Antilisterial peptides from Spanish dry-cured hams: Purification and identification

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ABSTRACT

The typical Spanish dry-cured ham has a particular sensory quality that makes it a distinctive food, highly appreciated for consumers worldwide. Its particular physicochemical properties, such as high salt content and reduced water activity contribute to their shelf-stability. However, post-processing actions carried out for the commercialization of these products such as slicing may increase the risk of development of pathogenic microorganisms as *Listeria monocytogenes*. During ripening, muscle proteins are hydrolyzed by muscle peptidases releasing peptides and free amino acids. Some of these peptides have been described to exert biological activities such as antioxidant and ACE-inhibition. In this study, a peptidomic strategy using mass spectrometry techniques has been used to identify and sequence those naturally generated peptides showing antilisterial activity. One hundred and five peptides have been identified in active fractions and some synthesized and their MIC calculated. Ten peptides were able to inhibit the growth of *L. monocytogenes*, being the pentapeptide RHGYM the most effective showing a MIC value of 6.25 mM. This study proves for the first time the potential antimicrobial action against *L. monocytogenes* of certain naturally generated peptides obtained from Spanish dry-cured ham.

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1. Introduction

Spanish dry-cured ham is produced from white pigs crossbreeds (Losantos et al., 2000). Basically, the preparation method involve four main stages: conditioning of pieces, salting, post-salting and dry-curing. During the salting step, a microbial reservoir is especially created on the surface of hams produced from microorganisms present in the salt and environment (Cornejo et al., 1992). Some of these microorganisms together with endogenous enzymes contribute to the changes occurred during dry-cured processing such as proteolysis, lipolysis and nitrate reduction influencing the sensory quality of hams (Toldrá, 1998; Sondergaard and Stahnke, 2002).

Dry-cured ham is a Ready-To-Eat (RTE) meat product, which is sliced and packaged under vacuum as an extra protection barrier prior to distribution and commercialization. However, these post-processing actions could favor the contamination of the product

due to the development of pathogenic organisms such as *Listeria monocytogenes* (Chaitiemwong et al., 2014). In fact, *L. monocytogenes* is difficult to eradicate owing to its resistance to drying and high salt concentrations, being its survival in dry-cured meats a major food safety concern (Nightingale et al., 2006). Immunocompromised patients, pregnant women, children and elderly people are primarily affected by listeriosis, a rare but extremely serious zoonosis (Giovannini et al., 2007). Low morbidity and very high mortality rates (20%) have been related to listeriosis (EFSA and ECDC, 2015), which makes the elimination or reduction of *L. monocytogenes* from food processing plants a constant challenge for RTE food producers.

The ripening process of Spanish dry-cured ham is very long and could be up to 24 months in drying chambers. During the processing, different chemical and biochemical changes occur obtaining, as a result, the characteristic color, flavor and taste of dry-cured ham (Toldrá et al., 1992). Among all the complex biochemical reactions occurring during dry-cured hams processing, proteolysis is one of the most important. In this process, muscle proteins are hydrolyzed by muscle endogenous enzymes with the

release of small peptides and free amino acids (Toldrá and Flores, 1998; Lametsch et al., 2003). The role of sarcoplasmic and myofibrillar proteins in the generation of small peptides have been previously described in the final product (Sentandreu et al., 2007; Mora et al., 2009, 2010; 2011), and during the dry-curing process at different times (Gallego et al., 2014; Mora et al., 2015). On the other hand, some of the generated peptides have shown good antihypertensive and antioxidant activity (Escudero et al., 2012a, b, 2013; Toldrá and Reig, 2011).

However, to our knowledge, there are no studies describing antimicrobial peptides in dry-cured ham. In this sense, the identification of antilisterial activity in naturally generated peptides during dry-curing of Spanish ham would increase the value of this product by improving its safety especially when commercialized in sliced form. Thus, in this study, a peptidomic strategy has been used to identify and sequence those naturally generated peptides showing antilisterial activity derived from dry-cured ham protein degradation.

2. Materials and methods

2.1. Spanish dry-cured ham material

Spanish dry-cured ham was produced from 6-months old pig (Landrace × Large White). As a pre-salting stage, hams were prepared according to traditional procedures. During salting, potassium nitrate was incorporated covering hams with NaCl and locating them in chambers at 1–3 °C and 80–90% of relative humidity. During post-salting stage hams were subjected to a temperature increase (3–5 °C) and relative humidity decrease (75–85%) for 60 days. Finally, the ripening-drying was carried out at temperatures in the range of 14–20 °C and lower relative humidity (up to 70%). The total processing time was 10 months. All analysis was done in *Biceps femoris* muscle.

