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**Proteomic characterisation of a size-exclusion chromatography
fraction containing antioxidant peptides from 400 to 2500Da generated
in Spanish dry-cured ham**

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ABSTRACT

The processing of dry-cured ham results in an intense proteolysis that leads to the generation of peptides of different sizes and composition, which have been shown to be biologically active. In this study, a total of ninety-three peptides mainly derived from actin, β -enolase, myosin heavy chain, and creatine kinase proteins have been identified from a size-exclusion chromatography (SEC) fraction that resulted antioxidant by using matrix-assisted laser desorption/ionization time-of-flight (MALDI-ToF) and nESI-ion trap mass spectrometry. Several of the identified peptides have been synthesised and their antioxidant activity tested *in vitro* by using DPPH radical-scavenging assay and reducing power analysis. The peptide with sequence SNAAC showed the best results with a $95.7\% \pm 0.3$ of antioxidant activity from a concentration of 0.1 mg/mL as well as the higher reducing power with 1.5 units of absorbance (UA) at 700 nm at a concentration of 0.2 mg/mL, very similar to the positive control 2,6-di-tert-butyl-4-methyl phenol (BHT) that showed a $93.5\% \pm 0.4$ antioxidant activity and 2.8 UA at 0.2 mg/mL. These results suggest that Spanish dry-cured ham represent an important source of powerful antioxidant peptides which due to their natural characteristics may represent a highly valuable alternative in human health.

Keywords: Size-exclusion chromatography, dry-cured ham, antioxidant peptides, mass spectrometry.

1. INTRODUCTION

The production of traditional Spanish dry-cured ham requires a very long and complex processing that could last up to 24 months and involves an intense degradation of the muscle proteins as a result of the action of endogenous muscle peptidases that results in the generation of numerous peptides of different sizes and composition as well as free amino acids (Toldrá & Flores, 1998; Toldrá, Aristoy & Flores, 2000).

The low molecular weight and assorted amino acids composition of the peptides that were naturally generated during dry-cured processing increase the chance for being potentially bioactive as well as to cross the intestinal barrier to exert their biological effects. In this sense, recent studies suggested that Spanish dry-cured ham contains peptides with antioxidant and antihypertensive activities (Escudero, Aristoy, Nishimura, Arihara & Toldrá, 2012) and pointed the potential of this product as a source of bioactive peptides naturally generated during dry-curing processes (Escudero, Mora, Fraser, Aristoy, & Toldrá, 2013a; Escudero, Mora, Fraser, Aristoy, Arihara, & Toldrá, 2013b).

The identification of the sequence of these small peptides requires good fractionation and isolation techniques for their subsequent mass spectrometry analysis and, frequently, different separation techniques or a combination of chromatography analysis are done to simplify the complex mixture of peptides. Size-exclusion chromatography (SEC), performed under low pressure, has been frequently used as an initial step for the analysis of dry-cured ham peptides (Mora, Sentandreu, Koistinen, Fraser, Toldrá & Bramley, 2009; Mora, Sentandreu, Fraser, Toldrá & Bramley, 2009; Mora, Sentandreu & Toldrá, 2010) which are fractionated according to their molecular size. Previous studies revealed that water soluble fractions of Spanish dry-cured ham exhibited strong DPPH radical-scavenging activity in the elution zone between 220 and 345 mL after

1 SEC (Escudero et al, 2012). Recently, main peptides responsible for such bioactivity
2 were identified in the fractions corresponding to elution volumes from 280 to 325 mL
3 (Escudero et al, 2013a) but the rest still remained unknown.

4 In this study, the proteomic identification of the peptides naturally generated from the
5 processing of dry-cured ham that were isolated in a size-exclusion chromatography
6 fraction showing antioxidant activity comprising SEC elution volumes from 200 to 275
7 mL has been done by using matrix-assisted laser desorption/ionization time-of-flight
8 (MALDI-ToF) and tandem mass spectrometry (nLC-MS/MS). Thus, this study
9 describes for the first time the sequence of new peptides which, following their
10 identification, were selected, synthesised, and assayed to verify their individual
11 antioxidant activity *in vitro*.

12 **2. MATERIALS AND METHODS**

13 **2.1. Spanish dry-cured ham preparation**

14 Three Spanish dry-cured hams were produced using raw hams from 6-months old pig
15 (Landrace x Large White) and prepared according to traditional procedures previously
16 described in Escudero et al (2012). Briefly, the pre-salting stage was done using a
17 mixture of curing ingredients (salt, 300 mg nitrate/kg ham and 100 mg nitrite/kg ham)
18 for 30 min; in the salting stage, hams were completely covered with solid salt in a cold
19 room (2-4 °C) and 90-95% relative humidity for 12 days; post-salting (4-5 °C, 75-85%
20 relative humidity for 60 days) and finally, the ripening period (14-20 °C, relative
21 humidity progressively reduced to 70% for 10 months). The muscle *Biceps femoris* in
22 the final dry-cured hams had pH values within the range 6.1-6.2, the water content 55.0-
23 55.5% and the salt content 5.8-6.1g/100g.

