Evidence of peptide oxidation from major myofibrillar proteins in dry-cured ham

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Abstract

In this study, a peptidomic approach has been used in the identification of naturally generated peptides during dry-curing process showing methionine (Met) oxidation in their sequence. A total of 656 peptides derived from major myofibrillar proteins in Protected Designation of Origin (PDO) Teruel dry-cured ham have been identified by nanoliquid chromatography coupled to tandem mass spectrometry (nLC-MS/MS), including 120 peptides showing methionine oxidation. The percentage of oxidised peptides in the studied proteins ranged from 6 to 35%, being peptides derived from nebulin, titin, myosin heavy chains, and troponin I proteins, those showing the highest number of oxidised methionine. The identification of the peptide sequence incorporating the oxidised amino acid provides valuable information of neighbouring amino acids, degree of hydrolysis of the sample, and characteristics of the peptide, which might be very useful for a future better understanding of the oxidation mechanisms occurring in dry-curing processing.

Keywords: Methionine, oxidation, peptides, dry-cured ham, proteomics, mass spectrometry.

1. Introduction

Teruel dry-cured ham was the first Spanish meat product with accepted Protected Designation of Origin (PDO). This denomination was created to control and guarantee the traditional manufacture that yields in high quality dry-cured ham with typical organoleptic and nutritional characteristics. The final product is the result of numerous and complex biochemical reactions which take place along the fourteen months of Teruel dry-cured ham processing. Proteolysis is the most important reaction that generates large amounts of peptides and free amino acids from the degradation of myofibrillar and sarcoplasmic proteins by the action of endogenous muscle peptidases (Toldrá, Rico, & Flores, 1993; Aristoy, & Toldrá, 1995; Toldrá, & Flores, 1998; Lametsch, Karlsson, Rosenvold, Andersen, Roepstorff, & Bendixen, 2003).

Oxidation of lipids and proteins is the main cause for quality deterioration during processing and storage of meat products, causing changes in nutritional value and sensory traits (Ladikos, & Lougovois, 1990; Lund, Heinonen, Baron, & Estévez, 2011). However, lipid oxidation is also essential in the maturation process and the desirable flavour of dry-cured hams, being this process extensively studied (Antequera, et al., 1992; Toldrá, & Flores, 1998; Ruiz, Ventanas, Cava, Andrés, & García, 1999; Gandemer, 2002). On the other hand, protein oxidation has been less studied in dry-cured hams, even though certain increase from the salting stage and tending to stabilise towards the ham maturation period have been reported (Armenteros, Heinonen, Ollilainen, Toldrá, & Estévez, 2009; Estévez, 2011; Wang, Xu, Zhang, Li, Lin, & Ma, 2011; Koutina, Jongberg, & Skibsted, 2012). In fact, the oxidation of muscle proteins comprises colour, flavour and texture deterioration as well as the loss of essential amino acids, and changes in conformation and functionality of proteins due to oxidative changes, that includes (i) formation of covalent intermolecular cross-linked protein

derivatives, (ii) cleavage of peptide bonds, and (iii) modification of amino acid side chains (Estévez, Ventanas, & Cava, 2005; Estévez, 2011; Lund, et al., 2011; Zhang, Xiao, & Ahn, 2013).

During the last decades, most of the studies about protein oxidation have been focused on the role played by oxidised proteins in different human diseases whereas the mechanisms of protein oxidation in food systems are largely unknown (Elias, Kellerby, & Decker, 2008). The oxidation of essential amino acids 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 oxidized proteins (Lund, et al., 2011). However, although the oxidation of proteins has been described, no information about the identification of oxidised peptides generated during dry-cured ham processing has been reported to date.

Methionine is one of the most susceptible amino acids to oxidation by almost all forms of reactive oxygen species because of its reactive sulfur atom, leading to modifications in structure of proteins and loss of enzyme activity (Zhang, et al., 2013). The first and the main oxidation product formed is methionine sulfoxide, which can be further oxidised, although a lesser amount, to methionine sulfone (Vogt, 1995; Shacter, 2000). However, the oxidation can be reversible due to the action of methionine sulfoxide reductases, which reduce methionine sulfoxides to methionine residues. Moreover, this cyclic oxidation-reduction process could act in antioxidant mechanisms and in regulation of cellular processes (Levine, Mosoni, Berlett, & Stadtman, 1996; Ghesquière, & Gevaert, 2014; Kim, Weiss, & Levine, 2014).

