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Intense Degradation of Myosin Light Chain Isoforms in Spanish Dry-Cured Ham

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S *Supporting Information*

ABSTRACT: One of the main biochemical changes that take place during the processing of dry-cured ham is the degradation of the muscle protein fraction, mainly due to the action of muscle enzymes. In the present study, the isolation and tentative identification of 137 fragments from myosin light chain 1 (MLC 1), together with 88 fragments originated from myosin light chain 2 (MLC 2), have been achieved for the first time in Spanish dry-cured ham, proving the intense proteolysis experienced by myofibrillar proteins after dry-cured processing. This study was carried out by use of proteomic technology for peptide identification, and the possible enzymes contributing to the degradation of these proteins were also further discussed.

KEYWORDS: dry-cured ham, myosin light chain, proteolysis, proteomics, mass spectrometry, muscle peptidases

INTRODUCTION

Myosin is the major myofibrillar protein in skeletal muscle, making up greater than one-third of the total protein in this tissue. This protein is very large (~500,000 Da) and contains two identical heavy chains that constitute the head and tail domains, together with 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.[1]

Many authors have reported the intense proteolysis suffered by myosin during dry-cured ham processing. In this way, some authors observed through the use of SDS-PAGE gels a progressive disappearance of myosin heavy chain, myosin light chains 1 and 2 (MLC 1 and MLC 2), and troponins C and I, as well as an appearance of numerous smaller fragments in the 50−100 and 20−45 kDa regions.[2] More recently, an intense proteolysis of actin, tropomyosin, and myosin light chains extracted in the myofibrillar fraction of dry-cured hams with different ripening times was also reported.[3] These authors concluded that after 12 months of ripening, most myofibrillar proteins were completely hydrolyzed.

Specific polypeptide sequences of actin,[4] titin and MLC 1,[5] creatine kinase,[6] pyruvate kinase,[7] and troponin proteins,[8] have been already identified in dry-cured ham using proteomic tools. Information derived from the identification of the protein fragments naturally generated during dry-curing would be very important to better understand proteolysis and flavor development mechanisms occurring during this process.

The main objective of the present work was to provide information about the intense degradation of skeletal muscle myosin during dry-curing by means of the massive identification of peptides generated from both MLC 1 and MLC 2. This study was carried out using a quadrupole/time-of flight mass spectrometer for the identification of peptides, and the possible enzymes responsible for the hydrolysis of these proteins have also been discussed.

MATERIALS AND METHODS

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

Biceps femoris muscles of Spanish dry-cured ham with no extramuscular fat were processed following same method described by Mora et al.[5] Peptides obtained after extraction were fractionated according to their molecular mass using size exclusion chromatography. For this purpose, a Sephadex G25 column (2.5 × 65 cm, Amersham Biosciences, Uppsala, Sweden) was used as described in ref 5. Those fractions corresponding to a molecular weight between 1000 and 3000 Da were pooled together, dried under vacuum, and redissolved in 5 mL of 0.1% trifluoroacetic acid in water/acetonitrile (95:5, v/v).

Isolation by Reversed-Phase Chromatography. A 100 μL aliquot of the pooled mixture of peptides was injected into an HPLC Agilent 1100 series system (Agilent Technologies, Palo Alto, CA). Separation of peptides was carried out using a 250 × 4.6 mm, 5 μm, Symmetry C18 column from Waters (Milford, MA) at 25 °C. Mobile phases consisted of solvent A, containing 0.1% trifluoroacetic acid (TFA) in water, and solvent B, containing 0.05% TFA in water/acetonitrile (5:95). Both mobile phases A and B were filtered through a 0.45 μm filter and degassed prior to any analytical run. The chromatographic separation consisted of a 5 min isocratic gradient with solvent A, followed by a linear gradient from 0 to 40% of solvent B in 70 min at a flow rate of 1 mL/min. The separation was monitored at a wavelength of 214 nm, and 1 mL fractions were collected and lyophilized for further identification using tandem mass spectrometry.

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Myosin light chain 1 (protein accession number A1XQT6_PIG in UniProtKB/TrEMBL database)

10 20 30 40 50 60
MAPKKDVKKP AAAAAPAPAP APAPAPAPAP PKEEKIDLSA IKIEFSKEQQ DEFKEAFLLF

70 80 90 100 110 120
DRTGECKITL **SQVGDVLRAL GTNPTNAEVK KVLGNPSNEE MNAKKIEFEQ** FLPMLQAISN

130 140 150 160 170 180
NKDQGSYEDF VEGL**RVFDKE GNGTVMGAEL RH**VL**ATLGEK MKEEEVEALM AGQED**SNGCI

190
NYEAFVKHIM SI

Myosin light chain 2 (protein accession number Q5XLD2_PIG in UniProtKB/TrEMBL database)

10 20 30 40 50 60
MAPKKAKRRA AAEGSSNVFS MFDQTQIQEF KEAF**TVIDQN RDGIIDKEDL RDT**FAAM**GRL**

70 80 90 100 110 120
NVKNEELDAM MKEASGPINF TVFLTM**FGEK LKGADPEDVI TGAFKVLDPE GKGTIKKHFL**

130 140 150 160
EELLTTQCDR FSQEEIKNMW AAFPPDVGGN VDYKNICYVI THGDAKDQE

Figure 1. Sequences of porcine myosin light chain 1 and 2 proteins (NCBInr database accession no. ABK55642 and NP_001006592). The same sequences also correspond to the entries A1XQT6_PIG and Q5XLD2_PIG, respectively, in the UniProtKB/TrEMBL protein database. Letters in bold indicate the position of the identified peptides within the sequence.

