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Naturally Generated Small Peptides Derived from Myofibrillar Proteins in Serrano Dry-Cured Ham

LETICIA MORA,[†,§] MIGUEL ANGEL SENTANDREU,[†,§] KAISA M. KOISTINEN,[†] PAUL D. FRASER,[†] FIDEL TOLDRÁ,[§] AND PETER M. BRAMLEY*[,†]

School of Biological Sciences, Royal Holloway, University of London, Egham TW20 0EX, United Kingdom, and Instituto de Agroquímica y Tecnología de Alimentos (CSIC), P.O. Box 73, 46100 Burjassot, Valencia, Spain

A proteomic approach has been carried out to investigate the extensive proteolysis occurring in the processing of Serrano ham. In this study, a total of 14 peptide fragments derived from myosin light chain I and titin have been identified for the first time. Nine of these peptides originated from myosin light chain I protein, with the loss of dipeptides at the N-terminal position observed in some of them. This suggests that dipeptidyl peptidases are involved in the generation of dipeptides, which contribute to the generation of the characteristic taste associated with Serrano ham. The other five peptides came from the PEVK region of the titin protein. This region is believed to confer elasticity to the sarcomere as well as the ability to bind calpains. The hypothetical action of μ-calpain and calpain 3 enzymes over this region would make these enzymes potentially responsible for protein breakdown during the early dry-curing stage.

KEYWORDS: Serrano ham; titin; myosin light chain I; proteomics; peptide sequencing

INTRODUCTION

Dry-cured ham is a high-quality, processed meat product that represents an important proportion (57%) of the total production of dry-cured products in Spain. Serrano ham is protected by the European Community as a Traditional Specialty Guaranteed, controlled by the Foundation Jamón Serrano that defines and regulates specific raw materials and processing conditions. The processing of dry-cured ham is very complex and involves numerous biochemical reactions, most of them related to the muscle structure. In fact, many studies have been carried out to understand the main biochemical changes that take place during the post-mortem period in relation to the initial breakdown of myofibrils by muscle endopeptidases. This proteolysis is directly related to the development of meat tenderness (*1*, *2*). The activity of muscle exopeptidases has been also studied due to the high increase in free amino acids and small peptides, which greatly influence the unique flavor characteristics of dry-cured ham at the end of processing (*3*, *4*).

Despite several studies describing the presence of amino acids and peptides at the end of the curing process (*5*−*7*), the identity of these peptides has remained elusive (*8*, *9*). The information derived from the knowledge of the protein fragments naturally generated during dry-curing would be very important for a better understanding of the texture changes and flavor development mechanisms occurring in this process.

Myosin is the major myofibrillar protein in skeletal muscle, making up more than one-third of the total protein in muscle. This protein is very large (∼500 kDa) and contains two identical heavy chains, which constitute the head and tail domains, and two sets of light chains (two per head), which bind the heavy chains in the region between the head and tail. Myosin plays a key role in muscle contraction, being responsible for producing the contractile force.

Titin, also known as connectin, is the third most abundant protein of vertebrate striated muscle, after myosin and actin. The titin molecule is formed by a single polypeptide chain with a molecular weight of up to ∼4 MDa, constituting the largest protein found so far in nature. The molecule is a flexible filament longer than 1 μm (*10*). Interestingly, several studies have now suggested that titin degradation is concurrent with the development of meat tenderness (*1*, *11*, *12*).

In the present work, we have carried out a proteomic analysis of peptides generated in dry-cured ham in order to understand better the mechanisms leading to the development of final texture and flavor characteristics of this product.

MATERIALS AND METHODS

Extraction and Deproteinization of Dry-Cured Ham. The three Serrano dry-cured hams used in this study were produced using raw material from intensively reared industrial genotype pigs (Landrace × Large White), which were 6 months old at the moment of slaughtering. These hams were processed in a local factory in Castellón (Spain) and were submitted to a ripening process of 9 months.

A 50 g sample of Serrano dry-cured ham (biceps femoris) was minced and then homogenized in 100 mL of 0.01 N HCl using a

* Corresponding author [telephone (+44) 01784 443555; fax (+44) 01784 414224; e-mail p.bramley@rhul.ac.uk].
† University of London.
§ Instituto de Agroquímica y Tecnología de Alimentos (CSIC).

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Table 1. Sequence of the Nine Myosin Light Chain I Fragments Identified by LC-MS/MS[a]

peptide	^{14}A	A	P	A	P	A	P	A	P	A	P	A	P	A	P	A	P	P	K	E	E	K^{35}
1	A	A	P	A	P	A	P	A	P	A	P	A	P	A	P	A	P	P	K	E	E	K
2			P	A	P	A	P	A	P	A	P	A	P	A	P	A	P	P	K	E	E	K
3				A	P	A	P	A	P	A	P	A	P	A	P	A	P	P	K	E	E	K
4					P	A	P	A	P	A	P	A	P	A	P	A	P	P	K	E	E	K
5							P	A	P	A	P	A	P	A	P	A	P	P	K	E	E	K
6								P	A	P	A	P	A	P	A	P	P	K	E	E	K
7									P	A	P	A	P	A	P	P	K	E	E	K
8										P	A	P	A	P	P	K	E	E	K
9											P	A	P	P	K	E	E	K

[a] Spectra were interpreted both manually and using the online form of the MASCOT program. Superscripts indicate the position of the fragments in the protein.