In the present work, a peptidomic strategy has been used to identify and sequence those naturally generated peptides showing methionine oxidation in PDO Teruel dry-cured

ham, focusing on those peptides derived from the degradation of the major myofibrillar proteins along the dry-curing process.

2. Materials and methods

2.1 Materials

Dry-cured hams manufactured according to the specifications of PDO Teruel (Spain) were used in this study. Hams were obtained from white-breed pigs (maternal line Landrace x Large White, and paternal line Duroc) and prepared according to the traditional process until a total time of processing of fourteen months.

2.2 Extraction of peptides

A total of 100 g of *Biceps femoris* muscle from the dry-cured hams were minced and homogenised with 500 mL of 0.01 N HCl for 5 min using an Ultra-Turrax[®] T-25 (IKA[®]-Werke, Germany). The homogenate was kept at 4 °C overnight for decanting, and the supernatant was filtered to retain the large pieces, firstly through a plastic mesh, and secondly through a qualitative filter paper (Whatman[™], UK). Afterwards, the sample was freeze-dried and desalted by solid phase extraction using an Oasis[®] HLB cartridge (35cc, Waters, Ireland) in which peptides were retained and then eluted with methanol:water (95:5, v/v). Finally, the sample was lyophilised for the following analysis by tandem mass spectrometry.

2.3 Peptide identification by nESI-LC-MS/MS

The identification of the peptides was performed by nanoliquid chromatography-tandem mass spectrometry using an Eksigent Nano-LC Ultra 1D Plus system (Eksigent of AB Sciex, CA, USA) and a quadrupole/time-of-flight (Q-ToF) TripleTOF[®] 5600+ system (AB Sciex Instruments, MA, USA) with a nanoelectrospray ionisation source (ESI).

Desalted and lyophilised samples were resuspended to 10 mg/mL with 0.1% of TFA and centrifuged in cold at 200 *g* for 3 min. Then, 15 µL of the sample were cleaned and concentrated using Zip-Tip C18 with standard bed format (Millipore Corporation, Bedford, MA) according to the manufacturer recommendations. Peptides eluted from Zip-Tip were dried and then resuspended in 20 µL of 0.1% of TFA.

Five microliters of the sample were injected into the nESI-LC-MS/MS system. Sample was 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 for 5 min and using 0.1% v/v TFA as mobile phase. Then, the trap column was automatically switched in-line onto a nano-HPLC capillary column (3µm, 75µm x 12.3 cm, C18; Nikkoy Technos Co, Ltd. Japan). Mobile phases consisted of solvent A, containing 0.1% v/v formic acid in water, and solvent B, containing 0.1% v/v formic acid in 100% acetonitrile. The LC 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 temperature of 30 °C.

The column outlet 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 100 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 - 5 charged ions.

2.4 Data analysis

Mascot Distiller v2.5.1. software (Matrix Science, Inc., Boston, MA, USA; <http://www.matrixscience.com>) has been used for automated spectral processing, peak list generation, and database search. The identification of peptides has been performed using UniProt database, the taxonomy parameter was designated as Mammalia, and oxidation of methionine (M) was selected as variable modification. Other amino acid

oxidations such as cysteine (C), histidine-tryptophan (HW), and proline (P) have been also tested with non-significant results. Generated MS/MS spectra were searched using a significant threshold of $p < 0.1$, a FDR of 1.5%, and a peptide tolerance on the mass measurements of 0.3 Da in MS mode and MS/MS ions.

3. Results and discussion

Teruel dry-cured ham peptide extracts have been analysed by nESI-LC-MS/MS to identify those peptides showing oxidation in their methionine residues. As a result of the intense proteolysis which takes place along the dry-curing process of ham, approximately 62,000 peptides have been identified, including more than 17,700 which contain oxidations of methionine. In this study, only peptides derived from the degradation of major myofibrillar proteins were selected, comprising 120 peptides with methionine oxidation from a total of 656 peptides resulting from the degradation of α -actin (ACTS), nebulin (NEBU), titin (TITIN), troponin I fast skeletal muscle (TNNI2), two types of myosin light chain proteins (myosin regulatory light chain 2 (MLRS) and myosin light chain (MYL1)), and two types of myosin heavy chain proteins (myosin-1 (MYH1) and myosin-7 (MYH7)).

