

Proteomic Identification of Small (<2000 Da) Myoglobin Peptides Generated in Dry-Cured Ham

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Summary

Myoglobin is a very important sarcoplasmic protein responsible for the colour of meat and meat products. This protein is degraded during *post-mortem* period (ageing) as well as during longer processes such as dry curing. In the present study, a total of eleven naturally generated fragments of myoglobin detected at the end of the processing of dry-cured ham have been identified for the first time and the intense proteolysis of myoglobin during dry curing has been confirmed. This study was carried out using one-dimensional reversed-phase liquid chromatography combined with nano liquid chromatography and an electrospray ionization source coupled to a quadrupole time-of-flight mass spectrometer. Paragon and Mascot search engines with the databases NCBI nr and Swiss-Prot were used for the identification based on raw MS/MS data from one or more peptides, and the possible enzymes involved in myoglobin hydrolysis have also been discussed.

Key words: myoglobin, proteolysis, dry-cured ham, peptides, MS/MS ion search, protein identification

Introduction

Dry-cured ham is a traditional high-quality Spanish product that experiences an intense proteolysis during its processing. Such proteolysis is directly related to the development of meat tenderness and is responsible for the unique texture and flavour characteristics of this product at the end of processing (1–3).

Many authors have reported structural alterations and progressive disappearance of some of the most abundant muscular proteins as well as the generation of small peptides during the processing of dry-cured ham. Proteomic techniques such as denaturing electrophoresis on polyacrylamide gels (SDS-PAGE) and matrix-assisted laser desorption/ionization (MALDI) mass spectrometry are commonly used for determination of these changes in meat proteins. In fact, the progressive disappearance of myofibrillar proteins such as myosin light chains 1 and 2, myosin heavy chain, troponin I and C, actin, and troponin (4,5) as well as sarcoplasmic proteins such as

myoglobin and different glycolytic enzymes (6–11) during the dry curing of ham has been widely reported. However, despite the current knowledge regarding the disappearance of proteins and the generation of novel peptides (12,13), it is essential to use the advanced proteomic techniques such as tandem mass spectrometry in order to elucidate the sequence of these small peptides. In fact, specific peptide sequences of the myofibrillar proteins actin (14), titin (15), myosin light chains (15,16), and troponin (17), as well as some sarcoplasmic proteins such as creatine kinase (18) and a group of glycolytic enzymes (19,20) have already been identified in dry-cured ham using quadrupole time-of-flight (QqTOF) and TOF/TOF mass spectrometers.

Myoglobin is presumably the most important protein of sarcoplasm because it is responsible for meat colour, which is associated with the product quality (21). This complex molecule consists of a globular protein portion (globin) and a non-protein portion called haeme

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ring. The haeme portion of the pigment plays a special role in meat colour determined by the oxidation state of iron within the haeme ring. Thus, muscle and meat colour are primarily determined by the concentration and chemical state of the haeme-iron-protein complex (22). In cured meat products, the cured red meat colour depends on the reaction of nitric oxide (NO) with myoglobin to produce nitrosomyoglobin, which gives the characteristic pinkish red colour to dry-cured meat. Nitric oxide is generated from the sodium or potassium nitrate/nitrite added to the curing mixture during dry curing.

The main objective of the present work is the proteomic analysis of small peptides with less than 2000 Da from dry-cured ham to identify degradation products of myoglobin protein as a way to improve the knowledge about the *post-mortem* proteolysis during dry curing of ham and the potential peptidases responsible for this action. The peptides that are generated during proteolysis in the process of dry curing of ham could be biomarkers of the process and, what is more, they could be acting as bioactive peptides such as antimicrobial or antihypertensive agents in the final product. The use of these peptides in both the assessment of the progression of dry curing and the development of their potential as ingredients of healthier food products requires a better knowledge of the mechanisms that control the generation of these peptides during proteolysis, and advanced techniques of proteomics such as tandem mass spectrometry are playing a crucial role in reaching this understanding. This study was carried out using a quadrupole time-of-flight mass spectrometer and the identification was done using Paragon and Mascot search engines and UniProt and NCBI nr databases.

