**Degradation of LIM domain-binding protein three during Spanish
dry-cured ham processing**

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Abstract

Extensive proteolysis takes places during the processing of dry-cured ham due to the action of muscle peptidases. The aim of this work was to study the degradation of LIM domain binding protein 3 (LDB3), which is located at the Z-lines of the sarcomere, at different times during the Spanish dry-cured ham processing (2, 3.5, 5, 6.5, and 9 months). A total of 107 peptides have been identified by mass spectrometry, most of them generated from the first region of the protein sequence (position 1 to 90) providing evidence for the complexity and variability of proteolytic reactions throughout the whole process of dry-curing. Methionine oxidation has been observed in several peptides by the end of the process. The potential of some of the identified peptides to be used as biomarkers of dry-cured ham processing has also been considered.

Keywords: Dry-cured ham, LIM domain-binding protein 3, peptides, proteolysis, mass spectrometry, biomarker, peptide oxidation.

1. Introduction

Spanish dry-cured ham is a high-quality product for its organoleptic and nutritional characteristics. During the dry-curing process of ham occurs a series of biochemical reactions among which proteolysis of muscle proteins is one of the most important. Peptides and free 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 dry-cured ham (Aristoy & Toldrá, 1995; Toldrá & Flores, 1998; Lametsch, Karlsson, Rosenvold, Andersen, Roepstorff & Bendixen, 2003).

LDB3 protein, also called Z-band Alternatively Spliced PDZ-motif protein (ZASP) or protein cypher, is located in the sarcomere and it is essential for maintaining Z line structure and muscle integrity (Zhou et al., 2001). Although it is a minor component of the Z line (about 1 per 400 α -actinin), this protein is very important for myofibrillar development and mechanotransduction during muscle contraction (Leung, Hitchen, Ward, Messer & Marston, 2013). Thus, LDB3 may act as an adapter in signaling of the striated muscle, as it binds protein kinase C via its three C-terminal LIM domains to the cytoskeleton, and it may also interact via its N-terminal PDZ domain with α -actinin-2, which helps anchor the myofibrillar actin filaments providing mechanical strength to the Z-lines (Klaavuniemi, Kelloniemi & Ylänné, 2004; Luther, 2009).

Enzymes involved in protein changes are muscle endopeptidases such as cathepsins and calpains, which hydrolyse sarcoplasmic and myofibrillar proteins giving rise to large polypeptides, and exopeptidases like aminopeptidases, carboxypeptidases, peptidyl peptidases and peptidases, which degrade polypeptides to smaller peptides and free amino acids (Toldrá & Flores, 1998; Toldrá, Aristoy & Flores, 2000).

Protein degradation as well as the generation of several peptides and free amino acids during dry-cured ham processing have been described in some studies, in which

electrophoretic techniques such as sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) or high performance liquid chromatography (HPLC) have been used to separate the protein fragments for their subsequent identification (Rodríguez-Núñez, Aristoy & Toldrá, 1995; Hansen-Møller, Hinrichsen & Jacobsen, 1997; Sentandreu, Stoeva, Aristoy, Laib, Voelter & Toldrá, 2003). However, the identification of naturally generated peptides has become possible only through the use of the latest generation proteomic techniques such as tandem mass spectrometry. In fact, recent proteomic studies have identified peptides derived from myofibrillar proteins such as actin, myosin light chain, titin, or troponin T (Luccia et al., 2005; Sentandreu, Armenteros, Calvete, Ouali, Aristoy & Toldrá, 2007; Mora, Sentandreu, Koistinen, Fraser, Toldrá & Bramley, 2009; Mora, Sentandreu & Toldrá, 2010), and sarcoplasmic proteins like creatine kinase, myoglobin or glycolytic enzymes (Mora, Sentandreu, Fraser, Toldrá & Bramley, 2009; Mora, Valero, Sánchez del Pino, Sentandreu & Toldrá, 2011; Mora & Toldrá, 2012). Some studies have investigated how some muscle compounds such as the ratio hypoxanthine/inosine could be possible markers of the ripening time (Escudero, Mora, Aristoy & Toldrá, 2011). However, according to our knowledge, to date there are no studies based on the evolution of naturally generated peptides from proteins degradation during the dry-curing process.

The main goal of this study is the proteomic characterization of LDB3 protein to identify the peptides naturally generated at different times (2, 3.5, 5, 6.5, and 9 months) during the dry-cured ham processing in order to study its evolution and describe some of the proteolytic changes taking place during the process. Additionally, the potential of some of the identified peptides as biomarkers for processing time has been also studied.

2. Materials and methods

2.1 Dry-cured ham preparation

This study was done by triplicate using raw hams from 6 months old pigs (Landrace x Large White) for the production of Spanish dry-cured ham. Hams were directly provided under refrigeration from the slaughterhouse (Vaquero Meat Industry, Madrid, Spain) at 24 hour post-mortem. Once received, hams were bled and prepared in our pilot plant according to traditional procedures consisting on the pre-salting stage using a mixture of salt, nitrate and nitrite for 30 min; the salting period where hams were entirely buried in salt, piled up, and placed in a cold room for 10-12 days at 2-4 °C and 90-95% relative humidity; the post-salting stage where hams were kept for 60 days at 4-5 °C and 75-85% relative humidity; and finally the ripening-drying period at temperatures increasing from 5 °C to 14-20 °C and relative humidity decreasing to 70%. The total length of the dry-curing process was 9 months.

Test samples were selected at different times of processing: 2 months (at the end of the post-salting stage), 3.5, 5 and 6.5 months (during the ripening-drying period) and 9 months (at the end of the dry-cured process).

2.2 Dry-cured ham extraction and deproteinisation

A total of 50 grams 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 °C and 12000 g for 20 min, filtered through glass wool and then the solution was deproteinised by adding 3 volumes of ethanol and maintaining the sample at 4 °C for 20 hours. Afterwards, the sample was centrifuged again at 4 °C and 12000 g for 10 min and the supernatant was dried in a rotatory evaporator. Finally, 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 °C until use.

