were fragmented using the enhanced product ion with the Q0 trapping option. Enhanced resolution was performed at 250 amu/s across the entire mass range, a scanning mode that enables a mass accuracy of less than 20 ppm, making charge state identification reliable up to charge state 5. The term “enhanced product ion” refers to the performance of the PE-SCIEX developed and patented LINAC (Q2) collision cell technology, which accelerates ions through the collision cell, correcting thereby the slow movement of ions due to high pressures existing within the chamber, and provides high sensitivity and improved resolution in MS/MS mode in comparison to triple quadrupoles without the LINAC collision cell. For MS/MS experiments, Q1 was operated at unit resolution, the Q1-to-Q2 collision energy was set to 35 eV, the Q3 entry barrier was 8 V, the LIT (linear ion trap) Q3 fill time was 250 ms, and the scan rate in Q3 was 1000 amu/s. Collision-induced dissociation (CID) spectra were interpreted manually or using the online form of the MASCOT program at <http://www.matrixscience.com>. Parent proteins were assigned by amino acid sequence similarity searches against available online protein databases using the BLAST search engine (<http://www.bork.embl-heidelberg.de>).



**Figure 2.** CID spectra of ions  $865.9^{2+}$  (peptide A) and  $658.3^{3+}$  (peptide B) of fraction 28 (peak 1 in **Figure 1**, top). Peptide sequences matching each of the product ion spectra are shown in capital letters.

## RESULTS

**Fractionation by Gel Filtration.** A deproteinized extract of Spanish Serrano dry-cured ham was fractionated on a Sephadex G-25 size exclusion column. Fractions eluting at 215–230 mL, which according to a calibration curve from 180 to 12400 Da corresponded to a molecular mass of around 1530 Da, were pooled together.

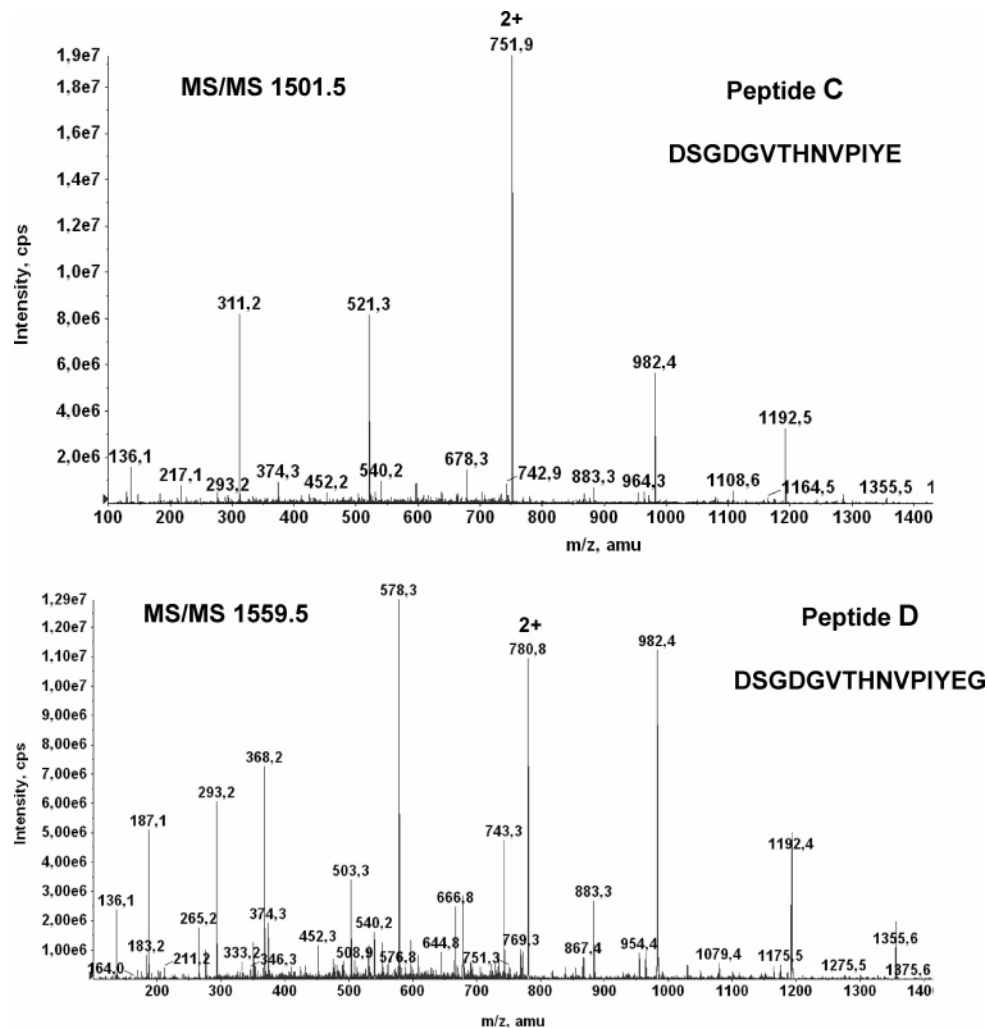
**Isolation of Peptides by Reversed-Phase HPLC.** Pooled fractions were concentrated and subjected to preparative C18 reversed-phase chromatography. Two fractions, exhibiting relevant absorbance values at  $\lambda = 214$  nm and eluting at a concentration of 12% (fraction 28) and 14% (fraction 31) ACN, respectively, were selected for the isolation and identification of peptides. To this end, the fractions were subsequently chromatographed on an analytical C18 reversed-phase column, generating the chromatograms displayed in **Figure 1**. Peaks detected at  $\lambda = 214$  nm were collected manually and analyzed by MALDI-TOF MS and CID-MS/MS.