Tables 1 and 2 show the sequences of the oxidised peptides, the protein of origin as well as the species in which it has been described, the observed and calculated masses together with the charge states, amino acid residues preceding and following the sequence, and the number of methionine oxidations detected in the peptide. Some of the identified peptides have been detected both with and without methionine oxidations (see those marked with an asterisk in Tables 1 and 2). This difference results on an earlier elution of the oxidised peptides from reversed-phase columns and a molecular mass increase of 16 Da for the respective non-oxidised peptides (Liu, Ponniah, Neill, Patel, &

176 [Andrien, 2013](#)). Three, two, four, and eight peptides derived from ACTS, MLRS,
177 MYH1, and MYL1 proteins, respectively, were present in both oxidised and non-
178 oxidised forms. Differences on both type of spectrums are described in [Figure 1](#), where
179 MS/MS spectrums of peptide DKEGNGTVMGAELR with and without methionine
180 oxidation from MYL1 protein are shown.

181 Cysteine and methionine are the most susceptible amino acids to oxidative changes
182 ([Zhang, et al., 2013](#)). Nevertheless, Cys, but also His-Trp, and Pro oxidations were also
183 included in the search with the result that only Met oxidations were detected.

184 [Table 3](#) lists the number of oxidised peptides compared to the total number of peptides
185 identified for each protein as well as the total percentage of methionine amino acid on
186 the studied sequences. Although theoretically, a high presence of Met residues on a
187 sequence could indicate a higher predisposition for oxidation, not all methionine
188 residues in a protein are susceptible in the same way because it depends on their
189 location within the protein structure and their accessibility to oxidants. In fact, the
190 highest percentages of Met are present in ACTS, TNNI2, and both myosin light chain
191 proteins (4.5, 5.5, 4.1, and 3.4 %, respectively), but corresponds to the lowest
192 percentages of identified oxidised peptides (11.6, 22.2, 5.9, and 7.4%, respectively).

193 The fact that these are the smallest of the studied proteins and those showing the highest
194 sequence coverage when identifying the sequence of the generated peptides, could be
195 due to heavier proteins such as TITIN, NEBU, or MYH also show more complex
196 structures that difficult the hydrolysis, showing lower percentages of sequence coverage.

197 TITIN, NEBU, and MYH showed the highest percentage of oxidation, comprising
198 between 35 and 17% of the total number of peptides identified from their sequences.

199 The neighbouring amino acid residues also determine methionine oxidation, being those
200 surrounded by Ala, Thr, Ser, and acidic amino acids such as Asp and Glu more

201 susceptible to oxidation than Met located close to basic amino acids ([Levine, et al.,](#)
202 [1996; Ghesquière, & Gevaert, 2014](#)). In this study, a total of 61 of the 120 oxidised
203 peptides identified in dry-cured ham extract show Asp, Glu, Ala, Thr and Ser residues
204 next to the oxidised Met. However, only 31 of the identified peptides showing oxidation
205 had any of the basic amino acids Arg, Lys, and His next to the oxidised Met.

206 Methionine oxidation can be caused by different factors, being influenced from the
207 oxidative stress suffered by the animal from the slaughtering up to the meat industry
208 processing and sample preparation and analysis. In fact, technological operations such
209 as pre-freezing, pre-mincing or packaging have been described to have a great impact
210 on the oxidation of muscle proteins ([Utrera & Estévez, 2013; Utrera, Parra & Estévez,](#)
211 [2014](#)). On the other hand, methionine amino acids have also been described to be
212 spontaneously oxidised during sample preparation, chromatography separation and
213 ionisation of MS analysis ([Liu, et al., 2013; Drazic, & Winter, 2014](#)), although it has
214 been established that these factors, when occur, do not represent more than 2% of total
215 amount of Met oxidations.