2.3 Size-exclusion chromatography

To fractionate deproteinised cured ham extracts according to molecular mass, a 5 mL aliquot of each extract was subjected to size-exclusion chromatography. A Sephadex G25 column (2.5 x 65 cm, Amersham Biosciences, Uppsala, Sweden), previously equilibrated with 0.01 N HCl and filtered through a 0.45 µm nylon membrane filter (Millipore), was employed for this purpose. The separation was performed using 0.01 N HCl as mobile phase, at a flow rate of 15 mL/h and 4 °C. 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.

2.4 Peptide identification by nanoliquid chromatography and mass spectrometry in tandem (nLC-MS/MS)

The nanoLC-MS/MS analysis was performed with an Ultimate 3000 RSLC nano system from Dionex (Thermo Fisher Scientific Ltd, Leicestershire, UK), coupled to an AmaZon ETD ion-trap mass spectrometer equipped with a nanoelectrospray ionization source (Bruker Daltonik GmbH, Bremen, Germany).

Lyophilised samples were resuspended in 100 µL of H₂O with 0.1% of formic acid (FA). Ten microliters of the solution were taken and 10 µL of H₂O: ACN (98:2, v/v) with 0.1% v/v of FA were added. Sample was kept in cold for 30 min and centrifuged at 10000g for 15 min. Two microliters of the supernatant were injected into the LC-MS system through the autosampler. Samples were then preconcentrated on a Dionex Acclaim® PepMap 100 column (100µm x 2 cm, C18, 5µm, 100Å) (Dionex Corporation, LC Packings), at a flow rate of 4 µL/min and using 0.1% v/v FA as mobile phase. After 3 min of preconcentration, the trap column was automatically switched in-line onto a

Dionex Acclaim® PepMap RSLC nano-column (75µm x 15cm, C18, 2µm, 100Å) (Dionex Corporation, LC Packings). 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 40% of solvent B over 70 min at a flow rate of 0.250 µL/min and running temperature of 30 °C. The column outlet was directly coupled to a nanoelectrospray ion source. The ion trap mass spectrometer was used in positive polarity mode. Typical ion spray voltage was in the range of 1.8 kV- 2.0 kV, and nitrogen was used as collision gas with a nebulizer pressure of 15 psi and a capillary temperature of 200 °C. The ion trap was used in MS2 in order to enhance scan mode resolution in the range from m/z 50 to 3000, at a speed of m/z 8100 per second. The first scan of MS scan was from m/z value 300 to 1500, with a maximum accumulation time of 50 ms and an average of 5. Other source parameters were optimised with a tryptic digest of BSA protein. Automated spectral processing, peak list generation, and database search were performed using Mascot Distiller v2.4.2.0 software (Matrix Science, Inc., Boston, MA) (<http://www.matrixscience.com>). Oxidation of methionine was used as variable modifications. The identification of protein origin of peptides was done using UniProt and NCBI nr protein databases, with a significance threshold $p < 0.05$, and a tolerance on the mass measurement of 100 ppm in MS mode and 0.3 Da for MS/MS ions.

3. Results and discussion

Each deproteinised sample extract was fractionated by size-exclusion chromatography, and fractions corresponding to the first peak eluted (a total of 7 fractions of 5 mL) were pooled together and lyophilised. The analysed fractions contained the highest size peptides that remained after protein precipitation with ethanol.

Fractions from each time of processing were analysed by nLC-MS/MS mass spectrometry for the identification of their peptidic content. A total of 107 peptides generated from the LDB3 protein have been identified at five different times of processing, as reported in [Tables 1, 2 and 3](#). These tables list the characterised amino acid sequences, the position of the identified peptides in the parent protein, amino acid residues preceding and following the sequence, the observed and calculated masses together with the charge states, and the process times when peptides have been identified.

All the peptide sequences identified in this work by using the protein database UniProt came according to this database from LIM domain-binding protein 3 of *Homo sapiens* species. All the identified peptides have been searched in BLAST and revealed 100% homology with a *Sus scrofa* uncharacterized protein. This is because LDB3 protein has not been characterised in porcine species, but it has in human and bovine (*Bos taurus*) species. So, [Figure 1](#) shows the sequence alignment of LDB3 protein between *Sus scrofa*, *Homo sapiens* and *Bos taurus* species. BLAST sequence similarity searches revealed 87% homology between LDB3 sequences of porcine and human (Uniprot database with accession numbers F1SEN8 and O75112, respectively). However, LDB3 has not been completely sequenced in bovine species (accession number Q3ZBC9 in Uniprot database), so *Sus scrofa* and *Bos taurus* only share a sequence fragment corresponding to the 42% of the full-length sequence of this protein in which BLAST revealed 93% homology between sequences of both species.

An extensive proteolysis has been reported during the processing of dry-cured ham and this comprises the action of endopeptidases and exopeptidases. Muscle endopeptidases are stable during the full process ([Toldrá, Rico & Flores, 1993](#)), except calpains that are inactivated after the salting stage ([Rosell & Toldrá, 1996](#)), and cathepsin D whose

activity disappears after 6 months of process (Toldrá et al., 1993). Regarding the activity of exopeptidases, aminopeptidases and dipeptidyl peptidases have shown good stability along the curing process (Toldrá, Flores & Sanz, 1997; Toldrá et al., 2000; Sentandreu & Toldrá, 2001).

Figure 2 shows the LDB3 protein sequence and the action of muscle peptidases at each processing time: 2, 3.5, 5, 6.5 and 9 months. The sequence region comprised between 1 and 90 amino acid residues is the one showing the highest amount of cleavage sites probably due to a more intense action of the proteolytic enzymes in this section of the sequence. There are evidences about the role of aminopeptidases and carboxypeptidases in the generation of the peptides identified from position 1 to 90 of the LDB3 protein sequence and the release of free amino acids (Table 1). However, cathepsins would be responsible for the protein breakdown and the generation of most of the peptides identified in the remaining sequence (position 91 to 715) at all times of processing tested, because calpains do not remain active after the salting stage (Rosell & Toldrá, 1996).

