Peptides naturally generated from ubiquitin-60S ribosomal protein as potential biomarkers of dry-cured ham processing time

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Abstract

Proteolysis is a complex and dynamic process which takes place throughout the whole dry-cured processing due to the action of endogenous muscle peptidases, and results in the generation of a high number of small peptides and free amino acids responsible for the final quality of dry-cured ham. In this study, a total of sixty-eight peptides derived from the ubiquitin-60S ribosomal protein have been identified in dry-cured ham at 2, 3.5, 5, 6.5, and 9 months of processing using various chromatographic separations and a quadrupole/time-of-flight mass spectrometer in tandem. Some of the identified peptides have been detected during the whole process, whereas a total of fourteen of them were exclusively identified at 9 months of curing. The presence of any of these peptides could be a good indicative that dry-cured ham pieces have reached a minimum curing process of 9 months. The study of the generated peptides has contributed both to a better knowledge of proteolysis evolution and the endogenous enzymes participating, and to determine their potential to be used as quality markers to control the processing time.

Keywords: Dry-cured ham, peptides, proteolysis, mass spectrometry, biomarker, ubiquitin protein.

1. Introduction

Protein degradation is a phenomena that occurs both *in vivo*, as one of the tactics employed by the cell for irreversible inactivating the proteins, and post-mortem, which has been widely reported during the last decades due to its importance in the final quality characteristics of meat and meat-derived products. There are two major pathways to control the destruction of intracellular proteins *in vivo*, the ubiquitin proteasome system (UPS), and the autophagy lysosome system (Wustrow, Zhou, & Rolfe, 2013). In the UPS, the proteins that are destined for degradation are targeted to the 26S proteasome by covalent attachment of a multi-ubiquitin chain. As a result, an enzymatic cascade that includes two ubiquitin-activating enzymes (E1s), approximately forty ubiquitin conjugating enzymes (E2s), and more than five hundred ubiquitin ligases (E3s) occurs. In the final step of this cascade, is the ubiquitin ligase the enzyme that controls the specificity of the ubiquitination reaction. Different studies suggested that the UPS plays an important role in the degradation of muscle proteins under various catabolic conditions, and the degradation of ubiquitin in *quadriceps femoris* beef muscle, that almost disappear at 10 days post-mortem, has been previously reported (Sekikawa, Seno, & Mikami, 1998).

The natural protein degradation occurred in the post-mortem period during the maturation or curing processes of meat has been widely reported during the last decade. In this respect, fragments of peptides and amino acids are generated in large amounts from the progressive enzymatic degradation of sarcoplasmic and myofibrillar proteins, contributing to flavour, texture, and final quality of meat-derived products such as dry-cured ham (Aristoy & Toldrá, 1995; Toldrá & Flores, 1998; Lametsch, Karlsson, Rosenvold, Andersen, Roepstorff & Bendixen, 2003).

The processing of dry-cured ham is very long, lasting up to 24 months in the highest quality pieces, and its final economic value is mainly given according to the porcine breed and the time of curing. Analytical methods to control the time of curing and the final quality of dry-cured ham would be very useful to assure that fraudulent or accidental mislabeling does not arise, especially when sold as sliced ham packages. The advances carried out during the last years in the techniques used in the separation of small peptides as well as the experimental design used in proteomic researches, have allowed an increase in the knowledge of the specific peptide sequences generated as well as its tracking during the whole curing process. This fact could permit the control of the dry-cured processing through the study of some of the identified peptide sequences as markers of the time of curing. Recently, a total of 107 sequences of peptides naturally generated from the degradation of the LIM domain-binding protein 3 at different times of curing during the processing of dry-cured ham have been identified using an ion trap mass spectrometer (Gallego, Mora, Fraser, Aristoy, & Toldrá, 2013) and its potential as markers of time of processing discussed.

