DOI:10.1021/jf901573t

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

Oligopeptides Arising from the Degradation of Creatine Kinase in Spanish Dry-Cured Ham

Leticia Mora,[†,‡] Miguel Angel Sentandreu,[‡] Paul D. Fraser,[†] Fidel Toldrá,[‡] and Peter M. Bramley*[,†]

[†]Center for Systems and Synthetic Biology, School of Biological Sciences, Royal Holloway, University of London, Egham TW20 0EX, United Kingdom, and [‡]Instituto de Agroquímica y Tecnología de Alimentos (CSIC), Post Office Box 73, 46100 Burjassot, Valencia, Spain

During the processing of dry-cured ham many biochemical changes occur, including the degradation of muscle proteins. These changes are due to the intense action of endogenous proteolytic enzymes. In the present study, the isolation and identification of a large number of peptides derived from creatine kinase has been achieved for the first time in dry-cured ham. A total of 58 peptides coming from different regions of the protein have been identified by mass spectrometry. This study provides evidence for the extensive degradation of creatine kinase during the processing of dry-cured ham as well as the role played by endo- and exopeptidases in the generation of small peptides and free amino acids from polypeptides. These peptides are important in the development of characteristic sensory properties associated with dry-cured ham.

KEYWORDS: Dry-cured ham; creatine kinase; proteolysis; aminopeptidases; proteomics; mass spectrometry; peptides

INTRODUCTION

Traditional Spanish dry-cured ham is a high-quality product that requires prolonged processing. During this process, many biochemical reactions responsible for the final characteristic traits of the product, such as texture and flavor, occur. One of the main changes arising in skeletal muscle during the dry-curing process is the degradation of protein (*1*, *2*). Muscle endopeptidases, such as cathepsins and calpains, degrade the muscle protein structure, giving rise to large polypeptides, that are further degraded to smaller peptides and free amino acids by exopeptidases, such as aminopeptidases and carboxypeptidases. The action of these enzymes during processing is of great importance because they are directly responsible for changes in postmortem muscle texture (*3*−*5*) and for the flavor development that occurs during the ripening of dry-cured meat products (*2*, *6*−*8*).

Creatine kinase (CK; EC 2.7.3.2) is a sarcoplasmic protein and, functionally, is an enzyme involved in energetic metabolism of tissues, such as skeletal muscle, with high and fluctuating energy demands *in vivo*. In this way, adenosine triphosphate (ATP) is continuously regenerated by the action of CK, which catalyzes the transphosphorylation reaction between phosphocreatine (PCr) and adenosine diphosphate (ADP). This enzyme is used as an indicator of pre-slaughter physical stress/muscle damage in animal production by the measurement of its levels in blood (*9*, *10*). Early postmortem, CK continues to exert important functions related to the conversion of muscle into meat. Numerous studies have related this enzyme to meat quality (*11*), establishing a negative correlation between the solubility of sarcoplasmic proteins (with CK being the major denatured protein) and drip loss (*12*, *13*) or pale color (*12*, *14*) in porcine meat. The texture of meat may also be affected somehow by the denaturation of sarcoplasmic proteins. In this way, the insolubilization of sarcoplasmic proteins, including CK, during vinegar curing of mackerel meat was hypothesized to be responsible for the texture changes caused by the curing process (*15*). Recently, some authors have established a positive correlation between the abundance of the full-length CK and firmness of fish meat, suggesting that limited proteolysis of CK in firm fish is possibly related to minimized oxidative conditions (*16*).

Many authors have reported the intense proteolysis suffered by sarcoplasmic proteins during dry-cured ham processing, reporting the degradation of CK occurred along the ripening period (*17*, *18*). Although there is evidence of the presence of CK and other protein fragments at the end of the curing period, little is known about the identity of specific peptide sequences coming from the degradation of this protein in dry-cured ham (*19*, *20*). A CK fragment was identified after characterization of proteolysis during the ripening of semi-dry fermented sausages (*21*), whereas a total of three CK fragments were isolated and identified during postmortem aging in bovine longissimus dorsi muscle (*22*).

Information derived from the knowledge of the protein fragments naturally generated during dry-curing of meat products would be very important to better understand both proteolysis and flavor development. Thus, the main objective of the present work was to elucidate some of the changes occurring in the sarcoplasmic fraction of Spanish dry-cured ham, through the identification of a large number of peptides generated from CK degradation.

*To whom correspondence should be addressed. Telephone: (+44) 01784-443555. Fax: (+44) 01784-414224. E-mail: p.bramley@rhul.ac.uk.

 Published on Web 09/04/2009

MATERIALS AND METHODS

Dry-Cured Ham Preparation. Three Spanish dry-cured hams were produced using raw material from 6-month-old pigs (Landrace x Large White). Selected hams were bled and prepared according to traditional procedures: (1) Hams were presalted using a mixture of curing ingredients (salt, nitrate, and nitrite) for 30 min to avoid microbiological spoilage. (2) In the salting stage, hams were placed fat side down, entirely buried in salt, and piled up without touching each other in salting chambers for 11−12 days. During this stage, curing agents slowly diffuse into the hams. The salting chambers were set at 4 °C and 85−90% relative humidity. (3) After salting, hams were brushed to remove remaining salt from their surfaces and transferred to the postsalting chamber, where a complete salt equalization takes place. The temperature was kept below 4 °C for 50 days, and the relative humidity in the chambers was established between 75 and 85%. (4) During the last stage, corresponding to the ripening period, hams were placed in air-conditioning chambers and subjected to different time, temperature, and relative humidity cycles. Higher temperatures (14−20 °C) and lower relative humidity (until 70%) were set during ripening to accelerate the drying process. The total length of the curing process was 9 months.