**Determination of the Peptide Sequence by Mass Spectrometry.** Peaks 1 (fraction 28) and 2 (fraction 31), shown in

**Figure 1**, yielded mass spectra showing molecular ions of appreciable intensity. MALDI-TOF MS of peak 1 yielded quasimolecular ions ( $M + H^+$ ) at  $m/z$  1729.5 (peptide A) and 1971.6 (peptide B), respectively, whereas peak 2 showed two major ions at  $m/z$  1501.5 (peptide C) and 1559.5 (peptide D), respectively (results not shown). These molecular masses were in accordance with the peptide size estimated by size exclusion chromatography.

The four peptide ions were subjected to ESI-ion trap mass spectrometry to elucidate their sequence. Collision-induced dissociation MS/MS spectra of the doubly charged ion 865.9 (corresponding to  $M + H^+$  at  $m/z$  1729.5) and the triply charged ion 658.3 ( $M + H^+$  at  $m/z$  1971.6), shown in **Figure 2**, matched the peptide sequence TKQEYDEAGPSIVHR for peptide A and ITKQEYDEAGPSIVHRK for peptide B, respectively. These two peptides share a common sequence except for the N- and C-terminal amino acids (I and K), present in peptide B but absent from peptide A.

The MS/MS spectra of **Figure 3** matched the sequence DSGDGVTHNPVIYE for peptide C, whereas that of peptide



**Figure 3.** CID-MS/MS spectra of ions 751.9<sup>2+</sup> (peptide C) and 780.8<sup>2+</sup> (peptide D) of fraction 31 (peak 2 in **Figure 1**, bottom). Peptide sequences matching each of the product ion spectra are shown in capital letters.