2.2. Bacterial strains and antimicrobial activity assay

A total of eight *Listeria* strains were used as test microorganisms for the determination of antilisterial activity. They include the type strain of the species *L. monocytogenes* CECT 4031[T] from the Spanish Type Culture Collection (CECT), five *L. monocytogenes* food isolates (14, 49, 75, 120, 127) from the IATA (Valencia Spain) that were identified in a previous study (Aznar and Elizaquível, 2008), clinical isolated *L. monocytogenes* FBUNT strain from Facultad de Bioquímica, Química y Farmacia, Universidad Nacional de Tucumán, (Argentina) (Castellano et al., 2004) and food isolated *Listeria innocua* 7 from the Unité de Recherches Laitieres et Genetique Appliqueé (INRA, France) (Castellano and Vignolo, 2006). All strains were grown overnight in tryptic soy broth (TSB; BBL, Cockeysville, MD) with 0.5% added yeast extract (YE) at 30 °C. *Lactobacillus curvatus* CRL705, used as a bacteriocin-producer positive control, was grown in MRS at 30 °C.

Listeria cells were added at a concentration of 10^7 CFU/mL in 10 mL of TSB + YE soft agar (0.7%) medium. The dry-cured ham fractions obtained from different steps of chromatographic purification were added (5 μL) onto the soft agar layer inoculated with different *Listeria* strains and incubated at 30 °C during 24 h. The antibacterial activity was measured as the diameter in mm of the clear zone of growth inhibition by comparison with the bacteriocin-containing supernatant from *L. curvatus* CRL705 as positive control and saline solution as negative control (Castellano and Vignolo, 2006).

2.3. Peptides extraction from dry-cured ham

Fifty grams of *Biceps femoris* muscle from Spanish dry-cured ham cured for 10 months were homogenized with 200 mL of 0.01 N HCl for 8 min at 4 °C. The homogenate was centrifuged (12,000 g during 20 min at 4 °C) and filtered. The deproteinization was done by adding ethanol (1:3; v:v) and keeping the mixture at 4 °C overnight. Then, sample was centrifuged (12,000 g during 20 min at 4 °C) and the supernatant lyophilized. Finally, the dried extract was dissolved in 25 mL of 0.01 N HCl, and stored at −20 °C until use.

2.4. Size-exclusion chromatography

The size exclusion chromatography was done using a previously equilibrated Sephadex G-25 column (2.5 × 65 cm, Amersham Biosciences, Uppsala, Sweden) with 0.01 N HCl at 4 °C and a flow rate of 15 mL/h. The collection of fractions (5 mL) was done using an automatic fraction collector and their absorbance was measured at 214, 254 and 280 nm (Agilent Cary 60 UV−Vis spectrophotometer, Agilent Technologies, Palo Alto, CA, USA). All fractions were assayed for *L. monocytogenes* inhibitory activities. Fractions showing the highest activities were put together freeze dried and dissolved in 1 mL of bidistilled water. A saline solution was used as negative control.
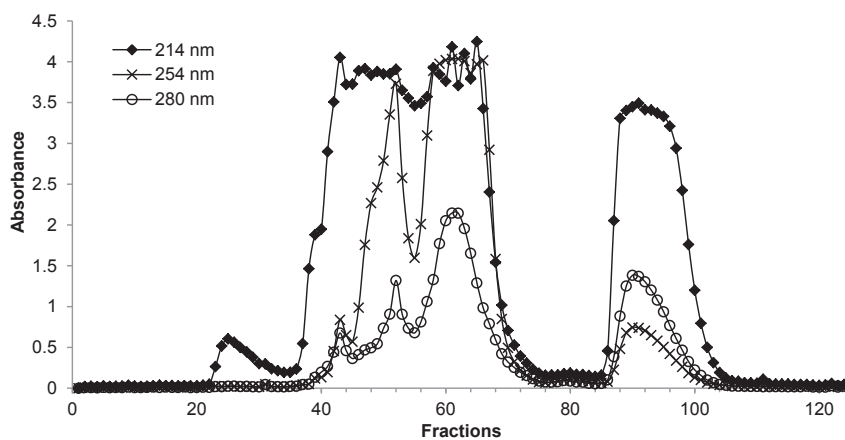