24 **2.2. Extraction and deproteinisation**

1 Fifty grams of *Biceps femoris* muscle from the processed Spanish dry-cured hams,
2 previously removed from their extramuscular fat, were minced and homogenised with
3 200 mL of 0.01N HCl in a stomacher (IUL Instrument, Barcelona, Spain) for 8 min in
4 cold. The homogenate was centrifuged (12000g for 20 min at 4 °C) and, after filtering
5 through glass wool, the supernatant was deproteinised by adding 3 volumes of ethanol
6 and maintaining the sample 20 h at 4 °C. Then, the sample was centrifuged again
7 (12000 g for 20 min at 4 °C) and the supernatant was dried in a rotatory evaporator.
8 Finally, the dried deproteinised extract was dissolved in 25 mL of 0.01N HCl, filtered
9 through a 0.45 µm nylon membrane filter (Millipore, Bedford, MA, USA), and stored at
10 -20 °C until use.

11 **2.3. Size-exclusion chromatography**

12 An aliquot (5 mL) of the deproteinised extract from the Spanish dry-cured ham was
13 subjected to SEC in order to fractionate the peptides according to their molecular mass.
14 For this purpose, a Sephadex G25 gel filtration column (2.5 x 65 cm, Amersham
15 Biosciences, Uppsala, Sweden), previously equilibrated with 0.01N HCl filtered
16 through a 0.45 µm nylon membrane filter (Millipore), was employed. The separation
17 was performed at 4°C using 0.01N HCl as eluent, at a flow rate of 15 mL/h. The first
18 195 mL were discarded, and then 5mL fractions were collected using an automatic
19 fraction collector and monitored by ultraviolet (UV) absorption at 214 nm (Agilent
20 8453 UV spectrophotometer, Agilent Technologies, Palo Alto, CA, USA). Fractions
21 corresponding to elution volumes from 200 mL to 275 mL were pooled together, dried
22 under vacuum, and dissolved in 3 mL of 0.1% trifluoroacetic acid (TFA) in bidistilled
23 water.

24 **2.4. Peptide sequencing using mass spectrometry**

1 The previously lyophilised sample was resuspended in 200 µl of 0.1% TFA and an
2 aliquot of 20 µl was taken and cleaned/concentrated with ZipTip C18 (Millipore,
3 Billerica, MA, USA) according to the manufacturer recommendations. Peptides were
4 eluted from ZipTip with 7 µl of H₂O:ACN (50:50, v/v) and 0.1% of formic acid (FA)
5 and subsequently analysed.

6 *2.4.1. MALDI ToF/ToF mass spectrometry.* One microlitre of the C18 ZipTip eluted
7 peptides (H₂O:ACN (50:50, v/v) and 0.1% of formic acid) was spotted onto an
8 AnchorChip target plate with transponder technology (MTP AnchorChip TM 600/384,
9 Bruker Daltonics, Bremen, Germany) in triplicate. After air-drying, one microlitre of a
10 saturated solution of α -cyano-4-hydroxycinnamic acid (HCCA) (201344, Bruker
11 Daltonics, Bremen, Germany) was added as a matrix solution. Mass spectra were
12 recorded in positive-reflectron mode using an Autoflex III MALDI-TOF/TOF mass
13 spectrometer from Bruker Daltonics equipped with delayed extraction by summing
14 3000 shots with a laser intensity of 45% in the MS mode. The mass spectrometer was
15 calibrated externally, once before each analysis, using a mixture of peptide calibration
16 standards for mass spectrometry with a mass range 1000-4000 Da (206195, Bruker
17 Daltonics, Bremen, Germany) containing Angiotensin II, Angiotensin I, Substance P,
18 Bombesin, ACTH clip 1-17, ACTH clip 18-39, and Somatostatin 28. Bradykinin
19 fragment 1-7 with a molecular weight of 756.4 Da (B4181, Sigma-Aldrich, MO, USA)
20 was also used as MALDI-ToF standard to cover the lowest range of m/z . The analysis
21 was done in the range of m/z from 350 to 2000.

22 *2.4.2. nESI-LC-MS/MS.* The nano-LC/ MS/MS analysis was performed in an Ultimate
23 3000 RSLC nano system from Dionex (Thermo Fisher Scientific Ltd, Leicestershire,
24 UK) coupled to an AmaZon ETD ion-trap mass spectrometer equipped with a
25 nanoelectrospray ionization source (Bruker Daltonik GmbH, Bremen, Germany).

Two microliters of the eluted solution including the peptides (H₂O:ACN (50:50, v/v) and 0.1% of formic acid) were injected into the LC-MS system by using the autosampler. Sample was preconcentrated on a Dionex Acclaim® PepMap 100 column (100 µm x 2 cm, C18, 5 µm, 100 Å) (Dionex Corporation, LC Packings) at a flow rate of 4 µL/min and using 0.1% of FA as mobile phase. After 3 minutes of preconcentration, the trap column was automatically switched in-line with a Dionex Acclaim® PepMap RSLC nano-column (75 µm x 15 cm, C18, 2 µm, 100Å) (Dionex Corporation, LC Packings). Mobile phases consisted of solvent A, containing 0.1% formic acid in water, and solvent B, containing 0.1% formic acid in 100% acetonitrile. Chromatographic conditions were a linear gradient from 95 to 60% of solvent A in 70 min at a flow rate of 0.250 µL/min at 30 °C.

The column outlet was directly coupled to a nanoelectrospray ion (nESI) source. Operating conditions for the ion trap mass spectrometer were positive polarity, capillary temperature of 200 °C, capillary voltage of 4.5 V, and nitrogen was used as collision gas. First scan even was full MS scan from *m/z* value 300 to 1500. The ion trap was used in MS(2) in Ultrascan mode with a maximum accumulation time of 200 ms and an average of 5.