Table 2. Myosin Light Chain (MLCI) Fragments Identified in the Biceps Femoris Muscle of a Dry-Cured Ham by NanoLC-MS/MS

peptide	obsd[a]	charged state	calcd[b]	score[c]	position[d]	MALDI-TOF[e]	LC-MS/MS[e]
1	682.74	(3+)	2045.09	83	14−35	X	X
2	635.38	(3+)	1903.02	89	16−35	X	X
3	603.03	(3+)	1805.97	91	17−35		X
4	579.32	(3+)	1734.93	78	18−35	X	X
5	523.28	(3+)	1566.84	69	20−35	X	X
6	467.28	(3+)	1398.75	59	22−35		X
7	411.24	(3+)	1230.66	54	24−35		X
8	532.31	(2+)	1062.57	50	26−35		X
9	448.25	(2+)	894.48	27	28−35		X

[a] Molecular ion mass observed in the LC-MS/MS system calculated in daltons. [b] Calculated relative molecular mass (Da) of the matched peptide. [c] Score obtained in Mascot using the NCBI nonredundant protein database. [d] Position of the peptides inside the partial MLCI sequence identified for porcine species (NCBI accession no. ABK55642). [e] Peptides marked with an "X" were observed using the corresponding mass spectrometric technique.

stomacher apparatus (Seward Laboratory Systems Inc.) for 8 min. The mixture was centrifuged at 4 °C and 24500*g* for 20 min. The resulting supernatant was filtered through glass wool; the solution was deproteinized by adding 3 volumes of ethanol and allowed to stand for 20 min at 4 °C. Finally, the sample was centrifuged (24500*g*) for 10 min at 4 °C, and the supernatant was dried in a rotatory evaporator. The residue remaining from the extract was finally redissolved in 25 mL of 0.01 N HCl. Finally, the sample was filtered through a 0.45 μm nylon membrane filter (Millipore, Bedford, MA) and stored at −20 °C until use.

Size Exclusion Chromatography. To fractionate the deproteinized cured ham extract on the basis of molecular weight, 5 mL of the extract was subjected to size exclusion chromatography on a Sephadex G25 column (2.5 × 65 cm, Amersham Biosciences, Uppsala, Sweden), at 5 °C. The degassed mobile phase was 0.01 N HCl, filtered through a 0.22 μm membrane filter. The separation was performed at a flow rate of 15 mL/h, and fractions of 5 mL were collected using an automatic fraction collector (model 2110, Bio-Rad Laboratories, U.K.). These fractions were monitored at 214, 254, and 280 nm with an UV spectrophotometer (Agilent 8453, Agilent Technologies, Palo Alto, CA). The fractions corresponding to an elution volume between 125 and 190 mL were pooled, then dried centrifuging under vacuum, and stored at −20 °C.

Reversed-Phase Chromatography. The dried sample was redissolved in 5 mL of 0.1% trifluoroacetic acid (TFA, v/v) in water/acetonitrile (95:5, v/v) and filtered through a 0.22 μm nylon membrane filter. After filtering, 500 μL of the redissolved sample was injected into a Bio-Cad workstation liquid chromatograph (Perseptive Biosystems, Framingham, MA). The chromatographic separation was developed using a Nucleosil C_{18} column (250 × 4.6 mm, 5 μm particle size) from Macherey-Nagel (Düren, Germany) at room temperature. Mobile phases comprised solvent A, containing 0.1% TFA in water (v/v), and solvent B, containing 0.08% TFA in water/acetonitrile (5:95). The degassed solvents were filtered through a 0.22 μm filter. The separation conditions consisted of solvent A isocratically for 10 min, followed by a linear gradient from 0 to 100% of solvent B over 90 min at a flow rate of 1 mL/min. The separation was monitored using a diode array detector at a wavelength of 214 nm, and 1 mL fractions were collected. Two of these fractions, containing myosin light chain I fragments and corresponding to the maximum level of absorbance, were pooled together, dried under vacuum, and stored at −20 °C. Another four fractions were selected and chromatographed again to improve the isolation of the titin fragments. To carry out this second chromatographic analysis, the same column and conditions described above were used but with 0.08% TFA in water/acetonitrile (40:60) as solvent B. Separation was monitored at 214 nm, and fractions corresponding to different maximal absorbances were collected manually, subsequently dried under vacuum, and stored at −20 °C.