**Table 1.** Actin Fragments Identified in Spanish Dry-Cured Ham at the End of Processing (Swiss-Prot Protein Entry Name P68137 ACTS\_PIG)

peptide	identified sequence	obsd mass (Da)	theoretical mass (Da)	N-terminal cleavage site	C-terminal cleavage site	homology (%)
A	<sup>360</sup> TKQEYDEAGPSIVHR <sup>374</sup>	1729.51	1729.85	<sup>359</sup> T <sup>360</sup>	R <sup>374</sup> -K <sup>375</sup>	100
B	<sup>359</sup> TKQEYDEAGPSIVHRK <sup>375</sup>	1971.63	1971.02	W <sup>358</sup> - <sup>359</sup>	K <sup>375</sup> -C <sup>376</sup>	100
C	<sup>156</sup> DSGDGVTHNVPIYE <sup>169</sup>	1501.51	1502.68	L <sup>155</sup> -D <sup>156</sup>	E <sup>169</sup> -G <sup>170</sup>	100
D	<sup>156</sup> DSGDGVTHNVPIYEG <sup>170</sup>	1559.46	1559.70	L <sup>155</sup> -D <sup>156</sup>	G <sup>170</sup> -Y <sup>171</sup>	100

D corresponded to the sequence DSGDGVTHNVPIYEG. Similarly to peptides A and B, the only difference between these sequences is the C-terminal glycine distinctly present in peptide D (**Figure 3**). BLAST sequence similarity searches revealed a 100% identity of peptides A, B, C, and D with two regions of porcine muscle actin (**Table 1** and **Figure 4**). Peptides A and B originated from the C-terminal region of the protein, whereas peptides C and D correspond to an internal region of the muscle actin molecule. The exact position of the identified peptides within the parent protein, their molecular masses (theoretical and experimental), and the nature of their N- and C-terminal cleavage sites are presented in **Table 1**.

## DISCUSSION

Results of the present work constitute clear evidence of the intense actin proteolysis occurring during dry-cured ham processing. Previous works have already reported a progressive

degradation of actin during meat aging (8), but especially during dry-curing when most myofibrillar proteins are extensively degraded (9). Other authors have reported a notable increase of the peptide content during dry-curing (10, 11), a phenomenon that is indicative of intense proteolysis and is closely related to the generation of the typical flavor in dry-cured meat products (1). However, to our knowledge, this is the first time that specific fragments from actin degradation have been isolated and fully identified in a dry-cured ham extract. Thus, peptides identified during postmortem muscle proteolysis by other groups had been mainly assigned to troponin T (12, 13) or some sarcoplasmic protein such as glyceraldehyde 3-phosphate dehydrogenase (14, 15), pyruvate kinase (16), or creatine kinase (15).

Proteolysis of muscle is a well-reported phenomenon occurring during both meat aging (2) and dry-curing, being especially more pronounced in this latter case due to a considerably longer duration of the process (1). However, despite being generally

P68137 ACTS_PIG	MCDEDETTAL VCDNGSGLVK AGFAGDDAPR AVFPSIVGRP RHQGVVMVGMG <sup>50</sup>
P68137 ACTS_PIG	QKDSYVGDEA QSKRGILTLK YPIEHGIITN WDDMEKIWHH TFYNELRVAP <sup>100</sup>
P68137 ACTS_PIG	EEHPTLLTEA PLNPKANREK MTQIMFETFN VPAMYVAIQ A VLSLYASGR <sup>150</sup>
Cath.D action (18)	-----DSGDG VTHNVPI--- -----
P68137 ACTS_PIG	TGIVLDSGDG VTHNVPIYEG YALPHAIMRL DLAGRDLTDY LMKILTERGY <sup>200</sup>
Peptide D	-----DSGDG VTHNVPIYEG -----
Peptide C	-----DSGDG VTHNVPIYE- -----
P68137 ACTS_PIG	SFVTTAEREI VRDIKEKLCY VALDFENEMA TAASSSSLEK SYELPDGQVI <sup>250</sup>
P68137 ACTS_PIG	TIGNERFRCP ETLFQPSFIG MESAGIHETT YNSIMKCDID IRKDLYANNV <sup>300</sup>
P68137 ACTS_PIG	MSGGTTMYPG IADRMQKEIT ALAPSTMKIK IIAPPERKYS VWIGGSILAS <sup>350</sup>
Cath.D action (18)	-----IT KQEYDEAGPS IVHRK--
P68137 ACTS_PIG	LSTFQQMWIT KQEYDEAGPS IVHRKCF <sup>377</sup>
Peptide B	-----IT KQEYDEAGPS IVHRK--
Peptide A	-----T KQEYDEAGPS IVHR---