In the present study, ubiquitin-60S ribosomal protein degradation has been studied at different times during the dry-cured ham processing (2, 3.5, 5, 6.5, and 9 months). The sequences of the identified peptides will allow to study its evolution through the process as well as to elucidate the possible proteolytic enzymes that are responsible for its generation. Finally, the potential of some of the identified peptides to be used as markers to control the processing time is discussed.**2. Materials and methods**

2.1 Spanish dry-cured ham processing

This study was done by triplicate using raw hams from 6 months old pig (Landrace x Large White) for the production of Spanish dry-cured ham according to the methodology described by Gallego et al. (2013). Briefly, hams were bled and prepared according to traditional

procedures consisting on the pre-salting stage for 30 min; the salting period where hams were entirely buried in salt and placed in a cold room for 10-12 days at 2-4 ºC and 90-95% relative humidity; the post-salting stage where hams were kept for 60 days at 4-5 ºC and 75-85% relative humidity; and the ripening-drying period at temperatures increasing from 5 ºC to 14-20 ºC and relative humidity decreasing to 70%. The total length of the dry-curing process was 9 months. Samples were taken at 2 months, 3.5 months, 5 months, 6.5 months, and 9 months of processing.

2.2 Extraction of peptides and deproteinisation

Fifty g samples of *Biceps femoris* muscle coming from each sampling time of the processed Spanish dry-cured hams were minced and homogenised with 200 mL of 0.01 N HCl for 8 minutes in a stomacher (IUL Instrument, Barcelona, Spain). The homogenate was centrifuged at 4 ºC and 12000 *g* for 20 min. The resulting supernatant was filtered through glass wool and deproteinised by adding 3 volumes of ethanol maintaining the sample at 4 ºC for 20 hours. Finally, the sample was centrifuged again at 4 ºC and 12000 *g* for 10 min and the supernatant was dried in a rotatory evaporator. The dried deproteinised extract was dissolved in 25 mL of 0.01 N HCl, filtered through a 0.45 µm nylon membrane filter (Millipore, Bedford, MA) and stored at -20 ºC until use.

2.3 Size-exclusion chromatography

Size-exclusion chromatography was used to fractionate deproteinised cured ham extracts according to molecular mass. A 5 mL aliquot of each extract filtered through a 0.45 µm nylon membrane filter (Millipore) was injected in a Sephadex G25 column (2.5 x 65 cm, Amersham Biosciencies, Uppsala, Sweden), previously equilibrated with 0.01 N HCl. The separation was performed using 0.01 N HCl as mobile phase, at a flow rate of 15 mL/h and 4 ºC, and fractions of 5 mL were collected using an automatic fraction collector and further monitored

by ultraviolet absorption at 214 nm (Ultrospec 3000 UV/Visible spectrophotometer, Pharmacia Biotech, Cambridge, England). Fractions corresponding to elution volumes from 125 to 160 mL were pooled together and aliquots of 100 μL were lyophilised for the mass spectrometry analysis.

2.4 Peptide identification by mass spectrometry in tandem (nESI-LC-MS/MS)

The nanoLC-MS/MS analysis was performed using an Eksigent Nano-LC Ultra 1D Plus system (Eksigent of AB Sciex, CA, USA) coupled to the quadrupole-time-of-flight (Q-ToF) TripleTOF® 5600+ system from AB Sciex Instruments (Framingham, MA, USA) that is equipped with a nanoelectrospray ionization source.

Lyophilised samples were resuspended in 100 μL of H_2O with 0.1% of trifluoroacetic acid (TFA). Twenty microlitres of each sample at different times of processing were cleaned and concentrated using Zip-Tip C18 with standard bed format (Millipore Corporation, Bedford, MA) according to manufacturer's instructions and kept at -20ºC until analysis. Five microlitres of the supernatant were injected into the LC-MS system through the autosampler.

Samples were then preconcentrated on an Eksigent C18 trap column (3μ, 350μm x 0.5mm) (Eksigent of AB Sciex, CA, USA), at a flow rate of 3 μL/min and using 0.1% v/v TFA as mobile phase. After 5 min of preconcentration, the trap column was automatically switched in-line onto a nano-HPLC capillary column (3μm, 75μm x 12.3 cm, C18) (Nikkyo Technos Co, Ltd. Japan). The mobile phases consisted of solvent A, containing 0.1% v/v FA in water, and solvent B, containing 0.1% v/v FA in 100% acetonitrile. Chromatographic conditions were a linear gradient from 5% to 35% of solvent B over 90 min, and 10 min from 35% to 65% of solvent B, at a flow rate of 0.30 μL/min and running temperature of 30 ºC.