Molecular Mass Determination and Peptide Identification by Mass Spectrometry. Dried fractions were redissolved in 40 μL of 0.1% TFA in water. A sample (1 μL) of each fraction was spotted onto an AnchorChip plate (Bruker Daltonik GmbH, Bremen, Germany) and mixed with 1 μL of 2,5-dihydroxybenzoic acid (DHB; Bruker Daltonik GmbH) in 0.1% TFA/acetonitrile (2:1, v/v). After air-drying, the plate was introduced in a Bruker Reflex III matrix-assisted laser desorption/ionization time-of-flight mass spectrometer (MALDI-TOF MS, Bruker Daltonik GmbH). The instrument was used in positive-ion reflector mode for mass analysis with an ion acceleration voltage of 20 kV. Spectra were obtained from the average of 300 laser shots. Mass calibration was performed using a peptide calibration standard in a mass range between 1000 and 3000 Da (code 206195, Bruker Daltonik GmbH). FlexAnalysis 2.4 software (Bruker Daltonik GmbH) was employed for data analysis.

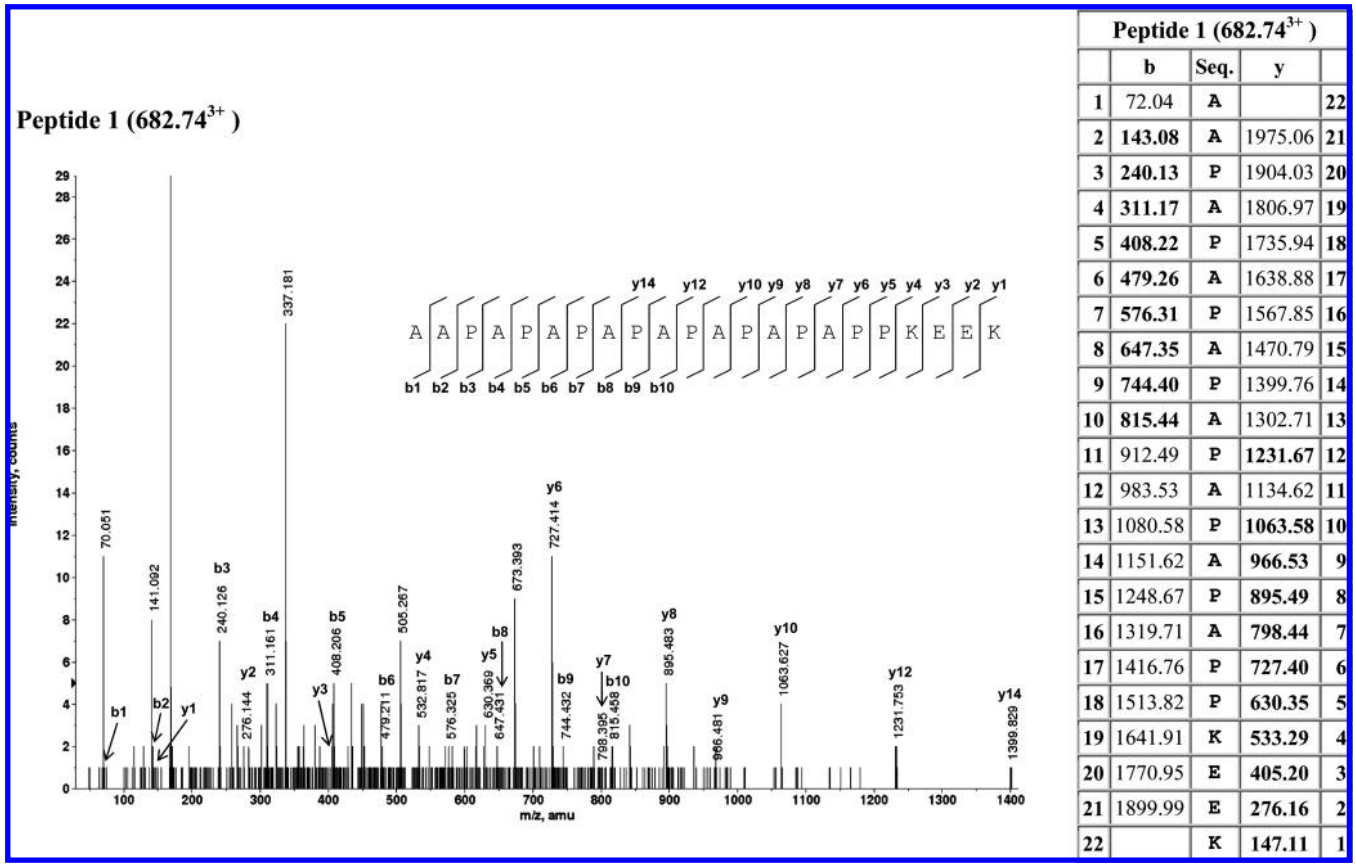
To identify the peptides by tandem mass spectrometry, they were separated using an Ultimate/Famos nano LC system (LC Packings, Amsterdam, The Netherlands). The sample was loaded onto a 0.2 × 5 mm PS-DVB monolithic trap column (Dionex, Sunnyvale, CA) at a flow rate of 10 μL/min of 0.1% TFA for 30 min. After preconcentration, the trap column was automatically switched in-line with a Dionex poly(styrene−divinylbenzene) (PS-DVB) monolithic column (0.1 × 50 mm) to elute peptides with a linear gradient starting from 95% solvent A (0.1% formic acid in water, v/v) to 40% solvent B (0.1% formic acid in CAN, v/v) for 120 min, at a flow rate of 100 nL/min. The column outlet was directly coupled to a nanoeletrospray ion source (Protana, Odense, Denmark) using a 10 μm PicoTip (New Objective, Woburn, MA). The positive TOF mass spectra were recorded on a QSTAR Pulsar *i* hybrid quadrupole TOF instrument (Applied Biosystems, Foster City, CA) using information-dependent acquisition (IDA). TOF MS survey scans were recorded over a mass range of *m*/*z* 400−1600, followed by MS/MS scans of the two most intense peaks. Typical ion spray voltage was in the range of 2.0−2.4 kV, and nitrogen was used as collision gas. Other source parameters and spray positions were optimized with a tryptic digest of bovine serum albumin.