Materials and Methods

Extraction of peptides from dry-cured ham

A total of 50 g of *biceps femoris* muscle from two 8-month-old dry-cured hams were minced and homogenised with 100 mL of 0.01 M HCl in a stomacher apparatus (IUL Instruments, Barcelona, Spain) for 8 min. The mixture was centrifuged at 4 °C and 12 000×g for 20 min and, after filtering through glass wool, the supernatant was deproteinised by adding 3 volumes of ethanol and maintaining the sample at 4 °C for 20 h. After that, the sample was centrifuged again at 12 000×g and 4 °C for 20 min and the supernatant was dried in a rotary evaporator. The remaining residue was redissolved in 25 mL of 0.01 M HCl, filtered through a 0.45-µm nylon membrane filter (Millipore, Bedford, MA, USA) and stored at -20 °C until use.

Isolation of the peptides

A 5-mL aliquot of the deproteinised dry-cured ham extract was subjected to size-exclusion chromatography using a Sephadex G25 column (2.5×65 cm) (Amersham Biosciences, Uppsala, Sweden), previously equilibrated with 0.01 M HCl. Separation was performed under isocratic conditions in 0.01 M HCl at a flow rate of 15 mL/h. Fractions of 5 mL were collected every 20 min using an automatic fraction collector and further monitored by ultraviolet (UV) absorption at 214 nm using an Agilent 8453 UV spectrophotometer (Agilent Technologies, Palo

Alto, CA, USA). Fractions corresponding to a molecular mass 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, by volume). Compounds of different molecular mass such as cytochrome c, bradykinin, tryptophane, and glycine, were used as reference material in the Sephadex G25 column to evaluate the size of the peptides that were eluting.

An aliquot of 100 µL of the redissolved mixture of peptides was injected into an HPLC Agilent 1100 Series system (Agilent Technologies). Separation of peptides was carried out using a Symmetry C18 column (250×4.6 mm, 5 µm particle size; Waters, Milford, MA, USA) 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, by volume). 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-minute 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 lyophilised for identification using tandem mass spectrometry.

Acquisition of mass spectrometry data

Peptides contained in the fractions obtained after reversed-phase chromatography were separated by liquid chromatography and subjected to MS/MS analysis to sequence the peptides using an Ultimate Plus/Famos nano LC system (LC Packings, Dionex, Thermo Scientific, Amsterdam, The Netherlands) and a QSTAR XL hybrid quadrupole TOF instrument (AB SCIEX, Foster CA, USA) equipped with a nanoelectrospray ion source (Protana, Odense, Denmark).

Fractions previously lyophilised were diluted in 60 µL of loading buffer (0.1 % of formic acid and 2 % of acetonitrile in water). Diluted samples were pre-concentrated on a PepMap C18 trap column (0.3×5 mm, 3 µm particle size and 100 Å pore size) from LC Packings (Dionex) at a flow rate of 40 µL/min and using 0.1 % of TFA as mobile phase. After three minutes of pre-concentration, the trap column was automatically switched in-line with a PepMap C18 column (0.075×150 mm, 3 µm particle size and 100 Å pore size). 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 nanoelectrospray ion source (Protana) using a 15-µm PicoTip emitter (SilicaTip needle, New Objective, Inc., Woburn, MA, USA). 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 from 2.5 to 3.0 kV, and nitrogen was used as collision gas at a pressure of 0.544 atm (8 psi). Other source parameters and spray position were optimized with a tryptic digest of protein mixture digest (LC Packings; P/N 161088).