Fig. 1. Gel filtration chromatography of 10 months of curing dry-cured ham extract using a Sephadex G-25 column. collection; [T]Type strain of the species; [*]Non inhibitory.
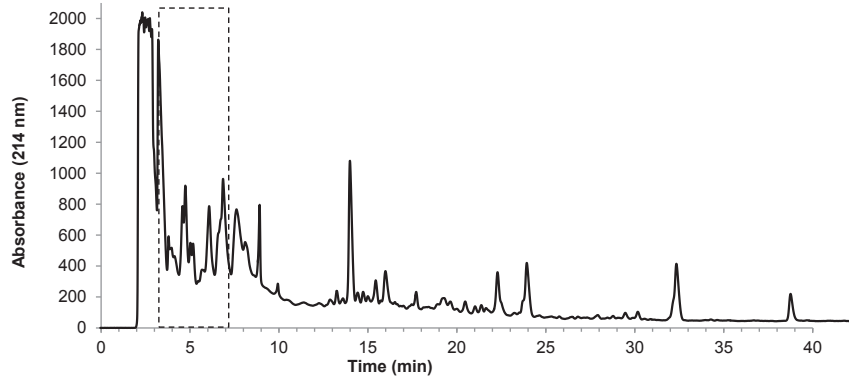
Fig. 2. Reversed-phase HPLC separation of fractions 41 and 42 obtained from gel filtration chromatography. Dotted line indicates fractions from 4 to 7 that showed *Listeria monocytogenes* inhibitory activity.
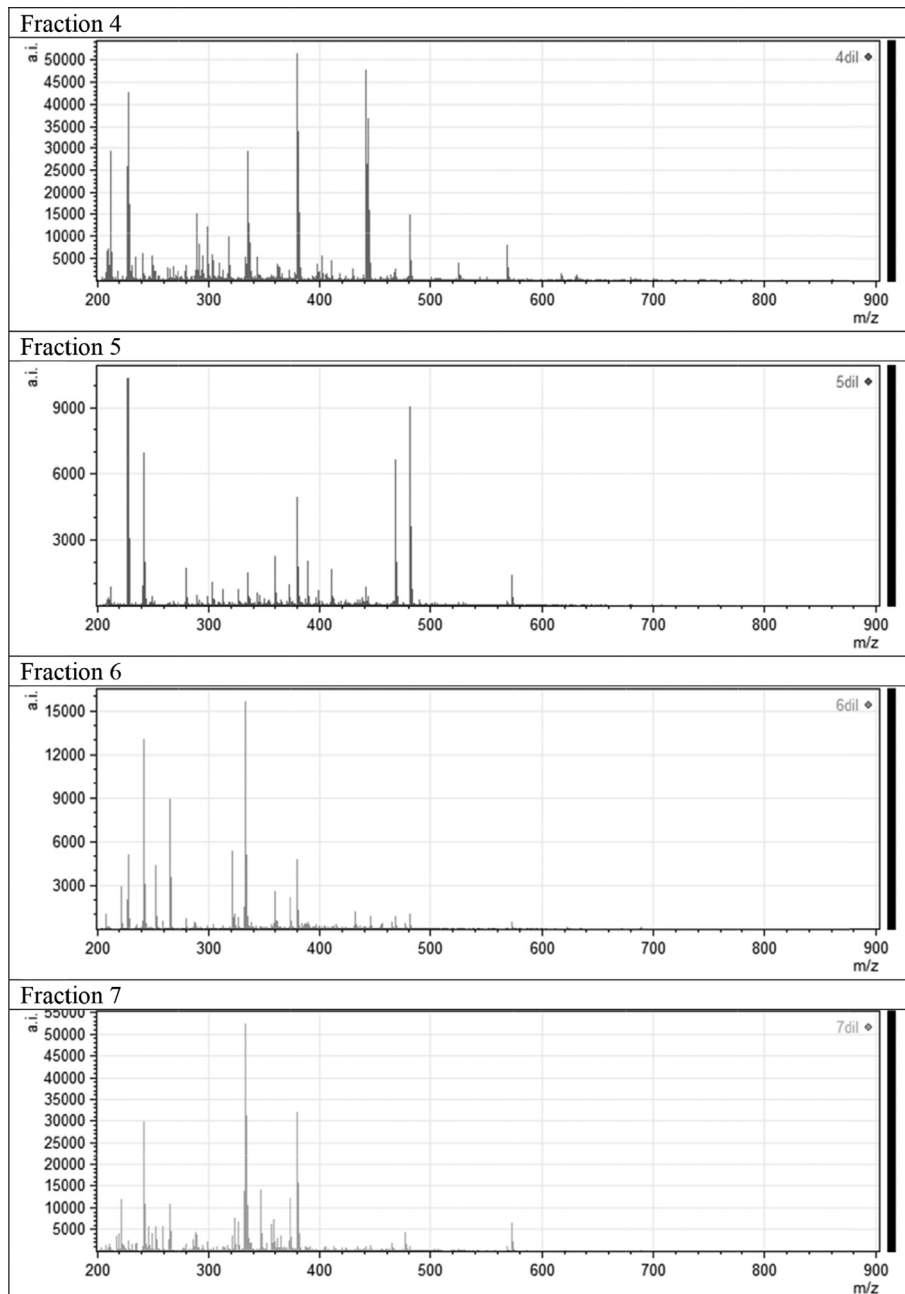
Fig. 3. MALDI-ToF spectra measured from 200 to 900 *m/z* [M−H+] of the most active fractions previously separated by RP-HPLC.

2.5. Reversed-phase high performance liquid chromatography

The reversed-phase chromatographic separation was done using an Agilent 1100 HPLC equipment (Agilent Tech., California, USA) with a Symmetry C18 column (4.6 × 250 mm, 5 μm) from Waters Co. (Milford, MA, USA). An aliquot of 200 μL of the most active fractions (fractions 41 and 42) was injected into system. Solvent A was 0.1% trifluoroacetic acid (TFA) in bidistilled water and solvent B contained acetonitrile (ACN) and bidistilled water (60:40, v/v) and 0.085% of TFA. The elution of the peptides was done using an isocratic gradient of 5 min with a 99% solvent A, followed by a linear gradient from 0% to 45% of solvent B in 40 min at a flow rate of 1 mL/min. The absorbance was measured at 214 nm and fractions of 1 mL were collected and assayed for *L. monocytogenes* inhibitory activity. Those fractions showing antilisteria activity were freeze dried and analysed by MS for the identification of the peptides.

