Degradation of myosin heavy chain and its potential as a source of natural bioactive peptides in dry-cured ham

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Abstract

Myofibrillar proteins are extensively degraded by muscle endo- and exopeptidases during the ageing of meat and the processing of meat products. One of the most studied products is dry-cured ham. This degradation implies changes in the product in terms of texture (mainly due to calpains and cathepsins endopeptidases) and flavour (due to the action of exopeptidases) and defines its final quality. During the last decade, naturally generated peptides from the myofibrillar proteins titin, myosin light chain, troponin T, LIM domain-binding protein 3, and actin have been identified using peptidomic approaches, showing also the potential to act as bioactives in the human body when ingested. In this study, a mass spectrometry in tandem approach has been used for the identification of peptides naturally generated during the proteolysis of myosin heavy chain protein occurred after 9 months of Spanish dry-cured ham processing. The potential of these peptides to act as bioactives has also been studied.

Keywords: mass spectrometry in tandem, peptidomics, dry-cured meat, proteolysis, bioactives, myofibrillar proteins.

1. Introduction

The processing of dry-cured meat products includes drying and ripening steps that result in the progressive reduction of water activity and the occurrence of proteolysis. This biochemical reaction implies the degradation of proteins by the action of endogenous peptidases and the generation of oligopeptides, small peptides, and free amino acids. Main enzymes of meat products involved in this process are endogenous muscle peptidases in loin and dry-cured ham, and the combined effect of muscle and microbial enzymes in products such as semidry- and dry-fermented sausages (Toldrá and Reig, 2015). The degradation of muscle proteins starts with the action of the muscle endopeptidases cathepsins and calpains and the generation of a large amount of polypeptides (Toldrá, 2002), and continues with the action of certain groups of muscle exopeptidases that are able to degrade protein fragments into small peptides such as di- and tripeptides, and free amino acids (Toldrá and Flores, 1998; Toldrá, 2006).

Myosins are a basic and majoritarian component of skeletal muscles that participate together with actin, tropomyosin, and troponin proteins in muscle contraction as well as in a wide variety of non-muscular cell movements. A myosin molecule is a hexamer consisting of two heavy chains (MHC) and two light chains (MLC) (Goll et al., 2008).

The intense proteolysis suffered by structural muscle proteins in general, and particularly by MHC, during the dry-curing process of ham has been extensively described. For that, methodologies such as electrophoresis on polyacrylamide gels (SDS-PAGE) to separate the proteins, and matrix-assisted laser desorption/ionisation (MALDI) mass spectrometry for the identification of proteins by peptide mass fingerprint have been used (Di Luccia et al., 2005; Fabbro et al., 2016). However, the use of mass spectrometry in tandem (MS/MS) is crucial to elucidate the sequence of the small peptides naturally generated during the processing.

Bioactive peptides are inactive when they are part of parent protein, but turn active when released due to the action of enzymes. In this respect, pork raw meat has been previously identified as a source of ACE-inhibitory peptides when digested under controlled conditions of hydrolysis as well as after *in vitro* gastrointestinal (GI) digestion (Arihara et al., 2001; Katayama et al., 2003; Escudero et al., 2010; Escudero et al., 2012). In a similar way, aged beef loin has been described to contain ACE-inhibitory and antioxidant peptides which remain active after cooking and simulated GI digestion (Mora et al., 2017). Finally, the generation of bioactive peptides such as ACE-inhibitory and antioxidant peptides from dry-cured meat products has also been in the focus of attention during the last years due to the importance of meat proteome as a source of functional biopeptides naturally generated during the ripening processes (Mora et al., 2016).

In this study, the identification of peptide sequences naturally hydrolysed from MHC in the fast-glycolytic *Biceps femoris* muscle during the processing of 9 months Spanish dry-cured ham has been achieved using mass spectrometry in tandem. The potential of bioactive peptides generation has also been discussed.

2. Material and methods

2.1. Chemicals and reagents

Trifluoroacetic acid (TFA) was purchased from Sigma Aldrich (St. Louis MO, USA), whereas the other reagents used in mass spectrometry analysis such as acetonitrile (ACN), formic acid (FA), and water were of MS grade and purchased from Scharlab (Barcelona, Spain). Reagents used in the peptide extraction were of analytical grade and purchased from Scharlab (Barcelona, Spain).

2.2. Extraction and fractionation of peptides using size-exclusion chromatography

A total of six dry-cured hams from pigs of industrial genotypes Landrace x Large White were processed until 9 months of curing in a local factory in Spain. Then, the extramuscular fat of *Biceps femoris* muscle was removed and the dry-cured muscle was processed according to Mora et al. (2011a). The extracted peptides were fractionated depending on their molecular weight using a Sephadex column (G25 stationary phase, 2.5 x 65 cm from Amersham Biosciences, Uppsala, Sweden) by size-exclusion chromatography according to Mora et al. (2011a). The fractions corresponding to the molecular weight peptides within the range 1000-3000 Da were pooled together and lyophilised. The mix was suspended in 5 ml of 0.1% TFA/ACN (95:5, v/v) for further analysis.

2.3. Reversed-phase high-performance liquid chromatography (RP-HPLC) of the selected peptide fractions

The RP-HPLC methodology was that described in Mora et al. (2011a). Briefly, a total of 100 µl of the isolated peptides was injected in a Symmetry column with C18 stationary phase (250 x 4.6 mm, 5 µm) from Waters (Milford, MA), and using an HPLC Agilent 1100 series system (Agilent Technologies, Palo Alto, CA). Mobile phases were solvent A, 0.1% TFA, and solvent B, 0.05% TFA/ACN (5:95, v/v). The chromatographic separation was 5 min with solvent A in isocratic gradient, and 70 min with a linear gradient from 0 to 40% of solvent B. The separation was done using a flow rate of 1 ml/min and at 25ºC of column temperature, and monitored at 214 nm to detect the peptides. Finally, fractions of 1 ml were collected with an automatic collector connected to the HPLC waste tube and lyophilised for its later identification by MS/MS.

2.4. Acquisition of mass spectrometry data and database search

Peptides previously obtained in RP-HPLC were analysed by MS/MS using a liquid chromatography system Ultimate Plus/Famos nano LC system (LC Packings,

129 Amsterdam, The Netherlands) and the QSTAR XL hybrid quadrupole-Time-of-Flight
130 mass spectrometer (ABSCIEX, CA, USA) with a nano-electrospray ion source.
131 Fractions of 1 ml collected from the RP-HPLC separation and previously lyophilised were
132 dissolved in 60 µl of loading buffer (0.1% of FA and 2% of ACN) and injected. Samples
133 were cleaned and concentrated on a C18 PepMap trap column (0.3 x 5 mm, 3 µm; LC
134 Packings, Dionex Company, Amsterdam, The Netherlands) at a flow rate of 40 µl/min
135 with 0.1% of TFA as mobile phase. After three minutes, the trap column was switched
136 in-line with a Dionex C18 PepMap column from LC Packings (0.075 x 150 mm, 3 µm;
137 Dionex Company, Amsterdam, The Netherlands). Mobile phases were solvent A, with
138 0.1% FA and solvent B, with 0.1% FA in 95% ACN. The LC separation of the peptides
139 was done during 30 minutes in a linear gradient from 95% to 50% of solvent A at a flow
140 rate of 0.2 µl/min. The column outlet was directly coupled to a nano-electrospray ion
141 source. QSTAR instrument was used in positive mode. TOF MS survey scan was
142 registered for mass range *m/z* 350 to 1800 followed by MS/MS scans of the three most
143 intense peaks. Source parameters and spray were optimised using a protein mixture
144 digestion with trypsin (LC Packings; P/N 161088; Dionex Company, Amsterdam, The
145 Netherlands).
146 Database search was done using Mascot 2.6 in combination with Mascot Daemon
147 interface 2.5 (Matrix Science, Inc., Massachusetts, USA)
148 (hppt://www.matrixscience.com), and the ProteinPilot™ software (ABSCIEX, CA,
149 USA). NCBInr protein database was used to identify the peptides. Main parameters
150 considered were unknown enzyme and *Sus scrofa* taxonomy. BLAST
151 (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was used in the alignment of the different
152 isoforms of MHC sequences.
153

3. Results and discussion

During the last decade, MS/MS has been the method of choice for the identification of the naturally generated peptides during meat processing (maturation, fermentation, dry-curing, …) and different studies in this respect have been published. The total number of identified peptides from different myofibrillar and sarcoplasmic proteins in dry-cured ham samples is compiled in **Table 1**. This table also includes the potential sequences of di- and tripeptides that could have been generated from the action of muscle exopeptidases on the identified peptides. The mass spectrometric approach used in these studies is optimal for the identification of peptides from 800 to 3000 Da that are the most common sizes after natural proteolytic processes.