Database search

The identification was done based on raw MS/MS data from the peptides contained in the RP-HPLC fraction. Search of Swiss-Prot (2011_03; 525997 sequences; 185874894 residues) and NCBIInr (20111103; 15916306 sequences; 5467648827 residues) databases was performed using Mascot v. 2.2 in combination with the Mascot Daemon interface v. 2.2.2 (Matrix Science, Inc., Boston, MA, USA, <http://www.matrixscience.com>), and the ProteinPilot v. 3.0 software (ABSCIEX). Mascot.dll v. 1.6b25 and ABSciex.DataAccess.WiffFileDataReader.dll were used for importing data to Mascot and ProteinPilot, respectively.

Results

A total of eleven degradation products corresponding to myoglobin protein were identified by LC-MS/MS in dry-cured ham. Peptides were rearranged and aligned according to their positions in the sequence of porcine myoglobin shown in Fig. 1 (23,24). Table 1 also shows the sequences as well as the observed and calculated masses of the identified peptides, and also the charged state of the corresponding ion detected in the positive ion mass spectra which were further used for MS/MS (low energy CID) experiments and the *post-mortem*

1 MGLSDGEWQL VLN[↓]VWGKVEA DVAGHGQEV[↓]L IRLFKGHPET LEKFDKFK[↓]HL
 51 **KSEDEM**KASE **DLK**KGNTVL TALGGIL**KKK** GHHEAEL**TPL** AQSHATKHKI
 101 PVKYLEFISE AIIQVLQSKH[↓] PGDFGADAQG AMSKALELFR NDMAAKYKEL
 151 GFQG

Fig. 1. Sequence corresponding to porcine myoglobin protein with accession number NP_999401 in NCBIInr database and P02189 (MYG_PIG) in Swiss-Prot database. Arrows indicate the fragmentation points of the peptides identified in previous works (23,24) and letters in bold indicate the peptides identified in this work

degradation modifications of the identified peptides. In this respect, six of the sequenced peptides numbered 1 to 6 show the oxidation of the methionine in position 56 of the sequence (see Fig. 1). Regarding the charge state of the identified peptides, six of them were double charged in the nanoelectrospray ionizator, four were triple charged, and only one was quadruply charged.

The sequenced fragments come from two different sections of the myoglobin sequence, as shown in Fig. 1 in bold letters. Thus, six of the identified fragments share the common sequence SEDEM**KASE**DLK (peptide 6, from position 52 to 63), whereas the other four peptides share the sequence **KKKG**HHEAEL. All identified peptides show important differences in their N- and C-terminal side and cover around 30 % of the total sequence of the myoglobin protein, as shown in Fig. 1. The MS/MS spectra with observed *y* and *b* ions of ion 506.29+ corresponding to peptide 7 GGIL**KKKG**HHEAEL and ion 888.43+ corresponding to peptide 2 H**LK**SEDEM**KASE**DLK are shown in Figs. 2 and 3, respectively.

BLAST sequence similarity searches revealed 100 % homology of the identified peptides to myoglobin sequence with protein accession number NP_999401 in NCBIInr database. Table 2 shows differences in the identification of the generated fragments using Paragon and Mascot search engines as well as NCBI non-redundant and UniProt databases.

Discussion

Muscle sarcoplasmic proteins constitute relevant substrates for proteolysis that occurs during the processing of dry-cured ham. The main enzymes responsible for such proteolysis are muscle endopeptidases and certain groups of exopeptidases. Regarding endopeptidases, as it has been described in previous studies (1,25), cathepsins B (EC 3.4.22.1) and L (EC 3.4.22.15) are still stable at the end of the dry curing process, showing activity even

Table 1. Degradation products of myoglobin protein identified in 8-month dry-cured ham