216 Recently, it has been described that oxidative protein modifications in dry-cured
217 products are accelerated during first steps of processing but stabilised towards the final
218 stages of maturation ([Koutina, et al., 2012](#)). Same authors also showed that oxidation
219 may change the susceptibility of proteins to the proteolytic enzymes in Parma ham,
220 influencing the proteolysis reactions ([Koutina, et al., 2012](#)). Proteolysis, protein
221 oxidation and peptidases activity have also been studied in Xuanwei ham, concluding
222 that although oxidation occurs during salting stage, it is decelerated due to the low
223 temperatures employed ([Wang, et al., 2011](#)). Finally, [Gallego, Mora, Fraser, Aristoy,](#)
224 [and Toldrá \(2014\)](#) reported the identification by LC-MS/MS of some peptides derived

from LIM domain-binding protein showing methionine oxidation in dry-cured hams at different times of ripening, identifying most of them at the end of the processing.

4. Conclusions

This study evidences the importance of methionine oxidation in dry-cured ham processing by identifying a total of 120 sequences of peptides including a minimum of one oxidised Met in their sequence. The described peptidomic approach gives a new point of view in the detection and identification of those peptides responsible for the oxidation and provides valuable information of neighbouring amino acids, degree of hydrolysis of the sample, proteins of origin affected, and characteristics of the peptide, which might be very useful to better understand and characterise the complex oxidation mechanisms occurring in dry-curing processing.

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References

- Antequera, T., López-Bote, C.J., Córdoba, J.J., García, C., Asensio, M.A., Ventanas, J.,
García-Regueiro, J.A., & Díaz, I. (1992). Lipid oxidative changes in the
processing of Iberian pig hams. *Food Chemistry*, 45 (2), 105-110.
- Aristoy, M.C., & Toldrá, F. (1995). Isolation of flavor peptides from raw pork meat and
dry-cured ham. In G. Charalambous (Ed.), *Food flavors: Generation, analysis
and processes influence* (pp. 1323-1344). Amsterdam: Elsevier Science.
- Armenteros, M., Heinonen, M., Ollilainen, V., Toldrá, T., & Estévez, M. (2009).
Analysis of protein carbonyls in meat products by using the DNPH-method,
fluorescence spectroscopy and liquid chromatography–electrospray
ionisation–mass spectrometry (LC–ESI–MS). *Meat Science*, 83, 104–112.
- Drazic, A., & Winter, J. (2014). The physiological role of reversible methionine
oxidation. *Biochimica et Biophysica Acta - Proteins and Proteomics*, 1844 (8),
1367-1382.
- Elias, R. J., Kellerby, S. S., & Decker, E. A. (2008). Antioxidant activity of proteins and
peptides. *Critical Reviews in Food Science and Nutrition*, 48, 430–441.
- Estévez, M., Ventanas, S., & Cava, R. (2005). Protein oxidation in frankfurters with
increasing levels of added rosemary essential oil: Effect on colour and texture
deterioration. *Journal of Food Science*, 70, C427–C432.
- Estévez, M. (2011). Protein carbonyls in meat systems: A review. *Meat Science*, 89 (3),
259–279.
- Gallego, M., Mora, L., Fraser, P.D., Aristoy, M.A., & Toldrá, F. (2014). Degradation of
LIM domain-binding protein three during processing of Spanish dry-cured ham.
Food Chemistry 149, 121–128.

274 Gandemer, G. (2002). Lipids in muscles and adipose tissues, changes during processing
 275 and sensory properties of meat products. *Meat Science*, 62 (3), 309–321.

276 Ghesquière, B., & Gevaert, K. (2014). Proteomics methods to study methionine
 277 oxidation. *Mass Spectrometry Reviews*, 33 (2), 147-156.

278 Kim, G., Weiss, S.J., & Levine, R.L. (2014). Methionine oxidation and reduction in
 279 proteins. *Biochimica et Biophysica Acta - General Subjects*, 1840 (2), 901-905.

280 Koutina, G., Jongberg, S., & Skibsted, L.H. (2012). Protein and lipid oxidation in Parma
 281 ham during production. *Journal of Agricultural and Food Chemistry*, 60 (38),
 282 9737-9745.