The outlet of the capillary column was directly coupled to a nano-electrospray ionisation system (nano-ESI). The Q/ToF was operated in positive polarity and information-dependent acquisition mode, in which a 0.25-s ToF MS scan from *m/z* of 300 to 1250 was performed, followed by 0.05-s product ion scans from *m/z* of 100 to 1500 on the 50 most intense 1 to 5 charged ions.

2.5 Data analysis

Automated spectral processing, peak list generation, and database search were performed using Mascot Distiller v2.4.2.0 software (Matrix Science, Inc., Boston, MA) (hppt://www.matrixscience.com). The identification of protein origin of peptides was done using UniProt protein database, with a significance threshold $p<0.05$ and a FDR of 0.5. The tolerance on the mass measurement was 0.3 Da in MS mode and 0.3 Da for MS/MS ions.

3. Results and discussion

Sample extracts were deproteinised by precipitation with ethanol and fractionated by size-exclusion chromatography. Those fractions corresponding to the first eluted peak (a total of 7 fractions of 5 mL) were pooled together and lyophilised for nESI-LC-MS/MS mass spectrometry identification of their peptidic content.

A total of sixty-eight peptides generated from ubiquitin-60S ribosomal protein have been identified at five different times of processing. The sequences of the identified peptides together with the *m/z* obtained in the detector as well as the molecular mass calculated according to the charge of ionisation are reported in **Table 1**. The time of processing when the peptides have been identified is also indicated in order to facilitate the tracking of the sequences.

Figure 1 shows the sequence of the ubiquitin-60S ribosomal protein, and peptides identified are indicated with black arrows. The identified peptides cover a 56% of the studied sequence with accession number P63053 according to UniProtKB/Swiss-Prot database. All of them have been identified between positions 3 and 74 of this sequence. Although the ubiquitin system of cellular protein degradation has been widely investigated in the fields of clinical medicine and cellular biology, there are not many studies about the state of this protein in post-mortem muscle (Sekikawa et al., 1998), and to our knowledge, no reports regarding naturally generated ubiquitin peptides have been previously published.

Enzymes involved in the extense proteolysis occurred during the processing of dry-cured ham are muscle endopeptidases such as cathepsins and calpains, which hydrolyse proteins giving rise to large polypeptides; and exopeptidases like aminopeptidases, carboxypeptidases, peptidyl peptidases and peptidases, which degrade polypeptides to small peptides and free amino acids (Toldrá & Flores, 1998; Toldrá, Aristoy & Flores, 2000). Cathepsins are stable during the full process (Toldrá, Rico & Flores, 1993), although cathepsin D activity disappears after 6 months of processing (Toldrá et al., 1993). Calpains are inactivated after the salting stage (Rosell & Toldrá, 1996), and regarding exopeptidases, only the activity of aminopeptidases and dipeptidyl peptidases have been studied along the curing process, showing good stability during this period of time (Toldrá, Flores & Sanz, 1997; Toldrá et al., 2000; Sentandreu & Toldrá, 2001).

Evidences about the activity of exopeptidases in the generation of the peptides identified from position 3 to position 74 of the ubiquitin-60S ribosomal protein sequence are shown in **Figure 1**, with the consecutive loss of amino acids, dipeptides, and tripeptides, from both terminal sites. These results are better appreciated in **Table 1**, where the sequences of the identified peptides have been aligned according to the sequence. In this sense, previous studies discuss the role of these exopeptidases in the generation of small peptides and amino acids at the end

of the dry-curing process in proteins such as actin (Sentandreu, Armenteros, Calvete, Ouali, Aristoy & Toldrá, 2007), myosin light chain and titin (Mora, Sentandreu, Koistinen, Fraser, Toldrá & Bramley, 2009), troponin T (Mora, Sentandreu & Toldrá, 2010), sarcoplasmic proteins like creatine kinase (Mora, Sentandreu, Fraser, Toldrá & Bramley, 2009), myoglobin (Mora & Toldrá, 2012) or glycolytic enzymes (Mora, Valero, Sánchez del Pino, Sentandreu & Toldrá, 2011). In this respect, the identification of the sequences of these small naturally generated peptides has become only possible through the use of advanced proteomic techniques such as tandem mass spectrometry like Time-of-Flight (ToF-ToF), ion trap (Q-Trap), and Quadrupole/Time-of-Flight mass spectrometers.