Peptide number	Sequence ^a	Observed <i>m/z</i> ^b	Modifications	Charged state	Expected <i>m/z</i>	Calculated <i>m/z</i> ^c	Conf. ^d %	Cov. ^e %	Position ^f
1	FKHL KSEDEM KASEDLK KKH	772.76	Met oxidation	(3+)	2315.16	2315.27	99	93	47–65
2	H LK SEDEM KASE DLK	888.43	Met oxidation	(2+)	1774.96	1774.84	95	93	49–63
3	L KSEDEM KASEDLK KKH	635.32	Met oxidation	(3+)	1902.97	1902.93	99	93	50–65
4	L KSEDEM KASEDLK	819.90	Met oxidation	(2+)	1637.89	1637.78	95	79	50–63
5	KSEDEM KASEDLK	763.36	Met oxidation	(2+)	1524.70	1524.70	99	79	51–63
6	SEDEM KASEDLK	699.32	Met oxidation	(2+)	1396.63	1396.60	95	93	52–63
7	GGIL KKKG HHEAEL	506.29		(3+)	1515.90	1515.85	96	93	74–87
8	KKKG HHEAEL TPL	744.42		(2+)	1486.83	1486.82	98	93	78–90
9	KKKG HHEAEL TPL AQSHATK	737.74		(3+)	2210.32	2210.19	96	93	78–97
10	KKKG HHEAEL TPL AQSHATK	553.49		(4+)	2210.32	2210.19	95	93	78–97
11	PL AQSHATK	476.70		(2+)	951.38	951.51	95		89–97

^asequence of the identified peptides that show 100 % homology with the sequences with accession numbers NP_999401 and P02189 of NCBIInr and UniProt databases, respectively; ^bmolecular ion mass observed in the LC-MS/MS system; ^ccalculated relative molecular mass of the matched peptide; ^dpercentage of confidence; ^epercentage of coverage of the identified peptide; ^fposition of the peptides inside the myoglobin sequence identified for porcine species