Automated spectral processing, peak list generation, and database searches were performed using Mascot search v1.6b21 script for Analyst QS 1.1 (Applied Biosystems) in combination with the Mascot interface (hppt://www.matrixscience.com). Identification of the proteins from which the peptides were derived was performed by homology blasting using the NCBI nonredundant protein database. Matches of MS/MS spectra against sequences of the database were verified manually.

To validate the results, two replicates of the technical analysis were done to confirm the myosin light chain I and titin sequences.

RESULTS

Peptide Isolation and Purification by Reversed-Phase HPLC. Fractions corresponding to an elution volume between 125 and 190 mL obtained after Sephadex G-25 size exclusion chromatography and containing peptides between 1000 and 3000 Da were pooled together and subjected to preparative reversed-phase chromatography. Peptides eluting at a concentration of acetonitrile (ACN) from 28 to 30% were pooled together, dried, and subjected to mass spectrometry for the identification of myosin light chain I fragments. Additionally, titin peptides were obtained in four fractions at an ACN concentration of 31−35%.



Peptide 1 (682.74^{3+})				
	b	Seq.	y	
1	72.04	A		22
2	**143.08**	A	1975.06	21
3	**240.13**	P	1904.03	20
4	**311.17**	A	1806.97	19
5	**408.22**	P	1735.94	18
6	**479.26**	A	1638.88	17
7	**576.31**	P	1567.85	16
8	**647.35**	A	1470.79	15
9	**744.40**	P	1399.76	14
10	**815.44**	A	1302.71	13
11	912.49	P	**1231.67**	12
12	983.53	A	1134.62	11
13	1080.58	P	**1063.58**	10
14	1151.62	A	**966.53**	9
15	1248.67	P	**895.49**	8
16	1319.71	A	**798.44**	7
17	1416.76	P	**727.40**	6
18	1513.82	P	**630.35**	5
19	1641.91	K	**533.29**	4
20	1770.95	E	**405.20**	3
21	1899.99	E	**276.16**	2
22		K	**147.11**	1

Figure 1. MS/MS spectrum of ion 682.74^{3+} (peptide 1). Peptide sequence matching the product ion spectrum is shown in capital letters, together with observed *b* and *y* ions. The spectrum is presented with its corresponding table showing the ions matched by MASCOT in bold letters.

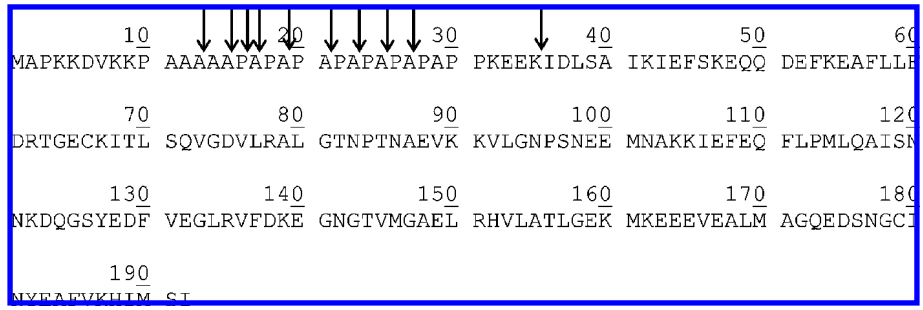


Figure 2. Sequence corresponding to porcine myosin light chain I (NCBI nonredundant database accession no. ABK55642). The same sequence also corresponds to entry A1XQT6_PIG in the UniProtKB/TrEMBL protein database. Cleavage sites of the peptides identified in the present work are indicated with black arrows.

A second reversed-phase chromatographic step was needed for further fractionation to improve the isolation of titin fragments. Thus, after this step, a main peak eluting at 38% ACN was collected for further peptide identification.

De Novo Peptide Sequencing Using Mass Spectrometry. *Myosin Light Chain I Fragments.* Fractions selected after the first reversed-phase chromatography were subjected to MALDI-TOF MS to determine peptide molecular masses. Four singly charged ions ($M + H^+$) were obtained at *m/z* values of 2046.07, 1903.99, 1735.88, and 1567.74. The same fraction was subjected to quadrupole time-of-flight tandem mass spectrometry along with a nanoelectrospray ionization source liquid chromatography (nanoLC-nESI-QTOF-MS) in order to elucidate the sequence of the peptides in this fraction. A total of nine peptide sequences were identified (see **Tables 1** and **2**). The molecular masses of peptides 1, 2, 4, and 5 were in accordance with the signals obtained from MALDI-TOF MS. **Figure 1** shows the MS/MS spectrum corresponding to peptide 1 (other spectra are not shown).