Dry-Cured Ham Extraction and Deproteinization. A total of 50 g of biceps femoris muscle coming from the processed dry-cured hams and previously removed from their extramuscular fat were minced and homogenized with 100 mL of 0.01 N HCl in a stomacher (IUL Instrument, Barcelona, Spain) for 8 min. The homogenate was centrifuged in the cold (24500*g* for 20 min at 4 °C), and after filtering through glass wool, the supernatant was deproteinized by adding 3 volumes of ethanol and maintaining the sample 20 min at 4 °C. After that, the sample was centrifuged again (24500*g* for 20 min at 4 °C), and the supernatant was dried in a rotatory evaporator. The dried deproteinized extract was redissolved in 25 mL of 0.01 N HCl, filtered through a 0.45 μm nylon membrane filter (Millipore, Bedford, MA), and stored at −20 °C until use.

Molecular Mass Fractionation. A 5 mL aliquot of the deproteinized dry-cured ham extract was subjected to size-exclusion chromatography to fractionate the peptides according to their molecular mass. For this purpose, a Sephadex G25 column (2.5 × 65 cm) (Amersham Biosciences, Uppsala, Sweden), previously equilibrated with 0.01 N HCl, was employed. The separation was performed using an isocratic gradient of 0.01 N HCl at a flow rate of 15 mL/h. Fractions of 5 mL were collected using an automatic fraction collector and further monitored by ultraviolet (UV) absorption at 214 nm using an Agilent 8453 UV spectrophotometer (Agilent Technologies, CA). Fractions corresponding to a molecular weight between 1000 and 4000 Da were pooled together, dried under vacuum, and redissolved in 5 mL of 0.1% trifluoroacetic acid in water/acetonitrile (95:5, v/v).

Reversed-Phase Chromatography. A 100 μL aliquot of the redissolved mixture of peptides was injected into a HPLC Agilent 1100 Series system (Agilent Technologies, Santa Clara, CA) equipped with an autosampler and a diode array detector, which was used in the range of 195−300 nm.

The 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 in water, and solvent B, containing 0.05% trifluoroacetic acid 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 using a diode array detector at a wavelength of 214 nm, and 1 mL fractions were collected and lyophilized separately.

Molecular Mass Determination by Matrix-Assisted Laser Desorption/Ionization Time-of-Flight (MALDI−TOF) Mass Spectrometry. The molecular mass of peptides contained in the reversed-phase HPLC fractions was determined using a Reflex III MALDI−TOF mass spectrometer (MS) (Bruker Daltonik GmbH, Bremen, Germany). By this way, fractions of interest were redissolved in 30−50 μL of 0.1% trifluoroacetic acid according to the concentration of peptides expected in each fraction. A total of 1 μL of each fraction was spotted onto an AnchorChip MALDI plate (Bruker Daltonik GmbH, Bremen, Germany) and mixed with 1 μL of 2,5-dihydroxybenzoic acid (Bruker Daltonik GmbH, Bremen, Germany) in 0.1% trifluoroacetic acid/acetonitrile (2:1, v/v). After air-drying, the plate was introduced in the MALDI−TOF MS. The instrument was used in positive-ion reflector mode, and ion acceleration voltage was 20 kV. Spectra were obtained from the average of 300 laser shots. Mass calibration was performed using a peptide calibration standard with code 206195 (Bruker Daltonik GmbH, Bremen, Germany) in a mass range between 1000 and 3000 Da. FlexAnalysis 2.4 software (Bruker Daltonik GmbH, Bremen, Germany) was employed for data analysis.

Peptide Identification by Tandem Mass Spectrometry. Peptides contained in the fractions obtained after reversed-phase chromatography were further separated and identified by liquid chromatography coupled to tandem mass spectrometry (LC−MS/MS) using an Ultimate/Famos nano LC system (LC Packings, Amsterdam, The Netherlands) and a QSTAR Pulsar *i* hybrid quadrupole-TOF instrument (Applied Biosystems, Foster City, CA) equipped with a nanoelectrospray ion source (Protana, Odense, Denmark).

Fractions previously resuspended for MALDI−TOF analysis were diluted 4 times using loading buffer (0.1% of formic acid and 2% of acetonitrile in water). A total of 10 μL of the diluted samples was preconcentrated on a 0.3 × 5 mm, 3 μm, C18 trap column from LC Packings (Dionex Company, Sunnyvale, CA) at a flow rate of 25 μL/min and using the loading buffer as the mobile phase. After 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 acetonitrile. Chromatographic conditions were a linear gradient from 95 to 60% solvent A in 70 min at a flow rate of 0.2 μL/min. The column outlet was directly coupled to a nanoeletrospray ion source (Protana, Odense, Denmark) using a 10 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). A TOF MS survey scan was recorded for mass range m/z 400−1600 followed by MS/MS scans of the two most intense peaks. A typical ion spray voltage was in the range of 1.8−2.0 kV, using nitrogen as collision gas. Other source parameters and spray position were optimized with a tryptic digest of bovine serum albumin.

Automated spectral processing, peak list generation, and database search were performed using both Mascot search v1.6b21 script for Analyst QS 1.1 (Applied Biosystems, Foster City, CA) in combination with the Mascot interface (Matrix Science, Inc., Boston, MA) (http://www.matrixscience.com) and the ProteinPilot 2.0.1. software (Applied Biosystems, Foster City, CA) with Paragon algorithm. Identification of the protein origin of peptides was performed using the National Center for Biotechnology Information (NCBI) nonredundant protein database. Matches of MS/MS spectra against sequences of the database were verified manually.

RESULTS AND DISCUSSION

Isolation and Purification of Peptides. A deproteinized dry-cured ham extract was submitted to size-exclusion chromatography to fractionate the peptide mixture according to their molecular mass. Fractions corresponding to elution volumes between 125 and 190 mL were pooled together, concentrated, and subjected to reversed-phase chromatography. During this chromatographic separation (**Figure 1**), 1 mL fractions were collected and analyzed separately by mass spectrometry.