2.6. MALDI ToF analysis and peptide identification by LC-MS/MS

The most active fractions obtained after the separation using reversed-phase HPLC were analysed by Matrix-Assisted Laser Desorption/Ionisation MS technique in order to obtain the *m/z* profile of the peptides in the mixture. The analysis was done in a 5800 MALDI ToF/ToF system (ABSciex) in positive reflectron mode (3000 shots at each position) in a range from 200 to 3000 Da. Plate model and acquisition method were calibrated by a peptide mass standards calibration mixture (ABSciex) in 13 positions. Dried hydrolysates were dissolved in 5% ACN; 0.1% TFA, and 1 μL of every sample was directly spotted on 10 positions in the MALDI plate and allowed to air dry. Once dried, 0.5 μL of matrix solution (5 mg/mL of
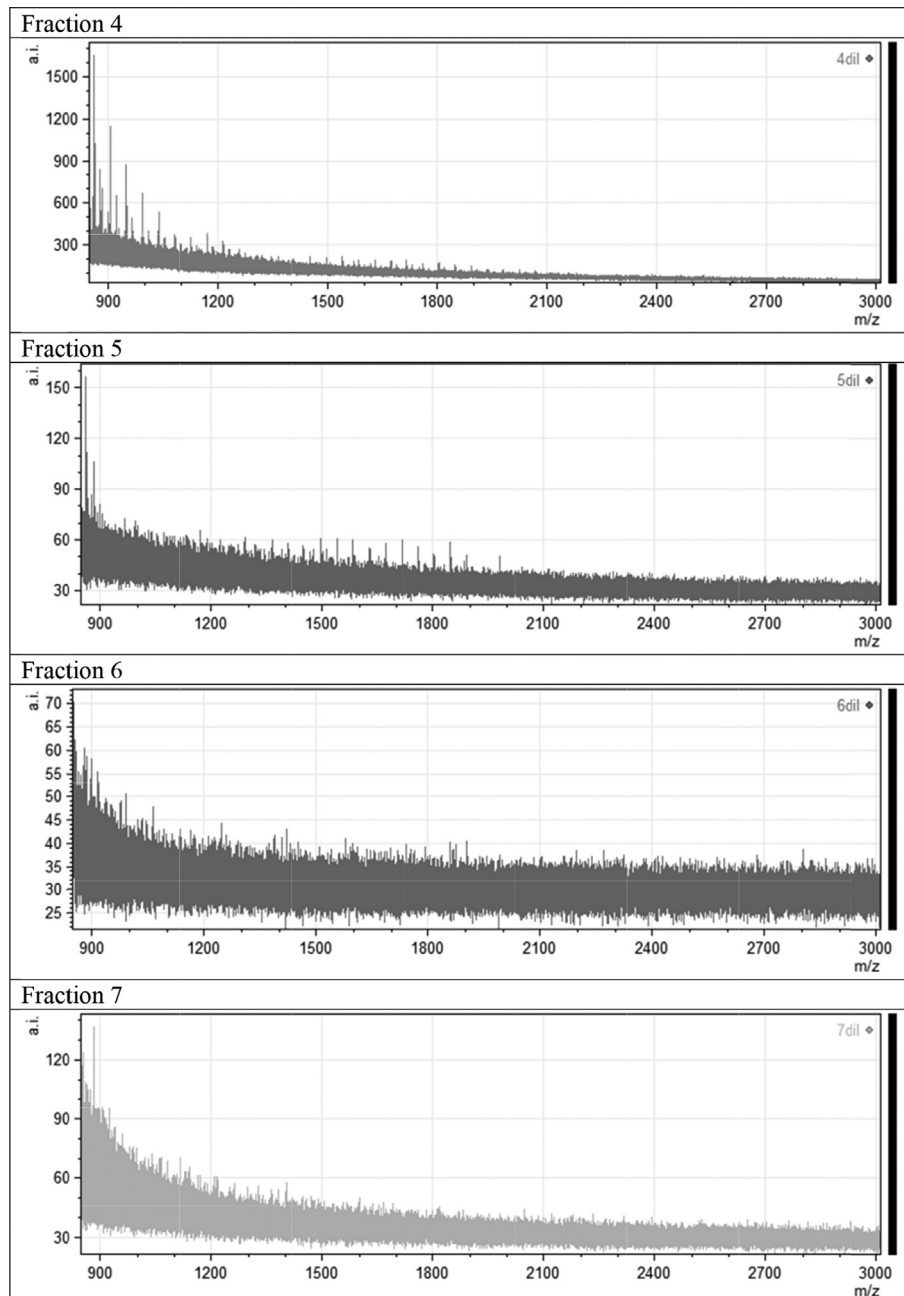

Fig. 4. MALDI-ToF spectra measured from 900 to 3000 *m/z* [M−H+] of the most active fractions previously separated by RP-HPLC.