Table 1 shows the time of processing, in months, when the different peptide sequences have been detected. This information reveals the complexity, dynamism, and variability of proteolysis phenomena as a result of the random action of muscle peptidases, that result in the generation of a complex mixture of peptides with unspecified cleavage sites (Gallego et al., 2013). The smallest peptides such as dipeptides or tripeptides that result from the degradation of ubiquitin protein are not able to be identified under the conditions specified in the MS analysis as it would be difficult to relate their sequence to a specific protein origin due to the high amount of possible matches when considering the whole pig proteome. This fact would explain the unidentification of the peptides MQ, MQI or MQIF from position 1-2, 1-3, and 1-4 of ubiquitin sequence, respectively, although it would be expected to have them in the extract of dry-cured ham.

A total of sixteen of the identified peptides remain stable during the 9 months of the studied processing period (**Table 1**). However, other peptides remain intact only during the first 5 months of processing and disappear at 6.5 and 9 months (peptides 17 and 18), whereas fourteen of the peptides (from peptide 52 to peptide 65) appear only at 9 months of processing. As an example, **Figure 2** shows the MS/MS spectra of peptides 52 and 53, where

it is shown the loss of the dipeptide KA due to the action of exopeptidases in the C-terminal site. Moreover, these peptides could be a good indicative for a minimum curing time of 9 months. This fact suggests the potential of some of the identified peptides, derived from the natural degradation of ubiquitin protein, to be used as biomarkers in this type of product with the aim to give a good estimation and control of the time of processing.

4. Conclusions

An increase in the knowledge of the specific peptide sequences generated from muscle proteolysis has been achieved during the last years. This situation has been possible through the use of modern advances carried out in separation techniques for small peptides, and proteomic experimental design, which have been used to identify natural peptides generated during the curing process. This fact could permit the control of the dry-cured processing through the study of some of the identified peptide sequences as markers of the time of curing. In fact, a better control of the processing chain of dry-cured ham would guarantee its safety and quality, making them more suitable for export trade, betting for the consumer protection. The ubiquitin peptides identified in this study provide an evidence of the action of aminopeptidases, carboxypeptidases, and di- and tripeptidyl peptidases to the release of peptides and free amino acids during the processing of dry-cured ham, and some of the identified sequences have resulted to be detected only at 9 months of processing, which indicates the potential of the peptides generated during the dry-curing to be used as markers of the time of processing.

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FIGURE CAPTIONS

Figure 1. Primary sequence of ubiquitin 60S ribosomal protein from *Sus scrofa* (UniprotKB/Swiss-Prot protein database accession number P63053). Cleavage sites of the identified peptides at each time of processing are indicated with black arrows.

Figure 2. MS/MS spectra of ions 616.29^{2+} and 387.72^{2+} that correspond to peptides 52 and 53, respectively, detected at 9 months of dry-cured ham processing.