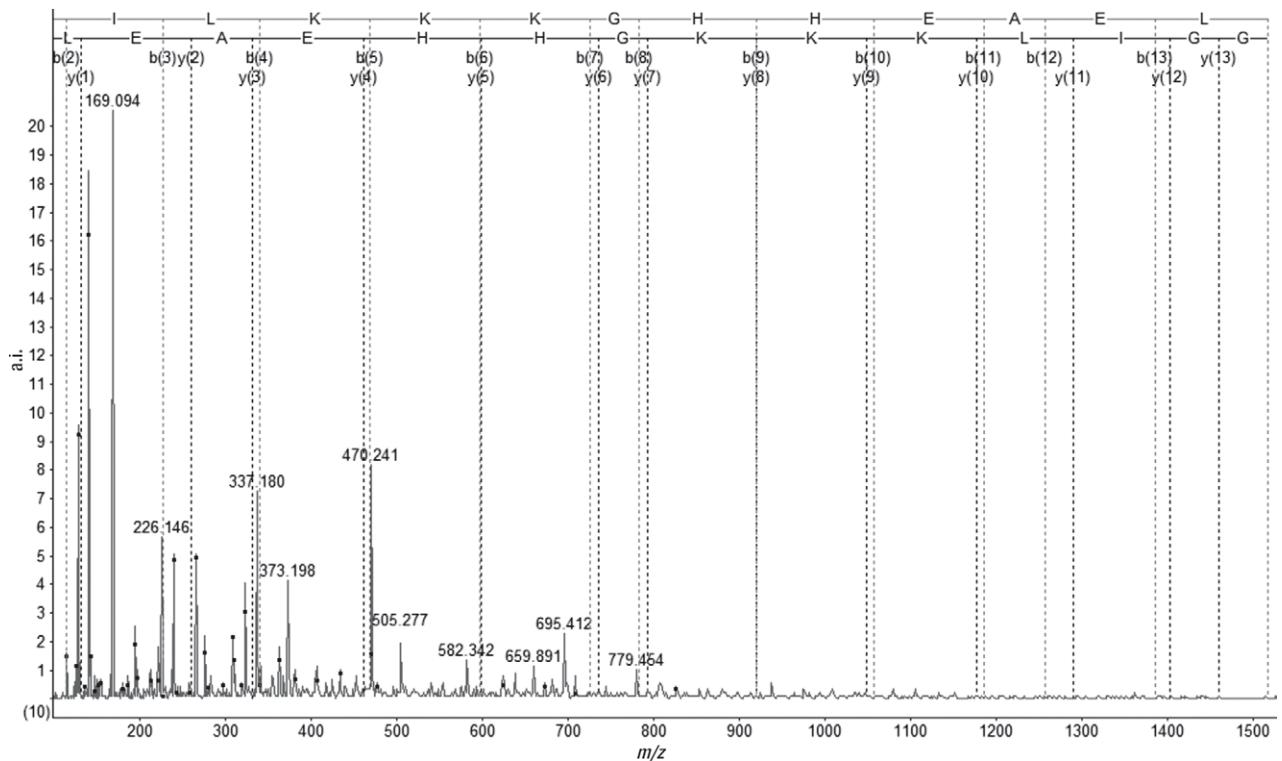


Fig. 2. MS/MS spectrum with the observed *y* and *b* ions of ion 506.29+ corresponding to peptide 7 GGILKKKGHHEAEL obtained using a quadrupole time-of-flight mass spectrometer

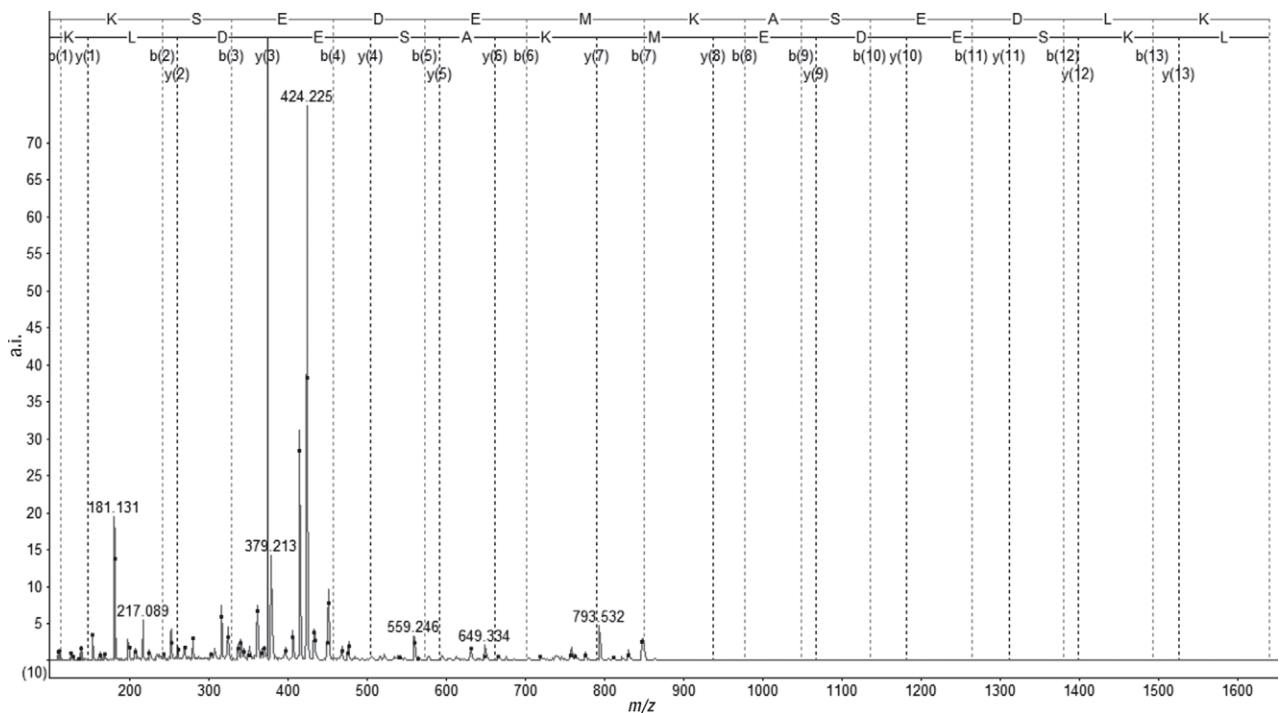


Fig. 3. MS/MS spectrum with the observed *y* and *b* ions of ion 888.43+ corresponding to peptide 2 HLKSEDEMKASEDLK obtained using a quadrupole time-of-flight mass spectrometer

after 15 months of processing. In the case of cathepsin D (EC 3.4.22.17), although its activity in the dry curing is restricted to the first few months of processing (4), its action on dry-cured ham proteolysis seems to be relevant (14).