2.4.3. Data analysis. Automated spectral processing and peak list generation was performed using Mascot Distiller v2.4.2.0 software (Matrix Science, Inc., Boston, MA) (<http://www.matrixscience.com>). The database search was done through Mascot Daemon software in combination with the Mascot interface 2.2 (Matrix Science, Inc., Boston, MA) (<http://www.matrixscience.com>). Regarding searching parameters, Mascot searches were done with none enzymatic specificity and a tolerance on the mass measurement of 100 ppm in MS mode and 0.6 Da for MS/MS ions. Oxidation of Met and the N-terminal transformation of Gln or Glu into pyro-Glu were used as variable

1 modifications. Identification of protein origin of peptides was done using UniProt
2 protein database. BLAST was used as a basic local alignment search tool to find regions
3 of local similarities between the identified peptides and the protein sequences of *Sus*
4 *scrofa* (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

5 **2.5. Peptide synthesis and preparation of solutions**

6 Twelve of the identified peptides were synthesised by Gen Script Corporation
7 (Piscataway, NJ, USA) and used for the *in vitro* assay of DPPH radical-scavenging
8 activity and ferric-reducing antioxidant power measurement.

9 Stock solutions of 200 mg/mL were prepared for all peptides. In the DPPH radical-
10 scavenging assay, peptides were assayed at 3, 2, 1.5, 1, and 0.5 mg/mL and peptide
11 showing the highest antioxidant activity (SNAAC) was also tested at smaller
12 concentrations (0.5, 0.25, 0.1, 0.05, 0.01, and 0.001 mg/mL). In the measurement of the
13 ferric-reducing antioxidant power, peptides were assayed at 1, 0.8, 0.6, 0.4, and 0.2
14 mg/mL, whereas SNAAC peptide, which showed the highest reducing power, was also
15 tested at 0.35, 0.17, 0.07, 0.035, 0.007, 0.0007 mg/mL. The synthetic antioxidant BHT
16 was used as positive control at same concentrations than the synthesised peptides. All
17 peptides were assayed individually.

18 **2.6. Antioxidant activity**

19 *2.6.1. DPPH radical-scavenging assay.* Synthesised peptides were analysed for the
20 DPPH radical-scavenging activity as described by Bersuder, Hole, & Smith (1998).
21 Briefly, 50 µL of the peptide sample prepared at the previously described concentrations
22 was mixed with 250 µL of ethanol and 62.5 µL of a DPPH solution prepared at 0.02%
23 of ethanol. The mixture was kept sixty minutes in the dark, and analysed measuring the
24 absorbance (Abs.) at 517 nm. The negative control was done using distilled water. All
25 analysis were done by triplicate. DPPH radical-scavenging activity was calculated as:

1 *DPPH radical scavenging activity = (Absorbance Control – Absorbance Sample) /*
2 *Absorbance Control x 100.*

3 2.6.2. *Ferric-reducing antioxidant power (reducing power).* The measurement of the
4 ferric-reducing antioxidant power is based on the ability of the peptide for reducing iron
5 from the Fe³⁺ (ferric) oxidation state to the Fe²⁺ (ferrous) oxidation state. The assay was
6 based on the method described by Huang, Tsai, & Mau (2006). Briefly, 70 µL of each
7 peptide solution prepared at the previously described concentrations was mixed with 70
8 µL of sodium phosphate buffer 200 mM, pH 6.6, and 70 µL of potassium ferricyanide
9 (10 mg/mL). The mixture was incubated for 20 min at 50 °C, and 70 µL of
10 trichloroacetic acid (100 mg/mL) was added. After a centrifugation for 10 min at 200g,
11 140 µL of the supernatant was taken and mixed with 140 µL of distilled water and 28
12 µL of ferric chloride (1 mg/mL). The absorbance was immediately measured at 700 nm.
13 The negative control was done using distilled water.

14 **3. RESULTS AND DISCUSSION**

15 A deproteinised Spanish dry-cured ham extract was submitted to size-exclusion
16 chromatography to fractionate the peptide mixture according to their molecular mass.
17 Fractions corresponding to elution volumes between 200 and 275 mL showing
18 antioxidant activity were pooled together, concentrated, and subjected to mass
19 spectrometry in tandem to determine molecular masses and sequences of respective
20 peptides. Size-exclusion chromatography is frequently used as an initial step for the
21 fractionation of the peptides generated during dry-cured ham processing and gives a
22 good estimation of the molecular weight of the analysed compounds. However,
23 MALDI-ToF mass spectrometry was used in this study to determine the exact mass
24 range (*m/z*) of the peptides contained in the SEC fractions comprised between 200 and
25 275 mL. **Figure 1** shows the MALDI-ToF mass spectrum of the studied fraction and