Figure 1

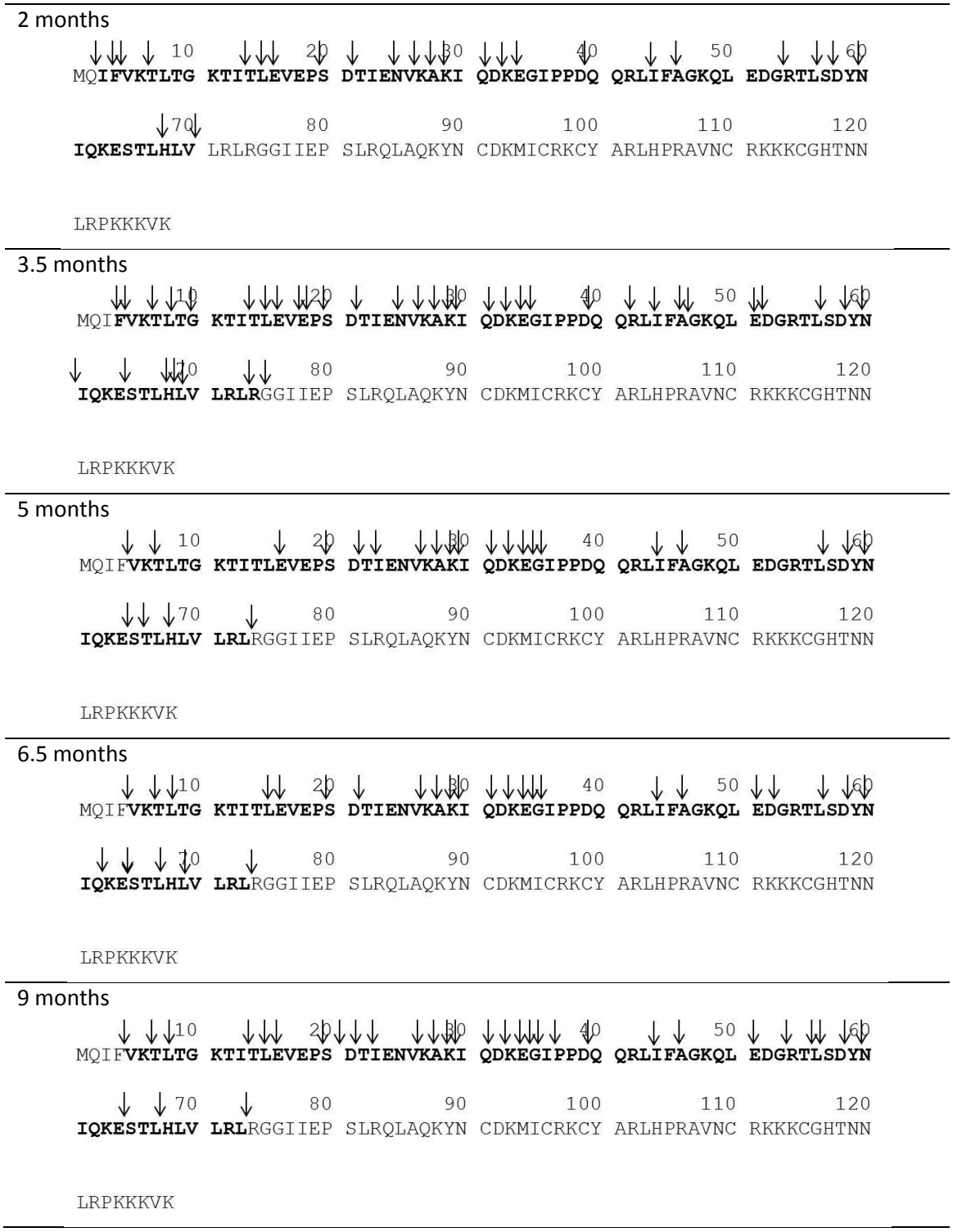


Figure 1. Mora et al.

Table 1

Table 1- Peptides identified by nESI-LC-MS/MS derived from ubiquitin 60S ribosomal protein at different times of dry-cured ham processing.

	Obtained	Charge	Calculated				Time of processing (months)[c]				
Pept.	(*m/z*)[a]	(+)	(*Mr*)[b]	P_0	Sequence	P_f	2	3.5	5	6.5	9
				3'	**IFVKTLTGKTITLEVEPSDTIENVKAKIQDKEGIPPDQQRLIFAGKQLEDGRTLSDYNIQKESTLHLVLRLR**	74'					
1	423.77	2	845.52	T	LTGKTITL	E					
2	587.87	2	1173.73	F	VKTLTGKTITL	E					
3	900.46	2	1798.93	L	EVEPSDTIENVKAKIQ	D					
4	957.98	2	1913.96	L	EVEPSDTIENVKAKIQD	K					
5	511.52	4	2042.05	L	EVEPSDTIENVKAKIQDK	E					
6	680.33	2	1358.66	L	EVEPSDTIENVK	A					
7	715.85	2	1429.69	L	EVEPSDTIENVKA	K					
8	487.59	3	1459.75	P	SDTIENVKAKIQD	K					
9	491.77	4	1963.09	V	KAKIQDKEGIPPDQQRL	I					
10	612.67	3	1834.99	K	AKIQDKEGIPPDQQRL	I					
11	514.28	3	1539.84	D	KEGIPPDQQRLIF	A					
12	576.80	2	1151.59	K	EGIPPDQQRL	I					
13	640.85	2	1279.69	D	KEGIPPDQQRL	I					
14	698.36	2	1394.72	Q	DKEGIPPDQQRL	I					
15	649.31	2	1296.62	L	SDYNIQKESTL	H					
16	548.29	2	1094.56	D	YNIQKESTL	H					
17	483.27	3	1446.78	L	IFAGKQLEDGRTL	S					
18	604.97	3	1811.91	L	IFAGKQLEDGRTLSDY	N					
19	636.13	5	3175.64	L	EVEPSDTIENVKAKIQDKEGIPPDQQRL	I					
20	661.40	2	1320.80	I	FVKTLTGKTITL	E					
21	726.41	3	2176.20	E	NVKAKIQDKEGIPPDQQRL	I					
22	367.27	2	732.44	K	TLTGKTI	T					
23	531.33	2	1060.65	F	VKTLTGKTIT	L					
24	661.45	2	1320.80	Q	IFVKTLTGKTIT	L					
25	719.78	2	1437.78	V	KAKIQDKEGIPPD	Q					
26	404.25	5	2016.06	G	RTLSDYNIQKESTLHLV	L					
27	321.24	3	960.55	L	TGKTITLEV	E					
28	495.19	2	988.54	T	GKTITLEVE	P					
29	588.99	3	1763.95	A	KIQDKEGIPPDQQRL	I					
30	587.74	2	1173.69	Q	RLIFAGKQLE	D					