α-Cyano-4-hydroxycinnamic acid (CHCA) in 0.1% TFA-ACN/H$_2$O (7:3, v/v) was spotted.

Those fractions obtained after RP-HPLC showing the highest values for antilisterial activity were analysed by nLC-MS/MS in order to identify the sequences of the peptides according with Gallego et al. (2015). Fractions were resuspended in H$_2$O with 0.1% of trifluoroacetic acid (TFA) to obtain a final concentration of 0.2 mg/mL. Five microlitres of the supernatant were analysed in the LC-MS/MS system.

The database search of peptides was done through the proteomic platform of Mascot Distiller v2.4.2.0 software (Matrix Science, Inc., Boston, MA) (http://www.matrixscience.com). The search parameters used in the identification were: UniProt as protein database, with a significance threshold $p < 0.1$ and a FDR of 1.5%, and a tolerance of 100 ppm in MS mode and 0.3 Da in MS/MS.

2.7. Peptide synthesis

The most promising sequences of identified peptides were synthesized by GenScript Corporation (Piscataway, NJ, USA) and their purity was certified by analytical LC-MS. The in vitro inhibition of *L. monocytogenes* and minimum inhibitory concentration (MIC) of synthetic peptides was determined by the critical dilution assay according to (Vignolo and Suriani, 1993). Serial two-fold dilutions of synthetic peptides in sterile water (from 100 to 0,78 mg/mL) were prepared and 5 μL volume was placed on a semi-solid TSB

overlay inoculated with *L. monocytogenes* CECT 4031^T and FBUNT strains (ca. 10^7 CFU/mL), as indicator organisms in separated experiments. The MIC value was determined after incubating with the diluted peptides for 24 h at 30 °C. All the analysis were done in triplicate.

2.8. Statistical analysis

The ANOVA procedure was used to determine significant differences in the obtained MIC value using the software Minitab Statistic Program, v8.21 (Minitab Inc., PA, USA). Each statistical analysis was done in triplicate (n = 3) and the normality of the data was tested before applying the ANOVA procedure.

3. Results and discussion

The application of antimicrobial natural compounds as a preservation approach has experienced a high interest during the last years due to the increase of consumer's demand for safe and fresh-tasting Ready-to-Eat products with low amounts of chemical preservatives. In this study, a peptidomic strategy has been used to identify and sequence those naturally generated peptides showing antilisterial activity derived from dry-cured ham protein degradation. The pathogen was chosen as the most frequently encountered on slices of dry-cured meat products (Vorst et al., 2006).

Samples of dry-cured ham were fractionated using SEC as

Table 1
Peptides identified in fraction 4 of RP-HPLC.

Accession number[a]	Protein name	Expected mass (m/z)	Mass Charge[b] (Da)	Calculated mass	Po	Identified sequence[c]	Pf	Modifications
TITIN_MOUSE	Titin	413.10	2	824.39	S	MDPKYR[c]	D	Oxidation (M)
KPYM_RABIT	Pyruvate kinase isozymes M1/M2	309.08	2	616.27	L	DIDSAP	I	
		821.93	2	1641.76	K	DPVQEAWAEDVDLR	V	
MYOM1_BOVIN	Myomesin-1	503.06	1	502.24	A	PTTGQ	I	Oxidation (M)
		509.22	1	508.28	Q	AHPGK	Y	
		525.44	1	524.31	G	VPGRP	R	
		533.15	1	532.29	I	KAISD	E	
		542.04	1	541.30	G	VPGRN	R	
		577.10	1	576.21	K	DSNNAG	V	
		579.44	1	578.24	P	SAPMTG	Q	
		362.14	2	722.33	N	NAGVHEP	E	
		376.12	3	1125.48	P	EETGGAEITGY	Y	
DYH3_HUMAN	Dynein heavy chain 3	521.09	1	520.18	S	CNPGM	K	Oxidation (M)
		525.44	1	524.15	Y	NDSSC	C	
		526.35	1	525.25	P	NPAPQ	W	
		528.36	1	527.31	S	NGIPK	L	
		559.23	1	558.26	F	PADEK	A	
		559.24	1	558.30	L	AQDIL	S	
		755.25	1	754.38	E	TRMGYK	P	
		476.14	2	950.45	L	NMPAKEVY	G	
		336.13	3	1005.48	F	SDFSLSHTL	G	
		471.28	3	1410.66	A	LDNPYPNPAPQW	L	
		668.59	3	2002.97	I	NMLLNTGDVPNIFPADEK	A	
ENOB_PIG	Beta-enolase	613.47	1	612.30	G	SHAGNK	L	
		506.05	3	1514.87	E	KKLSVVDQEKVDK	F	
		1034.08	2	2065.93	K	NYPVVSIEDPFDQDDWK	T	
TPM4_HUMAN	Tropomyosin alpha-4 chain	622.31	2	1242.65	R	IQLVEEELDR	A	
MARH4_HUMAN	E3 ubiquitin-protein ligase	579.08	3	1733.99	L	RLCKYRDILLSEIL	M	
KCRM_BOVIN	Creatine kinase	616.30	2	1230.62	K	DLFDPIIQDR	H	
HASP_HUMAN	Serine/threonine-protein kinase	323.10	2	644.32	R	RCPGGR[c]	V	
RL4_PIG	60S ribosomal protein L4	514.38	1	513.33	K	KPAAK	K	
		515.37	1	514.28	K	PAAEK	K	
		580.32	1	579.35	K	KPAHK[c]	K	
		580.33	1	579.35	K	PAHKK	P	
		670.53	1	669.45	K	KPAVKK[c]	P	