shows the molecular masses of the singly charged ions ($[M+H]^+$) present in the sample. Spectrum reveals molecular masses between 400 and 2000 Da for the studied peptides. The identification of the sequence of the generated peptides was done using a nESI-LC-MS/MS system and a total of 93 peptides derived from different pig muscle proteins were sequenced in the studied fraction. In this sense, **Figure 2A** shows the distribution in percentages of the identified peptides according to their origin protein. The sequence of the peptides identified in this study are shown in **Tables 1 to 5**, corresponding to twenty-seven, twenty-three, nineteen, and seventeen peptides that have been identified from actin, β -enolase, myosin heavy chain, and creatine kinase proteins, respectively. Other twenty-seven peptides from other origin proteins were also identified as shown in **Table 5**. In this sense, the protein with accession number ASPH1_BOVIN corresponding to aspartate β -hydroxylase has not been previously sequenced in *Sus scrofa*. However, the similarity between *Bos taurus* and *Sus scrofa* species and the presence of this protein in the cDNA of skeletal muscle (<http://www.ncbi.nlm.nih.gov/UniGene/>) make this assignment reliable. On the other hand, the sequence of the proteins GPAT1_BOVIN, TDIF1_BOVIN, and PTPRN_BOVIN (all in **Table 5**), show a similarity of 95, 97, and 93% with the uncharacterised proteins F1S5L4_PIG, F1SC86_PIG, F15R78_PIG, of *Sus scrofa*, respectively. The bovin protein carboxypeptidase E (CBPE_BOVIN) shares the 95% of its sequence with the porcine carboxypeptidase E (A1IU54_PIG). The Mascot server alignment of the elastin protein from bovine species instead of porcine species could be due to only a fragment of 133 amino acids has been sequenced in porcine (G8FUN3_PIG) while the sequence of elastin in bovine species included in the UniProt database has a total of 747 amino acids. Only 20 of the 93 peptides identified in the Sephadex fraction using nLC-MS/MS have also been identified using the MALDI-ToF

instrument. This fact could be due to differences in the ionization of the peptides present in the sample when different ionisation techniques such as MALDI and ESI are used.

Despite of this disadvantage, MALDI-ToF technology has given a good estimation of the singly charged ions ($[M+H]^+$) present in the sample.

Actin is an important protein in skeletal muscle responsible for cell movement and muscle contraction that helps to maintain the cytoskeleton in skeletal muscle. Previous works have already reported the progressive degradation of this protein during meat ageing (Morzel, Hamelin, Sante-Lhoutellier, Sayd, Monin, & Chambon, 2004) as well as during dry-curing (Di Luccia et al, 2005). Peptides DSGDGVTHNVPIYE and DSGDGVTHNVPIYEG described in this work in **Table 1** confirm two of the four oligopeptides previously isolated and identified in Spanish dry-cured ham by Sentandreu, Armenteros, Calvete, Ouali, Aristoy, & Toldrá (2007), which studied the identity of the generated peptides after using a Sephadex G-25 column and supported the relevant action of cathepsin D enzyme during the dry-curing process (Sentandreu et al, 2007).

Enolase 3 or β -enolase is a sarcoplasmic protein that participates in the ninth step of the glycolysis metabolic pathway. Its disappearance during the dry-curing of sausages (Picariello et al, 2006) and the early stages of salt-vinegar curing of fish (Toyohara, Murata, Ando, Kubota, Sakaguchi & Toyohara, 1999) have been described although it has been proved that remains visible until the end of curing in Bayonne hams (Monin et al, 1997). Mora, Valero, Del Pino, Sentandreu and Toldrá (2011) described for the first time forty-five specific fragments of glycolytic enzymes evidencing the intense proteolysis occurring in the sarcoplasmic fraction of dry-cured ham and eighteen of these peptides were derived from enolase 3 (Mora et al, 2011). Peptides DSRGNPTVEVDL and DSRGNPTVEVDLHTAKGR identified in **Table 2** of this

work share part of their sequence with the previously described peptide DSRGNPTVEVDLHTAKG. This difference of one amino acid (arginine, R) in the C-terminal side of the peptide DSRGNPTVEVDLHTAKGR could be due to the action of carboxypeptidases which has been proved to be responsible for the loss of C terminal amino acids in phosphoglycerate mutase and enolase 3 peptides (Mora et al, 2011).

Creatine kinase (CK) is a sarcoplasmic protein involved in the energetic metabolism of tissues with high and fluctuating demands of energy *in vivo* such as skeletal muscle. The degradation of CK occurs along all the ripening period (Picariello et al, 2006; Pérez, Ruiz, Contreras, & Ibáñez, 2003). A CK fragment was identified after characterization of proteolysis during the ripening of semi-dry fermented sausages (Hughes, Kerry, Arendt, Kenneally, McSweeney & O'Neill, 2002), whereas a total of three CK fragments were isolated and identified during postmortem aging in bovine *Longissimus dorsi* muscle (Stoeva, Byrne, Mullen, Troy & Voelter, 2000). More recently, fifty-eight peptides derived from CK in Spanish dry-cured ham were identified by mass spectrometry (Mora et al, 2009). Four of the peptides identified in this study that are shown in **Table 3** share their sequence with the previously identified DVIQTGVDPNPGHP, DDVIQTGVDPNPGHP, DVIQTGVDPNPGHPF, and DDVIQTGVDPNPGHP. Differences between the sequences of these peptides evidence the contribution of aminopeptidases and carboxypeptidases to the generation and release of free amino acids during dry-cured ham processing. On the other hand, a recent publication that describes the purification and identification of antihypertensive peptides in Spanish dry-cured ham (Escudero et al, 2013b) studied the fractions corresponding to an elution volume from 200 mL to 320 mL after a size-exclusion chromatographic separation which exhibited *in vitro* ACE inhibitory activity as well as *in vivo* antihypertensive activity. Peptides DVIQTGVDPNPGHP, KGGDDLDPNY,

KAEEEYPDL, DDVIQTGVDPNGHP, GDDLDPNYV, AEEEYPDL, EEYPDL, DKETPSGFTLD, DDVIQTGVDPNGHPF, and DVIQTGVDPNGHPF shown in **Table 3**, were also identified in the study, which confirms the obtained results.