Myoglobin protein has been found to be degraded during the *post-mortem* storage of pig meat (3) as well as during the processing of dry-cured ham. In this respect, electrophoretic bands for the sarcoplasmic proteins at different stages of the dry curing process of Iberian ham

Table 2. Comparison of the identification of myosin degradation products using different search engines and databases

Peptide number	Identification			
	Paragon ^a		Mascot ^b	
	NCBIInr	Swiss-Prot	NCBIInr	Swiss-Prot
1	—	X	—	—
2	X	X	X	X
3	X	X	—	X
4	—	X	—	X
5	—	X	—	—
6	—	—	—	X
7	X	X	—	—
8	X	X	—	—
9	X	X	X	X
10	—	—	X	—
11	—	—	X	—

^aParagon scores (S) are related to confidence (C) by the formula: $C=(1-10^{-S})\times 100$; ^bIon score is $-10\times \log P$, where P is the probability that the observed match is a random event

were reported until 17.5 months of curing, showing that the levels of myoglobin protein were undetectable after this period of time (11). A similar study was done on Bayonne hams cured for 8 months (6), and also a gradual decrease in the intensity of the electrophoretic band corresponding to myoglobin was detected until the end of curing. A 16-kDa electrophoretic band obtained after SDS-PAGE separation was identified as myoglobin (7) but this band disappeared after 11 months of dry curing and authors suggested that the reason could be that myoglobin protein bound to NO to form nitrosomyoglobin, which is not soluble in aqueous buffers (11). Other authors (8) have also described partial hydrolysis of myoglobin in 12-month dry-cured ham after the analysis of the bands using two-dimensional gel electrophoresis.

The complete sequence of the myoglobin peptides naturally generated during dry curing has not been described previously. However, two myoglobin peptides generated during the ripening of semi-dry fermented sausages (GKVEADVAGH and HAKHPSDFGA from porcine heart and cardiac muscles, respectively) (23), and a longer peptide from raw meat extracts (GLSDGEWQLVLNAWGKVE from bovine myoglobin protein) (24) were sequenced using Edman degradation. The position of these fragments is indicated in Fig. 1 by arrows.

Exopeptidases degrade the large polypeptides generated by endopeptidases, giving rise to small peptides and free amino acids that, together with volatile compounds, are responsible for the characteristic flavour of dry-cured ham (25). Dipeptidyl peptidases (DPP) are enzymes that release different dipeptides from the N-terminus of peptides and show high stability during the processing of dry-cured ham (26). DPP-I preferentially hydrolyzes N-terminal peptides with a penultimate basic residue, but those peptides containing a basic amino acid in the N-terminal position are not hydrolyzed by this enzyme, neither are those containing a proline residue on either side of the peptide bond. Thus, this enzyme could be responsible for the loss of the dipeptide Phe-Lys-X (FK) in peptide 1 due to the basic charge of lysine amino acid as well as the neutral character of phenylalanine amino acid (27). Regarding the other dipeptidyl pepti-

dases, DPP-II and DPP-IV, they preferentially hydrolyse substrates of type X-Pro-, followed by those of type X-Ala-, and DPP-III activity is assayed using the substrate Arg-Arg-AMC, although Ala-Ala-pNa is also hydrolysed by the enzyme (28). Therefore, it seems unlikely that DPP-II, DPP-III or DPP-IV are involved in the generation of the dipeptide Phe-Lys-X (FK) in peptide 1. This is due to the length of the peptide, because the degree of inhibition of DPP-III and DPP-IV depends on the length of the peptides, with the highest inhibition shown by peptides longer than 10 amino acids.