Thus, peptides from 11 to 17 show the sequential loss of phenylalanine (F), asparagine (N), methionine-proline (MP), leucine (L), threonine (T) and isoleucine-serine (IS) from their N-terminal end probably by the action of aminopeptidases and dipeptidyl peptidases that remain active during all dry-curing process. Moreover, carboxypeptidases generate free amino acids from C-terminal position of peptides, as occurs with the amino acids leucine (L; in peptide 27), aspartic acid (D; in peptide 30), and glycine (G; in peptide 31). In fact, these peptides share the common sequence FNMPLTISRITPGSKAAQSQLSQ (peptide 31), as it is shown in Table 1. On the other hand, tripeptidyl peptidases could be responsible for the N-terminal loss of tripeptides like serine-valine-threonine (SVT) and thus generate the peptides 2, 4, and 6 from the

peptides 1, 3, and 5 respectively, as well as the loss of the tripeptide asparagine-methionine-proline (NMP) from peptide 35 to generate peptide 36, or the loss of glutamic acid-alanine-glutamine (EAQ) from the peptides 58 and 60 to generate peptides 59 and 61, respectively. As an example, [Figure 1](#) of Supplementary material shows the MS/MS spectra of peptides 6 and 7 demonstrating the loss of arginine-leucine (RL) dipeptide from the C-terminal site, and [Figure 2](#) of Supplementary material shows the consecutive loss of the tripeptide serine-glutamine-glycine (SQG) (peptide 21) and the amino acid aspartic acid (D) (peptide 22) from the N-terminal site of peptide 20. The generation of most peptides shown in [Table 2](#) could be the result of the action of endopeptidases on LDB3 protein. For example, peptides like 66, 74, 77, 81, 90, and 95, or peptide 64 from [Table 1](#), which have been identified during dry-curing stage, would have been generated by the action of cathepsins. After the action of these enzymes, peptides may be further degraded by exopeptidases giving rise to smaller peptides and free amino acids, as happens in peptide 65 which is generated from peptide 64, or peptides 83 and 84 from peptide 82.

Proteolysis is a dynamic and variable process where muscle proteases carry out their action randomly during the process of dry-curing. The complexity of the proteolysis is remarkable as new peptides are continuously being generated and hydrolysed by the action of both endo- and exopeptidases to create new ones ([Rodríguez-Núñez et al., 1995; Toldrá et al., 1997](#)), as can be observed in [Tables 1 and 2](#). Thus, the elevated number of enzymes acting during proteolysis would result in the generation of a complex mixture of peptides with unspecified cleavage sites. Some of these peptides are present at certain times of processing at low concentrations due to the constant degradation and are not able to be detected by MS techniques. This fact would explain

the unidentification of some peptides at a certain time of processing but its identification again in later times, as occurs for peptides 33, 42, and 88, among others.

The oxidation of essential amino acids such as methionine has been a concern because of its effect on meat quality but also on the nutritive value due to the decreased availability of essential amino acids and poorer digestibility of oxidised proteins (Strange, 1984; Lund, Heinonen, Baron & Estévez, 2011). Most studies have been focused on protein oxidation mechanisms and the development of new analysis methodologies to assess the protein oxidation in dry-cured and cooked meat products (Armenteros, Heinonen, Ollilainen, Toldrá & Estévez, 2009). However, no studies have been described about the identification of naturally generated peptides showing amino acid modifications along the processing of meat products. In this study, methionine oxidation has been observed in twelve peptides (Table 3), and six of these peptides show such modification at the end of dry-cured ham processing (9 months) while others are oxidised a bit earlier. This indicates that the oxidation of methionine tends to occur mainly in the latest stages of ham dry-curing. So, our results are in agreement with a previous study showing that protein oxidation increases with increasing maturation time of hams (Koutina, Jongberg & Skibsted, 2012).

The fact that the identification of some peptides remains stable during the first stages of the dry-curing process such as the case of peptide 6 (MS/MS spectrum is shown in Figure 1 of Supplementary material), which has been identified from 2 to 6.5 months of processing, or peptide 11 that has been identified from 2 to 5 months (MS/MS spectrum is shown in Figure 3 of Supplementary material), as well as the fact that some peptides have only been detected after 9 months of processing (e.g. case of peptides 35, 36, and 37 or peptides from 43 to 46), would suggest the potential use of these peptides as a way to estimate and control the time of processing. Nevertheless, further studies would

be needed in order to consider and confirm these peptides as biomarkers of the dry-curing ham process.

4. Conclusions

Proteolysis is a complex and dynamic process which takes place throughout the whole process of dry-curing. The peptides generated through the degradation of LIM domain-binding protein 3 has been observed at different times of processing providing an evidence of the action of cathepsins, but more remarkable is the action of aminopeptidases, carboxypeptidases, di- and tripeptidyl peptidases to the release of peptides and free amino acids during dry-cured ham processing. Additional studies are required to assess these peptides as biological markers of dry-curing time.

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FIGURES CAPTIONS

Figure 1. Alignment of LIM domain binding protein 3 sequences of *Sus scrofa*, *Homo sapiens* and *Bos taurus* using UniprotKB/TrEMBL protein database (accession numbers F1SEN8, O75112, and Q3ZBC9, respectively). The same sequences also correspond to accession numbers XP_003359314.1 (*Sus scrofa*), NP_009009.1 (*Homo sapiens*) and NP_001030493.1 (*Bos taurus*) in the NCBI database.

Figure 2. Primary sequence of porcine LDB3 protein (UniprotKB/TrEMBL protein database accession number F1SEN8). Cleavage sites of the identified peptides at each time of processing are indicated with black arrows.