(Table 1 continued)

31	767.91	2	1533.82	L	IFAGKQLEDGRTLS	D					
32	659.83	2	1317.65	A	GKQLEDGRTLSD	Y					
33	449.24	3	1344.70	D	YNIQKESTLHL	V					
34	616.82	2	1231.62	D	YNIQKESTLH	L					
35	302.87	3	905.59	L	HLVLRLR	G					
36	375.75	2	749.49	L	HLVLRL	R					
37	397.71	2	793.40	D	YNIQKE	S					
38	779.90	2	1557.79	L	EVEPSDTIENVKAK	I					
39	512.28	2	1022.55	E	GIPPDQQRL	I					
40	483.77	2	965.53	G	IPPDQQRL	I					
41	449.25	3	1344.72	P	SDTIENVKAKIQ	D					
42	381.89	3	1142.67	D	TIENVKAKIQ	D					
43	629.85	2	1257.69	D	TIENVKAKIQD	K					
44	474.29	2	946.57	K	LTGKTITL	E					
45	736.87	2	1471.74	T	LEVEPSDTIENVK	A					
46	528.23	2	1054.46	L	EDGRTLSDY	N					
47	550.62	3	1648.84	L	IFAGKQLEDGRTLSD	Y					
48	466.75	2	931.50	Y	NIQKESTL	H					
49	348.21	3	1041.62	T	IENVKAKIQ	D					
50	462.93	3	1385.79	D	TIENVKAKIQDK	E					
51	367.22	2	732.44	L	TGKTITL	E					
52	616.29	2	1230.56	L	EVEPSDTIENV	K					
53	387.72	2	773.43	D	TIENVKA	K					
54	420.24	3	1257.69	S	DTIENVKAKIQ	D					
55	427.23	2	852.45	I	PPDQQRL	I					
56	613.85	2	1225.68	G	IPPDQQRLIF	A					
57	642.35	2	1282.70	E	GIPPDQQRLIF	A					
58	552.63	3	1654.87	Q	DKEGIPPDQQRLIF	A					
59	1020.00	2	2038.08	K	EGIPPDQQRLIFAGKQLE	D					
60	706.88	2	1411.75	K	EGIPPDQQRLIF	A					
61	584.31	2	1166.60	D	KEGIPPDQQR	L					
62	879.95	2	1757.95	D	QQRLIFAGKQLEDGR	T					