Despite the importance of MHC as responsible, together with MLC, for contractile properties in muscle, the identification of naturally generated fragments from MHC has been scarcely reported to date. In fact, several sequences of MYH1_PIG, MYH4_PIG and MYH7_PIG were previously described in Iberian dry-cured ham with 24 months of curing (Mora et al., 2015b), as well as a total of 11 and 7 oxidised peptides from MYH1_PIG and MYH7_PIG, respectively, were identified by nESI-LC-MS/MS in PDO Teruel dry-cured ham of 14 months of curing (Gallego et al., 2015b).

In mammalian skeletal muscles there are four MHC isoforms, the three fast (2a, 2x, and 2b) isoforms and the slow (1) isoform, that correspond to UniProt database entry names MYH1, MYH2, MYH4, and MYH7, respectively. Regarding pig muscle proteome, they share most of their proteomic sequence as shown in **Figure 1 of Supplementary material**. In this regard, **Table 2A** shows the identity percentage resulting from the comparison of the different sequences in BLAST database. These results show the high percentage of homology between isoforms that comprises from 96.5% to 94.2% between MYH1, MYH2, and MYH4. In **Table 2B**, the different nomenclatures used by UniProt

179 and NCBInr databases for same proteins are clarified. Regarding similarities between
180 sequences, MYH7_PIG presents the lowest homology with the other isomers as shown
181 in **Table 2A** (between 81.7 and 81.8%), but presents 100% homology with Q29107_PIG
182 protein, which is the partial sequence of slow myosin heavy chain-beta protein in UniProt
183 database, as well as with the NCBInr protein described as myosin heavy chain slow
184 isoform protein (gi|125987844).
185 In this study, peptides generated from *Biceps femoris* muscle in 9 months Spanish dry-
186 cured ham have been extracted, isolated, and analysed by MS/MS, identifying a total of
187 51 peptides originated from the different isoforms of MHC. The fragments identified
188 from MHC protein isoforms come from different sections of the sequences as it is shown
189 in grey shadow in **Figure 1 of Supplementary material,** whereas **Table 3** shows the
190 sequences identified by nLC-MS/MS from 9 month Spanish dry-cured ham. Twenty-
191 three of the identified peptides share homology with MYH1_PIG, twenty-seven peptides
192 with MYH2_PIG, and forty-five peptides with MYH4_PIG, whereas only five peptides
193 were identified from MYH7_PIG. The knowledge of the generated MHC sequences
194 contributes to elucidate the main enzymes acting during the curing period and proves the
195 intense hydrolysis occurred. In this sense, **Table 3** shows the action of endopeptidases
196 enzymes in the scission observed between the peptides SSDQEMAIFGEAAPYLR
197 (position 2-18) and KSEKERIEAQNKPFDAKTS (position 19-37), in both MYH1 and
198 MYH4 proteins. The action of endopeptidases at this stage of curing makes sense because
199 cathepsins B, H, and L have been previously reported to be stable even after 15 months
200 of dry-cured ham processing (Toldrá et al., 1993; Toldrá, 1998), and cathepsin D activity
201 remains up to 6 to10 months of processing (Rico et al.,1991). The optimal pH for
202 cathepsins B and L is around 6, whereas for cathepsin H is 6.8. Cathepsin D shows an
203 optimal pH range between 3.0 and 5.0. Finally, calpains are also able to participate in the

204 degradation of proteins but only during the first weeks of the curing processing because
205 of their low stability and optimal neutral pH of 7.5 (Toldrá and Flores, 1998). Different
206 studies have described the relation of cathepsins with the disappearance of myosin protein.
207 As an example, Hirao et al. (1984) incubated *in vitro* myosin from rabbit muscle with
208 cathepsin B and proved the hydrolysis of MHC and MLC 2. Also the progressive
209 disappearance of myofibrillar proteins like MHC, MLC 1 and MLC 2, and troponins C
210 and I, as well as the generation of fragments with sizes between 50-100 and 20-45 kDa
211 have been reported by Toldrá et al. (1993).
212 According to the observed sequences derived from this study, there is also a contribution
213 of exopeptidases to the generation of di- and tripeptides such as the dipeptides TS, TL,
214 FD, VK, AT and QT, and the tripeptides SRE, TVQ, NAS, KIE and GKM (Sentandreu
215 et al., 2003). However, the action and stability of dipeptidyl peptidases depends on
216 different factors such as the presence of salt or other peptides so it is difficult to determine
217 their participation (Gianelli et al., 2000; Sentandreu and Toldrá, 2001). More specifically,
218 the action of aminopeptidases and carboxypeptidases and their contribution to increase
219 the free amino acids content in the muscle has been reported to last more than 12 months
220 of processing and it is also proved with the release of G, K, D, T, A, L, S, I, and F free
221 amino acids (Toldrá et al., 2000).
222 The composition in MLC and MHC of muscle fibres can determine the functional
223 properties of muscles. In this respect, slow type fibres such as beta myosin heavy chain
224 (MYH7_PIG) are rich in slow oxidative type muscles, whereas fast type fibres such as
225 myosin heavy chain 2b (MYH4_PIG) are rich in fast glycolytic type muscles (Schiaffino
226 and Reggiani, 1996). *Biceps femoris* muscle has been classified as a glycolytic muscle by
227 different authors (Flores et al., 1996; Leseigneur-Meynier and Gandemer, 1991). This
228 fact could explain the high amount of peptides derived from MHY4_PIG that have been

229 identified in this study in comparison with peptides derived from MHY7_PIG. The
230 relation of MHC fibres to meat quality parameters such as pH, drip loss, colour, or yield
231 force in pork meat has been well documented considering that muscles with fast
232 glycolytic fibres (MHC 2x, 2b) are associated to better quality attributes than those
233 muscles rich in slow-oxidative fibres (Chang et al., 2003). Also the influence of MHC
234 isoforms on fatty acids composition and sensory quality (juiciness, off-flavour, and
235 tenderness attributes) has been proved (Kang et al., 2011). In relation to this, the type of
236 fibres has been described to influence the early postmortem glycolytic rate and thus, the
237 content in the different MHC isoforms may constitute a useful parameter for examining
238 the variations of pork quality in PSE (pale, soft and exudative) muscles (Choi et al., 2007)
239 as well as to differentiate between meat containing halothane gen (HAL)-negative and
240 halothane carrier (Nn) pigs (Eggert et al., 2002).
241 On the other hand, certain food processes such as curing or fermentation in cheese, wine,
242 or meats have been described as good sources of bioactive peptides (Corrêa et al., 2014;
243 Mohanty et al., 2016), which are generally short sequences of 2–20 amino acids in length
244 with molecular mass of approximately 400–3000 Da (Korhonen and Pihlanto, 2003). In
245 this study, the identified peptides show a molecular mass distribution from 1000 to 2500
246 Da (**Table 3**). As it has been previously described, the presence of several di- and
247 tripeptides can be elucidated from the identified sequences showed in **Table 3**. In this
248 respect, BIOPEP database (http://www.uwm.edu.pl/biochemia/index.php/ en/biopep)
249 was used to identify potential bioactive peptides generated from the MHC protein. The
250 dipeptides TS, TL, AT, VK, QT have been described as DPP IV inhibitors (Lan et al.,
251 2015), whereas the dipeptide VK, obtained from soya source, was identified as ACE
252 inhibitor showing an IC_{50} value of 13 μM (Wang and Gonzalez de Mejia, 2005). Previous
253 studies identified two ACE inhibitory pentapeptides (MNPPK and ITTNP) from

thermolysin digestion of myosin protein, both corresponding to MHC. Both peptides also resulted to be antihypertensive *in vivo* when tested in spontaneous hypertensive rats at a concentration of 1 mg/kg animal weight, resulting in a decrease in systolic blood pressure of 23.4 ± 3.0 mmHg and 21.0 ± 3.1 mmHg after 6 h, respectively (Arihara et al., 2001). On the other hand, the peptide SNAAC derived from MHC 1 and 4 has been reported as the most antioxidant peptide identified to date in Spanish dry-cured ham (Mora et al., 2014). This peptide showed an IC_{50} value of 75.2 μM in DPPH radical-scavenging assay and 205 μM in ferric-reducing antioxidant power analysis, which were similar values to those obtained for the positive control butylated hydroxytoluene (BHT) (Mora et al., 2014). Moreover, a recent study evidenced the stability of this potent antioxidant peptide to different processing conditions and its effectiveness to partially prevent lipid oxidation (Gallego et al., 2018).