283 Ladikos, D., & Lougovois, V. (1990). Lipid oxidation in muscle foods – a review. *Food*
 284 *Chemistry*, 35 (4), 295-314.

285 Lametsch, R., Karlsson, A., Rosenvold, K., Andersen, H. J., Roepstorff, P., & Bendixen,
 286 E. (2003). Postmortem proteome changes of porcine muscle related to
 287 tenderness. *Journal of Agricultural and Food Chemistry*, 51 (24), 6992-6997.

288 Levine, R.L., Mosoni, L., Berlett, B.S., & Stadtman, E.R. (1996). Methionine residues
 289 as endogenous antioxidants in proteins. *Proceedings of the National Academy*
 290 *of Sciences of the United States of America*, 93 (26), 15036-15040.

291 Liu, H., Ponniah, G., Neill, A., Patel, R., & Andrien, B. (2013). Accurate determination
 292 of protein methionine oxidation by stable isotope labeling and LC-MS analysis.
 293 *Analytical Chemistry*, 85 (24), 11705-11709.

294 Lund, M.N., Heinonen, M., Baron, C.P., & Estévez, M. (2011). Protein oxidation in
 295 muscle foods: A review. *Molecular Nutrition and Food Research*, 55 (1), 83-
 296 95.

297 Ruiz, J., Ventanas, J., Cava, R., Andrés, A., & García, C. (1999). Volatile compounds of
 298 dry-cured Iberian ham as affected by the length of the curing process. *Meat*
 299 *Science*, 52 (1), 19-27.

300 Shacter, E. (2000). Quantification and significance of protein oxidation in biological
 301 samples. *Drug Metabolism Reviews*, 32 (3-4), 307-326.

302 Toldrá, F., Rico E., & Flores, J. (1993). Cathepsin B, D, H and L activity in the
 303 processing of dry-cured ham. *Journal of the Science of Food and Agriculture*,
 304 62 (2), 157-161.

305 Toldrá, F., & Flores, M. (1998). The role of muscle proteases and lipases in flavor
 306 development during the processing of dry-cured ham. *Critical Reviews in Food*
 307 *Science and Nutrition*, 38 (4), 331-352.

308 Utrera, M., & Estévez, M. (2013). Oxidative damage to poultry, pork, and beef during
 309 frozen storage through the analysis of novel protein oxidation markers. *Journal*
 310 *of Agricultural and Food Chemistry*, 61 (33), 7987-7993.

311 Utrera, M., Parra, V., & Estévez, M. (2014). Protein oxidation during frozen storage and
 312 subsequent processing of different beef muscles. *Meat Science*, 96 (1), 812-820.

313 Vogt, W. (1995) Oxidation of methionyl residues in proteins: Tools, targets, and
 314 reversal. *Free Radical Biology and Medicine*, 18 (1), 93-105.

315 Wang, Z., Xu, Y., Zhang, J., Li, X., Lin, Z., & Ma, C. (2011). Proteolysis, protein
 316 oxidation and protease activity in dry-cured Xuanwei ham during the salting
 317 stages. *International Journal of Food Science and Technology*, 46 (7), 1370-
 318 1377.

319 Zhang, W., Xiao, S., & Ahn, D.U. (2013). Protein oxidation: basic principles and
 320 implications for meat quality. *Critical Reviews in Food Science and Nutrition*,
 321 53 (11), 1191-1201.

322 **FIGURES CAPTIONS**

323 **Figure 1.** MS/MS spectrum of peptide DKEGNGTVMGAELR from myosin light chain
324 protein (MYL1_BOVIN). B) Spectrum of same peptide showing the oxidised
325 methionine in blue.

Table 1. Oxidised peptides identified by nESI-LC-MS/MS from actin, titin and troponin I proteins in dry-cured ham.