Table 1

Table 1. Peptides identified by nLC-MS/MS from position 1 to 90 of the LIM domain-binding protein 3 in Spanish dry-cured ham (UniprotKB/TrEMBL protein database accession number F1SEN8).

No.	Position ^a	P ₀ ^b	Sequence	P _f ^c	Obs. ^d (<i>m/z</i>)	Charge (+)	Calc. ^e (<i>m/z</i>)	Identified ^f				
								2 mo.	3.5 mo.	5 mo.	6.5 mo.	9 mo.
1	4-22	Y	SVTLTGPGFWGFRLLQGKGD	F	658,23	3	1972,02		x			
2	7-22	T	LTGPGFWGFRLLQGKGD	F	562,49	3	1684,87		x			
3	4-19	Y	SVTLTGPGFWGFRLLQG	G	836,75	2	1671,87		x			
4	7-19	T	LTGPGFWGFRLLQG	G	693,40	2	1384,73		x			
5	4-17	Y	SVTLTGPGFWGFRL	Q	744,22	2	1486,79	x	x			
6	7-17	T	LTGPGFWGFRL	Q	600,73	2	1199,65	x	x	x	x	
7	7-15	T	LTGPGFWGF	R	466,12	2	930,46		x			
8	7-15	T	LTGPGFWGF	R	931,23	1	930,46	x	x			
9	7-12	T	LTGPGP	W	540,79	1	540,29		x			
10	11-23	P	GPWGFRLQGGKDF	N	488,84	3	1463,73	x				
11	23-53	D	FNMLTISRITPGSKAAQSQLSQGDLVVAID	G	1086,60	3	3256,71	x	x	x		
12	24-53	F	NMPLTISRITPGSKAAQSQLSQGDLVVAID	G	1037,66	3	3109,64		x			
13	25-53	N	MPLTISRITPGSKAAQSQLSQGDLVVAID	G	999,39	3	2995,60		x			
14	27-53	P	LTISRITPGSKAAQSQLSQGDLVVAID	G	923,60	3	2767,51	x	x	x	x	x
15	28-53	L	TISRITPGSKAAQSQLSQGDLVVAID	G	885,56	3	2654,42		x			x
16	29-53	T	ISRITPGSKAAQSQLSQGDLVVAID	G	851,94	3	2553,38					x
17	31-53	S	RITPGSKAAQSQLSQGDLVVAID	G	785,24	3	2353,26		x	x	x	x
18	39-53	A	AQSQLSQGDLVVAID	G	772,21	2	1542,79		x	x	x	x
19	42-53	S	QLSQGDLVVAID	G	629,20	2	1256,66					x
20	44-53	L	SQGDLVVAID	G	1016,27	1	1015,52					x
21	47-53	G	DLVVAID	G	744,23	1	743,41			x		x
22	48-53	D	LVVAID	G	629,26	1	628,38					x

23	23-51	D	FNMPLTISRITPGSKAAQSQLSQGDLVVA	I	1010,75	3	3028,60	x	x				x
24	47-51	G	DLVVA	I	516,01	1	515,30						x
25	23-50	D	FNMPLTISRITPGSKAAQSQLSQGDLVV	A	986,59	3	2957,56		x				
26	23-48	D	FNMPLTISRITPGSKAAQSQLSQGDL	V	920,90	3	2759,43	x	x	x	x		x
27	23-47	D	FNMPLTISRITPGSKAAQSQLSQGD	L	882,87	3	2646,34	x	x	x	x		x
28	27-47	P	LTISRITPGSKAAQSQLSQGD	L	719,97	3	2157,14	x					x
29	31-47	S	RITPGSKAAQSQLSQGD	L	581,96	3	1742,89						x
30	23-46	D	FNMPLTISRITPGSKAAQSQLSQG	D	844,41	3	2531,32		x				
31	23-45	D	FNMPLTISRITPGSKAAQSQLSQ	G	825,63	3	2474,30	x	x	x			x
32	31-45	S	RITPGSKAAQSQLSQ	G	524,49	3	1570,84						x
33	23-43	D	FNMPLTISRITPGSKAAQSQL	S	753,91	3	2259,20	x		x	x		x
34	23-43	D	FNMPLTISRITPGSKAAQSQL	S	1130,81	2	2259,20		x				x
35	24-43	F	NMPLTISRITPGSKAAQSQL	S	705,25	3	2112,14						x
36	27-43	P	LTISRITPGSKAAQSQL	S	590,86	3	1770,00						x
37	31-43	S	RITPGSKAAQSQL	S	678,71	2	1355,75						x
38	32-43	R	ITPGSKAAQSQL	S	401,25	3	1199,65			x			
39	23-42	D	FNMPLTISRITPGSKAAQSQ	L	716,44	3	2146,12		x				x
40	23-40	D	FNMPLTISRITPGSKAAQ	S	644,52	3	1931,03			x			x
41	23-38	D	FNMPLTISRITPGSKA	A	578,21	3	1731,93	x	x	x	x		x
42	23-38	D	FNMPLTISRITPGSKA	A	866,76	2	1731,93	x	x	x			x
43	27-38	P	LTISRITPGSKA	A	415,15	3	1242,73						x
44	29-38	T	ISRITPGSKA	A	515,18	2	1028,60						x
45	31-38	S	RITPGSKA	A	415,17	2	828,48						x
46	23-37	D	FNMPLTISRITPGSK	A	554,64	3	1660,90						x
47	32-37	R	ITPGSK	A	301,64	2	601,34			x			
48	23-30	D	FNMPLTIS	R	461,62	2	921,46						x
49	23-30	D	FNMPLTIS	R	922,24	1	921,46	x	x	x	x		x
50	23-28	D	FNMPLT	I	722,20	1	721,35						x