Aminopeptidase activity has been detected in dry-cured ham even after more than 24 months of processing, suggesting that these enzymes may be involved in the later stages of protein degradation, and may be responsible for the increase in free amino acid concentration observed during the dry curing process. Alanyl (AAP; EC 3.4.11.14) and arginyl aminopeptidases (RAP; EC 3.4.11.6), 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. Methionyl aminopeptidase (MAP; EC 3.4.11.18), whose activity is enhanced in the presence of low amounts of NaCl, can also generate free amino acids in cured pork meat products. This enzyme has broad substrate specificity, exhibiting maximal activity in the hydrolysis of N-terminal Met (100 %) and Lys (81 %) (29). Table 1 shows the loss of His (H) N-terminal amino acid in peptide 1 as well as the loss of Leu (L) amino acid in peptides 2 and 3, and the loss of Lys (K) in peptide 4. Considering the stability of aminopeptidases during dry curing, alanyl, arginyl and methionyl aminopeptidases could be responsible for the loss of these N-terminal amino acids.

Carboxypeptidases constitute another group of exopeptidases responsible for the hydrolysis of amino acids from the C-terminal side of peptides. This group of enzymes has not been studied so well as aminopeptidases and the effect of curing agents on their stability during processing remains quite unknown (30). Despite this, these enzymes could be responsible for the consecutive loss of C-terminal amino acids Lys (K), Leu (L), and His (H) in peptide 2, as it is shown in Table 1.

The identification of the peptides was done using Mascot and Paragon search engines with the database search approaches of NCBIInr and UniProt. The choice of Mascot search parameters requires some user expertise, and has to be previously evaluated with a control sample. When using ProteinPilot software it is not necessary to define mass tolerance or possible modifications because in Paragon algorithm, features such as modifications, substitutions, and cleavage events are modelled with probabilities rather than by discrete user-controlled settings to consider or not consider a feature. Thus, the algorithm removes the user expertise requirement (20). Results from Mascot and ProteinPilot were accepted at a confidence level greater than 95 %. Table 1 shows the level of confidence for the individual peptides together with their percentage of coverage. Many complementary results are obtained when using both engines and different databases such as NCBIInr and Swiss-Prot (see Table 2). According to the results observed in Table 2, the identification using Paragon search engine resulted

in the assignment of 8 myoglobin peptides, whereas the identification using Mascot resulted in the assignment of 7 peptides. A total of 10 peptides were identified with both search engines.

Regarding the protein databases, Paragon search engine was used in NCBIInr and Swiss-Prot databases, and a total of 5 sequences of myoglobin peptides were identified in NCBIInr, whereas 8 sequences were identified in Swiss-Prot database (see Table 2). All peptides identified in NCBIInr were also identified in Swiss-Prot. On the other hand, Mascot search engine was also tested, and the identification of 4 and 7 sequences of peptides using NCBIInr and Swiss-Prot databases, respectively, was also obtained. However, two sequences obtained with Mascot search engine coincided with those obtained with the NCBIInr and Swiss-Prot databases.

A previous work with glycolytic enzyme fragments derived from dry curing of ham compared the results obtained with Paragon and Mascot search engines and complementary results were obtained combining both search engines with different databases (17).

Conclusions

In this study, a total of eleven fragments generated from myoglobin protein have been identified for the first time in dry-cured ham, proving the intense proteolysis suffered by this protein during the processing of this product. The obtained results suggest the contribution of muscle cathepsins and exopeptidases such as dipeptidyl peptidase I (DPP-I) to the generation of the identified peptides as well as alanyl and arginyl amino peptidases to the increase of free amino acids during ripening. This study also showed that the use of different search engines in combination with different databases provides an increase of assigned peptides, resulting in more identifications.

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