Table 4 shows a total of 11, 9, 10, and 13 peptides from myosin heavy chain (MHC) 1, 2, 4, and 7, respectively. Despite the degradation of MHC protein during ageing and dry-curing of ham (Di Luccia et al, 2005; Picariello et al, 2006) has been widely described, little is known about the sequence of the generated peptides. Same occurs with the peptides described in **Table 5** that derived from less abundant proteins. The loss of the dipeptide KD is observed in the N-terminal site of peptide KDVYVPDDKEE with respect to peptides VYVPDDKEEF and VYVPDDKEEFV showed in **Table 4** probably due to the action of dipeptidyl peptidases. The antioxidant activity of this dipeptide has been previously described using ion-exchange chromatography and gel filtration in a dried bonito extract (Suetsuna, 1999).

Bioactive peptides usually contain between 3 and 20 amino acid residues (Kim, Je & Kim, 2007). The twenty percent of the peptides identified in this study correspond to peptides of 5 amino acid residues lower than 600 Da in size (**Figure 2B**), which increase the chance of crossing the intestinal barrier and exert their biological effects. On the other hand, the antioxidant activity of peptides is also related to their sequence and amino acid composition. It has been reported that the presence of hydrophobic amino acids and one or more residues of H, P, C, Y, W, F or M might enhance the antioxidant activity (Ren et al, 2008). Thus, a total of twelve of the identified peptides were chosen according to their length and amino acid composition, and synthesised with the aim to perform *in vitro* experiments to determine their antioxidant activity. The DPPH radical-scavenging activity and ferric-reducing antioxidant power of these peptides were determined, and the results are shown in **Figure 3**. The highest DPPH

radical-scavenging activity (**Figure 3A**) was found to be for the peptides SNAAC and AEEEEYPDL and result in $95.7\% \pm 0.3$ and $63.6\% \pm 0.2$ of antioxidant activity at 3 mg/mL, respectively. The peptide SNAAC was also tested using this assay at lower concentrations and results showed that a 95% of antioxidant activity was reached from a concentration of 0.1 mg/mL of the peptide (**Figure 4A**). In fact, DPPH radical-scavenging activity of the synthetic antioxidant BHT used as positive control resulted lower than the obtained for SNAAC peptide as it is shown in **Figure 4A**, being an indicative of the antioxidant strength of this peptide. In most of the analysed peptides, a dose-dependent increase in the DPPH radical-scavenging activity was observed.

On the other hand, the ferric-reducing antioxidant power of the synthesised peptides was also tested and results are shown in **Figure 3B**. According to this measurement, SNAAC and AEEEEYPDL peptides showed again the highest reducing power, with values of absorbance at 700 nm of 1.7 and 0.5 at 1 mg/mL of concentration, respectively. In this case, the absorbance value of the positive control BHT was 2.3. The peptide SNAAC was also tested using this assay at lower concentrations (**Figure 4B**) and results showed a dose-dependent increase in the absorbance reaching the highest value at 1.5 corresponding to a concentration of 0.35 mg/mL.

Figure 5 shows the MS/MS spectra of SNAAC and AEEEEYPDL peptides used in their identification and sequence. Results obtained in this study reveal a very high antioxidant activity for peptide SNAAC in comparison with the rest of the assayed sequences, being even higher than the synthetic peptide used as positive control (BHT) when DPPH radical-scavenging activity assay was used. In fact, the antioxidant activity percentages obtained for all tested peptides except for SNAAC and AEEEEYPDL sequences are similar to those previously reported in dry-cured ham naturally generated peptides by Escudero et al (2013a), which assayed peptides showed the highest DPPH radical-

1 scavenging activity at around 50% for a concentration of 3 mg/mL, similar results were
2 obtained after the ferric-reducing antioxidant power measurement.

3 **4. CONCLUSIONS**

4 This study reports the proteomic identification of the naturally generated peptides
5 resulted in the volumes corresponding from 200 to 275 mL obtained after a SEC
6 separation derived from Spanish dry-cured ham that showed antioxidant activity with
7 intense radical-scavenging activity in a previous work (Escudero et al, 2012). A total of
8 93 peptides derived from different pig muscle proteins have been sequenced in the
9 studied fraction. Twenty-seven, twenty-three, nineteen, and seventeen peptides from
10 actin, β -enolase, myosin heavy chain, and creatine kinase proteins, respectively, have
11 been identified together with twenty-seven peptides from other minor proteins. Several
12 of the identified peptides were synthesised, and their antioxidant activity tested *in vitro*
13 by using DPPH radical-scavenging assay and reducing power analysis. The peptide
14 SNAAC showed the best results with a percentage of $95.7\% \pm 0.3$ antioxidant activity
15 from a concentration of 0.1 mg/mL as well as the higher reducing power with 1.5 units
16 of absorbance (UA) at 700 nm at a concentration of 0.2 mg/mL, very similar to the
17 positive control 2,6-di-tert-butyl-4-methyl phenol (BHT).

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FIGURE CAPTIONS

Figure 1. MALDI-ToF spectra of the pull of fractions obtained after SEC separation corresponding from 200 to 275 mL.

Figure 2. A) Distribution in percentages of the identified peptides according to the origin protein in skeletal muscle of *Sus scrofa*. B) Distribution of percentages of the number of amino acid residues per identified peptide.