Protein name ^a	Species	Observed (m/z) ^b	Calculated (mr) ^c	Charge (+)	P _o ^d	Sequence	Pf ^e	No ox ^f
ACTS	BOVIN	293.21	876.45	3	G	IADRMQK	E	1
		530.25	1058.47	2	F	IGMESAGIHE*	T	1
		589.31	1176.61	2	K	EITALAPSTMK*	I	1
		608.31	1214.56	2	P	RHQGVVMGMGQ	K	1
		636.29	1270.55	2	M	VGMGQKDSYVGD*	E	1
		671.32	1340.60	2	M	SGGTTMYPGIADR	M	1
		464.24	1389.59	3	D	LYANNVMSGGTTM	Y	2
		477.82	1430.58	3	Y	ANNVMSGGTTMYPG	I	2
		615.32	1842.93	3	L	TEAPLNPKANREKMTQ	I	1
		716.33	2145.98	3	D	LYANNVMSGGTTMYPGIADR	M	1
		856.54	2566.35	3	H	AIMRLDLAGRDLTDYLMKILTE	R	1
TITIN	HUMAN	561.26	1120.51	2	A	RMSPARMSPG	R	2
		355.07	708.35	2	E	VKSQMT	E	1
		253.18	756.28	3	A	SMSAQSM	S	1
		405.26	808.37	2	K	ASDRLTM	K	1
		419.31	836.41	2	K	LTVLNMM	K	1
		432.24	862.47	2	P	VVTNRKM	C	1
		439.24	876.45	2	D	HGLYMIK	V	1
		318.52	952.41	3	S	VEVMETET	A	1
		325.20	972.49	3	G	RPVPAMTW	F	1
		327.22	978.51	3	R	IDLSVAMKS	L	1
		338.15	1011.46	3	R	TYIPVMSGE	N	1
		514.28	1026.48	2	I	DDYLAMKR	T	1
		531.26	1060.43	2	H	GERYQMDf	L	1
		359.19	1074.61	3	S	ASIMVKAINI	A	1
		575.29	1148.57	2	I	DLSTMPQKTI	H	1
		384.16	1149.59	3	R	VDKYKHMLT	I	1
		393.23	1176.57	3	P	IRMSPARMSP	A	2
		598.31	1194.54	2	N	ADISMGDVATLS	V	1
		399.22	1194.58	3	T	FQVMVNSAGR	S	1
		629.44	1256.67	2	K	EMRLNVLGRPG	P	1
		650.68	1299.49	2	T	CQATNDVGKDMC	S	1
		441.30	1320.64	3	R	IAAENSIGMGPFV	E	1
		669.37	1336.65	2	S	LEVNTIAKDSMT	V	1
		465.77	1394.55	3	T	EMKFASMSAQSM	S	3
		466.24	1395.63	3	S	SQSVQMSASKQEA	S	1
		469.25	1404.70	3	S	FVQKPDPMQDLT	G	1
		490.26	1467.81	3	R	LMVSVSGRPPPVIT	W	1
		746.85	1491.74	2	L	MRKTQASTVATGPE	V	1
		500.84	1499.66	3	L	ISQSERCSTMTTE	K	1
		504.28	1509.72	3	A	RLMIREAFAEDSG	R	1
		507.94	1520.82	3	T	LTVKNASGTKAVSVM	V	1
		510.31	1527.73	3	D	YELLMKSQQEML	Y	1
		523.34	1566.72	3	I	KHGERYQMDFLQ	D	1
		530.28	1587.78	3	F	QVMAVNSAGRSAPRE	S	1
		533.27	1596.83	3	K	VPVTMTRYRSTGLT	E	1
		543.28	1626.77	3	E	PPEIDMKNFSPSTV	Y	1
		545.30	1632.75	3	K	GSMLVSWTPPLDNGGS	P	1
		563.31	1686.78	3	E	SYVIELMTGTDEW	V	1
		564.29	1689.77	3	Q	WTKPVYDGGSMITGY	I	1
		567.53	1699.71	3	S	SFSSSSASSMTEMKFA	S	1
		871.45	1740.90	2	K	AMTLGVSYKVTGLIEGS	D	1
		590.49	1768.71	3	S	MTEMKFASMSAQSMSS	M	1
		607.59	1819.91	3	P	AVIVEKAGPMTVTGETC	T	1
		611.61	1831.82	3	G	LYICKAENMLGESTCAA	E	1
		616.33	1845.97	3	G	FIERKDAKMHTWR	Q	1
		985.06	1967.95	2	E	NLYGSDPLVSDSMKAKD	R	1
		657.37	1968.84	3	D	GMTLTWYPPEDDGGSQVT	G	1
		988.49	1974.77	2	S	ASSMTEMKFASMSAQSMS	S	4
		718.35	2152.09	3	A	NLKMELRDALCAIYEEI	D	1
		719.37	2155.18	3	D	KSAAVATVVAADMARVREPV	I	1
		778.47	2332.14	3	P	IRMSPARMSPARMSPARMSPA	R	2
		908.31	2722.09	3	S	SMTMTEMKFASMSAQSMSSMQESFVE	M	2
TNNI2	HUMAN	424.73	847.45	2	K	SVMLQIAA	T	1
		327.22	978.40	3	I	PGSMSEVQE	L	1