4. Conclusions

Peptidomic approaches using mass spectrometry in tandem have contributed to an advance in the identification of naturally generated peptides during the processing of meat products. Different myofibrillar proteins have been monitored at the end of the meat dry-curing process and peptides identified. This study shows a total of 23, 27, and 45 peptides from MYH1_PIG, MYH2_PIG, and MYH4_PIG, respectively, whereas only five peptides were identified from MYH7_PIG. The size, sequence, and previously described properties of some of the generated peptides would show the potential of MHC protein as a good source of bioactive peptides.

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281 **References**

282 Arihara, K., Nakashima, Y., Mukai, T., Ishikawa, S., & Itoh, M. (2001). Peptide
283 inhibitors for angiotensin I-converting enzyme from enzymatic hydrolysates of
284 porcine skeletal muscle proteins. *Meat Science,* 57(32), 319-324.
285 Chang, K.C., da Costa, N., Blackley, R., Southwood, O., Evans, G., Plastow, G., Wood,
286 J.D., & Richardson, R.I. (2003). Relationships of myosin heavy chain fibre types to
287 meat quality traits in traditional and modern pigs. *Meat Science,* 64(1), 93-103.
288 Choi, Y.M., Ryu, Y.C., & Kim, B.C. (2007). Influence of myosin heavy- and light chain
289 isoforms on early postmortem glycolytic rate and pork quality. *Meat Science,* 76(2),
290 281-288.
291 Corrêa, A.P.F., Daroit, D.J., Fontoura, R., Meira, S.M.M., Segalin,J., & Brandelli, A.
292 (2014). Hydrolysates of sheep cheese whey as a source of bioactive peptides with
293 antioxidant and angiotensin-converting enzyme inhibitory activities. *Peptides*, 6, 48-
294 55.
295 Di Luccia. A., Picariello, G., Cacace, G., Scaloni, A., Faccia, M., Liuzzi, V., Alviti, G.,
296 & Musso, S.S. (2005). Proteomic analysis of water soluble and myofibrillar protein
297 changes occurring in dry-cured hams. *Meat Science,* 69(3), 479-491.
298 Eggert, J.M., Depreux, F.F.S., Schinckel, A.P., Grant, A.L., Gerrard, D.E. (2002). Myosin
299 heavy chain isoforms account for variation in pork quality. *Meat Science*, 61(2), 117-
300 126.

301 Escudero, E., Sentandreu, M. A., Arihara, K., & Toldrá, F. (2010). Angiotensin I-
302 converting enzyme inhibitory peptides generated from in vitro gastrointestinal
303 digestion of pork meat. *Journal of Agricultural and Food Chemistry*, 58(5), 2895-2901.
304 Escudero, E., Aristoy, M. C., Nishimura, H., Arihara, K., & Toldrá, F. (2012).
305 Antihypertensive effect and antioxidant activity of peptide fractions extracted from
306 Spanish dry-cured ham. *Meat Science*, 91(3), 306-311.
307 Fabbro, A., Bencivenni, M., Piasentier, E., Sforza, S., Stecchini, M. L., & Lippe, G.
308 (2016). Proteolytic resistance of actin but not of myosin heavy chain during processing
309 of Italian PDO (protected designation of origin) dry-cured hams. *European Food*
310 *Research and Technology*, 242(6), 881-889.
311 Flores, M., Alasnier, C., Aristoy, M.C., Navarro, J.L., Gandemer, G., & Toldrá, F. (1996).
312 Activity of aminopeptidase and lipolytic enzymes in five skeletal muscles with various
313 oxidative patterns. *Journal of the Science of Food and Agriculture*, 70(1), 127-130.
314 Gallego, M., Mora, L., Fraser, P.D., Aristoy, M.C., & Toldrá, F. (2014). Degradation of
315 LIM domain-binding protein 3 during Spanish dry-cured ham processing. *Food*
316 *Chemistry*, 149, 121-128.
317 Gallego M, Mora L, Aristoy MC, & Toldrá F. (2015a). Titin-derived peptides as
318 processing time markers in dry-cured ham. *Food Chemistry*, 167, 326-339.
319 Gallego, M., Mora, L., Aristoy, M.C., & Toldrá, F. (2015b). Evidence of peptide
320 oxidation from major myofibrillar proteins in dry-cured ham. *Food Chemistry*, 187,
321 230-235.
322 Gallego, M., Mora, L., Reig, M., & Toldrá, F. (2018). Stability of the potent antioxidant
323 peptide SNAAC identified from Spanish dry-cured ham. *Food Research International*,
324 105, 873-879.

325 Gianelli, P., Flores, M., Moya, V.J., Aristoy, M.C., & Toldrá, F. (2000).Effect of
326 carnosine, anserine and other endogenous skeletal peptides on the activity of porcine
327 muscle alanyl and arginyl aminopeptidases. *Journal of Food Biochemistry,* 24, 69-78.
328 Goll, D.E., Neti, G., Mares, S.W., & Thompson, V.F. (2008). Myofibrillar protein
329 turnover: the proteasome and the calpains. Journal of Animal Science. 86(14), 19-35.
330 Hirao, T., Hara, K., & Takahashi, K. (1984). Purification and characterization of
331 cathepsin-B from monkey skeletal-muscle. *Journal of Biochemistry,*95(3), 871-879.
332 Kang, Y.K., Choi, Y.M., Lee, S.H., Choe, J.H., Hong, K.C., & Kim, B.C. (2011). Effects
333 of myosin heavy chain isoforms on meat quality, fatty acid composition, and sensory
334 evaluation in Berkshire pigs. *Meat Science,* 89(4), 384-389.
335 Katayama, K., Fuchu, H., Sakata, A., Kawahara, S., Yamauchi, K., Kawamura, Y., &
336 Muguruma, M. (2003). Angiotensin I-converting enzyme inhibitory activities of
337 porcine skeletal muscle proteins following enzyme digestion. *Asian-Australasian*
338 *Journal of Animal Sciences*, 16(3), 417-424.
339 Korhonen, H., & Pihlanto, A. (2003). Food-derived bioactive peptides-opportunities for
340 designing future foods. *Current Pharmaceutical Design*, 9(16), 1297-1308.
341 Lan, V.T.T., Ito, K., Ohno, M., Motoyama, T., Ito, S., & Kawarasaki, Y. (2015).
342 Analyzing a dipeptide library to identify human dipeptidyl peptidase IV inhibitor.
343 *Food Chemistry,* 175, 66-73.
344 Leseigneur-Meynier, A., & Gandemer, G. (1991). Lipid-composition of pork muscle in
345 relation to the metabolic type of the fibers. *Meat Science*, 29(3), 229-241.
346 Mohanty, D.P., Mohapatra, S., Misra, S., & Sahu, P.S. (2016). Milk derived bioactive
347 peptides and their impact on human health e a review. *Saudi Journal of Biological*
348 *Sciences,* 23(5), 577-583.

349 Mora, L., Sentandreu, M.A., Koistinen, K.M., Fraser, P.D., Toldrá, F., & Bramley, P.M.
350 (2009a). Naturally generated small peptides derived from myofibrillar proteins in
351 Serrano dry-cured ham. *Journal of Agricultural and Food Chemistry,* 57, 3228-3234.
352 Mora, L., Sentandreu, M.A., Fraser, P.D., Toldrá, F., & Bramley, P.M. (2009b).
353 Oligopeptides arising the degradation of creatine kinase in Spanish dry-cured ham.
354 *Journal of Agricultural and Food Chemistry*, 57, 8982-8988.
355 Mora, L., Sentandreu, M.A., & Toldrá, F. (2010). Identification of small troponin T
356 peptides generated in dry-cured ham. *Food Chemistry,* 123, 691-697.
357 Mora, L., Sentandreu, M.A., & Toldrá, F. (2011a). Intense degradation of myosin light
358 chain isoforms in Spanish dry-cured ham. *Journal of Agricultural and Food Chemistry*,
359 59, 3884-3892.
360 Mora, L., Valero, M.L., Del Pino, M.M.S., Sentandreu, M.A., & Toldrá, F. (2011b). Small
361 peptides released from muscle glycolytic enzymes during dry-cured ham processing.
362 *Journal of Proteomics*, 74, 442-450.
363 Mora, L., & Toldrá, F. (2012). Proteomic identification of small (<2000 Da) myoglobin
364 peptides generated in dry-cured ham. *Food Technology and Biotechnology,* 50(3),
365 343-349.
366 Mora, L., Escudero, E., Fraser, P. D., Aristoy, M. C., & Toldrá, F. (2014). Proteomic
367 identification of antioxidant peptides from 400 to 2500Da generated in Spanish dry-
368 cured ham contained in a size-exclusion chromatography fraction. *Food Research*
369 *International,* 56, 68-76.
370 Mora, L., Gallego, M., Aristoy, M.C., Fraser, P.D., & Toldrá, F. (2015a). Peptides
371 naturally generated from ubiquitin-60S ribosomal protein as potential biomarkers of
372 dry-cured ham processing time. *Food Control,* 48, 102-107.

