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Iberian dry-cured ham as a potential source of α-glucosidase-inhibitory peptides

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ABSTRACT

Iberian dry-cured ham is a meat product known as a source of different bioactive peptides due to its high protein content and intense hydrolysis during its processing. In this study, the potential of α-glucosidase inhibitory peptides generated in Iberian dry-cured ham was evaluated for the first time. After size-exclusion chromatography and high-performance liquid chromatography using different stationary phases, the characterization and identification of the peptide sequences contained in the most active fractions was done using MALDI-ToF mass spectrometry and Q-ToF mass spectrometry in tandem. A total of 16 and 47 sequences of peptides were identified in the two most active fractions. On the other hand, the α-glucosidase inhibitory activity of previously identified bioactive peptides in Spanish dry-cured ham such as AEEEYPDL and LGVGG was established, confirming their multifunctionality.

1. Introduction

The enzyme α-glucosidase (EC 3.2.1.20) is an important enzyme participating in the digestion of carbohydrates. Its specificity is focused on the hydrolysis of non-reducing α-(1 → 4), α-(1 → 3) and α-(1 → 2) linked D-glucose residues with the result of the release of D-glucose from oligosaccharides, with preference for the α-(1 → 4) linkage. Thus, it is an exoglycosidase that hydrolyses oligosaccharides such as maltose and sucrose and, more slowly, larger substrates as maltoheptaose or starch polysaccharides.

Due to its relation with the carbohydrate metabolism, this enzyme is frequently used as a target for the treatment of type 2 Diabetes mellitus (T2DM) disease, a serious global health problem (Mollica et al., 2019; Picot et al., 2017). Inhibitors of this enzyme are effective in retarding the carbohydrate digestion, reducing the impact of carbohydrates on blood glucose level and decreasing the chance for hyperglycemia (Bhandari, Jong-Anurakkun, Hong, & Kawabata, 2008; Zengin et al., 2019).

Traditionally, acarbose, voglibose and miglitol are used as α-glucosidase inhibitors to control glucose level in blood of diabetic patients. However, the side effects and long synthetic routes necessary to obtain them has promoted researchers to look for different heterocyclic molecules such as oxindole derivatives (Asadollahi-Baboli & Dehnavi, 2018), triazole derivatives (Avula et al., 2019;), pyrrolidine derivatives (Guazzelli et al., 2019), and oxadiazole derivatives (Khan, Zafar, Patel, Shah, & Bishayee, 2019) as well as pyrazole-triazolopyrimidine hybrids (Pogaku, Gangarapu, Basavoju, Tatapudi, & Katragadda, 2019) or thiosemicarbazide-triazole hybrids (Bakherad, Mohammadi-Khanaposhtani, Sadeghi-Aliabadi, Rezaei, Fassihi, Bakherad, Rastegar, Biglar, Saghaie, & Larijani, 2019), using kinetic and molecular modeling studies in their design and evaluation (Dhameja & Gupta, 2019).

On the other hand, the study of natural α-glucosidase inhibitors is also in the focus of attention of nutritionists, food researchers, and consumers due to the current trends to avoid synthetic compounds and the capability of natural compounds to inhibit α-glucosidase with fewer side effects. In this sense, phenolic compounds derived from plants such as flavonoids have been extensively studied (Rasouli, Hosseini-Ghazvini, Adibi, & Khodarahmi, 2017). *Retama raetam* (Ghani et al., 2019), *Camellia sinensis* tea (Hua et al., 2018), *Clinacanthus nutans* leaf (Murugesu et al., 2019), or *Samanea saman* (Vinodhini & Rajeswari, 2018) are some of the most recent plant extracts describing phenolic compounds with α-glucosidase inhibitory activity. Other studies have been carried out to obtain natural α-glucosidase inhibitory compounds using microbial conversion (Kang, Yi, & Lee, 2013; Li et al., 2019; Nguyen & Wang, 2018) as well as α-glucosidase inhibitory peptides generated under controlled conditions of enzymatic hydrolysis (Abdelhedi et al., 2019; Ben Slama-Ben Salem et al., 2018; Jemil et al., 2017; Lacroix & Li-Chan, 2013; Matsui, Oki, & Osajima, 1999; Vilcacundo, Martínez-Villaluenga, & Hernández-Ledesma, 2017).

During the last years, Spanish dry-cured hams have been described

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as a source of naturally generated peptides with different biological functions such as antioxidant (Escudero, Mora, Fraser, Aristoy, & Toldrá, 2013a; Mora, Escudero, Fraser, Aristoy, & Toldrá, 2014), antihypertensive (Escudero et al., 2013b; Escudero, Mora, & Toldrá, 2014), antimicrobial, DPP-IV inhibitory peptides with potential antidiabetic activity (Gallego, Aristoy, & Toldrá, 2014) and anti-inflammatory (Gallego et al., 2019a).