^aProtein name according to Uniprot database. ACTS: Actin, alpha skeletal muscle; TITIN: Titin; TNNI2: Troponin I, fast skeletal muscle. ^b Molecular ion mass observed in the nESI-LC-MS/MS analysis calculated in Daltons. ^cCalculated relative molecular mass of the matched peptide. ^dPosition of the amino acid residue preceding the peptide sequence. ^ePosition of the amino acid residue following the peptide sequence. ^fNumber of methionine oxidations in the identified peptide. *Sequences identified with and without methionine oxidation.

Table 2. Oxidised peptides identified by nESI-LC-MS/MS from myosin and nebulin proteins in dry-cured ham.

Protein		Observed	Calculated	Charge				
name ^a	Species	(m/z) ^b	(mr) ^c	(+)	P _o ^d	Sequence	Pf ^e	No ox ^f
MLRS	BOVIN	590.77	1179.53	2	D	AMMKEASGPIN	F	2
		605.81	1209.57	2	M	MKEASGPINFT*	V	1
		536.25	1605.66	3	R	AAAEAGSSSVFSMFDQ	T	1
		669.98	2006.93	3	N	VKNEELDAMMKEASGPIN*	F	2
MYH1	PIG	413.27	824.37	2	P	TSGKMQGT	L	1
		511.43	1020.57	2	E	KPMGIFSIL	E	1
		520.14	1038.47	2	R	VESMQSML	D	1
		550.26	1098.50	2	K	TPGAMEHELV	L	1
		646.33	1290.67	2	Y	QKMVERRESI	F	1
		652.32	1302.61	2	I	IPNETKTPGAME*	H	1
		668.34	1334.63	2	E	DQVFPMNPPKF*	D	1
		759.37	1516.72	2	L	TVKEDQVFPMNPP*	K	1
		554.29	1659.85	3	K	IEDEQALAMQLQKK	I	1
		636.65	1906.91	3	L	TVKEDQVFPMNPPKFD*	K	1
		488.76	1950.87	4	N	EVEDLMIDVERSNAACAA	L	1
MYH7	PIG	537.28	1072.46	2	C	MFPKATDMT	F	2
		404.26	1209.58	3	T	KGQNVQQVMY	A	1
		407.86	1220.63	3	R	KELEEKMSVL	L	1
		656.35	1310.63	2	V	TKGQNVQQVMY	A	1
		665.42	1328.68	2	A	LAKAVYEKMFN	W	1
		522.28	1563.71	3	R	LEEAGGATSVQIEMN	K	1
		524.27	1569.77	3	I	QLSHANRMAEAQK	Q	1
MYL1	BOVIN	557.25	1112.48	2	V	FDKEGNGTVM*	G	1
		585.76	1169.50	2	V	FDKEGNGTVMG*	A	1
		606.78	1211.55	2	R	VFDKEGNGTVM*	G	1
		635.29	1268.57	2	R	VFDKEGNGTVMG*	A	1
		670.81	1339.61	2	R	VFDKEGNGTVMGA*	E	1
		498.24	1491.70	3	F	DKEGNGTVMGAELR*	H	1
		543.92	1628.76	3	F	DKEGNGTVMGAELRH*	V	1
		554.29	1659.81	3	N	AEVKKVLGNPSNEEM	N	1
		592.29	1773.86	3	N	AEVKKVLGNPSNEEMN*	A	1
		615.94	1844.89	3	N	AEVKKVLGNPSNEEMNA	K	1
		696.54	2086.85	3	E	EVEALMAGQEDSNGCINYE	A	1
NEBU	HUMAN	818.40	2452.19	3	K	VLGNPSNEEMNAKKIEFEQFL	P	1
		401.22	800.39	2	V	KHAMEVA	K	1
		402.22	802.40	2	N	ALTMSKH	L	1
		452.23	902.48	2	S	PVDMLSIL	L	1
		302.20	903.45	3	L	SMLGRPDI	E	1
		911.62	910.41	1	K	FSSPVDML	G	1
		498.28	994.43	2	R	GKGLTEMED	T	1
		525.38	1048.51	2	H	TPLDMVSVTA	A	1
		351.22	1050.47	3	Y	TMSPDLPQF	L	1
		357.20	1068.48	3	K	SNYSIMLEP	P	1
		363.19	1086.50	3	Y	HTPADMLSVT	A	1
		551.30	1100.54	2	K	AHMLKTRND	Y	1
		408.90	1223.50	3	I	HTYNMLPDAM	S	2
		423.24	1266.68	3	G	KGKMVGFRSLE	D	1
		427.27	1278.62	3	L	LSRVNQITMSD	K	1
		714.42	1426.68	2	K	DSQLYKVMKDAN	N	1
		775.38	1548.70	2	E	KSMSYYETVLDTP	E	1
		535.62	1603.77	3	A	KMQSERYKKDF	E	1
		821.49	1640.79	2	A	DYADFMKGIGWLPL	G	1
		555.37	1662.82	3	D	MLSVTAAKDAQANITN	T	1
		779.41	2335.20	3	L	EQVLAKNNALNMNKRLYTEA	W	1
		797.50	2389.29	3	V	IRKKVDPSKFMTPYIAHSQK	M	1