Peptide Identification by Tandem Mass Spectrometry. Peptides contained in the fractions obtained after reversed-phase chromatography were separated by liquid chromatography coupled to MS/MS analysis to sequence the peptides using an Ultimate Plus/Famos nano LC system (LC Packings, Amsterdam, The Netherlands) and a QSTAR XL hybrid quadrupole-TOF instrument (AB Sciex Biosystems, Foster City, CA) equipped with a nanoelectrospray ion source (Protana, Odense, Denmark).

Fractions previously lyophilized using a VirTis Genesis 35EL lyophilizer (SP Scientific, Gardiner, NY) were diluted in 60 μL of loading buffer (0.1% of formic acid and 2% of acetonitrile in water) and preconcentrated on a 0.3 × 5 mm, 3 μm, C18 trap column from LC Packings PepMap (Dionex Co., Amsterdam, The Netherlands) at a flow rate of 40 μL/min and using 0.1% of TFA as mobile phase. After 3 min of preconcentration, the trap column was automatically switched in-line with a 0.075 × 150 mm, 3 μm, Dionex C18 PepMap column from LC Packings. Mobile phases consisted of solvent A, containing 0.1% formic acid in water, and solvent B, containing 0.1% formic acid in 95% acetonitrile. Chromatographic conditions were a linear gradient from 95 to 50% solvent A in 30 min at a flow rate of 0.2 μL/min. The column outlet was directly coupled to a nanoeletrospray ion source (Protana) using a 15 μm PicoTip EMITTER SilicaTip needle (New Objective, Woburn, MA). The positive TOF mass spectra were recorded on the QSTAR instrument using information-dependent acquisition (IDA). TOF MS survey scan was recorded for mass range m/z 350−1800 followed by MS/MS scans of the three most intense peaks. Typical ion spray voltage was in the range of 2.5−3.0 kV, and nitrogen was used as collision gas. Other source parameters and spray position were optimized with a tryptic digest of protein mixture digest (LC Packings; P/N 161088).

Database Search. Automated spectral processing, peak list generation, and database search was performed using Analyst QS 1.1 (Applied Biosystems, Carlsbad, CA) in combination with the Mascot interface 2.2 (Matrix Science, Inc., Boston, MA) (hppt://www.matrixscience.com), and the ProteinPilot 3.0 software (Applied Biosystems) with Paragon algorithm. Mascot.dll 1.6b25 and ABSciex.DataAccess. Wiff File DataReader.dll were used for importing data to Mascot and Protein Pilot, respectively.

Mascot searches were done using the enzyme option "none", and a tolerance on the mass measurement of 100 ppm in MS mode and 0.6 Da for MS/MS ions. Using ProteinPilot software is not necessary to fix mass tolerance or possible modifications because Paragon algorithm used preset values due to the fact that features such as modifications, substitutions, and cleavage events are modeled with probabilities rather than by discrete user-controlled settings.

Identification of protein origin of peptides was done using NCBI nonredundant protein database. Matches of MS/MS spectra against sequences of the database were verified manually.

RESULTS AND DISCUSSION

Dry-cured ham peptides were extracted and isolated, and a total of 225 peptides originated from myosin light chain have been tentatively identified. One hundred and thirty-seven peptides corresponded to MLC 1 fragments, whereas the remaining 88 showed identity with MLC 2. As can be observed in Figure 1, peptides identified as MLC 1 fragments covered around 68% of the total sequence of the MLC 1 protein, whereas the fragments identified from MLC 2 covered 47% of the respective sequence.

Fragments generated from MLC 1 and MLC 2 proteins came from different sections of the corresponding sequences. Tables 1−3 show the sequences of the MLC 1 fragments identified by LC-MS/MS from dry-cured ham. Peptides have been rearranged and aligned according to their positions in the sequence of the proteins. Figure 2 shows the MS/MS spectrum corresponding to peptide 69 from MLC 1 (Table 1). Table A of the Supporting Information shows the observed and calculated masses of the MLC 1 identified peptides, as well as the score obtained, the mass modifications suffered by the peptides, and

Table 1. Myosin Light Chain 1 Peptides Tentatively Identified in Spanish Dry-Cured Ham Located between Positions 1 and 60 in the Complete Sequence (Protein Accession No. A1XQT6_PIG in UniProtKB/TrEMBL)