Recently, a clinical study evaluated the cause-effect relationship between dry-cured ham consumption and cardiovascular effects, showing that consumption of dry-cured ham improves inflammatory responses and the regulation of the thrombogenic status (Martínez-Sánchez et al., 2017). A similar study also reported that a daily intake of 80 g of dry-cured ham per day did not impair blood pressure whereas total cholesterol, LDL and basal glucose levels dropped (Montoro-García et al., 2017), which suggests the potential of dry-cured ham as a source of natural antidiabetic peptides.

In this study, α-glucosidase inhibitory peptides obtained from an aqueous extract of Iberian dry-cured ham were evaluated using different chromatographic separations and mass spectrometry in tandem was used for the identification of the peptide sequences contained in the most active fractions. On the other hand, the α-glucosidase inhibitory activity of previously identified bioactive peptides in Spanish dry-cured ham was established, confirming their multifunctionality.

2. Materials and methods

2.1. Chemicals and reagents

α-Glucosidase activity assay kit (MAK123), α-glucosidase *from Saccharomyces cerevisiae*-Type I, > 10 U/mg protein (G5003), acarbose (PHR1253) were obtained from Sigma-Adrich (St Louis, MO, USA). Hydrochloric acid, trifluoroacetic acid (TFA), formic acid (FA), ethanol (EtOH), acetonitrile (ACN), ammonium acetate, monosodic phosphate, disodium phosphate, and dimethylsulfoxid were from Scharlab (Barcelona, Spain).

Iberian dry-cured ham used in this study was 50% Iberian and it was obtained from cross-breed pigs (Iberian females with Duroc males breed). Pigs were fed with cereals and pulses in an intensive farming system, and slaughtering was done at a minimum of 10 months age. Iberian hams were salted with sodium chloride and potassium nitrate and then dry-cured for a minimum of 24 months.

2.2. Peptides extraction

A total of 50 g of Biceps femoris muscle excised from 50% Iberian dry-cured hams obtained from a local market were minced and homogenized with 200 mL of 0.01 N HCl in a Stomacher (IUL Instrument, Barcelona, Spain) at 4 °C for 8 min, according to Escudero et al. (2013a). The homogenate was centrifuged in the cold (12,000*g* for 20 min at 4 °C) and filtered through glass wool. The obtained volume was measured and proteins were precipitated by adding 3 volumes of ethanol and keeping the sample at 4 °C during 20 h. After that, the sample was centrifuged again (12,000*g* for 20 min at 4 °C). The ethanol contained in the supernatant was evaporated and sample was lyophilized (SCANVAC CoolSafe, Labogene APS, Lynge, Denmark). A total of 1g of sample was resuspended with 5 mL of HCl 0.01 N and filtered through 0.45 μm nylon filters (Millipore, Bedford, MA) for further injection in size-exclusion chromatography. The analysis were done in triplicate in three different Iberian dry-cured hams.

2.3. Measurement of the α-glucosidase inhibitory activity

The assay was done using α-glucosidase activity assay kit (MAK123; Sigma-Aldrich) according to the manufacturer instructions with minor changes. In this assay, the activity of the enzyme is determined by a reaction where α-glucosidase hydrolyzes p-nitrophenyl- α-D-glucopyranoside (p-NPG) resulting on the formation of p-nitrophenol, a colorimetric product at 405 nm. Acarbose at a concentration of 5 mg/mL was used as positive control whereas 100 mM phosphate buffer pH 6.8 was used as negative control. α-glucosidase enzyme was prepared at a concentration of 0.5U/mL in 100 mM phosphate buffer pH 6.8. 50 μL of peptide solution, positive control and negative control were added in triplicate onto a microplate (96 wells) and incubated with 25 μL of α-glucosidase enzyme during 5 min at 37 °C for conditioning. Then, 25 μL of p-NPG 1.25 mM in 100 mM phosphate buffer pH 6.8 were added and the microplate was incubated again during 20 min at 37 °C. Then, the absorbance was measured at 405 nm using a spectrometer (Opsys MR Dynex technologies, UK). The analysis was done in triplicate. The obtained absorbances were used to calculate the inhibitory capacity of the enzyme according to the formula:

$$\%\ \text{Inhibition} = \left(1 - \frac{\text{Abs sample}}{\text{Abs control}}\right) \times 100$$

2.4. Fractionation using size-exclusion chromatography (SEC)

A Sephadex G25 column (2.5 × 65 cm; Amersham Biosciences, Uppsala, Sweden) was used for the separation of the peptides according to their molecular weight. The peptides were eluted using 0.01 N HCl at 4 °C with a flow rate of 0.250 mL/min. Fractions of 5 mL were collected using an automatic collector. Each fraction was monitored at 214, 254, and 280 nm using an Agilent UV spectrophotometer (Agilent 8453, Agilent Technologies, Palo Alto, CA, USA) and also the α-glucosidase inhibitory activity was measured. Later, samples were stored at −20 °C.