[a] Accession number according to UniProt protein database.
[b] Charge of the peptide according to the ionisation occured in nanoLC-MS/MS analysis.
[c] Sequences with an asterisc were chosen to be synthesized and tested in vitro.

Table 2
Peptides identified in fraction 5 of RP-HPLC.

Accession number[a]	Protein name	Expected mass (m/z)	Mass Charge[b] (Da)	Calculated mass	Po	Identified sequence[c]	Pf	Modifications
TITIN_MOUSE	Titin	763.63	1	762.44	Y	TKYRVP[c]	D	
		783.53	2	1564.86	E	IIADGLKYRIQEF[c]	K	
KPYM_RABIT	Pyruvate kinase isozymes M1/M2	551.16	1	550.31	G	FLGSK	K	
		340.14	2	678.30	R	PGSGFTN	T	
		689.12	1	688.32	V	CKDPVQ	E	
MYOM1_BOVIN	Myomesin-1	515.10	1	514.24	A	GVNEP	E	Oxidation (M)
		528.47	1	527.26	T	LPPSD	Y	
		530.37	1	529.25	S	APGTGQ	I	
		533.08	1	532.27	L	PVKCS	N	
		533.16	1	532.26	V	KASNN	A	
		305.05	2	608.26	V	KMSNN	A	
		615.25	1	614.41	Y	IIITR	K	
		639.26	1	638.29	P	EIQSY	R	
		702.20	1	701.43	Y	IIITDK	Q	
DYH3_HUMAN	Dynein heavy chain 3	505.06	1	504.27	S	ARTSA	N	
		506.05	1	505.20	R	SSSEP	M	
		511.43	1	510.32	G	PPIGK	K	
		515.10	1	514.28	V	KAAEP	G	
		519.10	1	518.23	R	EADGK	K	
		521.10	1	520.21	R	EQASS	L	
		525.43	1	524.27	D	LHAAN	Q	
		538.32	1	537.29	L	PITAH	P	
		546.38	1	545.32	V	AAKEK	P	
		547.39	1	546.34	E	VKTSL	T	
		559.24	1	558.23	S	ANEEP	S	
		578.10	1	577.25	M	NPGYAG	R	
		593.13	1	592.29	R	KDMIA	P	Oxidation (M)
		617.28	1	616.35	P	IGAAASK	E	
		623.28	1	622.26	M	EMESK	E	
		339.20	2	676.22	E	NDYYC	S	
		352.27	2	702.36	L	IIPTME	T	
		404.21	2	806.35	V	NRDTNTS	I	
		737.26	2	1472.68	D	VFFRNLIMGMDD	N	Oxidation (M)
		533.16	3	1596.75	V	FVDDLNMPAKEVYG	A	
		533.26	3	1596.78	T	SPIHLAFSMMRLY	S	2 Oxidation (M)
ENOB_PIG	Beta-enolase	486.30	3	1455.81	D	LVVGLCTGQIKTGAP	C	
MUC2_HUMAN	Mucin-2	621.28	3	1860.91	L	SLAGGSELQTEGRTRYH	G	
NEK10_HUMAN	Serine/threonine-protein kinase	684.20	2	1366.61	F	TSNRYHSYPWG[c]	T	
CYB_ORYME	Cytochrome b	352.27	2	702.28	I	HANGASM	F	Oxidation (M)
		359.09	2	716.32	I	GGQPVEM	P	
RL4_PIG	60S ribosomal protein L9	342.97	3	1025.59	K	PAAEKKPASK	K	

[a] Accession number according to UniProt protein database.
[b] Charge of the peptide according to the ionisation occured in nanoLC-MS/MS analysis.
[c] Sequences with an asterisc were chosen to be synthesized and tested in vitro.

Table 3
Peptides identified in fraction 6 of RP-HPLC.