Peptide No.	10 20 30 40 50 APKKDVKKPAAAAAPAPAPAPAPAPAPAPPKEEKIDLSAIKIEFSKEQQDE	Position
1	APAPAPAPAPAPAPAPPKEEKIDLSAIKIEFSKEQQDE	15-53
2	APAPAPAPAPAPAPAPPKEEKIDLSAIKIE	15-44
3	APAPAPAPAPAPPKEEKIDLSAIKIE	19-44
4	AAAPAPAPAPAPAPAPAPPKEEKIDLSAIK	13-42
5	AAPAPAPAPAPAPAPAPPKEEKIDLSAIK	14-42
6	APAPAPAPAPAPAPAPPKEEKIDLSAIK	15-42
7	PAPAPAPAPAPAPAPPKEEKIDLSAIK	16-42
8	APAPAPAPAPAPAPPKEEKIDLSAIK	17-42
9	PAPAPAPAPAPAPPKEEKIDLSAIK	18-42
10	APAPAPAPAPAPPKEEKIDLSAIK	19-42
11	PAPAPAPAPAPPKEEKIDLSAIK	20-42
12	APAPAPAPAPPKEEKIDLSAIK	21-42
13	APAPAPAPPKEEKIDLSAIK	23-42
14	AAAPAPAPAPAPAPAPAPPKEEKIDLSAI	13-41
15	AAPAPAPAPAPAPAPAPPKEEKIDLSAI	14-41
16	APAPAPAPAPAPAPAPPKEEKIDLSAI	15-41
17	PAPAPAPAPAPAPAPPKEEKIDLSAI	16-41
18	APAPAPAPAPAPAPPKEEKIDLSAI	17-41
19	PAPAPAPAPAPAPPKEEKIDLSAI	18-41
20	APAPAPAPAPAPAPAPPKEEKIDLSA	15-40
21	PAPAPAPAPAPAPAPPKEEKIDLSA	16-40
22	APAPAPAPAPAPAPPKEEKIDLSA	17-40
23	AAAPAPAPAPAPAPAPAPPKEEKIDLS	13-39
24	AAPAPAPAPAPAPAPAPPKEEKIDLS	14-39
25	APAPAPAPAPAPAPAPPKEEKIDLS	15-39
26	PAPAPAPAPAPAPAPPKEEKIDLS	16-39
27	APAPAPAPAPAPAPPKEEKIDLS	17-39
28	PAPAPAPAPAPAPPKEEKIDLS	18-39
29	APAPAPAPAPAPPKEEKIDLS	19-39
30	PAPAPAPAPAPPKEEKIDLS	20-39
31	APKKDVKKPAAAAAPAPAPAPAPAPAPAPPKEEKIDL	1-38
32	AAAPAPAPAPAPAPAPAPPKEEKIDL	13-38
33	AAPAPAPAPAPAPAPAPPKEEKIDL	14-38
34	APAPAPAPAPAPAPAPPKEEKIDL	15-38
35	PAPAPAPAPAPAPAPPKEEKIDL	16-38
36	APAPAPAPAPAPAPPKEEKIDL	17-38
37	PAPAPAPAPAPAPPKEEKIDL	18-38
38	APAPAPAPAPAPPKEEKIDL	19-38
39	PAPAPAPAPAPPKEEKIDL	20-38
40	APAPAPAPAPPKEEKIDL	21-38
41	APPKEEKIDL	28-38
42	APKKDVKKPAAAAAPAPAPAPAPAPAPAPPKEEKID	1-37
43	KKPAAAAAPAPAPAPAPAPAPAPPKEEKID	8-37
44	AAAPAPAPAPAPAPAPAPPKEEKID	13-37
45	AAPAPAPAPAPAPAPAPPKEEKID	14-37
46	APAPAPAPAPAPAPAPPKEEKID	15-37
47	PAPAPAPAPAPAPAPPKEEKID	16-37
48	APAPAPAPAPAPAPPKEEKID	17-37
49	PAPAPAPAPAPAPPKEEKID	18-37
50	APAPAPAPAPPKEEKID	21-37
51	PAPAPAPAPPKEEKID	22-37
52	APAPAPAPPKEEKID	23-37
53	PAPAPAPPKEEKID	24-37
54	PAPAPPKEEKID	26-37
55	PPKEEKID	30-37
56	AAAPAPAPAPAPAPAPAPPKEEKI	13-36
57	AAPAPAPAPAPAPAPAPPKEEKI	14-36
58	APAPAPAPAPAPAPAPPKEEKI	15-36
59	PAPAPAPAPAPAPAPPKEEKI	16-36
60	APAPAPAPAPAPAPPKEEKI	17-36
61	PAPAPAPAPAPAPPKEEKI	18-36
62	APAPAPAPAPAPPKEEKI	19-36
63	APKKDVKKPAAAAAPAPAPAPAPAPAPAPPKEEK	1-35
64	KKPAAAAAPAPAPAPAPAPAPAPPKEEK	8-35
65	AAAAPAPAPAPAPAPAPAPPKEEK	12-35