**Figure 4.** Primary structure of porcine skeletal muscle actin (Swiss-Prot accession code P68137 ACTS\_PIG), indicating the position of peptides A, B, C, and D isolated and identified in the present work at the end of ham dry-curing. Related peptide sequences obtained by Hughes et al. (18) by hydrolyzing bovine actin with spleen cathepsin D are also displayed.

accepted that this degradation is of enzymatic nature, the role and relevance of the involved enzymes remains elusive and subject to controversy in the field of meat science. The groups of endogenous muscle enzymes that have been traditionally studied in relation to postmortem muscle proteolysis are calpains and cathepsins and, more recently, also the proteasome (2). It is thought that the action of calpains (cysteine peptidases with an optimal functioning neutral pH) would be mainly restricted to the first hours postmortem before the pH fall (1). On the other hand, lysosomal cathepsins exhibit an optimal working acidic pH and have been shown to remain active during both the aging of meat and dry-curing (17). Besides calpains, cathepsins, and the proteasome, muscle tissue contains a large battery of other enzymes which, although less studied, could also contribute to postmortem muscle protein degradation (2). In any case, there is a need for better knowledge about the action of the different endogenous enzymes on degradation of muscle proteins and their cleavage specificity so that even if some protein fragments have been identified in meat and/or cured meat products, in most cases the identification of the enzymes generating these fragments becomes a very difficult task, if not impossible. For the peptides identified in the present work, at least a partial answer about how these fragments have been originated could be obtained from the work carried out by Hughes et al. (18). This group incubated in vitro bovine F-actin with cathepsin D (EC 3.4.23.5) at pH 5.5, a value that is not strange in postmortem muscle, and reported extensive degradation of actin into fragments too small to be observed by SDS-PAGE. In that study, they identified 15 peptide fragments and 28 cleavage sites. Interestingly, cathepsin D generated the peptide ITKQEYDEAGPSIVHRK, which is identical to peptide B found in the present work (Table 1). Further, cathepsin D was able to generate the cleavage site L<sup>155</sup>-D<sup>156</sup> corresponding to the N-terminal side of peptides C and D (Table 1 and Figure 4). However, the C-terminal cleavage site observed at I<sup>167</sup>-Y<sup>168</sup> reported by Hughes et al. (18) departs from that determined here for peptides C and D at E<sup>169</sup>-G<sup>170</sup> and G<sup>170</sup>-Y<sup>171</sup>, respec-

tively (Figure 4). Further work is required to assess whether cathepsin D is also able to reproduce the C-terminal cleavage sites of peptides C and D. Anyway, the fact that in vitro proteolysis of actin by cathepsin D generates peptides that can be naturally found at the end of dry-cured ham processing highlights the potential importance of this lysosomal enzyme in the biochemistry of dry-cured ham.

The work of Hughes et al. (18), together with some other contributions (19, 20), leaves no doubt about the ability of cathepsin D to hydrolyze muscle actin. Some other related enzymes, such as cathepsins B and L, have also been reported to degrade actin (20–22). However, considering to date available data, cathepsin D is the only peptidase able to explain some of the fragments and cleavage sites found in the present work for actin degradation at the end of curing. Hughes et al. (23) also incubated bovine F-actin with cathepsin B at pH 5.5, but in that case none of the 20 characterized cleavage sites coincided with our dry-cured ham experimental results (Table 1). Taken together, these findings led us to reconsider the role of cathepsin D in postmortem proteolysis during dry-cured ham processing.