2.5. Isolation of the peptides of interest using high-performance liquid chromatography

Fractions showing the highest α-glucosidase inhibitory activity were further isolated using high-performance liquid chromatography. Thus, a total of 200 μL of each of the most active fractions were pooled together in an Eppendorf and lyophilized. Before injection, they were suspended in 150 μL of MilliQ water and filtered through 0.45 μm nylon filters (Millipore, Bedford, MA). This procedure was done in duplicate as samples were injected using reversed-phase chromatography and also hydrophilic interaction chromatography using an Agilent 1100 HPLC system (Agilent Tech., California, USA).

2.5.1. Reversed-phase chromatography (RP-HPLC)

A Waters Symmetry C18 column (250 × 4.6 mm, 5 μm; Waters Co. Milford, MA, USA) was used in the separation. The volume of injection was 100 μL. The mobile phases consisted of solvent A: 0.1% v/v TFA and solvent B: 0.1% v/v TFA in ACN (40:60, v/v). Peptides were diluted using the following gradient: 1% B from 0 to 5 min, linearly increasing to 70% B until 60 min and up to 100% of B until 65 min, using a flow rate of 1 mL/min. Absorbance was monitored at 214 nm and fractions were collected every minute. These fractions were freeze-dried and stored at − 20 °C until further analysis. Fractions were collected at 1 mL/min, which is the flow rate of the separation methodology.

2.5.2. Hydrophilic interaction chromatography (HILIC-HPLC)

A TSK Gel amide 80 column (150 × 4.6 mm, 3 μm; Tosoh Bioscience GmbH, Griesheim, Germany) was used in the separation. The volume of injection was 100 μL. The mobile phases consisted of solvent A: Ammonium acetate 10 mM pH 6 with ACN (20:80, v/v) and solvent B: Ammonium acetate 10 mM with ACN (70:30, v/v). Peptides were diluted using the following gradient: starting with 100% A and decreasing to 50% A from 0 to 15 min and up to 100% B until 20 min, using a flow rate of 1 mL/min. Absorbance was monitored at 214 nm and fractions were collected every minute. These fractions were freeze-dried and stored at −20 °C until further analysis. Fractions were

collected at 1 mL/min, which is the flow rate of the separation methodology.

α-Glucosidase inhibitory activity was measured in each collected fraction for both chromatographic separations.

2.6. Mass spectrometry analysis

2.6.1. Analysis by Matrix-Assisted Laser Desorption/Ionisation mass spectrometry (MALDI-ToF MS)

The most active fractions obtained after the separation using HPLC were analysed by MALDI-ToF mass spectrometry 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, CA, USA) in positive reflectron mode (3000 shots at each position) in a range from 400 to 3000 *m/z*. Plate model and acquisition method were calibrated by a peptide mass standards calibration mixture (ABSciex, CA, USA). 1 μL of every fraction of interest was directly spotted on the MALDI plate and allowed to air dry. Once dried, 1 μL of matrix solution (10 mg/mL of a-Cyano-4-hydroxycinnamic acid (CHCA) in 0.1% TFA-ACN (30:70, v/v) was also spotted and let to air dry.

2.6.2. Identification of peptides by nESI–LC–MS/MS

Those fractions obtained after HPLC separation showing the highest α-glucosidase inhibitory activity were analysed by nLC-MS/MS in order to identify the sequences of the peptides using an Eksigent nano-LC Ultra 1D Plus system (Eksigent of AB Sciex, CA, USA) with nanoelectrospray ionization source quadrupole/time-of-flight (nanoESI-Q-ToF) TripleTOF® 5600 system (AB Sciex Instruments, MA, USA).

The lyophilized sample was re-dissolved in 0.1% TFA:ACN (98:2, v/v). A total 5 μL of sample was loaded onto an C18-CL Eksigent trap column (3 μm, 350 μm × 0.5 mm; Nikkyo Technos Co, Ltd. Japan) for 5 min at 3 μL/min using 0.1%TFA as mobile phase.

Later, sample was automatically loaded into the analytical column C18-CL (3 μm, 75 μm × 120 mm; Nikkyo Technos Co, Ltd. Japan). The mobile phases consisted of solvent A: 0.1% FA in milliQ water and solvent B: 0.1% FA in ACN. Peptides were diluted linearly from 5% to 35% solvent B during 60 min, under a flow rate of 0.3 μL/min at 30 °C.