Table 1. Continued

Peptide No.	APKKDVKKPAAAAAPAPAPAPAPAPAPAPPKEEKIDLSAIKIEFSKEQQDE	Position
66	AAAPAPAPAPAPAPAPAPPKEEK	13-35
67	AAPAPAPAPAPAPAPAPPKEEK	14-35
68	APAPAPAPAPAPAPAPPKEEK	15-35
69	PAPAPAPAPAPAPAPPKEEK	16-35
70	APAPAPAPAPAPAPPKEEK	17-35
71	PAPAPAPAPAPPKEEK	20-35
72	PAPAPAPAPPKEEK	22-35
73	APAPAPAPPKEEK	23-35
74	PAPAPAPPKEEK	24-35
75	APAPAPPKEEK	25-35
76	PAPPKEEK	28-35
77	APKKDVKKPAAAAAPAPAPAPAPAPAPAPAPPKEE	1-34
78	AAAPAPAPAPAPAPAPAPPKEE	13-34
79	AAPAPAPAPAPAPAPAPPKEE	14-34
80	APAPAPAPAPAPAPAPPKEE	15-34
81	PAPAPAPAPAPAPAPPKEE	16-34
82	APAPAPAPAPAPAPPKEE	17-34
83	PAPAPAPAPAPAPPKEE	18-34
84	APAPAPAPAPAPPKEE	19-34
85	APKKDVKKPAAAAAPAPAPAPAPAPAPAPAPPKE	1-33
86	AAAPAPAPAPAPAPAPAPPKE	13-33
87	AAPAPAPAPAPAPAPAPPKE	14-33
88	APAPAPAPAPAPAPAPPKE	15-33
89	PAPAPAPAPAPAPAPPKE	16-33
90	APAPAPAPAPAPAPPKE	17-33
91	PAPAPAPAPAPAPPKE	18-33
92	APAPAPAPAPAPPKE	19-33
93	APAPAPAPAPPKE	21-33
94	APKKDVKKPAAAAAPAPAPAPAPAPAPAPAPPK	1-32
95	APAPAPAPAPAPAPAPPK	15-32
96	PAPAPAPAPAPAPAPPK	16-32
97	APAPAPAPAPAPAPPK	17-32
98	APKKDVKKPAAAAAPAPAPAPAPAPAPAPAPP	1-31
99	AAPAPAPAPAPAPAPAPP	14-31
100	APAPAPAPAPAPAPAPP	15-31
101	PAPAPAPAPAPAPAPP	16-31
102	APAPAPAPAPAPP	19-31
103	PAPAPAPAPAPP	20-31
104	APAPAPAPAPAPAPAP	15-30
105	PAPAPAPAPAPAPAP	16-30
106	APAPAPAPAPAPAP	15-28
107	APKKDVKKPAAAAA	1-14

the charge state of the corresponding ion detected in MS/MS analysis. A total of 69 of the 137 fragments identified using the Paragon algorithm were also identified using Mascot. Sequences of peptides 67, 69, 70, 72, 74, and 76 originated from MLC 1 (see Table 1) have been previously described by Mora et al.[5] Tables 4 and 5 show MLC 2 sequences tenatitively identified in this study, whereas Table B of the Supporting Information describe the most relevant data related to these fragments. With respect to MLC 2, a total of 63 of the 88 fragments identified using the Paragon algorithm were also identified using Mascot search engine.

The muscle myofibrillar protein fraction is involved in the numerous changes that take place due to the enzymatic proteolysis occurring during the processing of dry-cured ham. Mainly responsible for the degradation of myosin light chain during dry-cured ham processing are muscle endopeptidases, which generate protein fragments, and certain groups of exopeptidases degrading the previously generated polypeptides into smaller ones. Exopeptidases are also responsible for the generation of dipeptides and free amino acids, which have been proved to contribute to flavor development in dry-cured products.

Role of Endopeptidases in the Degradation of Myosin Light Chains. Cathepsin stability during dry-cured ham processing has been widely studied, with the conclusion that cathepsins B, H, and L are quite stable, showing activity even after 15 months of processing,[2,9] whereas cathepsin D is not so stable, its activity disappearing after 6−10 months of processing.[2,10]

Several studies relate the action of cathepsins with the progressive disappearance of myosin. In this way, Hirao et al.[11] incubated rabbit skeletal muscle myosin with cathepsin B and proved the degradation of myosin heavy chain and MLC 2 in the presence of this enzyme. On the other hand, Etherington[12] 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 active in degrading myosin, although to a lesser extent than other proteins such as titin. In contrast, Okitani et al.[13] reported that myosin was not

Table 2. Myosin Light Chain 1 Peptides Tentatively Identified in Spanish Dry-Cured Ham Located between Positions 61 and 120 in the Complete Sequence (Protein Accession No. A1XQT6_PIG in UniProtKB/TrEMBL)