All of the identified myosin light chain I peptides share the common sequence PAPPKEEK (peptide 9), which corresponds to the C-terminus of the peptides. However, as can be observed in **Table 1**, the identified peptides show remarkable differences at their N-termini. Peptide 1 contains two additional amino acids at the N-terminus compared with peptide 2, whereas peptide 2 has an additional proline relative to peptide 3. Finally, a consecutive loss of the dipeptide Pro-Ala is observed from peptide 4 to peptide 8. BLAST sequence similarity searches revealed 100% homology of the nine peptides to porcine myosin light chain I (accession no. ABK55642 in NCBI nonredundant database), as shown in **Figure 2**.

Titin Fragments. The main peak obtained after the second chromatographic step, analyzed by MALDI-TOF MS, showed the molecular ions in **Figure 3**. Among the peaks showing the

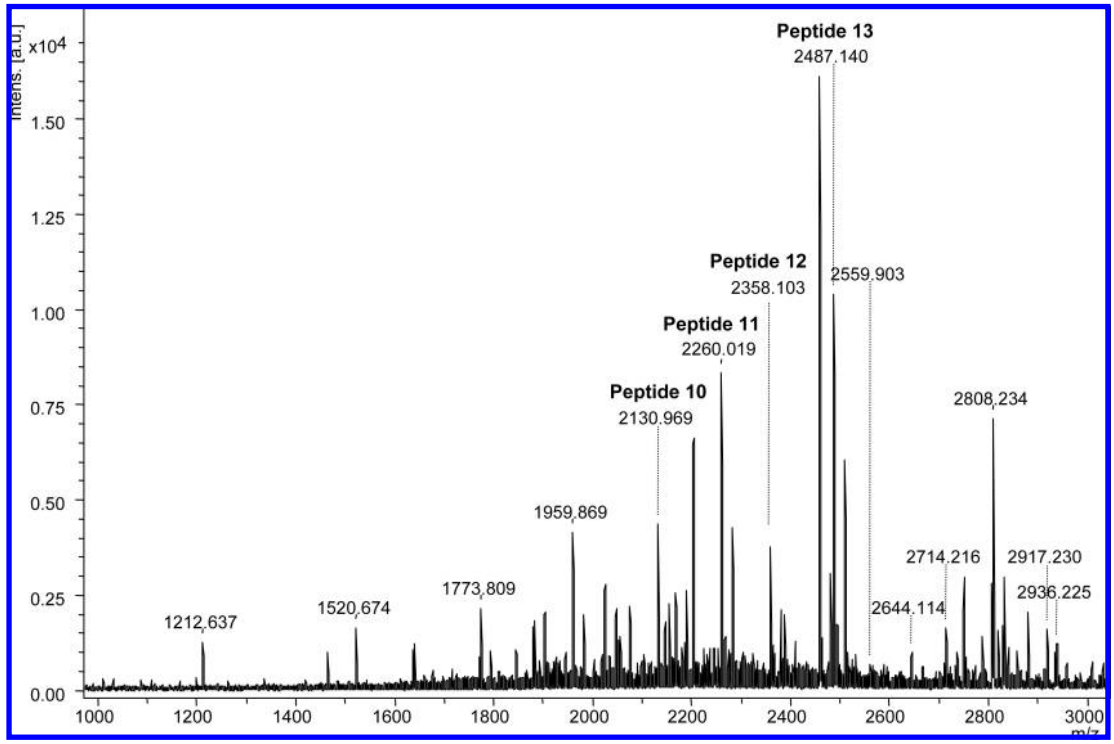


Figure 3. MALDI-TOF mass spectrum of the titin fraction collected manually after the second reversed-phase chromatography.

Table 3. Sequences of the Five Titin Fragments Identified by LC-MS/MS[a]

peptide	^{95}K	V	P	E	I	K	P	A	I	P	L	P	G	P	E	P	K	P	K	P	E	P	E^{117}
10			P	E	I	K	P	A	I	P	L	P	G	P	E	P	K	P	K	P	E	P	
11			P	E	I	K	P	A	I	P	L	P	G	P	E	P	K	P	K	P	E	P	E
12	K	V	P	E	I	K	P	A	I	P	L	P	G	P	E	P	K	P	K	P	E	P	
13	K	V	P	E	I	K	P	A	I	P	L	P	G	P	E	P	K	P	K	P	E	P	E
14												P	G	P	E	P	K	P	K	P	E	P	E

[a] Spectra were interpreted both manually and using the online form of the MASCOT program. Superscripts indicate the position of the fragments in the protein.