51	23-27	D	FNMPL	T	621,18	1	620,30				x
52	28-58	L	TISRITPGSKAAQSQLSQGDLVVAIDGVNTD	T	1047,57	3	3140,63			x	
53	38-58	A	AQSQLSQGDLVVAIDGVNTD	T	677,36	3	2029,00	x			
54	42-57	L	SQGDLVVAIDGVNT	D	694,58	2	1386,70				x
55	48-63	D	LVVAIDGVNTDTMTHL	E	849,72	2	1697,87	x			
56	61-67	M	THLEAQN	K	406,61	2	811,38	x			
57	67-77	Q	NKIKSASYNLS	L	612,68	2	1223,65	x			
58	64-75	L	EAQNKIKSASYN	L	676,67	2	1351,67	x			
59	67-75	Q	NKIKSASYN	L	512,64	2	1023,53	x			
60	64-74	L	EAQNKIKSASY	N	619,68	2	1237,63	x			
61	67-74	Q	NKIKSASY	N	455,64	2	909,49	x	x	x	x
62	68-74	N	KIKSASY	N	398,64	2	795,45	x	x		x
63	68-74	N	KIKSASY	N	796,25	1	795,45	x			
64	78-90	S	LTLQKSKRPIPIIS	T	494,10	3	1479,91			x	
65	78-86	S	LTLQKSKRP	I	535,72	2	1069,66	x			

^a Position of the identified peptides inside the LDB3 sequence.

^b Amino acid residue preceding the peptide sequence.

^d Molecular ion mass observed in the nLC-MS/MS system calculated in Daltons.

^fPeptides identified at each time of ham processing.

Table 2

Table 2. Peptides identified by nLC-MS/MS from position 91 to 715 of the LIM domain-binding protein 3 in Spanish dry-cured ham (UniprotKB/TrEMBL protein database accession number F1SEN8).

No.	Position ^a	P ₀ ^b	Sequence	P _f ^c	Obs. ^d (<i>m/z</i>)	Charge (+)	Calc. ^e (<i>m/z</i>)	Identified ^f				
								2 mo.	3.5 mo.	5 mo.	6.5 mo.	9 mo.
66	92-97	T	AVPPIQ	S	625,34	1	623,36		x			
67	112-128	L	DANSSLAAPSPHPEARA	S	845,71	2	1689,81				x	
68	112-116	L	DANSS	L	493,68	1	492,18	x				
69	126-140	E	ARASPGTPGTPEHGL	T	723,77	2	1446,72			x		
70	187-191	P	LGSSQ	P	490,82	1	490,24	x				
71	221-226	G	KASGAG	L	490,85	1	489,25				x	
72	212-225	A	QMYQMSLRGKASGA	G	509,76	3	1526,73	x				
73	226-230	A	GLLG	S	417,12	1	415,24		x			
74	239-243	T	VDSAS	P	478,56	1	477,21		x			
75	262-271	D	EWARRSSNLQ	S	623,43	2	1245,62		x			
76	304-308	E	HAPVC	T	525,05	1	525,24			x		
77	311-319	S	QAATPLLPA	S	440,88	2	880,50			x		
78	320-324	A	SAQPP	A	498,51	1	498,24			x		
79	322-326	A	QPPAA	A	482,34	1	482,25			x		
80	346-351	T	ASAAAP	A	487,77	1	486,24				x	
81	361-366	R	PQASAY	S	637,25	1	635,29		x			
82	384-395	E	APAAPAPKPRVV	T	587,61	2	1172,70					x
83	387-391	A	APAPK	P	482,29	1	482,29	x				
84	385-390	A	PAAPAP	K	522,70	1	522,28		x			x
85	398-402	T	ASIRP	S	542,95	1	542,32	x				
86	406-410	Y	QPVPA	S	511,14	1	510,28	x				
87	408-413	P	VPASTY	S	637,12	1	636,31					x

88	435-444	Y	TPSPAPTYSP	S	340,20	3	1016,48	x		x	x
89	446-450	S	PAPAY	T	518,14	1	517,25	x	x		x
90	467-472	G	PAESAS	R	560,19	1	560,24				x
91	499-510	Q	SLPRGAPAYTPP	L	409,20	3	1225,65		x		
92	506-511	P	AYTPPL	Q	662,40	1	660,35				x
93	508-513	Y	TPPLQG	P	612,78	1	611,33	x			
94	596-604	A	PVCAKCNTK	I	963,77	1	962,47		x		
95	665-669	V	EAGDK	F	519,97	1	518,23		x		

^a Position of the identified peptides inside the LDB3 sequence.

^b Amino acid residue preceding the peptide sequence.

^c Amino acid residue following the peptide sequence.

^d Molecular ion mass observed in the nLC-MS/MS system calculated in Daltons.

^e Calculated relative molecular mass in Daltons of the matched peptide.

^f Peptides identified at each time of ham processing.

Table 3

Table 3. Peptides identified by nLC-MS/MS from LIM domain-binding protein 3 in Spanish dry-cured ham with a methionine oxidation (UniprotKB/TrEMBL protein database accession number F1SEN8).

No.	Position ^a	P ₀ ^b	Sequence	P _f ^c	Obs. ^d (<i>m/z</i>)	Charge (+)	Calc. ^e (<i>m/z</i>)	Identified ^f				
								2 mo.	3.5 mo.	5 mo.	6.5 mo.	9 mo.
96	23-53	D	FNMPLTISRITPGSKAAQSQLSQGDLVVAID	G	1091,71	3	3272,71		x	x		x
97	23-47	D	FNMPLTISRITPGSKAAQSQLSQGD	L	888,30	3	2662,34					x
98	23-45	D	FNMPLTISRITPGSKAAQSQLSQ	G	830,88	3	2490,29					x
99	23-43	D	FNMPLTISRITPGSKAAQSQL	S	759,28	3	2275,20					x
100	23-40	D	FNMPLTISRITPGSKAAQ	S	649,77	3	1947,02					x
101	23-38	D	FNMPLTISRITPGSKA	A	583,53	3	1747,93					x
102	59-63	D	TMTHL	E	309,63	2	617,28			x	x	
103	59-67	D	TMTHLEAQN	K	353,97	3	1059,47		x			
104	197-219	N	NPIGLYSAETLREMAQMYQMSLR	G	906,56	3	2717,30		x			
105	207-226	T	LREMAQMYQMSLRGKASGAG	L	733,99	3	2200,06	x				
106	593-611	Q	FFAPVCAKCNTKIMGEVMH ^g	A	1079,59	2	2156,99		x			
107	602-613	C	NTKIMGEVMHAL	R	680,83	2	1358,67			x		

^a Position of the identified peptides inside the LDB3 sequence.