Peptide Sequencing using Mass Spectrometry. Fractions obtained after reversed-phase chromatography were subjected to MALDI−TOF mass spectrometry to determine peptide molecular masses. By this way, 18 singly charged ions ($M + H^+$) corresponding to CK fragments were obtained. To elucidate the sequence of the peptides, samples were further analyzed by liquid chromatography coupled to a quadrupole time-of-flight mass spectrometer equipped with a nanoelectrospray ionization source (nanoLC−nESI−QTOF MS). The molecular masses of peptides 4−7, 13, 15, 19, 21, 42, 43, 47−49, 51, 53, and 55 were in accordance with the signals previously obtained by MALDI−TOF MS (**Table 1**).

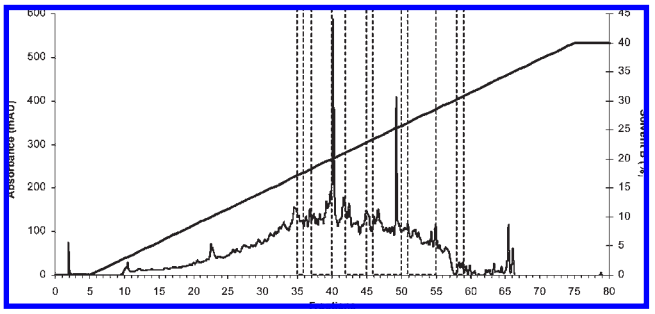


Figure 1. Reversed-phase chromatographic separation of the selected pooled fractions obtained from size-exclusion chromatography. Dotted lines indicate the fractions where CK fragments have been detected. Bold line indicates the solvent gradient of the chromatographic separation.

As can be seen in **Table 2**, a total of 58 peptide sequences have been identified. Peptides have been arranged according to their position in the sequence of porcine CK. Thus, peptide 1 has its initial position in Lys^{15}, whereas peptide 58 finishes in Lys^{381}. **Table 2** also shows the reversed-phase fraction (**Figure 1**) from where each peptide has been identified. BLAST sequence similarities searches revealed 100% homology of the whole peptides to the sequence of porcine M-type CK (accession number NP_01123421 in the NCBI nonredundant database), which is shown in **Figure 2**. All spectra were interpreted using the online form of the Mascot program, the Paragon algorithm, and also manually. **Figure 3** shows the MS/MS spectrum corresponding to peptide 36 as an example of a mass spectrometric fragmentation.

Peptides 6−12 share the common sequence VIQTGVDNPG-HPF (peptide 12). However, at the N-terminal site, these peptides show important differences. Peptide 6 contains an additional leucine in this position with respect to peptide 7, whereas peptides 8, 9, 10, and 11 show a consecutive loss of the amino acids threonine, leucine, and two aspartic acids, respectively. A similar profile is also observed in peptides 13−18. This group shares the common sequence DVIQTGVDNPGHP (peptide 18). With regard to the N-terminal side of this group of peptides, peptides 5, 12, and 16 and peptides 6, 13, and 17, show the same profile.

Methionine is a common amino acid in the first and last section of the CK sequence (**Table 2**), and all of the peptides identified in these sections present at least an oxidation of this amino acid. In fact, peptide 49 has been identified without modification but also with one, two, and three methionine oxidations (**Table 1**). On the other hand, peptides 50, 51, and 52 show just two methionine oxidations, whereas peptides 5, 53, 55, 56, and 57 present one. Peptide 58 was identified in two ways, with a methionine oxidation and also presenting an additional modification of glutamic acid to pyroglutamic acid in its N-teminal site. Other modifications are glutamine (peptide 33) and asparagine (peptides 27, 29, 30, 31, and 45) deamidation and aspartic acid dehydration in peptides 31 and 40.

As shown in **Table 1**, peptides have been sequenced matching the MS/MS spectra of 2, 3, 4, and 6 times charged ions. In this way, 27 peptides were sequenced from 2 times charged ions, whereas 19 and 13 peptides were sequenced from 3 and 4 times charged ions, respectively. Four peptides containing a high proportion of basic amino acids were sequenced from 6 times charged ions (peptides 1−3 and 32). **Table 1** also indicates the exact position of the identified peptides within the CK sequence, as well as their observed and calculated masses.

The 58 peptides identified in this study cover around 44% of the total sequence of CK protein, and a total of 27 peptides have been identified in at least two of the three studied hams.

Sarcoplasmic proteins correspond to approximately 30% of the total muscle protein and are generally more susceptible to denaturation than myofibrillar proteins (*23*). Structural studies of rabbit and chicken CK based on the identification of the major products obtained after digestion by trypsin, chymotrypsin, and endoproteinase Glu-C showed that the C-terminal region (positions 215−380) was more resistant to digestion than the N-terminal region (positions 1−133), whereas the central tract of 81 residues (positions 134−214) was particularly sensitive to digestion by all three peptidases (*24*). Contrary to that, most CK fragments identified in this study correspond to the C- and N-terminal portions of the protein (**Figure 2**). In the present study, a total of 34 and 22 peptides from C- and N-terminal sections, respectively, have been identified, whereas only 2 of the total 58 CK fragments sequenced in this study come from the central section of the CK sequence. A possible explanation would be an increased proteolysis suffered by the central area of CK during ripening, which could have generated fragments smaller than 1000 Da, and thus out of the peptide mass range studied in the present work.

Muscle sarcoplasmic proteins constitute relevant substrates for proteolysis occurring during the processing of dry-cured ham. The main enzymes responsible for the degradation of these proteins during this period are muscle endopeptidases and certain groups of exopeptidases.