373 Mora, L., Escudero, E., Arihara, K. & Toldrá, F. (2015b). Antihypertensive effect of
374 peptides naturally generated during Iberian dry-cured ham processing. *Food Research*
375 *International*, 78, 71-78.
376 Mora, L., Escudero, E., & Toldrá, F. (2016). Characterization of the peptide profile in
377 Spanish Teruel, Italian Parma and Belgian dry-cured hams and its potential bioactivity.
378 *Food Research International*, 89, 638-646.
379 Mora, L., Bolumar, T., Heres, A., & Toldrá, F. (2017). Effect of cooking and simulated
380 gastrointestinal digestion of aged beef meat on the activity of generated bioactive
381 peptides. *Food & Function*, 8, 4347–4355.
382 Picariello, G., De Martino, A., Mamone, G., Ferranti, P., Addeo, F., Faccia, M.,
383 SpagnaMusso, S., & Di Luccia, A. (2006). Proteomic study of muscle sarcoplasmic
384 proteins using AUT-PAGE/SDS-PAGE as two-dimensional gel electrophoresis.
385 *Journal of Chromatography B*, 833, 101-108.
386 Rico, E., Toldrá, F., & Flores, J. (1991). Assay of cathepsin D activity in fresh pork
387 muscle and dry-cured ham. *Meat Science*, 29, 287-293.
388 Schiaffino, S., & Reggiani, C. (1996). Molecular diversity of myofibrillar proteins: gene
389 regulation and functional significance. *Physiological Reviews,* 76, 371-423.
390 Sentandreu, M.A., & Toldrá, F. (2001). Dipeptidylpeptidase activities along the
391 processing of Serrano dry-cured ham. *European Food Research and Technology,* 213,
392 83-87.
393 Sentandreu, M.A., Stoeva, S., Aristoy, M.C., Laib, K., Voelter, W., & Toldrá, F. (2003).
394 Identification of taste related peptides in Spanish Serrano dry-cured hams. *Journal of*
395 *Food Science,* 68, 64-69

396 Sentandreu, M.A., Armenteros, M., Calvete, J.J., Ouali, A., Aristoy, M.C., & Toldrá, F.
397 (2007). Proteomic identification of actin-derived oligopeptides in dry-cured ham.
398 *Journal of Agricultural and Food Chemistry,* 55(9), 3613-3619.
399 Toldrá, F., Rico, E., & Flores, J. (1993). Cathepsin-B, cathepsin-D, cathepsin-H and
400 cathepsin-l activities in the processing of dry-cured ham. *Journal of the Science of*
401 *Food and Agriculture*, 62, 157-161.
402 Toldrá, F. (1998). Proteolysis and lipolysis in flavour development of dry-cured meat
403 products. *Meat Science,* 49, S101-S110.
404 Toldrá F, & Flores M. (1998). The role of muscle proteases and lipases in flavor
405 development during the processing of dry-cured ham. *Critical Reviews in Food*
406 *Science and Nutrition*, 38, 331-352.
407 Toldrá, F., Aristoy, M.C., & Flores, M. (2000). Contribution of muscle aminopeptidases
408 to flavor development in dry-cured ham. Food Research International, 33, 181-185.
409 Toldrá, F. (2002). *Dry-cured meat products*. Ames, IO: Wiley-Blackwell. ISBN:
410 9780917678547.
411 Toldrá, F. (2006). The role of muscle enzymes in dry-cured meat products with different
412 drying conditions. *Trends in Food Science & Technology*, ., 17, 164-168.
413 Toldrá, F., & Reig, M. (2015). The biochemistry of meat and fat. In F. Toldrá, Y.H. Hui,
414 I. Astiasarán, J.G. Sebranek, R. Talon, (Eds.), *Handbook of Fermented Meat and*
415 *Poultry.* Ames, IO: Wiley–Blackwell. p. 49-54.
416 Wang, W., & Gonzalez de Mejia, E. (2005). A new frontier in soy bioactive peptides that
417 may prevent age-related chronic diseases. *Comprehensive Reviews in Food Science*
418 *and Food Safety,* 4, 63-77.
419

SUPPLEMENTARY MATERIAL

Figure 1. MHC isoforms of mammal skeletal muscles. The three fast (2a, 2x, and 2b) isoforms and the slow (1) isoform, that correspond to UniProt database entry names MYH1 (Q9TV61), MYH2 (Q9TV63), MYH4 (Q9TV62), and MYH7 (P79293), respectively. Grey shadow shows the identified fragments in this study.

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Q9TV61 MSSDQEMAIFGEAAPYLRKSEKERIEAQNKPFDAKTSVFVAEPKESFVKGTVQSREGGKV 60
Q9TV62 MSSDQEMAIFGEAAPYLRKSEKERIEAQNKPFDAKTSVFVAEPKESFVKGTVQSREGGKV 60
Q9TV63 MSSDQEMAIFGEAAPYLRKSEKERIEAQNRPFDAKTSVFVAEPKESFVKGTIQSREGGKV 60
P79293 -MVDAEMAAFGEAAPYLRKSEKERLEAQTRPFDLKKDVYVPDDKEEFVKAKILSREGGKV 59
          * *** ***************:***.:*** *..*:*.: **.***..: *******

Q9TV61 TVKTEAGATLTVKEDQVFPMNPPKFDKIEDMAMMTHLHEPAVLYNLKERYAAWMIYTYSG 120
Q9TV62 TVKTEAGATLTVKEDQVFPMNPPKFDKIEDMAMMTHLHEPAVLYNLKERYAAWMIYTYSG 120
Q9TV63 TVKTEAGATLTVKEDQVFPMNPPKFDKIEDMAMMTHLHEPGVLYNLKERYAAWMIYTYSG 120
P79293 TAETEHGKTVTVKEDQVLQQNPPKFDKIEDMAMLTFLHEPAVLYNLKERYASWMIYTYSG 119
       *.:** * *:*******:  *************:*.****.**********:********

Q9TV61 LFCVTVNPYKWLPVYNAEVVTAYRGKKRQEAPPHIFSISDNAYQFMLTDRENQSILITGE 180
Q9TV62 LFCVTVNPYKWLPVYNAEVVTAYRGKKRQEAPPHIFSISDNAYQFMLTDRENQSILITGE 180
Q9TV63 LFCVTVNPYKWLPVYNPEVVTAYRGKKRQEAPPHIFSISDNAYQFMLTDRENQSILITGE 180
P79293 LFCVTINPYKWLPVYNAEVVAAYRGKKRSEAPPHIFSISDNAYQYMLTDRENQSILITGE 179
       *****:**********.***:*******.***************:***************

Q9TV61 SGAGKTVNTKRVIQYFATIAVTGEKKKEEPTSGKMQGTLEDQIISANPLLEAFGNAKTVR 240
Q9TV62 SGAGKTVNTKRVIQYFATIAVTGEKKKEEPTPGKMQGTLEDQIISANPLLEAFGNAKTVR 240
Q9TV63 SGAGKTVNTKRVIQYFATIAVTGEKKKEEPTSGKMQGTLEDQIISANPLLEAFGNAKTVR 240
P79293 SGAGKTVNTKRVIQYFAVIAAIGDRSKKEQTPG--KGTLEDQIIQANPALEAFGNAKTVR 237
       *****************.**. *::.*:* *.*  :********.*** ***********

Q9TV61 NDNSSRFGKFIRIHFGTTGKLASADIETYLLEKSRVTFQLKAERSYHIFYQIMSNKKPEL 300
Q9TV62 NDNSSRFGKFIRIHFGTTGKLASADIETYLLEKSRVTFQLKAERSYHIFYQIMSNKKPEL 300
Q9TV63 NDNSSRFGKFIRIHFGTTGKLASADIETYLLEKSRVTFQLKAERSYHIFYQITSNRKPEL 300
P79293 NDNSSRFGKFIRIHFGATGKLASADIETYLLEKSRVIFQLKAERDYHIFYQILSNKKPEL 297
       ****************:****************** *******.******* **:****

Q9TV61 IEMLLITTNPYDYAFVSQGEITVPSIDDQEELMATDSAIEILGFTSDERVSIYKLTGAVM 360
Q9TV62 IEMLLITTNPYDYAFVSQGEITVPSIDDQEELMATDSAIEILGFTSDERVSIYKLTGAVM 360
Q9TV63 IEMLLITTNPYDYPFISQGEISVASIDDQEELIATDSAIDILGFTNEEKVSIYKLTGAVM 360
P79293 LDMLLITNNPYDYAFISQGETTVASIDDAEELMATDNAFDVLGFTSEEKNSMYKLTGAIM 357
       ::*****.*****.*:**** :*.**** ***:***.*:::****.:*: *:*******:*