Table 4. Titin Fragments Identified in the Biceps Femoris Muscle of a Dry-Cured Ham by NanoLC-MS/MS

peptide	obsd[a]	charged state	calcd[b]	score[c]	position[d]	MALDI-TOF[e]	LC-MS/MS[e]
10	533.57	(4+)	2130.21	62	97−116	X	X
11	565.83	(4+)	2259.25	47	97−117	X	X
12	590.40	(4+)	2357.37	70	95−116	X	X
13	622.62	(4+)	2486.41	76	95−117	X	X
14	651.35	(2+)	1300.67	43	106−117		X

[a] Molecular ion mass observed in the LC-MS/MS system calculated in daltons. [b] Calculated relative molecular mass (Da) of the matched peptide. [c] Score obtained in Mascot using the NCBI nonredundant protein database. [d] Position of the peptides inside the partial titin sequence identified for porcine species (NCBI accession no. AAD00528). [e] Peptides marked with an "X" were observed using the corresponding mass spectrometric technique.

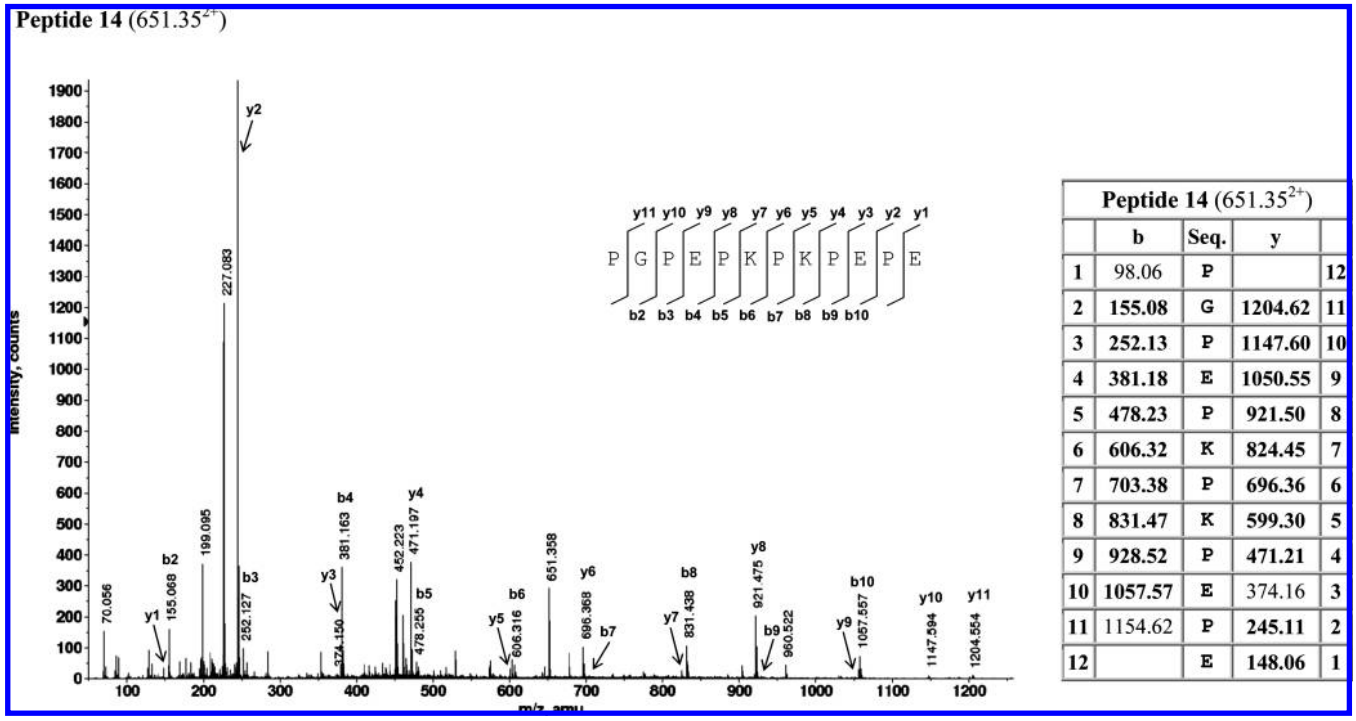
highest intensities, singly charged ions ($M + H^+$) were obtained at *m*/*z* values of 2130.97, 2260.02, 2358.10, and 2487.14. The same fraction was also subjected to nanoLC-MS/MS to elucidate the sequences of the peptides in it. A total of five peptide sequences were identified (see **Tables 3** and **4**), their molecular masses being in accordance with the most intense signals obtained in MALDI-TOF MS (**Figure 3**). The MS/MS spectra of the tetra-charged ions 533.57, 565.83, 590.40, and 622.62 matched the peptide sequence PEIKPAIPLPGPEPKPKPEP for peptide 10, PEIKPAIPLPGPEPKPKPEPE for peptide 11, KVPEIKPAIPLPGPEPKPKPEP for peptide 12, and KVPEIKPAIPLPGPEPKPKPEPE for peptide 13. A fifth peptide not detected previously by MALDI-TOF MS was also identified. The MS/MS spectrum for the doubly charged precursor matched the sequence PGPEPKPKPEPE (peptide 14), which corresponds to the C-terminal side of peptides 11 and 13. **Figure 4** shows the MS/MS spectrum of peptide 14.