Accession number[a]	Protein name	Expected mass (m/z)	Mass Charge[b] (Da)	Calculated mass	Po	Identified sequence[c]	Pf	Modifications
KPYM_RABIT	Pyruvate kinase isozymes M1/M2	683.22	1	682.35	Q	TARQAH[c]	L	Oxidation (M)
		371.25	2	740.33	V	ARMNFS	H	
MYOM1_BOVIN	Myomesin-14	306.02	2	610.28	S	NNAGVH	E	
DYH3_HUMAN	Dynein heavy chain 3	503.06	1	502.28	G	LGGEK	D	
		514.15	1	513.12	N	DSSCC	A	
		301.13	2	600.27	N	CHLAAS	W	
		624.55	1	623.32	N	IIEYS	R	
		332.21	2	662.38	G	IMKQK[c]	K	Oxidation (M)
		339.03	2	676.34	M	VPDYAL	I	
		713.25	1	712.24	L	IMGMDD	N	2 Oxidation (M)
		329.20	3	984.55	V	ILRCLRPD	K	
		333.07	3	996.47	W	ALMIDPHGQ	A	Oxidation (M)
KCRM_BOVIN	Creatine kinase	522.10	1	521.23	K	GGNMK	E	Oxidation (M)

[a] Accession number according to UniProt protein database.
[b] Charge of the peptide according to the ionisation occured in nanoLC-MS/MS analysis.
[c] Sequences with an asterisc were chosen to be synthesized and tested in vitro.

shown in Fig. 1. A total of 128 fractions were tested for antilisterial activity in Petri plates against the eight *Listeria* strains, including seven *L. monocytogenes*. Results showed that fractions 41 and 42 inhibited the growth of all assayed *Listeria* strains which would

Table 4
Peptides identified in fraction 7 of RP-HPLC.

Accession number[a]	Protein name	Expected mass (m/z)	Mass Charge[b] (Da)	Calculated mass	Po	Identified sequence[c]	Pf	Modifications
KPYM_RABIT	Pyruvate kinase isozymes M1/M2	619.45	1	618.26	A	WAEDV	D	
		621.47	1	620.28	S	DGIMVA	R	Oxidation (M)
		666.33	1	665.29	V	ICATQM	L	
MYOM1_BOVIN	Myomesin-1	308.13	2	614.34	L	PVKASN	N	
		459.11	2	916.38	K	YGSEISDF	T	
DYH3_HUMAN	Dynein heavy chain 3	503.06	1	502.24	L	VAADQ	P	
		503.06	1	502.31	R	AVKSV	L	
		533.14	1	532.29	Q	IAKSD	S	
		326.13	2	650.38	N	LPITAH	P	
		340.12	2	678.29	V	RHGYM*	I	Oxidation (M)
		362.14	2	722.29	E	TVMENN	P	Oxidation (M)
		656.41	2	1310.59	R	HCNKKYRSEM[c]	E	Oxidation (M)
MARH4_HUMAN	E3 ubiquitin-protein ligase	532.24	2	1062.57	R	ELVMRVTTV	–	Oxidation (M)
HASP_HUMAN	Serine/threonine-protein kinase	394.18	3	1179.60	R	REHQEASVPK	G	
CYB_ORYME	Cytochrome b	622.39	2	1242.57	G	YVLPWGQMSF	W	Oxidation (M)

[a] Accession number according to UniProt protein database.
[b] Charge of the peptide according to the ionisation occured in nanoLC-MS/MS analysis.
[c] Sequences with an asterisc were chosen to be synthesized and tested in vitro.

Table 5
MIC values (mM) of the synthetised antibacterial peptides tested against *L. monocytogenes* CECT 4031[T] and FBUNT strains.

Peptide	MIC (mM)	
	FBUNT	CECT 4031[T]
TKYRVP	100	100
TSNRYHSYPWG	100	100
IIADGLKYRIQEF	100	100
IPAVF	n.i.	n.i.
MDPKYR	50	50
RCPGGR	50	50
HCNKKYRSEM	50	50
IMKQK	n.i.	n.i.
TARQAH	50	50
KPAHK	50	50
RHGYM	6.25	6.25
KPAVKK	50	50

CECT, Spanish Type Culture Collection; [T]Type strain of the species.
n.i. Means non−inhibitory at any of the tested concentrations.

indicate antilisterial activity; the remaining collected fractions failed to exert inhibitory effect against the tested strains. These results are in agreement with previously described antioxidant and ACE-inhibitory activity results of dry-cured ham samples after their fractionation using a Sephadex G-25 column in which the highest ACE inhibitory activity (80% of ACE inhibition) was also detected from fraction 40 (Escudero et al., 2013). Subsequent *in vivo* studies using Spontaneously Hypertensive Rats (SHR) showed antihypertensive activity with a decrease of 40 mmHg in systolic blood pressure (Escudero et al., 2012a, b). Regarding antioxidant activity, a similar increase in DPPH radical-scavenging and ferric reducing power was observed from fraction 40 in a Sephadex G25 in the same study (Escudero et al., 2012a, b). This fact would be related to the size of sequences of the peptides contained on that elution volume as it has been widely described that sequences comprised between 2 and 20 amino acids were the most characteristic for bioactive peptides. However, proteolysis is very dynamic and causes changes in the generated peptide sequences which can be created and hydolysed depending on the action of a wide distribution of endogenous enzymes that are acting during dry-cured ham processing.