Q9TV61 HYGNLKFKQKQREEQAEPDGTEVADKAAYLQGLNSADLLKALCYPRVKVGNEFVTKGQTV 420
Q9TV62 HYGNLKFKQKQREEQAEPDGTEVADKAAYLQGLNSADLLKALCYPRVKVGNEFVTKGQTV 420
Q9TV63 HYGNLKFKQKQREEQAEPDGTEVADKAAYLQSLNSADLLKALCYPRVKVGNEYVTKGQTV 420
P79293 HFGNMKFKLKQREEQAEPDGTEEADKSAYLMGLNSADLLKGLCHPRVKVGNEYVTKGQNV 417
       *:**:*** ************* ***:*** .********.**:********:*****.*

Q9TV61 QQVYNAVGALAKAVYDKMFLWMVTRINQQLDTKQPRQYFIGVLDIAGFEIFDFNSLEQLC 480
Q9TV62 QQVYNAVGALAKAVYDKMFLWMVTRINQQLDTKQPRQYFIGVLDIAGFEIFDFNSLEQLC 480
Q9TV63 EQVTNAVGALAKAVYEKMFLWMVTRINQQLDTKQPRQYFIGVLDIAGFEIFDFNSLEQLC 480
P79293 QQVMYATGALAKAVYEKMFNWMVTRINTTLETKQPRQYFIGVLDIAGFEIFDFNSFEQLC 477
       :**  *.********:*** *******  *:************************:****

Q9TV61 INFTNEKLQQFFNHHMFVLEQEEYKKEGIEWEFIDFGMDLAACIELIEKPMGIFSILEEE 540
Q9TV62 INFTNEKLQQFFNHHMFVLEQEEYKKEGIEWEFIDFGMDLAACIELIEKPMGIFSILEEE 540
Q9TV63 INFTNEKLQQFFNHHMFVLEQEEYKREGIEWTFIDFGMDLAACIELIEKPMGIFSILEEE 540
P79293 INFTNEKLQQFFNHHMFVLEQEEYKKEGIEWEFIDFGMDLQACIDLIEKPMGIMSILEEE 537
       ************************:***** ******** ***:********:******

Q9TV61 CMFPKATDTSFKNKLYEQHLGKSNNFQKPKPAKGKVEAHFSLIHYAGTVDYNITGWLDKN 600
Q9TV62 CMFPKATDTSFKNKLYEQHLGKSNNFQKPKPAKGKAEAHFSLIHYAGTVDYNITGWLDKN 600
Q9TV63 CMFPKATDTSFKNKLYEQHLGKSANFQKPKPAKGKVEAHFSLIHYAGTVDYNITGWLDKN 600
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P79293 CMFPKATDMTFKAKLYDNHLGKSNNFQKPRNIKGRPEAHFALIHYAGTVDYNIIGWLQKN 597
       ******** :** ***::***** *****:  **: ****:************ ***:**

Q9TV61 KDPLNETVVGLYQKSSVKTLAFLFTGAAGADAEAGGGKKGGKKKGSSFQTVSALFRENLN 660
Q9TV62 KDPLNETVVGLYQKSSVKTLAFLFAERQ--SSEEGGTKKGGKKKGSSFQTVSALFRENLN 658
Q9TV63 KDPLNDTVVGLYQKSALKTLAFLFSGAQTGEAEAGGTKKGGKKKGSSFQTVSALFRENLN 660
P79293 KDPLNETVVDLYKKSSLKLLSNLFANYAGADTPVEKGK-GKAKKGSSFQTVSALHRENLN 656
       *****:***.**:**::* *: **:     .:     * *  ************.*****

Q9TV61 KLMTNLRSTHPHFVRCIIPNETKTPGAMEHELVLHQLRCNGVLEGIRICRKGFPSRILYA 720
Q9TV62 KLMTNLRSTHPHFVRCIIPNETKTPGAMEHELVLHQLRCNGVLEGIRICRKGFPSRILYA 718
Q9TV63 KLMTNLRSTHPHFVRCIIPNETKTPGAMEHELVLHQLRCNGVLEGIRICRKGFPSRILYA 720
P79293 KLMTNLRSTHPHFVRCIIPNETKSPGVIDNPLVMHQLRCNGVLEGIRICRKGFPNRILYG 716
       ************************:**.::: **:********************.****.

Q9TV61 DFKQRYKVLNASAIPEGQFIDSKKASEKLLGSIDIDHTQYKFGHTKVFFKAGLLGLLEEM 780
Q9TV62 DFKQRYKVLNASAIPEGQFIDSKKASEKLLGSIDIDHTQYKFGHTKVFFKAGLLGTLEEM 778
Q9TV63 DFKQRYKVLNASAIPEGQYIDSKKASEKLLASIDIDHTQYKFGHTKVFFKAGLLGLLEEM 780
P79293 DFRQRYRILNPAAIPEGQFIDSRKGAEKLLGSLDIDHNQYKFGHTKVFFKAGLLGLLEEM 776
       **:***::**.:******:***:*.:****.*:****.**************** ****

Q9TV61 RDEKLAQLITRTQARCRGFLARVEYQKMVERRESIFCIQYNIRAFMNVKHWPWMKLYFKI 840
Q9TV62 RDEKLAQLITRTQAMCRGFLMRVEFRKMMERRESIFCIQYNIRAFMNVKHWPWMKLYFKI 838
Q9TV63 RDDKLAQLITRTQARCRGFLARVEYQKMVERRESIFCIQYNIRAFMNVKHWPWMKLFFKI 840
P79293 RDERLSRIITRIQAQSRGVLSRMEFKKLLERRDSLLIIQWNIRAFMSVKNWPWMKLYFKI 836
       **::*:::*** ** .**.* *:*::*::***:*:: **:******.**:******:***

Q9TV61 KPLLKSAETEKEMANMKEEFEKTKESLAKAEAKRKELEEKMVALMQEKNDLQLQVQAEAD 900
Q9TV62 KPLLKSAETEKEMANMKEEFEKTKEDLAKSEAKRKELEEKMVALMQEKNDLQLQVQAEAD 898
Q9TV63 KPLLKSAESEKEMATMKEEFQKTKDELAKSEAKRKELEEKMVTLLKEKNDLQLQVQAEAE 900
P79293 KPLLKSAETEKEMATMKEEFGRLKEALEKSEARRKELEEKMVSLLQEKNDLQLQVQAEQD 896
       ********:*****.***** : *: * *:**:*********:*::************ :

Q9TV61 SLADAEERCDQLIKTKIQLEAKIKEVTERAEDEEEINAELTAKKRKLEDECSELKKDIDD 960
Q9TV62 GLADAEERCDQLIKTKIQLEAKIKEVTERAEDEEEINAELTAKKRKLEDECSELKKDIDD 958
Q9TV63 GLADAEERCDQLIKTKIQLEAKIKEVTERAEDEEEINAELTAKKRKLEDECSELKKDIDD 960
P79293 NLADAEERCDQLIKNKIQLEAKVKEMTERLEDEEEMNAELTAKKRKLEDECSELKRDIDD 956
       .*************.*******:**:*** *****:********************:****

Q9TV61 LELTLAKVEKEKHATENKVKNLTEEMAGLDETIAKLTKEKKALQEAHQQTLDDLQAEEDK 1020
Q9TV62 LELTLAKVEKEKHATENKVKNLTEEMAGLDENIAKLTKEKKALQEAHQQTLDDLQAEEDK 1018
Q9TV63 LELTLAKVEKEKHATENKVKNLTEEMAGLDETIAKLTKEKKALQEAHQQTLDDLQAEEDK 1020
P79293 LELTLAKVEKEKHATENKVKNLTEEMAGLDEIIAKLTKEKKALQEAHQQALDDLQAEEDK 1016
       ****************************** ****************:**********

Q9TV61 VNTLTKAKTKLEQQVDDLEGSLEQEKKLRMDLERAKRKLEGDLKLAQESTMDIENDKQQL 1080
Q9TV62 VNTLTKAKTKLEQQVDDLEGSLEQEKKLRMDLERAKRKLEGDLKLAQESTMDIENDKQQL 1078
Q9TV63 VNTLTKAKTKLEQQVDDLEGSLEQEKKLRMDLERAKRKLEGDLKLAQESIMDIENEKQQL 1080
P79293 VNTLTKAKVKLEQHVDDLEGSLEQEKKVRMDLERAKRKLEGDLKLTQESIMDLENDKQQL 1076
       ********.****:**************:*****************:*** **:**:****