Cathepsins B (EC 3.4.22.1), H (EC 3.4.22.16), and L (EC 3.4.22.15) have been reported to be stable during dry-cured ham processing, showing activity even after 15 months of processing (*8*, *25*). Several studies relate the action of cathepsins with the disappearance of CK. In fact, sea bass muscle sarcoplasmic extract was incubated with a mixture of cathepsins L, B, and D (EC 3.4.23.5), showing the degradation of the CK-aldolase doublet (*26*). Studies carried out with these enzymes separately established that both cathepsins B and L were especially active against CK, whereas cathepsin D was not able to degrade any sarcoplasmic protein (*16*). However, calpains (EC 3.4.22.17) might participate in the postmortem muscle proteolysis only during the first stages of curing processes because both the high salt content and low pH values found in subsequent steps would completely inhibit their activity. In this way, it has been summarized that CK is an *in vitro* substrate of calpain, suggesting that *m*-calpain might regulate the activity of this protein as well as its postmortem degradation (*27*).

Cathepsins B and L, together with *m*-calpain, seem to be the main endopeptidases responsible for the hydrolysis of muscle CK during the first stages of dry-curing. Observing the results obtained in this study, peptides 4 and 5 (**Table 2**) appear consecutively in the sequence of CK. Peptide 5 expands from position 15 to position 32, whereas peptide 4 expands from position 33 to position 50. Observing **Table 2**, it seems that peptide 1 could be the origin of peptides 4 and 5. Thus, it would be possible that any of these endopeptidases were responsible for the cleavage suffered between positions 32−33, although more studies would be necessary to assign the contribution of these enzymes to the generation of these peptides.

Aminopeptidase activity has been detected in dry-cured ham even after more than 12 months of processing. This procedure has been associated with the remarkable increase in free amino acid concentration observed during dry-cured ham processing. Glutamic and aspartic acids, alanine, leucine, lysine, valine, and threonine appear to be some of the amino acids experiencing larger increases (*8*, *28*, *29*).

CK peptides identified in this study constitute important evidence of the contribution of aminopeptidases to the remarkable increase in free amino acids observed during dry-cured ham

Table 1. CK Fragments Identified in Biceps Femoris Muscle of Spanish Dry-Cured Ham

peptide	observed[b]	charged state	calculated[c]	score[a]		position[d]	MALDI−TOF[e]	LC−MS/MS[e]	identified[f]
				Mascot	Paragon				
1	698.36	(6+)	4183.14		14	15−50		×	×
2	676.86	(6+)	4055.04		14	16−50		×	
3	504.61	(6+)	3021.62		14	25−50		×	
4	687.99	(3+)	2061.15	29		33−50	×	×	
5[g]	539.94	(4+)	2155.99	42		15−32	×	×	×
6	739.62	(4+)	2954.46	54		42−68	×	×	
7	711.35	(4+)	2841.36	32		43−68	×	×	
8	912.85	(2+)	1823.87	34		52−68		×	
9	862.3	(2+)	1722.82	33		53−68		×	
10	805.77	(2+)	1609.73	54		54−68		×	×
11	748.25	(2+)	1494.71	39		55−68		×	×
12	690.71	(2+)	1379.68	49		56−68		×	
13	847.05	(3+)	2538.19	49		44−67	×	×	
14	808.71	(3+)	2423.16	63		45−67		×	
15	839.41	(2+)	1676.8	56		52−67	×	×	
16	788.79	(2+)	1575.75	61		53−67		×	×
17	732.25	(2+)	1462.67	52		54−67		×	×
18	674.84	(2+)	1347.64	55		55−67		×	×
19	693.83	(2+)	1385.67	50		52−64	×	×	×
20	643.24	(2+)	1284.62	47		53−64		×	×
21	832.89	(2+)	1663.79	44		44−58	×	×	
22	516.26	(3+)	1545.96		13	31−43		×	
23	599.24	(4+)	2393.12	41		106−126		×	×
24	589.26	(3+)	1764.79		19	109−124		×	×
25	584.89	(3+)	1751.8	61		108−123		×	×
26	551.57	(3+)	1650.75	72		109−123		×	×
27[h]	712.72	(2+)	1423.62		14	111−123		×	
28	655.23	(2+)	1308.59		14	112−123		×	
29[h]	552.84	(3+)	1655.73		16	108−122		×	
30[h]	519.25	(3+)	1554.68		15	109−122		×	
31[h,i]	655.21	(2+)	1308.56		14	111−122		×	
32	434.73	(6+)	2602.3		23	95−115		×	
33[j]	617.82	(4+)	2468.21		16	89−109		×	
34	552.28	(4+)	2205.13		14	91−109		×	
35	630.2	(2+)	1258.68	44		153−164		×	
36	586.71	(2+)	1171.64	69		153−163		×	
37	495.61	(3+)	1483.81		17	303−314		×	
38	479.23	(3+)	1434.76	37		299−310		×	
39	417.82	(3+)	1250.64		14	301−310		×	
40[i]	842.41	(3+)	2524.28		13	335−358		×	
41	808.32	(2+)	1614.85	79		344−358		×	
42	973.36	(2+)	1944.94	46		335−352	×	×	
43	917.27	(2+)	1831.85	68		335−351	×	×	×
44	859.31	(2+)	1716.83	62		336−351		×	
45[h]	766.77	(2+)	1531.71		15	338−351		×	
46	969.94	(2+)	1937.9	54		332−349		×	
47	803.27	(2+)	1604.73	61		335−349	×	×	×
48	739.26	(2+)	1476.67	40		335−348	×	×	
49	637.75	(4+)	2547.27	46		360−381	×	×	×
49[g]	641.75	(4+)	2563.27	25		360−381	×	×	×
49[k]	645.8	(4+)	2579.26	42		360−381	×	×	×
49[l]	649.75	(4+)	2595.26	45		360−381		×	×
50[k]	612.94	(4+)	2448.22	48		361−381		×	
51[k]	588.22	(4+)	2349.15	41		362−381	×	×	×
52[k]	555.97	(4+)	2220.11	49		363−381		×	×
53	558.23	(3+)	1671.85	46		367−381		×	×
53[g]	563.57	(3+)	1687.84	45		367−381	×	×	×
54	697.36	(3+)	2089.04		15	361−378		×	×
55[g]	626.58	(3+)	1876.93	36		363−378	×	×	
56[g]	582.88	(3+)	1745.89	44		364−378		×	×
57[g]	539.94	(3+)	1616.84	38		365−378		×	
58[m]	808.33	(2+)	1614.83	36		364−377		×	×
58[g,m]	816.4	(2+)	1630.82	36		364−377		×	×