Figure 3. A) DPPH radical-scavenging activity at different concentrations of the synthesised peptides. B) Reducing power of different concentrations of synthesised peptides. Values represent means of three independent replicates (n=3). *The synthetic compound 2,6-di-tert-butyl-4-methylphenol (BHT) was used as positive control.

Figure 4. A) DPPH radical-scavenging activity at different concentrations of the synthesised peptide SNAAC. B) Reducing power of different concentrations of synthesised peptide SNAAC. Values represent means of three independent replicates (n=3). * The synthetic compound 2,6-di-tert-butyl-4-methylphenol (BHT) was used as positive control.

Figure 5. MS/MS spectra of the synthesised peptides showing the highest antioxidant activity, A) Ion 466.18+ corresponding to peptide sequence SNAAC from myosin heavy chain showed in Table 4. B) Ion 965.24+ corresponding to peptide sequence AEEEEYPDL from creatine kinase protein showed in Table 3. The peptide sequences matching the product ion spectra are shown in capital letters, together with observed y and b ions.

Figure 1

Figure 1.

Figure 2

Figure 2.

Figure 3

Figure 3.

Figure 4

Figure 4.

Figure 5

Figure 5.

Table 1

Peptides from the protein actin identified in pork dry-cured ham using an ion trap mass spectrometer with an electrospray ionisation source. Peptides identified using MALDI-TOF are indicated with an asterisk.

Accession Number: ACTS_PIG						
Protein N α Actin, alpha skeletal muscle						
P ₀	Peptide sequence	P _f	Observed ^a m/z	Expected ^b m/z	Charge ^c	Calculated ^d m/z
W	IGGSI*	W	445.09	444.08	1	445.25
I	ITNWDDM	I	894.23	893.22	1	893.36
P	DGQVITIGN	P	916.31	915.30	1	915.47
G	IITNWDDM	G	1007.28	1006.27	1	1006.44
S	YELPDGQVI	S	1033.35	1032.34	1	1032.51
H	GIITNWDDM	H	1064.29	1063.28	1	1063.46
H	GIITNWDDM	H	532.66	1063.31	2	1063.46
C	DNGSGLVKAGF	C	532.72	1063.42	2	1063.53
E	YDEAGPSIVH	E	1087.31	1086.30	1	1086.50
S	YELPDGQVIT*	S	1134.35	1133.34	1	1133.56
D	NGSGLVKAGFAGDD	D	654.51	1307.00	2	1306.62
H	GIITNWDDMEK	H	661.23	1320.44	2	1320.60
S	YELPDGQVITIGN	S	709.75	1417.49	2	1417.71
C	DNGSGLVKAGFAGDD	C	711.73	1421.45	2	1421.64
E	KSYELPDGQVITI	E	731.80	1461.59	2	1461.77
L	DSGDGVTHNVPIYE	L	751.74	1501.46	2	1501.67
Y	ELPDGQVITIGNER*	Y	770.78	1539.55	2	1539.79
S	YELPDGQVITIGNE	S	774.25	1546.49	2	1546.75
L	DSGDGVTHNVPIYEG	L	780.20	1558.39	2	1558.69
E	KSYELPDGQVITIGN	E	817.28	1632.55	2	1632.84
Y	ELPDGQVITIGNERF	Y	844.84	1687.66	2	1686.86
S	YELPDGQVITIGNER	S	852.26	1702.51	2	1702.85
S	YELPDGQVITIGNER	S	568.88	1703.63	3	1702.85
L	DSGDGVTHNVPIYEGY	L	861.74	1721.46	2	1721.75
E	KSYELPDGQVITIGNE	E	881.79	1761.57	2	1761.88
L	EKSYELPDGQVITIGN	L	881.85	1761.69	2	1761.88
E	KSYELPDGQVITIGNE	E	588.26	1761.75	3	1761.88
K	SYELPDGQVITIGNER	K	895.79	1789.56	2	1789.88
E	KSYELPDGQVITIGNER	E	959.84	1917.66	2	1917.98
E	KSYELPDGQVITIGNER	E	640.25	1917.72	3	1917.98

a. Observed peptide mass in the detector after ionisation using an ESI source.
b. Expected mass calculated from the Observed mass according to the charge state of the ion.
c. Charge state of the identified peptide.
d. Predicted mass.
*. Peptides IGGSI, YELPDGQVI, and ELPDGQVITIGNER were identified in MALDI-TOF MS.

Table 2

Peptides from the protein beta-enolase identified in pork dry-cured ham using an ion trap mass spectrometer with an electrospray ionisation source. Peptides identified using MALDI-TOF are indicated with an asterisk.