Q9TV61 DEKLKKKEFEMSNLQSKIEDEQALAMQLQKKIKELQARIEELEEEIEAERASRAKAEKQR 1140
Q9TV62 DEKLKKKEFEMSNLQSKIEDEQALAMQLQKKIKELQARTEELEEEIEAERASRAKAEKQR 1138
Q9TV63 DEKLKKKEFEISNLQSKIEDEQALAIQLQKKIKELQARIEELEEEIEAERASRAKAEKQR 1140
P79293 DERLKKKDFELNALNARIEDEQALGSQLQKKLKELQARIEELEEELEAERTARAKVEKLR 1136
       **:****:**:. *:::******. *****:****** ******:****::***.** *

Q9TV61 SDLSRELEEISERLEEAGGATSAQIEMNKKREAEFQKMRRDLEEATLQHEATAATLRKKH 1200
Q9TV62 SDLSRELEEISERLEEAGGATSAQIEMNKKREAEFQKMRRDLEEATLQHEATAAALRKKH 1198
Q9TV63 SDLSRELEEISERLEEAGGATSAQIEMNKKREAEFQKMRRDLEEATLQHEATAAALRKKH 1200
P79293 SDLSRELEEISERLEEAGGATSVQIEMNKKREAEFQKMRRDLEEATLQHEATAAALRKKH 1196
       **********************.*******************************:*****

Q9TV61 ADSVAELGEQIDNLQRVKQKLEKEKSEMKMEIDDLASNMETVSKAKGNLEKMCRTLEDQL 1260
Q9TV62 ADSVAELGEQIDNLQRVKQKLEKEKSELKMEIDDLASNMETVSKAKGNLEKMCRTLEDQL 1258
Q9TV63 ADSVAELGEQIDNLQRVKQKLEKEKSEMKMEIDDLASNMETVSKAKGNLEKMCRTLEDQL 1260
P79293 ADSVAELGEQIDNLQRVKQKLEKEKSEFKLELDDVTSNMEQIIKAKANLEKMCRTLEDQM 1256
       ***************************:*:*:**::**** : ***.************:
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Q9TV61 SELKTKEEEQQRLINDLTAQRARLQTESGEYSRQLDEKDTLVSQLSRGKQAFTQQIEELK 1320
Q9TV62 SEVKTKEEEHQRLINELSAQKARLQTESGEFSRQLDEKEALVSQLSRGKQAFTQQIEELK 1318
Q9TV63 SELKSKEEEQQRLINDLTAQRGRLQTESGEFSRQLDEKEALVSQLSRGKQAYTQQIEELK 1320
P79293 NEHRSKAEETQRSVNDLTSQRAKLQTENGELSRQLDEKEALISQLTRGKLTYTQQLEDLK 1316
       .* ::* ** ** :*:*::*:.:****.** *******::*:***:*** ::***:*:**

Q9TV61 RQLEEEIKAKSALAHAVQSSRHDCDLLREQYEEEQEAKAELQRAMSKANSEVAQWRTKYE 1380
Q9TV62 RQLEEETKAKSALAHAVQSSRHDCDLLREQYEEEQEAKAELQRAMSKANSEVAQWRTKYE 1378
Q9TV63 RQLEEEIKAKNALAHALQSSRHDCDLLREQYEEEQESKAELQRALSKANTEVAQWRTKYE 1380
P79293 RQLEEEVKAKNALAHALQSARHDCDLLREQYEEETEAKAELQRVLSKANSEVAQWRTKYE 1376
       ****** ***.*****:**:************** *:******.:****:**********

Q9TV61 TDAIQRTEELEEAKKKLAQRLQDAEEHVEAVNAKCASLEKTKQRLQNEVEDLMIDVERSN 1440
Q9TV62 TDAIQRTEELEEAKKKLAQRLQDAEEHVEAVNAKCASLEKTKQRLQNEVEDLMLDVERSN 1438
Q9TV63 TDAIQRTEELEEAKKKLAQRLQAAEEHVEAVNAKCASLEKTKQRLQNEVEDLMLDVERTN 1440
P79293 TDAIQRTEELEEAKKKLAQRLQDAEEAVEAVNAKCSSLEKTKHRLQNEIEDLMVDVERSN 1436
       ********************** *** ********:******:*****:****:****:*

Q9TV61 AACAALDKKQRNFDKILAEWKQKYEETHAELEASQKESRSLSTELFKVKNAYEESLDQLE 1500
Q9TV62 AACAALDKKQRNFDKILAEWKHKYEETQAELEASQKESRSLSTELFKVKNAYEESLDQLE 1498
Q9TV63 AACAALDKKQRNFDKILAEWKQKYEETHAELEASQKEARSLGTELFKMKNAYEESLDQLE 1500
P79293 AAAAALDKKQRNFDKILAEWKQKYEESQSELESSQKEARSLSTELFKLKNAYEESLEHLE 1496
       **.******************:****:::***:****:***.*****:********::**

Q9TV61 TLKRENKNLQQEISDLTEQIAEGGKRIHELEKIKKQVEQEKSEIQAALEEAEASLEHEEG 1560
Q9TV62 TLKRENKNLQQEISDLTEQIAEGGKHIHELEKVKKQIEQEKSELQAALEEAEASLEHEEG 1558
Q9TV63 TLKRENKNLQQEISDLTEQIAEGGKRIHELEKIKKQVEQEKSEIQAALEEAEASLEHEEG 1560
P79293 TFKRENKNLQEEISDLTEQLGSSGKTIHELEKVRKQLEAEKLELQSALEEAEASLEHEEG 1556
       *:********:********:...** ******::**:* ** *:*:**************

Q9TV61 KILRIQLELNQVKSEVDRKIAEKDEEIDQLKRNHVRVVESMQSMLDAEIRSRNDAIRLKK 1620
Q9TV62 KILRIQLELNQVKSEIDRKIAEKDEEIDQMKRNHIRVVESMQSTLDAEIRSRNDALRIKK 1618
Q9TV63 KILRIQLELNQVKSEVDRKIAEKDEEIDQLKRNHVRVVESMQSMLDAEIRSRNDAIRLKK 1620
P79293 KILRAQLEFNQIKAEMERKLAEKDEEMEQAKRNHLRVVDSLQTSLDAETRSRNEALRVKK 1616
       **** ***:**:*:*::**:******::* ****:***:*:*: **** ****:*:*:**

Q9TV61 KMEGDLNEMEIQLNHANRMAAEALRNYRNTQGILKDTQIHLDDALRSQEDLKEQLAMVER 1680
Q9TV62 KMEGDLNEMEIQLNHANRQATEAIRNLRNTQGVLKDTQLHLDDAIRGQDDLKEQLAMVER 1678
Q9TV63 KMEGDLNEMEIQLNHANRMAAEALRNYRNTQGILKDTQIHLDDALRGQEDLKEQLAMVER 1680
P79293 KMEGDLNEMEIQLSHANRMAAEAQKQVKSLQSLLKDTQIQLDDAVRANDDLKENIAIVER 1676
       *************.**** *:** :: :. *.:*****::****:*.::****::*:***

Q9TV61 RANLLQAEIEELRATLEQTERSRKVAEQELLDASERVQLLHTQNTSLINTKKKLETDISQ 1740
Q9TV62 RANLMQAEIEELRASLEQTERSRRVAEQELLDASERVQLLHTQNTSLINTKKKLETDISQ 1738
Q9TV63 RANLLQAEIEELRATLEQTERSRKVAEQELLDASERVQLLHTQNTSLINTKKKLETDISQ 1740
P79293 RNNLLQAELEELRAVVEQTERSRKLAEQELIETSERVQLLHSQNTSLINQKKKMEADLSQ 1736
       * **:***:***** :********::*****:::********:******* ***:*:*:**

Q9TV61 IQGEMEDIIQEARNAEEKAKKAITDAAMMAEELKKEQDTSAHLERMKKNLEQTVKDLQHR 1800
Q9TV62 IQGEMEDIVQEARNAEEKAKKAITDAAMMAEELKKEQDTSAHLERMKKNMEQTVKDLQHR 1798
Q9TV63 MQGEMEDILQEARNAEEKAKKAITDAAMMAEELKKEQDTSAHLERMKKNMEQTVKDLQHR 1800
P79293 LQTEVEEAVQECRNAEEKAKKAITDAAMMAEELKKEQDTSAHLERMKKNMEQTIKDLQHR 1796
       :* *:*: :**.**************************************:***:******

Q9TV61 LDEAEQLALKGGKKQIQKLEARVRELEGEVESEQKRNVETVKGLRKHERRVKELTYQTEE 1860
Q9TV62 LDEAEQLALKGGKKQIQKLEARVRELENEVENEQKRNVEAVKGLRKHERRVKELTYQTEE 1858
Q9TV63 LDEAEQLALKGGKKQIQKLEARVRELEGEVESEQKRNAEAVKGLRKHERRVKELTYQTEE 1860
P79293 LDEAEQIALKGGKKQLQKLEARVRELENELEAEQKRNAESVKGMRKSERRIKELTYQTEE 1856
       ******:********:***********.*:* *****.*:***:** ***:*********