[a] Scores obtained in Mascot (absolute scores) and Paragon (relative scores) using the NCBInr database. Results present extensive homology ($p < 0.05$). [b] Molecular ion mass observed in the LC−MS/MS system calculated in Daltons. [c] Calculated relative molecular mass (Da) of the matched peptide. [d] Position of the peptides inside the CK sequence identified for porcine species (NCBI accession number NP_001123421). [e] Peptides marked with a "×" were observed using the corresponding mass spectrometric technique. [f] Peptides identified in at least two of the three hams. [g] One methionine oxidation. [h] Peptides presenting an asparagine deamidation. [i] Aspartic acid dehydratation. [j] Glutamine deamidation. [k] Two methionine oxidations. [l] Three methionine oxidations. [m] A modification of glutamic acid to pyroglutamic in its N-terminal site.

Table 2. Sequences and Fractions of the CK Peptides Identified in Spanish Dry-Cured Ham after Separation by Reversed-Phase Chromatography

Peptide	Identified sequence	RP Fraction*
1	^{15}KAEEEYPDLSKHNNHMAKALTLEIYKKLRDKETPSG^{50}	55
2	^{16}AEEEYPDLSKHNNHMAKALTLEIYKKLRDKETPSG	55
3	^{25}KHNNHMAKALTLEIYKKLRDKETPSG	53
4	^{33}ALTLEIYKKLRDKETPSG	52
5	^{15}KAEEEYPDLSKHNNHMAK^{32}	36
6	^{42}LRDKETPSGFTLDDVIQTGVDNPGHPF^{68}	59
7	^{43}RDKETPSGFTLDDVIQTGVDNPGHPF	59
8	^{52}TLDDVIQTGVDNPGHPF	54
9	^{53}LDDVIQTGVDNPGHPF	52
10	^{54}DDVIQTGVDNPGHPF	48
11	^{55}DVIQTGVDNPGHPF	48
12	^{56}VIQTGVDNPGHPF	45
13	^{44}DKETPSGFTLDDVIQTGVDNPGHP^{67}	55
14	^{45}KETPSGFTLDDVIQTGVDNPGHP	55
15	^{52}TLDDVIQTGVDNPGHP	47
16	^{53}LDDVIQTGVDNPGHP	44
17	^{54}DDVIQTGVDNPGHP	39
18	^{55}DVIQTGVDNPGHP	38
19	^{52}TLDDVIQTGVDNP^{64}	48
20	^{53}LDDVIQTGVDNP	43
21	^{44}DKETPSGFTLDDVIQ^{58}	55
22	^{31}AKALTLEIYKKLR^{43}	44
23	^{106}HKTDLNHENLKGGDDLDPNYV^{126}	43
24	^{109}DLNHENLKGGDDLDPN^{124}	47
25	^{108}TDLNHENLKGGDDLDP^{123}	40
26	^{109}DLNHENLKGGDDLDP	40
27	^{111}NHENLKGGDDLDP	36
28	^{112}HENLKGGDDLDP	36
29	^{108}TDLNHENLKGGDDLD^{122}	38
30	^{109}DLNHENLKGGDDLD	47
31	^{111}NHENLKGGDDLD	36
32	^{95}DRHGGYKPTDKHKTDLNHENL^{115}	40
33	^{89}FDPIIQDRHGGYKPTDKHKTD^{109}	52
34	^{91}PIIQDRHGGYKPTDKHKTD	52
35	^{153}AVEKLSVEALNS^{164}	45
36	AVEKLSVEALN^{163}	45
37	^{303}SKHPKFEEILTR^{314}	48
38	^{299}LAHLSKHPKFEE^{310}	40
39	^{301}HLSKHPKFEE	36
40	^{335}DVSNADRLGSSEVEQVQLVVDGVK^{358}	55
41	^{344}SSEVEQVQLVVDGVK	53
42	^{335}DVSNADRLGSSEVEQVQL^{352}	49
43	DVSNADRLGSSEVEQVQ^{351}	40
44	^{336}VSNADRLGSSEVEQVQ	38
45	^{338}NADRLGSSEVEQVQ	36
46	^{332}SVFDVSNADRLGSSEVEQ^{349}	48
47	^{335}DVSNADRLGSSEVEQ	36
48	DVSNADRLGSSEVE^{348}	36
49	^{360}MVEMEKKLEKGQSIDDMIPAQK^{381}	44,48
50	^{361}VEMEKKLEKGQSIDDMIPAQK	45
51	^{362}EMEKKLEKGQSIDDMIPAQK	44
52	^{363}MEKKLEKGQSIDDMIPAQK	43
53	^{367}LEKGQSIDDMIPAQK	44
54	^{361}VEMEKKLEKGQSIDDMIP^{378}	50
55	^{363}MEKKLEKGQSIDDMIP	50
56	^{364}EKKLEKGQSIDDMIP	49
57	^{365}KKLEKGQSIDDMIP	48
58	^{364}EKKLEKGQSIDDMI^{377}	48,49