As can be observed (**Table 3**), the identified titin peptides share a common sequence but show differences at both N- and C-termini. Thus, peptides 11 and 13 have an additional glutamic acid at the C-terminus compared to peptides 10 and 12. On the other hand, peptides 12 and 13 contain two additional amino acids (Lys-Val) at the N-terminal position. BLAST sequence similarity searches revealed 100% homology of the five peptides to a partial sequence of porcine muscle titin (entry AAD00528 in NCBI nonredundant protein database), shown in **Figure 5**.

DISCUSSION

Extensive proteolysis has been reported during the processing of dry-cured ham (*13*, *14*). This implies the degradation of both sarcoplasmic and myofibrillar proteins by muscle endopeptidases (mainly by cathepsins and calpains) and the further degradation of the generated polypeptides by the action of certain groups of exopeptidases such as dipeptidyl peptidases (*15*).

Possible Role of Endopeptidases. Cathepsins are lysosomal enzymes that play an important role in both the development of meat tenderness and during dry-cured ham processing (*14*, *16*). They have been implicated in the breakdown of most myofibrillar proteins and connective tissue, playing an important role in the development of meat texture (*17*). Several studies have related the action of cathepsins with the progressive disappearance of myosin. For example, incubation of rabbit skeletal muscle myosin with cathepsin B proved the degradation of myosin heavy chain and one of the myosin light chains, L2, by this enzyme (*18*). Etherington also established that both cathepsins B and D were especially active against myosin. This author tested purified liver cathepsin L against intact myofibrils and found the enzyme degraded myosin, although to a lesser extent than other proteins such as titin (*19*). In contrast, other



Peptide 14 (651.35^{2+})				
	b	Seq.	y	
1	98.06	P		12
2	155.08	G	1204.62	11
3	252.13	P	1147.60	10
4	381.18	E	1050.55	9
5	478.23	P	921.50	8
6	606.32	K	824.45	7
7	703.38	P	696.36	6
8	831.47	K	599.30	5
9	928.52	P	471.21	4
10	1057.57	E	374.16	3
11	1154.62	P	245.11	2
12		E	148.06	1

Figure 4. MS/MS spectrum of ion 651.35^{2+} (peptide 14) of the main peak obtained after the second reversed-phase chromatography. The peptide sequence matching the product ion spectrum is shown in capital letters, together with observed *b* and *y* ions. The spectrum is presented with its corresponding table showing the ions matched by MASCOT in bold letters.

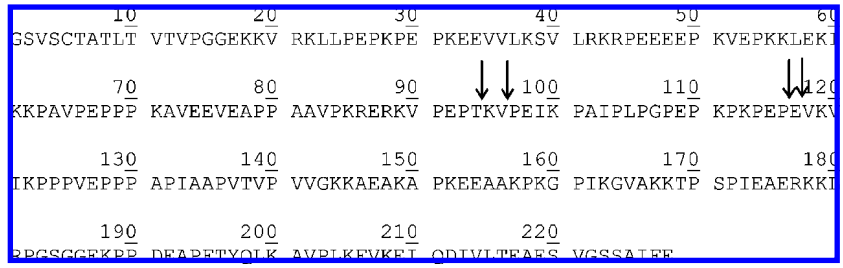


Figure 5. Partial sequence of porcine titin (NCBI protein accession no. AAD00528). The same sequence also corresponds to entry O97771 in the UniProtKB/TrEMBL protein database. Cleavage sites of the peptides identified in the present work are indicated with black arrows.

authors reported that myosin was not degraded by rabbit skeletal muscle cathepsin B, whereas cathepsin L, isolated from rabbit skeletal muscle, was able to hydrolyze myosin at pH 4.1 (*20*). In this way, a progressive disappearance of myosin light chains 1 and 2 during the dry-curing process was suggested (*21*). According to these authors, the disappearance of myosin light chains 1 and 2 could be due to cathepsins L and D, despite cathepsin D activity being notably reduced after 4−6 months of processing, due to its inhibition in the presence of high levels of NaCl.

The action of cathepsin D in the degradation of myofibrillar proteins has been widely studied during meat aging (*21*). Cathepsin D has been found to be especially active against myosin heavy chains, titin, proteins M and C, actin, tropomyosin, troponins I and II, and myosin light chains as well as to alter the Z disk structure (*22*). The degradation of myosin light chains by cathepsin D has also been studied by incubating purified cathepsin D with myofibrils at pH 5.5 and 37 °C (*23*). According to these authors, myosin light chains were degraded at a much slower rate than myosin heavy chains. The peptide sequences corresponding to the hydrolysis products were not identified.

Calpains constitute another relevant group of endopeptidases, even though many authors have established that they do not degrade myosin and actin (*24*). The proteolytic activity of calpains has been reported by many authors (*11*, *25*). In fact, binding assays using two titin regions showed that, in the presence of inhibitors, a titin fragment external to the N1 line that bears the PEVK/N_2 region tightly bound μ-calpain enzyme, whereas, in their absence, both fragments were cleaved by the enzyme (*26*). The titin PEVK region has been suggested to be directly involved in the mechanism of elasticity, contributing to passive force development of relaxed skeletal muscle during stretch (*27*). In relation to this, it is worth noting that the five titin fragments identified in our study belong to the last part of the PEVK region, situated in the extreme C-terminal region of titin (*28*). This finding would suggest that both μ-calpain and calpain 3 may be responsible for the protein breakdown at this level, thus contributing to the intense protein breakdown that occurs during the early steps of dry-cured processing (*29*).