Accession Number: ENOB_PIG						
Protein Name: Beta-enolase						
P ₀	Peptide sequence	P _f	Observed ^a m/z	Expected ^b m/z	Charge ^c	Calculated ^d m/z
P	DLVLPVP	P	752.37	751.36	1	751.45
P	DLVLPVPA	P	823.38	822.37	1	822.49
R	GNPTVEVDL	R	943.31	942.30	1	942.47
S	IEDPFDQD	S	978.23	977.22	1	977.40
A	GNPDLVLPVP	A	1020.40	1019.39	1	1019.57
S	IEDPFDQDD	S	1093.21	1092.21	1	1092.42
S	IEDPFDQDD	S	547.16	1092.30	2	1092.42
Y	PVVSIEDPFD	Y	1117.34	1116.33	1	1116.53
V	SIEDPFDQDD*	V	590.58	1179.15	2	1179.46
V	SIEDPFDQDD*	V	1180.25	1179.24	1	1179.46
P	VVSIEDPFDQD	P	632.23	1262.44	2	1262.57
I	EDPFDQDDWK	I	647.67	1293.33	2	1293.52
L	DSRGNPTVEVDL	L	651.25	1300.49	2	1300.63
Y	PVVSIEDPFDQD	Y	680.72	1359.43	2	1359.62
P	VVSIEDPFDQDD	P	1378.33	1377.32	1	1377.59
P	VVSIEDPFDQDD	P	689.70	1377.38	2	1377.59
Y	PVVSIEDPFDQDD	Y	1475.36	1474.36	1	1474.65
Y	PVVSIEDPFDQDD	Y	738.23	1474.45	2	1474.65
V	SIEDPFDQDDWK	V	747.70	1493.39	2	1493.63
K	NYPVVSIEDPFDQD	K	819.71	1637.40	2	1636.73
N	YPVVSIEDPFDQDD	N	547.16	1638.46	3	1637.71
K	NYPVVSIEDPFDQDD	K	877.20	1752.39	2	1751.75
I	KNYPVVSIEDPFDQD	I	883.25	1764.49	2	1764.82
I	KNYPVVSIEDPFDQD	I	589.20	1764.58	3	1764.82
Y	PVVSIEDPFDQDDWK	Y	895.27	1788.52	2	1788.82
I	KNYPVVSIEDPFDQDD	I	940.76	1879.51	2	1879.85
I	KNYPVVSIEDPFDQDD	I	627.54	1879.60	3	1879.85
L	DSRGNPTVEVDLHTAKGR	L	651.25	1950.74	3	1950.99
I	KNYPVVSIEDPFDQDDWKTW	I	828.17	2481.50	3	2481.15

a. Observed peptide mass in the detector after ionisation using an ESI source.

b. Expected mass calculated from the Observed mass according to the charge state of the ion.

c. Charge state of the identified peptide.

d. Predicted mass.

*. Peptide SIEDPFDQDD was identified in MALDI-TOF mass spectrometer.

Table 3

Peptides from the protein creatine kinase identified in pork dry-cured ham using an ion trap mass spectrometer with an electrospray ionisation source. Peptides identified using MALDI-TOF are indicated with an asterisk.

Accession Number: KCRM_PIG						
Protein Name: Creatine kinase						
P ₀	Peptide sequence	P _f	Observed ^a m/z	Expected ^b m/z	Charge ^c	Calculated ^d m/z
I	DDMIP + Ox (M)*	I	606.18	605.17	1	605.24
E	EEYPDL	E	765.22	764.22	1	764.32
A	EEEYPDL	A	894.20	893.19	1	893.37
K	DLFDPIIQ	K	960.36	959.35	1	959.50
K	DLFDPIIQ	K	480.69	959.37	2	959.50
K	AEEEYPDL	K	965.24	964.23	1	964.40
G	GDDLDPNYV	G	1007.25	1006.24	1	1006.42
G	GDDLDPNYV	G	504.17	1006.33	2	1006.42
K	GGDDLDPNYV	K	1064.26	1063.25	1	1063.45
V	FDVSNADRLG	V	547.18	1092.35	2	1092.52
F	KAEEEYPDL	F	547.19	1092.37	2	1092.50
L	KGGDDLDPNYV	L	596.70	1191.38	2	1191.54
R	DKETPSGFTLD	R	605.25	1208.48	2	1208.56
K	DLFDPIIQDR	K	616.24	1230.46	2	1230.62
D	DVIQTGVDPNPGHP	D	674.34	1346.67	2	1347.64
L	DDVIQTGVDPNPGHP	L	732.21	1462.40	2	1462.67
K	DLFDPIIQDRHGG	K	741.77	1481.53	2	1481.73
D	DVIQTGVDPNPGHPF*	D	748.26	1494.51	2	1494.71
D	DVIQTGVDPNPGHPF*	D	499.19	1494.56	3	1494.71
L	DDVIQTGVDPNPGHPF	L	805.72	1609.43	2	1609.74

a. Observed peptide mass in the detector after ionisation using an ESI source.

b. Expected mass calculated from the Observed mass according to the charge state of the ion.

c. Charge state of the identified peptide.

d. Predicted mass.

*. Peptides DDMIP and DVIQTGVDPNPGHPF were identified in MALDI-TOF mass spectrometer.

Table 4

Peptides from the proteins myosin heavy chain 1, 2, 4, and 7, with accession number MYH1_PIG, MYH2_PIG, MYH4_PIG, and MYH7_PIG, respectively, identified in pork dry-cured ham using an ion trap mass spectrometer with an electrospray ionisation source. Peptide also identified using MALDI-TOF is indicated with an asterisk.