#### Role of Cathepsin D in Postmortem Muscle Proteolysis.

Previous reports support a relevant role for cathepsin D in postmortem meat tenderization (20, 24), even at refrigeration temperatures (25, 26). In the case of dry-fermented sausages, cathepsin D appears to play a central role in proteolysis as the main peptidase responsible for the initial protein breakdown and the generation of peptide substrates that would be further degraded by the action of bacterial enzymes (27, 28). In the case of dry-cured ham, there is evidence that cathepsins remain active during a large part of the curing period (17, 29). Despite its possible relevance, cathepsin D has been traditionally underestimated in this process because (a) the usually found pH value in dry-cured ham (around 6.0) is too high compared to the optimal pH value of cathepsin D (3.0–5.0), so this enzyme would express a low percentage of activity (30), and (b) in vitro experiments have indicated that NaCl, at the

concentrations normally employed during dry-cured ham processing, is a strong inhibitor of cathepsin D (31–33). However, these evidences must be regarded with reservation. Thus, even if the pH normally found in dry-cured ham is higher than the optimal pH range of cathepsin D, several authors have reported that the enzyme remains remarkably active in the pH range 5.5–6.0 (18, 19, 23, 25, 26). Concerning the inhibition of cathepsin D by high NaCl concentrations, it must be noticed that results were obtained by in vitro experiments in which cathepsin D and curing agents were both present in solution (31, 33). This experimental model is quite far from the real conditions of the dry-cured ham processing where salt slowly diffuses from the outside to the internal parts of hams. Thus, the conditions of this model only reflect the situation after 2 months of the curing process, where salt has completely diffused to the inner part of the ham. Relevant to this point, Deng and Lillard (34) have suggested that the NaCl concentrations present in the interior of hams, at least during the initial steps of the curing process, may be unlikely be able to drastically reduce cathepsin D activity. Similar to cathepsin D, alanyl aminopeptidase has been shown to remain active during dry-curing (35), though in vitro studies indicated a strong inhibition of its activity in the presence of NaCl (36). However, this enzyme is believed to play a major role in the intense generation of free amino acids occurring during dry-curing (6). Clearly, further research with improved experimental models would be necessary for a more accurate evaluation of the real effect of NaCl and other curing agents on the activity of muscle enzymes during dry-curing. Another important factor that has notably limited an adequate study of cathepsin D activity relates to the type of enzyme assay employed. Traditionally, cathepsin D activity has been determined by using denatured hemoglobin as the substrate and measuring the absorbance generated by trichloroacetic acid-soluble peptides (37). This experimental setup contrasts the more specific and sensitive colorimetric and fluorescent peptide derivative-based assays currently employed for measuring the activity of other peptidases, such as cathepsin B or H (38).

**Conclusion.** Results obtained in the present work show that small peptide fragments coming from actin are present at the end of dry-cured ham processing, indicating an extensive hydrolysis of this muscle protein. Some of the cleavage sites obtained for these fragments have been reproduced by incubation of intact actin with cathepsin D (18), clearly pointing to a relevant action of this enzyme during ham dry-curing. Thus, arguments contrary to the action of cathepsin D in dry-cured ham postmortem proteolysis should be reconsidered with new experimental models capable of better explaining the real action of endogenous muscle enzymes. As suggested for dry-fermented sausages (27, 28), cathepsin D could develop a relevant action at least during the initial steps of dry-curing of hams, generating peptides that would be further hydrolyzed by other enzyme groups such as exopeptidases. This would explain the occurrence of peptides differing only in their N- and/or C-termini (Table 1). Finally, it must be kept in mind that whereas the action of cathepsins B, H, and L, considered to play an important role during postmortem proteolysis, is strongly regulated by the presence of endogenous inhibitors (cystatins) in muscle (2), cathepsin D, one of the major components of lysosomes (37), is an aspartyl-type endopeptidase regulated neither by the action of cystatins nor by the presence of other specific endogenous inhibitors in muscle (39). Further research is eagerly awaited to check this hypothesis and to advance the biochemistry of postmortem muscle proteolysis.

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