Q9TV61 DRKNILRLQDLVDKLQAKVKSYKRQAEEAEEQSNVNLSKFRKLQHELEEAEERADIAESQ 1920
Q9TV62 DRKNVLRLQDLVDKLQSKVKAYKRQAEEAEEQSNVNLSKFRKLQHELEEAEERADIAESQ 1918
Q9TV63 DRKNILRLQDLVDKLQAKVKSYKRQAEEAEEQSNTNLSKFRKLQHELEEAEERADIAESQ 1920
P79293 DRKNLLRLQDLVDKLQLKVKAYKRQAEEAEEQANTNLSKFRKVQHELDEAEERADIAESQ 1916
       ****:*********** ***:***********:*.*******:****:************

Q9TV61 VNKLRVKSREVHTKIISEE 1939
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Q9TV62 VNKLRVKSREVHTKVISEE 1937
Q9TV63 VNKLRVKSREVHTKVISEE 1939
P79293 VNKLRAKSRDIGTKGLNEE 1935
       *****.***:: ** :.**
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Table 1. Peptides naturally generated during the processing of dry-cured ham identified using mass spectrometry in tandem and sequences of the most probable di- and tripeptides generated according with the peptidases action and the identified peptides.

Protein Name	Identified Peptides	Elucidated di- and tripeptides from the identified sequences	References
Actin (ACT)	4	-	Sentandreu et al. (2007)
Myosin Light Chain 1 (MLC 1)	9 137	AA, PA AP, IE, PAP, EEM, NP, LG, VK, NAE, TN, TNP, VF, RVF, AL	Mora et al. (2011a) Mora et al. (2009a)
Myosin Light Chain 2 (MLC 2)	88	RD, LR, IN, SGP, IK, VLD, FK, PE, KV, AD, LKG, EK, EKL	Mora et al. (2011a)
Titin (TTN)	5 320	KV EP, SV, SVL, RKK, PK, PKE, AK, AKK, VE, KA, TPK, KAV, EE, EP, PA, EI, KER, PE, KP, EPE, VK, PPI, PTP, KA, PP, EA, KDE, KAV, EAK, GP, IKG, PSP, IEA, APF, DE, VKF, DEI, DAV, STS, MLK	Picariello et al. (2006) Gallego et al. (2015a)
Creatine kinase (CK)	58	DL, LA, VS, VE, GHP, HKT, YV, PD, VQ, SVF, AQK	Mora et al. (2009b)
Slow Troponin T (TnT)	2	NK	Mora et al. (2010)
Fast Troponin T (TnT)	25	NK, IP, AP	Mora et al. (2010)
Glycogen phosphorylase (PYGM)	2	-	Mora et al. (2011b)
Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)	8	CLA, VK	Mora et al. (2011b)
Phosphoglycerate kinase 1 (PGK)	4	-	Mora et al. (2011b)
Phosphoglycerate mutase 2 (PGAM)	7	VRK	Mora et al. (2011b)
Enolase (ENO)	18	RDG, VY, LYK	Mora et al. (2011b)
Piruvate kinase 3 isoform 2 (PK)	2 1	-	Mora et al. (2011b)
Lactate dehydrogenase (LDH)	4	LL	Mora et al. (2011b)
LIM domain-binding 3 (LDB3)	107	SVT, GKD, QG, RL, WGF, VV, SQ, AQ, MP, AQS, QL, SQG, NMP, LT, IS, LS, EAQ, SA, APA, PA, QP, STY, AY, QG, GD, SQ, SQL, AL	Gallego et al. (2014)
Myoglobin (MYO)	11	FK, KH, TPL	Mora and Toldrá (2012)
Ubiquitin-60S (UBI)	68	VKT, IF, SD, SDY, VK, IFA, DK, KA, KIQ, DKE, RT, GRT	Mora et al. (2015a)

Table 2. Myosin Heavy Chain isoforms 1, 2x, 2a and 2b. A) Percentage of homology between the isoforms. B) Accession number and definition of the different isoforms according to the main free access databases Uniprot and NCBInr.

A)

	MYH1_PIG MHC2x	**MYH2_PIG** MHC2a	**MYH4_PIG** MHC2b	**MHY7_PIG** MHC1
MYH1_PIG	100%	96.0%	96.5%	81.7%
MYH2_PIG	96.0%	100%	94.2%	81.8%
MYH4_PIG	96.5%	94.2%	100%	81.8%
MHY7_PIG	81.7%	81.8%	81.8%	100%

B)

Protein name	**UniProt database**		**NCBInr database**	
	Accession	**Definition**	**Accession**	**Definition**
Myosin heavy chain 1	Q9TV61	MYH1_PIG	gi|75056480	Myosin heavy chain, skeletal muscle, adult 1
			gi|5360750	**Myosin heavy chain 2x** [Sus scrofa]
			gi|157279731	Myosin-1 [Sus scrofa]
Myosin heavy chain 2	Q9TV63	MYH2_PIG	gi|75056482	Myosin heavy chain, skeletal muscle, adult 2
			gi|5360746	**Myosin heavy chain 2a** [Sus scrofa]
			gi|55741490	Myosin-2 [Sus scrofa]
Myosin heavy chain 4	Q9TV62	MYH4_PIG	gi|75056481	Myosin heavy chain, skeletal muscle, fetal
			gi|5360748	**Myosin heavy chain 2b** [Sus scrofa]
			gi|178056718	Myosin-4 [Sus scrofa]
Myosin heavy chain 7	P79293	MHY7_PIG	gi|55741486	Myosin-7 [Sus scrofa]
			gi|125987844	**Myosin heavy chain slow isoform [Sus scrofa]**
			gi|1698895	Beta-myosin heavy chain [Sus scrofa]

Table 3. Myosin heavy chain peptides identified in 9 months Spanish dry-cured ham corresponding to the different isoforms.

Position[a]	Identified sequence	Modifications[b]	Obs.[c] (m/z)	Exp.[d] (m/z)	Ch. (+)	Calc.[e] (MW)	C.[f] (%)	MYH[g] 1	2	4	7
2-24	SSDQEMAIFGEAAPYLRKSEKER	Prot Terminal Acetyl_N-term; Ox(M)_6	900.78	900.77	3	2699.33	99		X	X	
2-18	SSDQEMAIFGEAAPYLR	Prot Terminal Acetyl_N-term	963.96	963.95	2	1925.91	99	X	X	X	
19-37	KSEKERIEAQNKPFDAKTS	Methyl(K)_17	740.74	740.73	3	2219.20	99	X		X	
19-37	KSEKERIEAQNKPFDAKTS	Deamidated(Q)_10; Methyl(K)_17	741.07	741.05	3	2220.18	99	X		X	
19-37	KSEKERIEAQNKPFDAKTS	Methyl(T)_18	740.72	740.73	3	2219.15	99	X		X	
19-35	KSEKERIEAQNKPFDAK	-	673.38	673.36	3	2017.10	99	X		X	
19-35	KSEKERIEAQNKPFDAK	Methyl(D)_15	678.05	678.03	3	2031.13	99	X		X	
19-35	KSEKERIEAQNKPFDAK	Deamidated(N)_11	673.70	673.69	3	2018.07	99	X			
40-57	VAEPKESFVKGTVQSREG	-	650.02	650.01	3	1947.04	99	X	X	X	
40-57	VAEPKESFVKGTVQSREG	Deamidated(Q)_14	650.35	650.34	3	1948.02	99		X	X	
40-57	VAEPKESFVKGTVQSREG	Methyl(E)_17	654.69	654.68	3	1961.05	99			X	
40-57	VAEPKESFVKGTVQSREG	Methyl(E)_6	654.69	654.68	3	1961.03	99		X	X	
40-57	VAEPKESFVKGTVQSREG	Methyl(E)_3	654.69	654.68	3	1961.03	99		X	X	
40-57	VAEPKESFVKGTVQSREG	Methyl(S)_7	654.69	654.68	3	1961.04	99		X	X	
40-57	VAEPKESFVKGTVQSREG	Ox(P)_4; Methyl(E)_17	660.03	660.01	3	1977.08	99			X	
40-57	VAEPKESFVKGTVQSREG	Formyl(K)_5	659.36	659.34	3	1975.07	99		X	X	
40-57	VAEPKESFVKGTVQSREG	Dimethyl(R)_16	659.70	659.35	3	1976.09	96			X	
40-56	VAEPKESFVKGTVQSRE	-	631.02	631.00	3	1890.04	99			X	
40-56	VAEPKESFVKGTVQSRE	Methyl(E)_3	635.68	635.67	3	1904.03	99		X	X	
40-53	VAEPKESFVKGTVQ	Deamidated(Q)_14	760.43	760.40	2	1518.84	99	X		X	
40-50	VAEPKESFVKG	-	595.84	595.82	2	1189.66	99	X	X	X	
41-57	AEPKESFVKGTIQSREG	-	621.66	621.66	3	1861.97	99		X		