Peptide No.	SQVGDVLRALGTNPTNAEVKKVLGNPSNEEMNAKKIEFEQ (80, 90, 100, 110)	Position
108	NPSNEEMNAKKIEFEQ	95-110
109	AEVKKVLGNPSNEEM	87-101
110	GTNPTNAEVKKVLGNPSN	81-98
111	TNPTNAEVKKVLGNPSN	82-98
112	AEVKKVLGNPSN	87-98
113	GTNPTNAEVKKVLGNPS	81-97
114	TNPTNAEVKKVLGNPS	82-97
115	TNAEVKKVLGNPS	85-97
116	AEVKKVLGNPS	87-97
117	GTNPTNAEVKKVLGNP	81-96
118	GTNPTNAEVKKVLG	81-94
119	TNPTNAEVKKVLG	82-94
120	NPTNAEVKKVLG	83-94
121	QVGDVLRALGTNPTNAEVKKV	72-92
122	SQVGDVLRALGTNPTNAEVKK	71-91
123	QVGDVLRALGTNPTNAEVKK	72-91
124	QVGDVLRALGTNPTNAEVK	72-90
125	QVGDVLRALGTNPTNAE	72-88
126	QVGDVLRALGTNPT	72-85
127	SQVGDVLRALG	71-81

Table 3. Myosin Light Chain 1 Peptides Tentatively Identified in Spanish Dry-Cured Ham Located between Positions 134 and 175 in the Complete Sequence (Protein Accession No. A1XQT6_PIG in UniProtKB/TrEMBL)

Peptide No.	RVFDKEGNGTVMGAELRHVLATLGEKMKEEEVEALMAGQED (140, 150, 160, 170)	Position
128	RVFDKEGNGTVMGAELRH	138-152
129	VFDKEGNGTVMGAELRH	139-152
130	DKEGNGTVMGAELRH	141-152
131	TVMGAELRH	147-152
132	RVFDKEGNGTVMGAELR	138-151
133	DKEGNGTVMGAELR	141-151
134	MKEEEVEALMAGQED	161-175
135	ATLGEKMKEEEVEAL	155-169
136	EKMKEEEVEAL	159-169
137	ATLGEKMKEEEVE	155-167

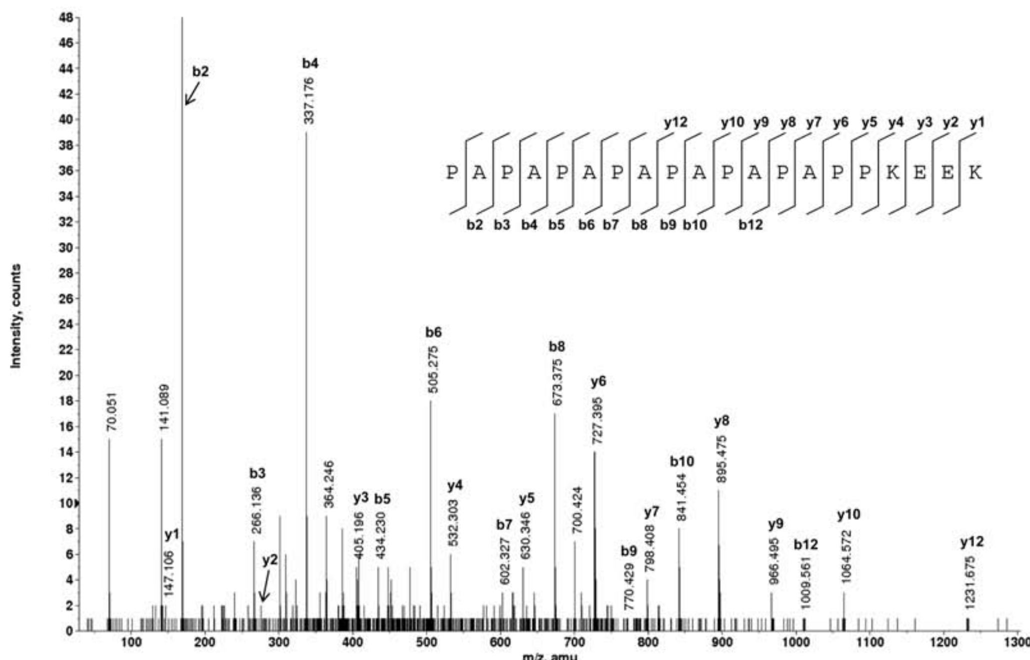
degraded by rabbit skeletal muscle cathepsin B, whereas cathepsin L isolated from rabbit skeletal muscle could hydrolyze myosin at pH 4.1.