* Fractions corresponding to the reversed-phase chromatogram (**Figure 2**).

processing. As can be seen in **Table 2**, peptides 8, 15, 19, 25, and 29 show the loss of threonine at the N-terminal site, whereas peptides 10, 11, 13, 17, 26, 30, and 43 show the loss of aspartic acid. **Table 2** also illustrates the degradation of CK in the release of free lysine, leucine, asparagine, methionine, valine, and glutamic acid from the N-terminal end. Peptides 6, 9, and 16 show the loss of a N-terminal leucine, whereas it is possible to appreciate the loss of methionine in peptides 49 and 55, glutamic acid in peptides 51 and 56, lysine in peptide 1, valine in peptide 50, and asparagine in peptide 27. Peptides 8−12, 15−18, 49−52, and 55−57 show the sequential losses of N-terminal amino acids, proving the important role of muscle aminopeptidases.

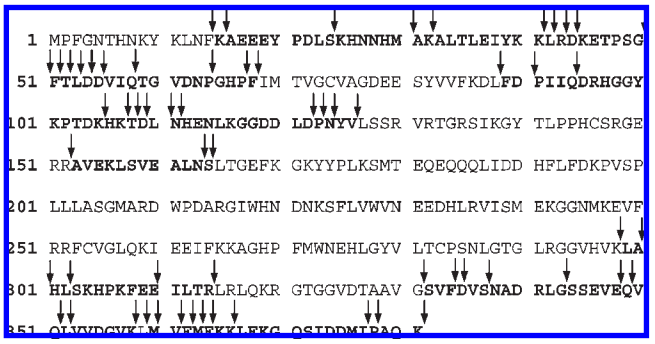
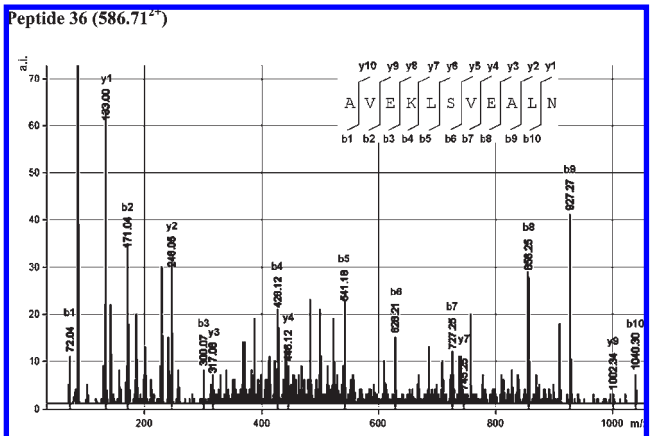


Figure 2. Primary sequence of porcine CK (NCBI nonredundant database accession number NP_001123421). The same sequence also corresponds to the entry KCRM_PIG in the UniProtKB/Swiss-Prot protein database. Cleavage sites of the peptides identified in the present work are indicated with black arrows.



Peptide 36 (586.71^{2+})				
	b	Sequence	y	
1	**72.04**	**A**		11
2	**171.04**	**V**	1101.61	10
3	**300.07**	**E**	**1002.34**	9
4	**428.12**	**K**	873.50	8
5	**541.18**	**L**	**745.25**	7
6	**628.21**	**S**	632.32	6
7	**727.25**	**V**	545.29	5
8	**856.25**	**E**	**446.12**	4
9	**927.27**	**A**	**317.08**	3
10	**1040.30**	**L**	**246.05**	2
11		**N**	**133.00**	1

Figure 3. MS/MS spectrum of ion 586.71^{2+} (peptide 36). 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.

It has been reported that alanyl aminopeptidase (AAP; EC 3.4.11.14) is the most relevant aminopeptidase found in porcine skeletal muscle. It accounts for as much as 83% of the total aminopeptidase activity, showing a broad substrate specificity hydrolyzing aromatic, aliphatic, and basic amino acids from the N-terminal end of peptides. This enzyme hydrolyzes unblocked N-terminal Ala residues as well as Leu, Arg, Phe, Tyr, and Met. Arginyl aminopeptidase (RAP; EC 3.4.11.6), also named aminopeptidase B, is the second most important aminopeptidase existing in skeletal muscle, showing substrate specificity against basic aminoacyl bonds (*30*). Alanyl and arginyl aminopeptidase, which show good stability during dry-curing and have an optimal neutral pH, appear to be the main contributors to the generation of free amino acids during the processing of dry-cured ham (*6*). Two other aminopeptidases existing in skeletal muscle are leucyl aminopeptidases (LAP; EC 3.4.11.1) and pyroglutamyl aminopeptidases (PGAP; EC 3.4.19.3). LAP catalyzes the release of leucine and methionine as well as other hydrophobic amino acids, such as valine, from the N terminus of polypeptides and, despite its optimum basic pH, keeps some of the initial activity at the pH found at the end of the curing process (pH 6). PGAP activity is negligible at about 40 days of processing. However, because of its high specificity toward pyroglutamic acid, it could contribute to the increase in free glutamic acid because of the transformation from pyroglutamic to glutamic acid. Methionyl aminopeptidase (MAP; EC 3.4.11.18), whose activity is enhanced in the presence of NaCl, can also generate free amino acids in cured pork meat products. Methionyl aminopeptidase has a broad substrate specificity, exhibiting maximal activity against Met and good activity against Leu (*31*).

Recently, a novel glutamyl aminopeptidase (aminopeptidase A) has been purified from chicken meat (*32*). This enzyme liberates glutamic or aspartic acid from the N terminus of peptides. However, no information related to its stability during dry-cured processing has been reported to date.