^aProtein name according to Uniprot database. MLRS: Myosin regulatory light chain 2, skeletal muscle; MYH1: Myosin-1; MYH7: Myosin-7; MYL1: Myosin light chain 1/3, skeletal muscle isoform; NEBU: Nebulin. ^b Molecular ion mass observed in the nESI-LC-MS/MS analysis calculated in Daltons. ^cCalculated relative molecular mass of the matched peptide. ^dPosition of the amino acid residue preceding the peptide sequence. ^ePosition of the amino acid residue following the peptide sequence. ^fNumber of methionine oxidations in the identified peptide. *Sequences identified with and without methionine oxidation.

Table 3

Table 3. Characteristics of studied proteins in relation to the Met oxidation identified from the dry-cured ham generated peptides.

Protein name ^a	Molecular mass (kDa)	Protein lenght (aa)	Number of Methionine	Percentage of Methionine (%)	Oxidised peptides ^b	Non-oxidised peptides ^c	Total number of peptides ^d	Percentage oxidation (%)	Sequence coverage ^e (%)
TITIN	3816.03	34350	398	1.15	52	240	292	17.81	30
NEBU	772.914	6669	185	2.77	21	39	60	35.00	23
MYH1	223.173	1939	52	2.68	11	51	62	17.74	30
MYH7	223.298	1935	52	2.68	7	22	29	24.14	32
ACTS	42.051	377	17	4.5	11	84	95	11.58	66
TNNI2	21.339	182	10	5.49	2	7	9	22.22	21
MYL1	19.505	176	6	3.4	12	150	162	7.41	80
MLRS	19.013	170	17	4.11	4	63	67	5.97	73

^a Protein name according to Uniprot database. ^b Number of peptides showing Met oxidation identified in the protein. ^c Number of non-oxidised peptides identified in the protein. ^d Total number of peptides identified. ^e Percentage of the sequence of origin protein covered by the identified peptides.

Figure 1

A) DKEGNGTVMGAELR (3+)

B) DKEGNGTVMGAELR (3+)

Figure 1. A) MS/MS spectrum of peptide DKEGNGTVMGAELR from myosin light chain protein (MYL1_BOVIN). B) Spectrum of same peptide showing the oxidised methionine in blue.