Toldrá et al.[2] published electrophoretic profiles of Biceps femoris muscle showing a progressive disappearance of MLC 1 and MLC 2 during the dry-curing process. According to these authors, the disappearance of these proteins could be due to cathepsins L and D, even if cathepsin D activity is minimal after some months of processing due to its inhibition in the presence of a high concentration of salt.[14] The action of cathepsin D on myosin has been widely studied by many authors, being considered responsible for the major breakdown of the main myofibrillar proteins such as myosin, actin, or troponin during meat aging.[14] In this way, Robbins et al.[15] established that cathepsin D from muscle degrades myofibrils under post-mortem pH conditions (5.1−5.3), causing an alteration of Z disk structure and breakdown of myosin heavy and light chains. The degradation of myosin light chains by cathepsin D has also been studied by incubating purified cathepsin D with myofibrils at pH 5.5 (37 °C); however, according to these authors, they are degraded at a much slower rate than that for myosin heavy chain.[16]

Calpains participate in proteolytic events only during the first stages of curing processes because an excess of salt together with the pH drop completely inhibits their activity. In fact, many authors have established that calpains do not degrade myosin and actin,[17] although others established that MLC 1 could be degraded in vitro by μ-calpain, suggesting MLC 1 as a novel substrate of μ-calpain-mediated regulation.[18]

Cathepsins and calpains have been the main focus of research regarding post-mortem proteolysis, considering the calpain system as the major contributor to meat tenderization.[19] However, it has been suggested during recent years that meat tenderization could be also due to the action of proteolytic enzymes such as caspases and the proteasome.[20,21] In fact, some authors investigated the potential role of recombinant caspase 3 in meat tenderization, observing MLC 3 degradation products with increasing concentrations of recombinant caspase 3.[22,23] On the other hand, the proteasome action against MLC was proved when the inhibition of proteasome chymotrypsin and trypsin-like activity results in the lack of degradation of this protein.[24]

Role of Exopeptidases in the Degradation of Myosin Light Chain. Dipeptidyl peptidases (DPP I−IV) are a group of



Peptide (635.36³⁺)				
	b	Seq.	y	
1	98.06	P		20
2	**169.10**	A	1806.97	19
3	**266.15**	P	1735.94	18
4	**337.19**	A	1638.88	17
5	**434.24**	P	1567.88	16
6	**505.28**	A	1470.79	15
7	**602.33**	P	1399.76	14
8	**673.37**	A	1302.71	13
9	**770.42**	P	**1231.67**	12
10	**841.46**	A	1134.62	11
11	938.51	P	**1063.58**	10
12	**1009.55**	A	**966.53**	9
13	1106.60	P	**895.49**	8
14	1177.64	A	**798.44**	7
15	1274.69	P	**727.40**	6
16	1371.74	P	**630.35**	5
17	1499.87	K	**533.29**	4
18	1628.88	E	**405.20**	3
19	1757.92	E	276.16	2
20		K	**147.11**	1

Figure 2. Example of MS/MS spectrum of ion 635.38^{3+} that corresponds to peptide 69 from MLC 1. Peptide sequence matching with the fragmentation ion spectrum is shown in capital letters, together with observed *b* and *y* ions. The spectrum is presented with its corresponding table with the matched ions in bold letters.

Table 4. Myosin Light Chain 2 Peptides Tentatively Identified in Spanish Dry-Cured Ham Located between Positions 34 and 79 in the Complete Sequence (Protein Accession No. Q5XLD2_PIG in UniProtKB/TrEMBL)

Peptide No.	40 50 60 70 T V I D Q N R D G I I D K E D L R D T F A A M G R L N V K N E E L D A M M K E A S G P I N	Position
1	T V I D Q N R D G I I D K E D L R D T	35-53
2	R D G I I D K E D L R D T	41-53
3	T V I D Q N R D G I I D K E D L R D	35-52
4	V I D Q N R D G I I D K E D L R D	36-52
5	I D Q N R D G I I D K E D L R D	37-52
6	D Q N R D G I I D K E D L R D	38-52
7	Q N R D G I I D K E D L R D	39-52
8	N R D G I I D K E D L R D	40-52
9	R D G I I D K E D L R D	41-52
10	G I I D K E D L R D	42-52
11	I I D K E D L R D	43-52
12	T V I D Q N R D G I I D K E D L R	35-51
13	V I D Q N R D G I I D K E D L R	36-51
14	I D Q N R D G I I D K E D L R	37-51
15	D Q N R D G I I D K E D L R	38-51
16	G I I D K E D L R	43-51
17	I I D K E D L R	44-51
18	T V I D Q N R D G I I D K E D	35-19
19	V I D Q N R D G I I D K E D	36-49
20	N R D G I I D K E	40-48
21	T V I D Q N R D G I I D K	35-47
22	V I D Q N R D G I I D K	36-47
23	T V I D Q N R D G	35-42
24	T V I D Q N R D	35-41
25	N V K N E E L D A M M K E A S G P I N	61-79
26	V K N E E L D A M M K E A S G P I N	62-79
27	A M M K E A S G P I N	69-79
28	V K N E E L D A M M K E A S G P	62-77
29	V K N E E L D A M M K E A	62-74
30	N V K N E E L D A M M K E	61-73
31	V K N E E L D A M M K E	62-73
32	G R L N V K N E E L D A M	58-70