Taking into account all of the experimental data obtained in this study, alanyl aminopeptidase could be one of the enzymes responsible for the loss of N-terminal leucine and methionine in peptides 6, 9, 16, 49, and 55 (**Table 2**). Leucyl aminopeptidase could be responsible for the N-terminal hydrolysis in these peptides as well as the threonine and valine losses in peptides 8, 15, 19, 25, 29, and 50, but this possibility seems to be less likely because of its low activity in the late steps of the dry-curing. On the other hand, methionyl aminopeptidase could also be responsible for the loss of leucine and methionine in the mentioned peptides.

Carboxypeptidases constitute another group of exopeptidases responsible for the hydrolysis of amino acids from the C-terminal site of peptides. The most studied carboxypeptidases are tissue carboxypeptidases A and B and lysosomal carboxypeptidases A and B, but only carboxypeptidases A have been previously studied in skeletal muscle (*33*, *34*). Tissue carboxypeptidase A (EC 3.4.17.1) is most active on hydrophobic C-terminal residues, such as phenylalanine and leucine, although it is unable to hydrolyze proline. At neutral pH, it can also hydrolyze threonine and glutamine. Lysosomal carboxypeptidase A (EC 3.4.16.1) also has a preference for substrates with hydrophobic amino acid residues at their C-terminal site, with phenylalanine and leucine being the most preferred substrates at pH 5.0−5.2. Although little is known about the effect of curing agents on the activity of these enzymes and their stability during the ripening period, it seems that these enzymes could be responsible for the C-terminal loss of phenylalanine in peptides 6−12, leucine in peptide 42, glutamine in peptides 46 and 47 (tissue carboxypeptidase A), and proline in peptides 54−57 (lysosomal carboxypeptidase A).

In conclusion, 58 CK fragments have been found for the first time in Spanish dry-cured ham, proving the intense proteolysis suffered by this protein during the processing of this product. Observing the obtained sequences, there is evidence of the contribution of aminopeptidases and carboxypeptidases to the generation of peptides and the release of free amino acids during dry-cured ham processing. The generated free amino acids will contribute, together with other compounds, to the development of the characteristic flavor in Spanish dry-cured ham. Further studies dealing with the enzymatic action that takes place during dry-curing would be needed to provide a better understanding of muscle proteolysis and flavor generation in these types of products.

ACKNOWLEDGMENT

We give special thanks to the Centro de Investigación Príncipe Felipe (CIPF) Proteomics core facility (Proteored member), Valencia, Spain, for its valuable contribution in the ProteinPilot data analysis.

LITERATURE CITED

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

(2) Toldrá, F.; Flores, M. The role of muscle proteases and lipases in flavor development during the processing of dry-cured ham. *Crit. Rev. Food Sci. Nutr*. **1998**, *38* (4), 331–352.

(3) Nagaraj, N. S.; Santhanam, K. Effects of muscle proteases, endogenous protease inhibitors and myofibril fragmentation on postmortem aging of goat meat. *J. Food Biochem*. **2006**, *30* (3), 269–291.

(4) Lametsch, R.; Karlsson, A.; Rosenvold, K.; Andersen, H. J.; Roepstorff, P.; Bendixen, E. Postmortem proteome changes of porcine muscle related to tenderness. *J. Agric. Food Chem*. **2003**, *51* (24), 6992–6997.

(5) Sentandreu, M. A.; Coulis, G.; Ouali, A. Role of muscle endopeptidases and their inhibitors in meat tenderness. *Trends Food Sci. Technol*. **2002**, *13* (12), 400–421.

(6) Toldrá, F.; Aristoy, M. C.; Flores, M. Contribution of muscle aminopeptidases to flavor development in dry-cured ham. *Food Res. Int*. **2000**, *33* (3−4), 181–185.

(7) Toldrá, F. Proteolysis and lipolysis in flavour development of dry-cured meat products. *Meat Sci*. **1998**, *49*, S101–S110.

(8) Toldrá, F.; Flores, M.; Sanz, Y. Dry-cured ham flavour: Enzymatic generation and process influence. *Food Chem*. **1997**, *59* (4), 523–530.

(9) Fábrega, E.; Manteca, X.; Font, J.; Gispert, M.; Carrión, D.; Velarde, A.; Ruiz-De-La-Torre, J. L.; Diestre, A. Effects of halothane gene and pre-slaughter treatment on meat quality and welfare from two pig crosses. *Meat Sci*. **2002**, *62* (4), 463–472.

(10) Tuomola, M.; Vainio, J.; Lovgren, T. Rapid time-resolved immunofluorometric assay for the measurement of creatine kinase in serum and whole blood samples. *J. Agric. Food Chem*. **2002**, *50* (23), 6659–6662.

(11) Daroit, D. J.; Brandelli, A. Implications of skeletal muscle creatine kinase to meat quality. *J. Anim. Feed Sci*. **2008**, *17* (3), 285–294.

(12) Ryu, Y. C.; Choi, Y. M.; Kim, B. C. Variations in metabolite contents and protein denaturation of the longissimus dorsi muscle in various porcine quality classifications and metabolic rates. *Meat Sci*. **2005**, *71* (3), 522–529.

(13) van de Wiel, D. F. M.; Zhang, W. L. Identification of pork quality parameters by proteomics. *Meat Sci*. **2007**, *77* (1), 46–54.

(14) Sayd, T.; Morzel, M.; Chambon, C.; Franck, M.; Figwer, P.; Larzul, C.; Le Roy, P.; Monin, G.; Cherel, P.; Laville, E. Proteome analysis of the sarcoplasmic fraction of pig semimembranosus muscle: Implications on meat color development. *J. Agric. Food Chem*. **2006**, *54* (7), 2732–2737.