41-57	AEPKESFVKGTIQSREG	-	621.66	621.66	3	1861.97	98		X		
58-70	GKVTVKTEAGATL	-	637.89	637.87	2	1273.76	99	X	X	X	
58-68	GKVTVKTEAGA	-	530.81	530.80	2	1059.60	98	X	X	X	
71-90	TVKEDQVFPMNPPKFDKIED	-	793.07	793.06	3	2376.19	99		X	X	
71-90	TVKEDQVFPMNPPKFDKIED	Ox(M)_10; Deamidated(N)_11	798.75	798.72	3	2393.23	99		X	X	
71-90	TVKEDQVFPMNPPKFDKIED	Deamidated(Q)_6; Ox(M)_10	798.74	798.72	3	2393.20	99		X	X	
71-90	TVKEDQVFPMNPPKFDKIED	Deamidated(N)_11	793.41	793.39	3	2377.20	99		X	X	
71-90	TVKEDQVFPMNPPKFDKIED	Deamidated(Q)_6; Deamidated(N)_11	793.74	793.72	3	2378.21	99		X	X	
71-90	TVKEDQVFPMNPPKFDKIED	Methyl(D)_20	797.75	797.73	3	2390.22	99		X	X	
71-86	TVKEDQVFPMNPPKFD	Ox(M)_10	954.48	954.46	2	1906.95	99		X	X	
71-86	TVKEDQVFPMNPPKFD	-	946.48	946.47	2	1890.95	99		X	X	
71-86	TVKEDQVFPMNPPKFD	-	631.32	631.31	3	1890.95	99		X	X	
71-86	TVKEDQVFPMNPPKFD	Ox(M)_10; Deamidated(N)_11	636.99	636.97	3	1907.95	97		X	X	
71-84	TVKEDQVFPMNPPK	Ox(M)_10	823.43	823.42	2	1644.84	99	X	X	X	
71-83	TVKEDQVFPMNPP	-	751.38	751.37	2	1500.75	99	X	X	X	
72-90	VKEDQVFPMNPPKFDKIED	-	759.39	759.38	3	2275.15	99		X	X	
72-90	VKEDQVFPMNPPKFDKIED	Ox(P)_8	764.70	764.71	3	2291.09	99		X	X	
72-90	VKEDQVFPMNPPKFDKIED	Ox(M)_9	764.73	764.71	3	2291.16	99		X	X	
72-90	VKEDQVFPMNPPKFDKIED	Ox(M)_9; Deamidated(N)_10	765.07	765.04	3	2292.18	99		X	X	
72-90	VKEDQVFPMNPPKFDKIED	Deamidated(Q)_5; Ox(M)_9	765.05	765.04	3	2292.14	99		X	X	
72-90	VKEDQVFPMNPPKFDKIED	Deamidated(N)_10	759.73	759.71	3	2276.16	99		X	X	
72-90	VKEDQVFPMNPPKFDKIED	Deamidated(Q)_5	759.72	759.71	3	2276.13	99		X	X	
72-90	VKEDQVFPMNPPKFDKIED	Deamidated(N)_10; Methyl(D)_15	764.40	764.38	3	2290.18	97		X	X	
72-89	VKEDQVFPMNPPKFDKIE	-	721.04	721.04	3	2160.11	99		X	X	
72-86	VKEDQVFPMNPPKFD	-	895.95	895.94	2	1789.89	99		X	X	
72-86	VKEDQVFPMNPPKFD	Deamidated(Q)_5	896.45	896.43	2	1790.89	99		X	X	

72-86	VKEDQVFPMNPPKFD	Ox(M)_9; Deamidated(N)_10	904.46	904.43	2	1806.90	97		X	X	
72-86	VKEDQVFPMNPPKFD	Ox(M)_9	903.96	903.94	2	1805.91	99		X	X	
74-90	EDQVFPMNPPKFDKIED	Ox(M)_7	689.00	688.99	3	2063.99	99		X	X	
82-90	PPKFDKIED	-	544.79	544.78	2	1087.57	98		X	X	
145-155	GKKRQEAPPHI	-	630.88	630.86	2	1259.74	99	X	X	X	
168-182	TDRENQSILITGESG	-	810.40	810.40	2	1618.79	99	X	X	X	X
177-194	ITGESGAGKTVNTKRVIQ	-	620.36	620.35	3	1858.06	99	X	X	X	X
197-216	ATIAVTGEKKKEEPTPGKMQ	Ox(M)_19; Deamidated(Q)_20	720.74	720.71	3	2159.20	99			X	
199-221	IAVTGEKKKEEPTPGKMQGTLED	Ox(M)_17	834.79	834.76	3	2501.35	99			X	
199-216	IAVTGEKKKEEPTPGKMQ	Ox(M)_17	663.03	663.02	3	1986.06	99			X	
199-215	IAVTGEKKKEEPTPGKM	Ox(M)_17	620.35	620.34	3	1858.02	99			X	
199-212	IAVTGEKKKEEPTP	-	763.93	763.92	2	1525.85	99			X	
199-212	IAVTGEKKKEEPTP	-	509.62	509.62	3	1525.84	99			X	
405-422	PRVKVGNEFVTKGQTVQQ	-	672.38	672.37	3	2014.11	99			X	
405-417	PRVKVGNEFVTKG	-	715.91	715.91	2	1429.81	99			X	
405-416	PRVKVGNEFVTK	-	687.40	687.40	2	1372.78	99			X	
624-650	FTGAAGADAEAGGGKKGGKKKGSSFQT	-	838.45	838.43	3	2512.33	99	X			
624-648	FSGAQTGEAEAGGTKKGGKKKGSSF	-	805.77	805.75	3	2414.28	99		X		
628-648	YAGADTPVEKGKGKAKKGSSF	-	709.40	709.38	3	2125.18	99				X
638-648	TKKGGKKKGSSF	-	626.89	626.87	2	1251.76	99			X	
638-648	TKKGGKKKGSSF	-	626.89	626.87	2	1251.76	99		X		
678-690	IPNETKTPGAMEH	Ox(M)_11	720.86	720.84	2	1439.70	96		X	X	
730-744	NASAIPEGQFIDSKK	Deamidated(N)_1	803.42	803.41	2	1604.82	99	X		X	
733-748	AIPEGQFIDSKKASEK	-	874.47	874.46	2	1746.93	99			X	
733-749	AIPEGQFIDSKKASEKL	-	621.01	621.01	3	1860.01	99	X		X	
734-744	IPEGQFIDSKK	-	631.35	631.34	2	1260.68	99			X	

739-749	FIDSKKASEKL	-	633.37	633.36	2	1264.72	99			X	
751-766	GSIDIDHTQYKFGHTK	Methyl(H)_7	620.98	620.98	3	1859.93	99	X		X	
752-766	SIDIDHTQYKFGHTK	Methyl(H)_6	601.98	601.97	3	1802.90	99	X	X	X	
753-766	IDIDHTQYKFGHTK	Methyl(H)_5	572.97	572.96	3	1715.88	99	X	X	X	
754-766	DIDHTQYKFGHTK	Methyl(H)_4	535.27	535.27	3	1602.79	99		X	X	
755-766	IDHTQYKFGHTK	Methyl(H)_3	496.93	496.92	3	1487.77	99	X	X	X	
751-766	GSLDIDHNQYKFGHTK	Deamidated(N)_8	620.99	620.97	3	1859.94	99				X
837-853	FKIKPLLKSAETEKEM	Ox(M)_16	636.69	636.69	3	1907.06	99	X		X	
838-853	KIKPLLKSAETEKEM	Ox(M)_15	587.67	587.67	3	1759.99	99	X		X	
839-853	IKPLLKSAETEKEM	Ox(M)_14	544.98	544.97	3	1631.90	96	X		X	
1907-1919	LDEAEERADIAESQVNK	-	639.66	639.64	3	1915.97	99				X

[a]- Position of the peptides inside the corresponding MYH sequence. [b]- Modifications observed in some amino acids of the peptides. The number indicates the position of the modification in the peptide sequence. [c]- Observed peptide mass in the detector after ionisation using nESI. [d]- Expected mass calculated from the observed mass according to the charge state of the ion. [e]- Predicted mass. [f]- Percentage of confidence. [g]- Peptides showing an 'X' in 1, 2, 4, or 7, have been identified in the protein MYH1_PIG, MYH2_PIG, MYH4_PIG, or MYH7_PIG, respectively.