^b Amino acid residue preceding the peptide sequence.

^c Amino acid residue following the peptide sequence.

^d Molecular ion mass observed in the nLC-MS/MS system calculated in Daltons.

^e Calculated relative molecular mass in Daltons of the matched peptide.

^f Peptides identified at each time of ham processing.

^g Peptide identified with two methionine oxidations.

Figure 1

```

1  MSYSUTLTGPGPWGFRLLQGKDFNMPLTISRITPGSKAAQSLSQGDLVVAIDGUNTDTM 60 F15EN8 F15EN8_PIG
1  MSYSUTLTGPGPWGFRLLQGKDFNMPLTISRITPGSKAAQSLSQGDLVVAIDGUNTDTM 60 075112 LDB3_HUMAN
1  MSYSUTLTGPGPWGFRLLQGKDFNMPLTISRITPGSKAAQSLSQGDLVVAIDGUNTDTM 60 Q32BC9 Q32BC9_BOVIN
*****

61  THLEAQNKIKAASYNLSTLQKSKRP IP ISTAVPP IQSPLPVIPHQKDPALDANSSLAAP 120 F15EN8 F15EN8_PIG
61  THLEAQNKIKAASYNLSTLQKSKRP IP ISTAPPVQTPLPVIPHQKDPALDTNGLVAP 120 075112 LDB3_HUMAN
61  THLEAQNKIKAASYNLSTLQKSKRP IP ISTAAPF IQSPLPVIPHQKDPALDTNSSLAAA 120 Q32BC9 Q32BC9_BOVIN
*****;_*;*;*****;_*;_*;_*

121  SPHPERARASPGTPGTPENGLTFSSSFSTSVFSSHTASDLGPPRGSPGAKTSLEGALDS 180 F15EN8 F15EN8_PIG
121  SPSPERARASPGTPGTPELRPTTFSPAFSRPSAFSSSLAEASDPGPPRASLRKTSPEGARDL 180 075112 LDB3_HUMAN
121  SPHPERARASPGTPGTPELGQIFSSSFSTSVFSSHTASDPGPPRGSPGAKISLEGALDS 180 Q32BC9 Q32BC9_BOVIN
** ***** **;*;_*;_*;_*;_*;_*;_*

181  LSLKGLPGSSQPRQYNNP IGLYS AETLREMAQMYQMSLRGKASGAGLLGGSLP IKDLTVD 240 F15EN8 F15EN8_PIG
181  LGPKALPGSSQPRQYNNP IGLYS AETLREMAQMYQMSLRGKASGUGLPGGSLP IKDLAVD 240 075112 LDB3_HUMAN
181  LSPKALPGSSQPRQYNNP IGLYS AETLREMAQMYQMSLRGKASGAGLLGGSLP IKDLTVD 240 Q32BC9 Q32BC9_BOVIN
*_* *****;_*;_*;_*;_*;_*;_*

241  SASPVYQAVIKNQKPEDEADEWARRSSNLQSRSPR ILAQMTGTEYMQDPDEEALRRSST 300 F15EN8 F15EN8_PIG
241  SASPVYQAVIKNQKPEDEADEWARRSSNLQSRSPR ILAQMTGTEYMQDPDEEALRRSST 300 075112 LDB3_HUMAN
241  SASPVYQAVIKNQKLEDEADWARRSSNLQSRSPR ILAQMTGTEYMQDPDEEALRRSRE 300 Q32BC9 Q32BC9_BOVIN
*****;_*;_*;_*;_*;_*;_*;_*

301  P IENAPVCTSQAAATPLLP A--SAQPPAAASPSTASPLATAAATATAS--AAAPASSPA 356 F15EN8 F15EN8_PIG
301  P IENAPVCTSQAAATPLLP A--SAQPPAAASPSTASPLATAAATATAS--AAAPASSPA 356 075112 LDB3_HUMAN
301  P IENAPVCTSQAAATPLLP A--SAQPPAAASPSTASPLATAAATATAS--AAAPASSPA 356 Q32BC9 Q32BC9_BOVIN
*****;_*;_*;_*;_*;_*;_*

357  DSPRPQASAYSPAVATSPAPAAH-TYSEAPAAPAPKPRVUTTASIRPSUYQPVAPSTYSP 415 F15EN8 F15EN8_PIG
357  DSPRPQASAYSPAVATSPAPAAH-TYSEAPAAPAPKPRVUTTASIRPSUYQPVAPSTYSP 415 075112 LDB3_HUMAN
357  DSPRPQASAYSPAVATSPAPAAH-TYSEAPAAPAPKPRVUTTASIRPSUYQPVAPSTYSP 415 Q32BC9 Q32BC9_BOVIN
*****;_*;_*;_*;_*;_*;_*

416  SPGANYSPPTPTPSPAPAYTPSPAPTYTPSPAPAYT-----PSPAPSYNPT---PYS 464 F15EN8 F15EN8_PIG
416  SPGANYSPPTPTPSPAPAYTPSPAPAYTPSPAPTYTPSPAPAYT-----PSPAPSYNPT---PYS 464 075112 LDB3_HUMAN
416  SPGANYSPPTPTPSPAPAYTPSPAPAYTPSPAPTYTPSPAPAYT-----PSPAPSYNPT---PYS 464 Q32BC9 Q32BC9_BOVIN
*****;_*;_*;_*;_*;_*;_*

465  GGPASASRPPWATDDSFQKFPAGKSTTSISKQSLPRGAPAYTPPLQGPQVSPLARGTV 524 F15EN8 F15EN8_PIG
465  GGPASASRPPWATDDSFQKFPAGKSTTSISKQSLPRGAPAYTPPLQGPQVSPLARGTV 524 075112 LDB3_HUMAN
465  GGPASASRPPWATDDSFQKFPAGKSTTSISKQSLPRGAPAYTPPLQGPQVSPLARGTV 524 Q32BC9 Q32BC9_BOVIN
*****;_*;_*;_*;_*;_*;_*

525  QRAERFPASSRTPLCGHCHNS IIRGPFVAMGRSWMHPEEFNCAYCKTSLADVCFVEEQNNV 584 F15EN8 F15EN8_PIG
525  QRAERFPASSRTPLCGHCHNS IIRGPFVAMGRSWMHPEEFNCAYCKTSLADVCFVEEQNNV 584 075112 LDB3_HUMAN
525  QRAERFPASSRTPLCGHCHNS IIRGPFVAMGRSWMHPEEFNCAYCKTSLADVCFVEEQNNV 584 Q32BC9 Q32BC9_BOVIN
*****;_*;_*;_*;_*;_*;_*

585  YCERCYEQFFAPVCAKCNKIMGEVMHALRQTWHTTCFVCAACKKPFQNSLFHMDGEPY 644 F15EN8 F15EN8_PIG
585  YCERCYEQFFAPVCAKCNKIMGEVMHALRQTWHTTCFVCAACKKPFQNSLFHMDGEPY 644 075112 LDB3_HUMAN
585  YCERCYEQFFAPVCAKCNKIMGEVMHALRQTWHTTCFVCAACKKPFQNSLFHMDGEPY 644 Q32BC9 Q32BC9_BOVIN
*****;_*;_*;_*;_*;_*;_*

645  CEKDYUMLFSTKCHGCDFFVEAGDKFIEALGHTWMDTCFICAVCHUMLEGQPFYSKKDKP 704 F15EN8 F15EN8_PIG
645  CEKDYUMLFSTKCHGCDFFVEAGDKFIEALGHTWMDTCFICAVCHUMLEGQPFYSKKDKP 704 075112 LDB3_HUMAN
645  CEKDYUMLFSTKCHGCDFFVEAGDKFIEALGHTWMDTCFICAVCHUMLEGQPFYSKKDKP 704 Q32BC9 Q32BC9_BOVIN
*****;_*;_*;_*;_*;_*;_*

705  LCKKHAAHAINV 715 F15EN8 F15EN8_PIG
717  LCKKHAAHAINV 727 075112 LDB3_HUMAN
717  LCKKHAAHAINV 727 Q32BC9 Q32BC9_BOVIN