Possible Role of Exopeptidases. Dipeptidyl peptidases (DPP I, II, III, and IV) are enzymes that release different dipeptides from the N-terminus of peptides. Considering the high stability of dipeptidyl peptidases during the processing of dry-cured ham (*30*) and the consecutive loss of dipeptides observed in the N-terminal side of the peptides identified in this work (see **Tables 1** and **2**), these enzymes could be the responsible for the loss of N-terminal dipeptides from myosin light chain I and

titin peptides. **Table 1** shows the loss of Ala-Ala in peptide 1 to obtain peptide 2, as well as the consecutive loss of Pro-Ala, giving rise to peptides 5−9. These dipeptides could contribute, together with amino acids and other nonvolatile compounds, to flavor development in dry-cured ham (*7*).

DPP I preferentially hydrolyzes N-terminal peptides with a penultimate basic residue, even though the synthetic derivative Ala-Ala-pNa was also hydrolyzed by porcine DPP. On the other hand, those peptides containing a basic amino acid in the N-terminal position are not hydrolyzed by this enzyme, as well as those containing a proline residue on either side of the peptide bond (*31*). Thus, it does not seem likely that DPP I could be responsible for the sequential loss of the dipeptide Pro-Ala in peptides 4−8, due to the presence of proline in the C-terminus of the scissile bond and at the P1 position of these peptides. On the other hand, the action of DPP I with peptides 1, 12, and 13 also seems unlikely, due to the presence of Pro in the scissile bond and the presence of Lys in an N-terminal position, respectively.

DPP II from porcine skeletal muscle preferentially hydrolyzes the synthetic substrates of type X-Pro-, followed by those of type X-Ala- in both fluorescent [dipeptidyl 7-amido-4-methylcoumarin (AMC)] and colorimetric (dipeptidyl-pNa) derivatives (*32*). Some authors have established that basic, neutral, and acidic amino acids are accepted in the N-terminal position of peptides, decreasing their relative activities in this order (*33*). Thus, the presence of Ala, Pro, and Lys in this position could make peptides 1, 4−8, 12, and 13 potential substrates for this enzyme, although these authors also established that rat brain DPP II activity decreases drastically with increasing chain length of the peptides.

Arg-Arg-AMC is the most commonly used substrate to determine DPP III activity, although Ala-Ala-pNa is also hydrolyzed by the enzyme (*34*). No activity was detected against peptide sequences containing more than 10 amino acids or a proline residue on either side of the scissile bond using DPP III purified from guinea-pig brain (*35*). Therefore, it seems unlikely that DPP III is involved in the generation of any of the fragments identified in this study because of their length (more than 10 amino acids in peptides from 1 to 8 and from 10 to 14) and the presence of a Pro residue in the scissile bond.

Substrates for DPP IV are those peptides containing a proline residue in the N-penultimate position and, to a lower degree, those containing alanine in this position (*36*). These authors also established that peptides were hydrolyzed by DPP IV even if they contained a proline residue also in the third position from the N-terminus. Other authors disagree, observing an inhibition of DPP IV with a number of nonsubstrate oligopeptides containing an N-terminal X-X-Pro- structure (*37*). The degree of inhibition by these peptides depends on their length, with the most potent inhibition shown by the longest peptides tested (peptides between 13 and 86 amino acids). A wide number of natural peptides contain this X-X-Pro- structure and, thus, according to these authors, it is conceivable that the DPP IV activity is regulated by such peptides in vivo.

In summary, any dipeptidyl peptidase with the exceptions of DPP I and DPP III could be responsible for the loss of the dipeptide Ala-Ala in peptide A. On the other hand, and due to the lack of literature related to the DPP specificity to the substrates Pro-Ala- and Lys-Val-, it is difficult to elucidate the enzymes involved in the loss of these dipeptides, although the action of DPP II and/or DPP IV would be feasible.

In conclusion, the peptides identified in this work provide clear evidence of the extensive hydrolysis of proteins during dry-cured ham processing. The myosin light chain I fragments identified could constitute evidence of the contribution of dipeptidyl peptidases in the generation of dipeptides during dry-cured ham processing. These dipeptides could contribute, together with other nonvolatile compounds, to flavor development in dry-cured ham. Some peptide fragments belong to the PEKV region of titin, a protein that is directly implicated in the elasticity of the sarcomere. The hypothetical action of μ-calpain and calpain 3 over this region would make these enzymes potentially responsible for the protein breakdown that occurs during the early dry-curing stage. Better knowledge about the enzymatic action that takes place during dry-cured ham processing is needed to provide a thorough understanding of the main enzymes involved and how their action influences the texture changes and flavor generation during processing.

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