Table 5. Myosin Light Chain 2 Peptides Tentatively Identified in Spanish Dry-Cured Ham Located between Positions 86 and 124 in the Complete Sequence (Protein Accession No. Q5XLD2_PIG in UniProtKB/TrEMBL)

Peptide No.	90 100 110 120 MFGEKLKGADPEDVITGAFKVLDPEGKGTIKKHFLEELL	Position
33	VLDPEGKGTIKKHFLEEL	106-123
34	PEGKGTIKKHFLEEL	109-123
35	GKGTIKKHFLEEL	111-123
36	KGTIKKHFLEEL	112-123
37	FGEKLKGADPEDVITGAFKVLDPEGKGTIKKHFLEE	87-122
38	LKGADPEDVITGAFKVLDPEGKGTIKKHFLEE	91-122
39	PEDVITGAFKVLDPEGKGTIKKHFLEE	96-122
40	VLDPEGKGTIKKHFLEE	106-122
41	PEGKGTIKKHFLEE	109-122
42	GKGTIKKHFLEE	111-122
43	KGTIKKHFLEE	112-122
44	FGEKLKGADPEDVITGAFKVLDPEGKGTIKKHFLE	87-121
45	LKGADPEDVITGAFKVLDPEGKGTIKKHFLE	91-121
46	PEDVITGAFKVLDPEGKGTIKKHFLE	96-121
47	VLDPEGKGTIKKHFLE	106-121
48	PEGKGTIKKHFLE	109-121
49	KVLDPEGKGTIKKHFL	105-120
50	GADPEDVITGAFKVLDPEGKGTIKKHF	93-119
51	FKVLDPEGKGTIKKHF	104-119
52	KVLDPEGKGTIKKHF	105-119
53	LDPEGKGTIKKHF	107-119
54	DPEGKGTIKKHF	108-119
55	PEGKGTIKKHF	109-119
56	FKVLDPEGKGTIKKH	104-118
57	KVLDPEGKGTIKKH	105-118
58	VLDPEGKGTIKKH	106-118
59	PEGKGTIKKH	109-118
60	FKVLDPEGKGTIKK	104-117
61	KVLDPEGKGTIKK	105-117
62	VLDPEGKGTIKK	106-117
63	PEGKGTIKK	109-117
64	FGEKLKGADPEDVITGAFKVLDPEGKGTIK	87-116
65	LKGADPEDVITGAFKVLDPEGKGTIK	91-116
66	ADPEDVITGAFKVLDPEGKGTIK	94-116
67	PEDVITGAFKVLDPEGKGTIK	96-116
68	KVLDPEGKGTIK	105-116
69	PEGKGTIK	109-116
70	KGADPEDVITGAFKVLDPEGKGT	92-114
71	KVLDPEGKG	105-113
72	VLDPEGKG	106-113
73	FGEKLKGADPEDVITGAFKVLD	87-108
74	LKGADPEDVITGAFKVLD	91-108
75	ADPEDVITGAFK	94-105
76	GEKLKGADPEDVITGA	88-103
77	EKLKGADPEDVITGA	89-103
78	LKGADPEDVITGA	91-103
79	KGADPEDVITGA	92-103
80	GEKLKGADPEDVITG	88-102
81	EKLKGADPEDVITG	89-102
82	GEKLKGADPEDVIT	88-101
83	EKLKGADPEDVIT	89-101
84	KGADPEDVIT	92-101
85	FGEKLKGADPEDVI	87-100
86	FGEKLKGAD	87-95
85	GEKLKGAD	88-95
86	EKLKGAD	89-95

enzymes able to release different dipeptides from the N-terminal site of peptides. The stability of dipeptidyl peptidases during the processing of Spanish dry-cured ham has been previously studied.[25] According to these authors, DPP I and DPP III kept their activity until the end of the process, whereas DPP II activity was almost negligible at 240 days. The activity of DPP IV decreased to very low values at 240 days, although its activity remained until the end of the process. Considering the stability of

dipeptidyl peptidases along the processing of dry-cured ham and its mode of action, these exopeptidases could be responsible for the consecutive loss of dipeptides observed in the N-terminal side of peptides 12, 71, 92, 115, and 129 of MLC 1 (Tables 1−3) and peptides 9, 34, 41, 52, 66, and 77 of MLC 2 (Tables 4 and 5). DPP I preferentially hydrolyzes N-terminal peptides with a penultimate basic residue.[26] Thus, this enzyme could be responsible for the loss of EK dipeptide from peptide 77 in MLC 2 protein. The action of DPP I in peptides 12, 71, and 92 of MLC 1 seems unlikely because this enzyme does not hydrolyze peptides containing a proline residue on the scissile bond. However, due to DPP II enzyme from porcine skeletal muscle preferentially hydrolyzing the synthetic substrates of type X-Pro,[27] this enzyme could be responsible for the loss of the dipeptide Ala-Pro from peptides 12 and 92 of MLC 1. It seems unlikely that DPP III has been involved in the generation of any of the fragments identified in this study because no activity of the enzyme was detected against peptide sequences containing >10 amino acids.[28] Finally, DPP IV activity is controversial. Some authors established that this enzyme is active against those peptides containing a proline residue in the N-penultimate position and, to a lower degree, against those containing alanine in this position.[29] However, other authors disagree, observing an inhibition of DPP IV with a number of nonsubstrate oligopeptides containing an N-terminal X-X-Pro- structure.[30]