Subsequently, these two active fractions (numbers 41 and 42) were pooled together and analysed by RP-HPLC and fractions of 1 mL collected (showed in Fig. 2). After measuring the antilisterial activity, those peaks eluted at 4, 5, 6, and 7 min showed inhibitory activity against *Listeria* (0.8 ± 0.2 mm in diameter). The size of the peptides included in these RP-HPLC fractions was elucidated using MALDI-ToF mass spectrometry at two different levels of *m/z* (from 200 to 900 *m/z* as it is shown in Fig. 3 and from 900 to 3000 *m/z* in Fig. 4) and resulted very useful in the characterization of the peptide profile of fractions 4, 5, 6, and 7. These results showed that most of the peptides contained in these fractions showed medium-low molecular weights, being the peptide profile showed on Fig. 4 less crowded than profiles of Fig. 3. These results are in agreement with previously published information about the size of the most active peptides in terms of antioxidant and ACE-inhibitory activities, despite many of the recently described antimicrobial sequences showed a very wide range of chain length (McClean et al., 2014; Trindade et al., 2015).

Lately, the identification of peptide sequences was done using a peptidomic approach based on nanoLC-MS/MS. The database search was carried out using Mascot as search engine and UniProt as protein database, which contains 549215 sequences and 195767212 residues. A total of 105 peptide sequences were identified from the most active fractions showing molecular masses between 502 and 2065 Da and from 5 to 18 amino acids in length (Tables 1–4). Regarding the length of bioactive peptides, numerous antibacterial peptides have been described as long amino acid chains, which can adopt an α-helical linear or circular structure organized in a β-sheet. This conformation is often essential considering the mechanism of action of active peptides against the microorganisms (Nicolas and Mor, 1995). On the other hand, one group of antimicrobial peptides produced by lactic acid bacteria and referred as bacteriocins, particularly Class IIa bacteriocins, constitute a large cluster of peptides with lengths between 36 and 49 amino acids that have been described to be active against *Listeria* species (Nishie et al., 2012). Regarding this, some bacteriocins loose the activity in meat products due to the action of specific meat ingredients such as salt and nitrite and conditions such as proteolytic degradation that destabilize their biological activity. According to the previously described as optimum molecular mass and sequence for antilisterial inhibition, some of the identified peptides were synthesized and their MIC calculated against *L. monocytogenes* CECT 4031[T] and FBUNT strains (Table 5). Among the twelve peptides synthesized, two of them failed to exert antilisterial activity. The remaining ten peptides were able to inhibit *L. monocytogenes* growth, being the pentapeptide RHGYM

identified in fraction 7 of RP-HPLC (see Table 4) the most effective with a MIC value of 6.25 mM. Non-similar sequences showing antimicrobial activity have been found but the tripeptide RHG have been previously described as a potential antioxidant sequence by Saito et al. (2003). Previous studies of bovine hemoglobin hydrolysates resulted into some peptides showing antimicrobial activity against *E. coli*, *Salmonella* Enteritidis, *L. innocua* and *Micrococcus luteus*. The peptide β114-145 and its peptic derivatives containing the RYH sequence exhibited the highest antimicrobial activity (85 μM) (Catiau et al., 2011a). In addition (Catiau et al., 2011b), determined that KYR was the minimal sequence of hemoglobin alpha-chain necessary to exert antibacterial activity. On the other hand, the important role of Tyr (Y) in the interaction with membranes together with the amino acid residues Arg (R) and Lys (K), which are known to act as peptide anchors in membranes by interacting with negatively charged membrane phospholipids was reported (Lopes et al., 2005). In the present study, six of the synthesized and tested peptides presented tyrosine (Y) and two positive charged amino acids such as histidine (H) or K in their sequences, which would be required for antibacterial activity. Also several of the identified peptides include the previously described antimicrobial sequences KYR and RYH although only MDPKYR and HCNKKYRSEM peptides showed MIC values of 50 mM. In fact, RHGYM the most active peptide described in this study with a MIC value of 6.25 mM shows in its sequence tyrosine, arginine and histidine amino acids.

The potential of dry-cured ham as carrier of antilisterial peptides is an added-value for this type of product as the joint action of these bioactive peptides could influence the development of possible cross-contaminations with *L. monocytogenes* during slicing and packaging. This would be a way to develop future natural strategies in the control of safer products as the use of natural antilisterial peptides as preservatives is an interesting alternative to chemicals compounds in the food area.

4. Conclusions

In this study a highly active antilisterial peptide has been identified among the naturally generated peptides in Spanish dry-cured ham. A total of 105 peptide sequences were identified from the most active fractions against eight *Listeria* strains after RP-HPLC separation, showing molecular masses between 502 and 2065 Da and from 5 to 18 amino acids in length. From them, a total of twelve peptides were chosen, synthesized and tested to calculate their MIC against *L. monocytogenes* CECT 4031[T] and FBUNT strains. The pentapeptide RHGYM resulted to be the most effective with a MIC value of 6.25 mM. Thus, according with the nowadays interest in food safety and food protection given by natural products, the results derived from this study prove the presence of peptides naturally generated during the processing of dry-cured ham and their antilisterial potential as preservative during storage and distribution of the sliced product.

Conflicts of interest

Authors declare that there are no conflicts of interest.

Acknowledgements

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