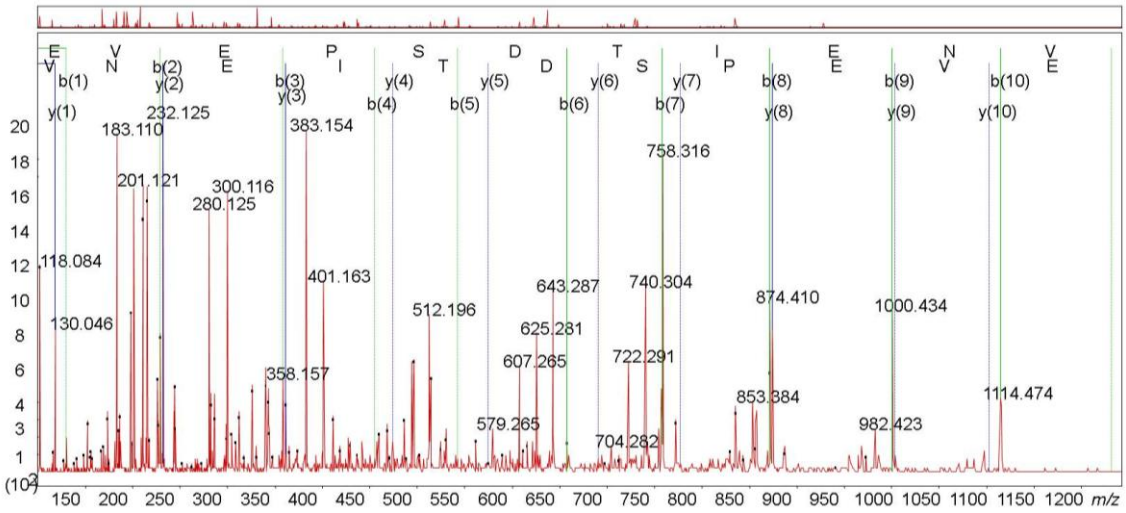
(Table 1 continued)

63	518.27	3	1551.75	F	AGKQLEDGRTLSDY	N					
64	555.27	2	1108.54	T	LSDYNIQKE	S					
65	377.69	2	753.37	G	RTLSDY	N					
66	406.20	2	810.39	D	GRTLSDY	N					
67	414.23	2	826.45	Q	KESTLHL	V					
68	441.22	2	880.43	D	YNIQKES	T					

a. Molecular ion mass observed in the nESI-LC-MS/MS analysis; b. Calculated molecular mass in Da of the matched peptide; c. Peptides identified at each time of curing.

Table 2

Peptide 52: **EVEPSDTIENV** (616.29, 2+)



Peptide 53: **TIENVKA** (387.72, 2+)

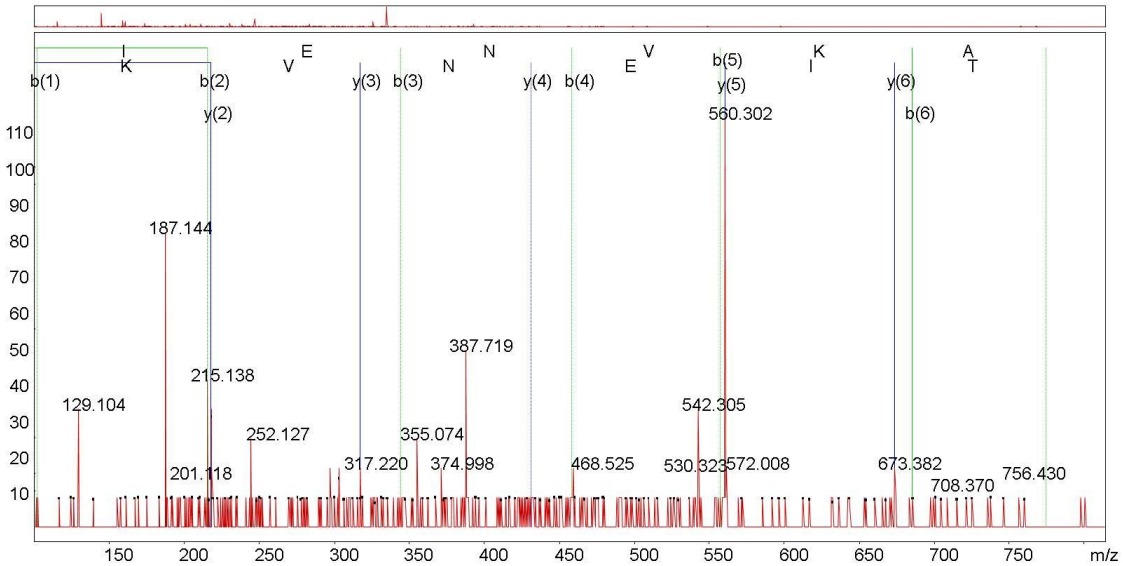


Figure 2. Mora et al.

Highlights

Novel peptides extracted, purified and identified in Spanish dry-cured ham

Ham peptides analysed through advanced proteomics tools

Natural generated peptides as potential biomarkers of dry-cured ham processing