```

Figure 1.

Figure 2

2 months	
1	MSYSVTLTGP GPWGFRLLGG KDFNMPLTIS RITPGSKAAQ SQLSQGDLVV AIDGVNTDTM THLEAQNKIK SASYNLSLTL QSKSRPIPIIS TAVPPIQSPL
101	FVIPHQKDKPA LDANSSLAAP SPHPEARASP GTPGTPEHGL TFSSSFQS TS VSSSHTEASD LGPPRGSPGA KTSLEGALDS LSLKGPLGSS QPRQYNNPIG
201	LYSAETLREM AQMYQMSLRG KASGAGLLGG SLPIKDLTVD SASPVYQAVI KNQNKPEDEA DEWARRSSNL QSRSFRI LAQ MTGTEYMQDP DEEALRRSST
301	PIEHAPVCTS QAATPLL PAS AQPPAAASPS TASPPLATAA AHTATASAAA PASSPADSPR PQASAYSPAV ATSPAPAAHT YSEAPAAPAP KPRVVTTASI
401	RPSVYQPVPA STYSPSPGAN YSPTPYTPSP APAYTPSPAP TYSPSPAPAY TSPSPASYNP TPYSGGPAES ASRPPVWTD D SFSQKFAPGK STTSISKQSL
501	PRGAPAYTTP LQGPQVSPLA RGTVQRAERF PASSRTPLCG HCNSIIRGPF LVAMGRSWHP EEFNCAYCKT SLADVC FVEE QNNVYCERCY EQFFAPVCAK
601	CNTKIMGEVM HALRQTWHTT CFVCAACKKP FGNSLFHMED GEPECKDYV NLFSTKCHGC DFPVEAGDKF IEALGHTWHD TCFICAVCHV NLEGQPFYSK
701	KDKPLCKKHA HAINV
3.5 months	
1	MSYSVTLTGP GPWGFRLLGG KDFNMPLTIS RITPGSKAAQ SQLSQGDLVV AIDGVNTDTM THLEAQNKIK SASYNLSLTL QSKSRPIPIIS TAVPPIQSPL
101	FVIPHQKDKPA LDANSSLAAP SPHPEARASP GTPGTPEHGL TFSSSFQS TS VSSSHTEASD LGPPRGSPGA KTSLEGALDS LSLKGPLGSS QPRQYNNPIG
201	LYSAETLREM AQMYQMSLRG KASGAGLLGG SLPIKDLTVD SASPVYQAVI KNQNKPEDEA DEWARRSSNL QSRSFRI LAQ MTGTEYMQDP DEEALRRSST
301	PIEHAPVCTS QAATPLL PAS AQPPAAASPS TASPPLATAA AHTATASAAA PASSPADSPR PQASAYSPAV ATSPAPAAHT YSEAPAAPAP KPRVVTTASI
401	RPSVYQPVPA STYSPSPGAN YSPTPYTPSP APAYTPSPAP TYSPSPAPAY TSPSPASYNP TPYSGGPAES ASRPPVWTD D SFSQKFAPGK STTSISKQSL
501	PRGAPAYTTP LQGPQVSPLA RGTVQRAERF PASSRTPLCG HCNSIIRGPF LVAMGRSWHP EEFNCAYCKT SLADVC FVEE QNNVYCERCY EQFFAPVCAK
601	CNTKIMGEVM HALRQTWHTT CFVCAACKKP FGNSLFHMED GEPECKDYV NLFSTKCHGC DFPVEAGDKF IEALGHTWHD TCFICAVCHV NLEGQPFYSK
701	KDKPLCKKHA HAINV
5 months	
1	MSYSVTLTGP GPWGFRLLGG KDFNMPLTIS RITPGSKAAQ SQLSQGDLVV AIDGVNTDTM THLEAQNKIK SASYNLSLTL QSKSRPIPIIS TAVPPIQSPL
101	FVIPHQKDKPA LDANSSLAAP SPHPEARASP GTPGTPEHGL TFSSSFQS TS VSSSHTEASD LGPPRGSPGA KTSLEGALDS LSLKGPLGSS QPRQYNNPIG
201	LYSAETLREM AQMYQMSLRG KASGAGLLGG SLPIKDLTVD SASPVYQAVI KNQNKPEDEA DEWARRSSNL QSRSFRI LAQ MTGTEYMQDP DEEALRRSST
301	PIEHAPVCTS QAATPLL PAS AQPPAAASPS TASPPLATAA AHTATASAAA PASSPADSPR PQASAYSPAV ATSPAPAAHT YSEAPAAPAP KPRVVTTASI
401	RPSVYQPVPA STYSPSPGAN YSPTPYTPSP APAYTPSPAP TYSPSPAPAY TSPSPASYNP TPYSGGPAES ASRPPVWTD D SFSQKFAPGK STTSISKQSL