P ₀	Peptide sequence	P _f	Myosin Heavy Chain*				Observed m/z ^a	Expected m/z ^b	Charge ^c	Calculated m/z ^d	Oxidation Methionine	MALDI TOF ^e
			1	2	4	7						
R	SNAAC	A	X		X		466.18	465.17	1	464.17		X
Q	AEEDK	V	X	X	X	X	590.58	589.57	1	590.25		
S	EMKME	I	X	X			341.90	681.79	2	682.27	Ox (M)	
R	EEQAEP	D	X	X	X	X	702.24	701.24	1	701.29		X
L	GEQIDNL	Q	X	X	X	X	788.26	787.25	1	787.37		X
D	QEMAIIGE	A	X	X	X		314.01	939.01	3	939.40	Ox (M)	
A	IPEGQFIDS	K	X		X	X	1005.33	1004.32	1	1004.48		
A	ELGEQIDNL	Q	X	X	X	X	1030.34	1029.33	1	1029.50		
A	ELGEQIDNL	Q	X	X	X	X	515.70	1029.38	2	1029.50		
E	DQVFPMPNP	K	X	X			1044.32	1043.32	1	1043.47		
N	YRNTQGILKDT	Q	X	X	X		654.75	1307.49	2	1307.68		
A	DSVAELGEQIDNLQ	R	X	X	X	X	765.77	1529.53	2	1529.72		
E	DIVQEA	R			X		674.34	673.33	1	673.33		X
Y	KLTGAIM	H				X	748.26	747.26	1	748.42	Ox (M)	
L	TEQLGSSGK	T				X	453.29	904.56	2	905.45		X
E	QHVDDLEGL	E				X	371.25	1110.72	3	1111.51		
D	VYVPDDKEEF	V				X	620.72	1239.43	2	1239.57		
K	KDVYVPDDKEE	F				X	669.25	1336.49	2	1335.62		
D	VYVPDDKEEFV	K				X	670.24	1338.46	2	1338.63		
K	DVYVPDDKEEF	V				X	678.21	1354.42	2	1354.59		

* Peptides with a 'X' in 1, 2, 4, or 7, have been identified in the protein MYH1_PIG, MYH2_PIG, MYH4_PIG, or MYH7_PIG, respectively.

a. Observed peptide mass in the detector after ionisation using an ESI source.

b. Expected mass calculated from the Observed mass according to the charge state of the ion.

c. Charge state of the identified peptide.

d. Predicted mass.

e. Peptides identified in MALDI-TOF mass spectrometer.

Table 5

Peptides identified in pork dry-cured ham using an ion trap mass spectrometer with an electrospray ionisation source. Peptide also identified using MALDI-TOF is indicated with an asterisk.

Accession Number/ /Protein	P ₀	Peptide sequence	P _i	Observed m/z ^a	Expected m/z ^b	Charge ^c	Calculated m/z ^d	Modification	MALDI TOF ^e
FETA_PIG	A	QGVAL	Q	486.61	485.60	1	486.28		
Alpha-fetoprotein	N	QMVKDVL	V	916.26	915.26	1	915.47	Gln->pyro-Glu (N-term Q)	
	N	QMVKDVL	V	458.64	915.27	2	915.47	Gln->pyro-Glu (N-term Q)	
	L	NQHMCIVMRQFGARTFRAITVTK	L	904.98	2711.91	3	2711.36	Ox (M)	
ASPH1_BOVIN	M	EGTGG	E	402.23	401.22	1	401.15	Glu->pyro-Glu (N-term E)	X
Aspartate b-hydroxylase domain-containing prot 1	G	GPSPG	G	414.26	413.25	1	413.19		X
	G	GRPGG	C	443.27	442.26	1	442.23		
	I	MPAWPL	P	357.64	713.27	2	713.36		
	R	SFMSANTF	G	453.19	904.36	2	903.38		
GPAT1_BOVIN	K	RGPVG	P	443.27	442.26	1	442.23		
Glycerol-3-phosphate acyltransferase 1	Y	IASGN	N	462.23	461.22	1	460.23		
	G	NNLNI	P	587.26	586.26	1	586.31		
	E	GTDSMI	N	320.16	638.31	2	638.26	Ox (M)	
CBPE_BOVIN	A	RGPVG	P	443.27	442.26	1	442.23		
Carboxypeptidase	D	NPGVH	E	523.35	522.35	1	522.26		X
	R	RRPQEDGISF	E	402.21	1203.59	3	1203.60		
TDIF1_BOVIN	P	RGPVG	A	443.27	442.26	1	442.23		
Deoxynucleotidyltransferase terminal-interacting prot	S	QMTTSFTDPAISMDLLRA	V	677.17	2028.48	3	2028.95	2 Ox (M)	
PTPRN_BOVIN	C	SDGAG	R	406.53	405.52	1	405.15		
Receptor-type tyrosine protein phosphatase-like N	P	GRPGG	P	443.27	442.26	1	442.23		
	Q	ESQVP	S	542.24	541.23	1	540.25	Glu->pyro-Glu (N-term E)	X
ANGL4_BOVIN	A	ATAGL	L	432.20	431.19	1	431.24		X
Angiopoietin-related protein 4	L	VNPGG	G	443.27	442.26	1	442.22		
	L	AGPAH	N	453.19	452.18	1	451.22		X
ELN_BOVIN	V	GGLGP	G	401.14	400.13	1	399.21		
Elastin	G	LGVGG	L	402.23	401.22	1	401.23		X
	F	FGAGG	G	407.21	406.21	1	407.18		X
	L	GVGGK	P	418.10	417.09	1	416.24		
	P	GGVPGG	V	443.27	442.26	1	442.22		
	A	AAAYK	A	523.33	522.32	1	522.28		X
	A	TAKAAKAAK	F	466.17	930.32	2	929.57		

a. Observed peptide mass in the detector after ionisation using an ESI source.

b. Expected mass calculated from the Observed mass according to the charge state of the ion.

c. Charge state of the identified peptide.

d. Predicted mass.

e. Peptides identified in MALDI-TOF mass spectrometer.