(15) Toyohara, M.; Murata, M.; Ando, M.; Kubota, S.; Sakaguchi, M.; Toyohara, H. Texture changes associated with insolubilization of sarcoplasmic proteins curing salt−vinegar curing of fish. *J. Food Sci*. **1999**, *64* (5), 804–807.

(16) Godiksen, H.; Morzel, M.; Hyldig, G.; Jessen, F. Contribution of cathepsins B, L and D to muscle protein profiles correlated with texture in rainbow trout (*Oncorhynchus mykiss*). *Food Chem*. **2009**, *113* (4), 889–896.

(17) Pérez, A. S.; Ruiz, A. G.; Contreras, C. M.; Ibanez, M. D. C. Separation and identification of sarcoplasmic proteins from hams from three white pig crosses containing Duroc. *Eur. Food Res. Technol*. **2003**, *216* (3), 193–198.

(18) Picariello, G.; De Martino, A.; Mamone, G.; Ferranti, P.; Addeo, F.; Faccia, M.; SpagnaMusso, S.; Di Luccia, A. Proteomic study of muscle sarcoplasmic proteins using AUT−PAGE/SDS−PAGE as two-dimensional gel electrophoresis. *J. Chromatogr., B: Anal. Technol. Biomed. Life Sci*. **2006**, *833* (1), 101–108.

(19) Sentandreu, M. A.; Armenteros, M.; Calvete, J. J.; Ouali, A.; Aristoy, M. C.; Toldrá, F. Proteomic identification of actin-derived oligopeptides in dry-cured ham. *J. Agric. Food Chem*. **2007**, *55* (9), 3613–3619.

(20) Sforza, S.; Boni, M.; Ruozi, R.; Virgili, R.; Marchelli, R. Identification and significance of the N-terminal part of swine pyruvate kinase in aged Parma hams. *Meat Sci*. **2003**, *63* (1), 57–61.

(21) Hughes, M. C.; Kerry, J. P.; Arendt, E. K.; Kenneally, P. M.; McSweeney, P. L. H.; O'Neill, E. E. Characterization of proteolysis during the ripening of semi-dry fermented sausages. *Meat Sci*. **2002**, *62* (2), 205–216.

(22) Stoeva, S.; Byrne, C. E.; Mullen, A. M.; Troy, D. J.; Voelter, W. Isolation and identification of proteolytic fragments from TCA soluble extracts of bovine M-longissimus dorsi. *Food Chem*. **2000**, *69* (4), 365–370.

(23) Farouk, M. M.; Wieliczko, K.; Lim, R.; Turnwald, S.; MacDonald, G. A. Cooked sausage batter cohesiveness as affected by sarcoplasmic proteins. *Meat Sci*. **2002**, *61* (1), 85–90.

(24) Webb, T.; Jackson, P. J.; Morris, G. E. Protease digestion studies of an equilibrium intermediate in the unfolding of creatine kinase. *Biochem. J*. **1997**, *321*, 83–88.

(25) Toldrá, F.; Rico, E.; Flores, J. Cathepsin B, D, H and L activities in the processing of dry-cured ham. *J. Sci. Food Agric*. **1993**, *62* (2), 157–161.

(26) Delbarre-Ladrat, C.; Verrez-Bagnis, V.; Noël, J.; Fleurence, J. Relative contribution of calpain and cathepsins to protein degradation in muscle of sea bass (*Dicentrarchus labrax* L.). *Food Chem*. **2004**, *88* (3), 389–395.

(27) Purintrapiban, J.; Wang, M. C.; Forsberg, N. E. Identification of glycogen phosphorylase and creatine kinase as calpain substrates in skeletal muscle. *Int. J. Biochem. Cell Biol*. **2001**, *33* (5), 531–540.

(28) Rodríguez-Nuñez, E.; Aristoy, M. C.; Toldrá, F. Peptide generation in the processing of dry-cured ham. *Food Chem*. **1995**, *53* (2), 187–190.

(29) Sentandreu, M. A.; Stoeva, S.; Aristoy, M. C.; Laib, K.; Voelter, W.; Toldrá, F. Identification of small peptides generated in Spanish dry-cured ham. *J. Food Sci*. **2003**, *68* (1), 64–69.

(30) Flores, M.; Aristoy, M. C.; Toldra, F. HPLC purification and characterization of porcine muscle aminopeptidase B. *Biochimie* **1993**, *75* (10), 861–867.

(31) Flores, M.; Marina, M.; Toldrá, F. Purification and characterization of a soluble methionyl aminopeptidase from porcine skeletal muscle. *Meat Sci*. **2000**, *56* (3), 247–254.

(32) Maehashi, K.; Abe, T.; Yasuhara, T.; Yamasato, K.; Yamamoto, Y.; Udaka, S. Purification and characterization of a novel glutamyl aminopeptidase from chicken meat. *Meat Sci*. **2003**, *64* (2), 163–168.

(33) Bodwell, J. E.; Meyer, W. L. Purification and characterization of carboxypeptidase A from rat skeletal muscle. *Biochemistry* **1981**, *20* (10), 2767–2777.

(34) Iodice, A. A.; Leong, V.; Weinstock, I. M. Separation of cathepsins A and D of skeletal muscle. *Arch. Biochem. Biophys*. **1966**, *117* (3), 477.

Received for review May 11, 2009. Revised manuscript received July 16, 2009. Accepted July 24, 2009. Food Standards Agency project Q 01R006 (U.K.), a FPU scholarship from the Ministerio de Educación y Ciencia (Spain) to L.M., Grant AGL2007-65379-C02-01/ALI from the Ministry of Education and Science (Madrid, Spain), and FEDER are fully acknowledged.