A remarkable increase in free amino acid concentration has been reported during dry-cured ham processing.[31−33] This accumulation of free amino acids, which is relevant for the development of the characteristic dry-cured flavor, has been attributed to muscle aminopeptidases, responsible for the release of amino acids from the N-terminus of peptides and proteins. Aminopeptidase activity has been detected in meat products even after >8 months of processing, suggesting that these enzymes are involved in the later stages of protein degradation. Many factors, such as curing agent or the presence of other peptides, can modulate the activity of these enzymes.[34,35]

According to Toldrá et al.,[33] alanyl and arginyl aminopeptidases, which show good stability during dry-cured processing and act at optimal neutral pH, appear to be the main contributors to the generation of free amino acids during the processing of dry-cured ham. Alanyl aminopeptidase (AAP, EC 3.4.11.14) is considered to be the major aminopeptidase in skeletal muscle with a broad range of specificity. This enzyme hydrolyzes unblocked N-terminal Ala residues as well as Leu, Arg, Phe, Tyr, and Met. However, peptides containing Pro in the N-terminal or subterminal position (P1 or P1′) seem to be totally resistant to hydrolysis by alanyl aminopeptidase,[36] although Flores et al.[37] detected a residual activity of this enzyme when acting on a Pro-AMC substrate. Thus, although this enzyme could be responsible for the loss of Ala in peptides 4, 5, 14, 15, 23, 24, 32, 33, 44, 45, 56, 57, 65−67, 78, 79, 86, 87, and 99 from MLC 1, it seems unlikely that this enzyme was responsible for the consecutive loss of Ala and Pro shown in the N-terminal side of subsequent peptides contained in Table 1. This aminopeptidase could also be responsible for the loss of Arg in peptide 128 from MLC 1 and the loss of Phe in peptides 51, 56, 60 and 86, and Leu in peptides 53 and 78 from MLC 2.

The activity of arginyl aminopeptidase (RAP, EC 3.4.11.6) is more restricted to a few terminal amino acids mainly of basic nature (Arg, Lys, and His), although according to Flores et al.,[38] Pro-MCA and Ala-MCA derivatives were also cleaved by the enzyme. Thus, RAP could be responsible for the loss of Lys observed in peptides 57, 61, and 71 as well as for the consecutive loss of Ala and Pro shown in the N-terminal side of peptides contained in Table 1. Similar to AAP, RAP enzyme could be also responsible for the loss of Arg in peptide 128 from MLC 1. Methionyl aminopeptidase (MAP, EC 3.4.11.18) might have a similar role as AAP, although at a lower activity.

Other aminopeptidases existing in skeletal muscle are leucyl aminopeptidase (LAP, EC 3.4.11.1) and pyroglutamyl aminopeptidase (PGAP, EC 3.4.19.3). These enzymes could also contribute to the loss of amino acids in peptides identified in this study, although the activity of these enzymes during dry-curing is lower than AAP and RAP due to the effect of pH and ripening agents.

Carboxypeptidases constitute another group of exopeptidases responsible for the hydrolysis of amino acids from the C-terminal site of peptides. Only tissue and lysosomal carboxypeptidase A have been previously studied in skeletal muscle. The effect of curing agents on the activity of these enzymes, together with their stability during the ripening period, has not been studied yet. Both carboxypeptidases are most active on hydrophobic C-terminal residues, with Phe and Leu as most preferred substrates. They could be responsible for the loss of Phe in peptides 50−55 from MLC 2 and of Leu in peptides 31−41 from MLC 1 and in peptides 33−36 and 49 from MLC 2, as well as for the loss of hydrophobic Ile (peptides 14−19 and 56−62 from MLC 1), Ala (peptides 20−22 from MLC 1 and peptides 76−79 from MLC 2), and Val (peptide 121 from MLC 1). Tissue carboxypeptidase A is not able to hydrolyze Pro, but it can hydrolyze Thr at neutral pH (peptides 1, 2, 70, and 82−84 from MLC 2).

In the present work, a total of 137 MLC 1 protein fragments and 88 peptides from MLC 2 have been identified, proving the intense proteolysis suffered by these proteins during dry-cured ham processing. The obtained results suggest the contribution of dipeptidyl peptidases, together with aminopeptidases and carboxypeptidases, to the generation of dipeptides and free amino acids during the dry-cured ham processing. These generated compounds can contribute, together with other nonvolatile and volatile compounds, to the development of the characteristic flavor of dry-cured ham.

■ ASSOCIATED CONTENT

Supporting Information. Tables A and B.This material is available free of charge via the Internet at http://pubs.acs.org.

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