501	PRGAPAYTTP LQGPQVSPLA RGTVQRAERF PASSRTPLCG HCNSIIRGPF LVAMGRSWHP EEFNCAYCKT SLADVC FVEE QNNVYCERCY EQFFAPVCAK
601	CNTKIMGEVM HALRQTWHTT CFVCAACKKP FGNSLFHMED GEPECKDYV NLFSTKCHGC DFPVEAGDKF IEALGHTWHD TCFICAVCHV NLEGQPFYSK
701	KDKPLCKKHA HAINV
6.5 months	
1	MSYSVTLTGP GPWGFRLLGG KDFNMPLTIS RITPGSKAAQ SQLSQGDLVV AIDGVNTDTM THLEAQNKIK SASYNLSLTL QSKSRPIPIIS TAVPPIQSPL
101	FVIPHQKDKPA LDANSSLAAP SPHPEARASP GTPGTPEHGL TFSSSFQS TS VSSSHTEASD LGPPRGSPGA KTSLEGALDS LSLKGPLGSS QPRQYNNPIG
201	LYSAETLREM AQMYQMSLRG KASGAGLLGG SLPIKDLTVD SASPVYQAVI KNQNKPEDEA DEWARRSSNL QSRSFRI LAQ MTGTEYMQDP DEEALRRSST
301	PIEHAPVCTS QAATPLL PAS AQPPAAASPS TASPPLATAA AHTATASAAA PASSPADSPR PQASAYSPAV ATSPAPAAHT YSEAPAAPAP KPRVVTTASI
401	RPSVYQPVPA STYSPSPGAN YSPTPYTPSP APAYTPSPAP TYSPSPAPAY TSPSPASYNP TPYSGGPAES ASRPPVWTD D SFSQKFAPGK STTSISKQSL
501	PRGAPAYTTP LQGPQVSPLA RGTVQRAERF PASSRTPLCG HCNSIIRGPF LVAMGRSWHP EEFNCAYCKT SLADVC FVEE QNNVYCERCY EQFFAPVCAK
601	CNTKIMGEVM HALRQTWHTT CFVCAACKKP FGNSLFHMED GEPECKDYV NLFSTKCHGC DFPVEAGDKF IEALGHTWHD TCFICAVCHV NLEGQPFYSK
701	KDKPLCKKHA HAINV
9 months	
1	MSYSVTLTGP GPWGFRLLGG KDFNMPLTIS RITPGSKAAQ SQLSQGDLVV AIDGVNTDTM THLEAQNKIK SASYNLSLTL QSKSRPIPIIS TAVPPIQSPL
101	FVIPHQKDKPA LDANSSLAAP SPHPEARASP GTPGTPEHGL TFSSSFQS TS VSSSHTEASD LGPPRGSPGA KTSLEGALDS LSLKGPLGSS QPRQYNNPIG
201	LYSAETLREM AQMYQMSLRG KASGAGLLGG SLPIKDLTVD SASPVYQAVI KNQNKPEDEA DEWARRSSNL QSRSFRI LAQ MTGTEYMQDP DEEALRRSST
301	PIEHAPVCTS QAATPLL PAS AQPPAAASPS TASPPLATAA AHTATASAAA PASSPADSPR PQASAYSPAV ATSPAPAAHT YSEAPAAPAP KPRVVTTASI
401	RPSVYQPVPA STYSPSPGAN YSPTPYTPSP APAYTPSPAP TYSPSPAPAY TSPSPASYNP TPYSGGPAES ASRPPVWTD D SFSQKFAPGK STTSISKQSL
501	PRGAPAYTTP LQGPQVSPLA RGTVQRAERF PASSRTPLCG HCNSIIRGPF LVAMGRSWHP EEFNCAYCKT SLADVC FVEE QNNVYCERCY EQFFAPVCAK
601	CNTKIMGEVM HALRQTWHTT CFVCAACKKP FGNSLFHMED GEPECKDYV NLFSTKCHGC DFPVEAGDKF IEALGHTWHD TCFICAVCHV NLEGQPFYSK
701	KDKPLCKKHA HAINV

Figure 2.

Supplementary Material fig 1

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Supplementary Material fig 2

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Supplementary Material fig 3

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