The flow from the LC system was directly injected into the mass spectrometry system and ionized applying 2.8 kV. The Q-ToF was operated in positive polarity and information-dependent acquisition mode (DAD). MS1 scan was acquired from 350 to 1250 *m/z* for 250 ms, while MS2 scan was required from 100 to 1500 *m/z* for 50 ms in 'high resolution' mode. The charge was established from +1 to +5 with 70 counts per second. Up to 25 ions were selected for fragmentation after each survey scan. Dynamic exclusion was set to 15 s. The sensitivity and accuracy of the system was controlled using 2 fmol of standard proteins (LC Packings).

The database searching of peptides was performed using the Mascot Distiller v 2.7.1 software (Matrix Science, Inc., Boston, MA), and Mascot search engine with a significance threshold $p < 0.01$ using Chordata taxonomy, none enzyme digestion, and Uniprot database. The tolerance on the mass measurement was 50 ppm for MS and 0.3 Da for MS/MS.

2.7. Peptide synthesis

The peptides AD, PP, PE, EA, VE, GGLGP, LGVGG, and AEEEYPDL were selected from a range of previously identified peptides in Spanish dry-cured hams according to potential bioactivity based on (i) length, (ii) molecular weight, (iii) sequence, and (iv) composition in amino acids. The selected peptides were synthesised (GenScript Corporation, Piscataway, NJ, USA) and the purity checked through liquid chromatography-mass spectrometry (LC-MS) analysis. Peptide solutions (1 mM) were prepared for α-glucosidase inhibitory bioactivity assays.

2.8. In silico analysis of the identified and synthesized peptides

In order to evaluate the potential of the identified peptides to cross the intestinal barrier, the probability that the peptide will be cell penetrating was studied with CPPpred tool (http://distilldeep.ucd.ie/CPPpred/). The potential bioactivity of all the identified peptides was predicted using the Peptide Ranker software (http://distilldeep.ucd.ie/PeptideRanker/), and values higher than 0.5 were considered for further *in silico* analysis simulating gastrointestinal digestion. Peptides were scored from 0 to 1 and the higher score means higher probability to be bioactive (Tu et al., 2019). The BIOPEP-UWM database (Minkiewicz, Iwaniak, and Darewicz, 2019). (http://www.uwm.edu.pl/biochemia/index.php/en/biopep) was used to simulate the gastrointestinal digestion using chymotrypsin (EC 3.4.21.1), trypsin (EC 3.4.21.4), and pepsin (EC 3.4.23.1) enzymes. This database was also used to determine the resulting active fragments as well as to describe their bioactivity.

Regarding the analysis of synthesized peptides, the potential peptide allergenicity was predicted using the AllerTOP v. 2.0 software (http://www.ddg-pharmfac.net/AllerTOP/index.html). Peptide toxicity and physicochemical properties (i.e., hydrophobicity, amphipathicity, steric hindrance, and pI) were studied using the ToxinPred software (http://crdd.osdd.net/raghava/toxinpred/) (Gupta et al., 2013), together with other peptide property calculator tools such as Pepcalc from Innovagen (https://pepcalc.com/).

2.9. Statistical analysis

One-way analysis of variance (ANOVA) and Turkey multiple range tests were performed using the software XLSTAT 2011 v5.01 (Addinsoft, Barcelona, Spain). Results were expressed as the mean of 3 replicates ± standard deviations, and differences were considered significantly at $P < 0.05$.

3. Results and discussion

3.1. Fractionation using size-exclusion chromatography (SEC) of the Iberian dry-cured ham extract

The deproteinised Iberian dry-cured ham extract was injected in size-exclusion chromatography to separate peptides according to their molecular mass. Thus, higher molecular masses were firstly eluted whereas smaller peptides were longer retained in the column and later eluted. Size-exclusion chromatography has been frequently used as an initial step for the fractionation of peptides generated during dry-cured ham processing obtaining similar profiles in Spanish, Italian Parma, and Belgium dry-cured ham (Mora, Escudero, & Toldrá, 2016). Fig. 1 shows the absorbance of the peptides profile at 214 nm, which is the wavelength of maximum absorption for the peptide bonds, and the obtained percentage of α-glucosidase inhibitory activity, showing a maximum of 70% between fractions 41–55 (volume between 205 and 275 mL). These results are very similar to those obtained in previous studies for antioxidant activity as well as for ACE-inhibitory activity in different European dry-cured hams after a size-exclusion chromatography separation using a Sephadex G-25 column, where the maximum levels of antioxidant activity were determined to be in fractions corresponding to elution volumes from 200 mL to 250 mL in Spanish, Italian Parma, and Belgian dry-cured ham (Mora et al., 2014, 2016), and maximum levels for ACE-inhibitory activity were observed between 195 and 250 mL in all European and Iberian dry-cured hams (Mora et al., 2016; Mora, Escudero, Arihara, & Toldrá, 2015). According to these previous publications, the molecular mass range of the peptides contained in the most active fractions would be between 400 and 2500 Da (Mora et al., 2016).

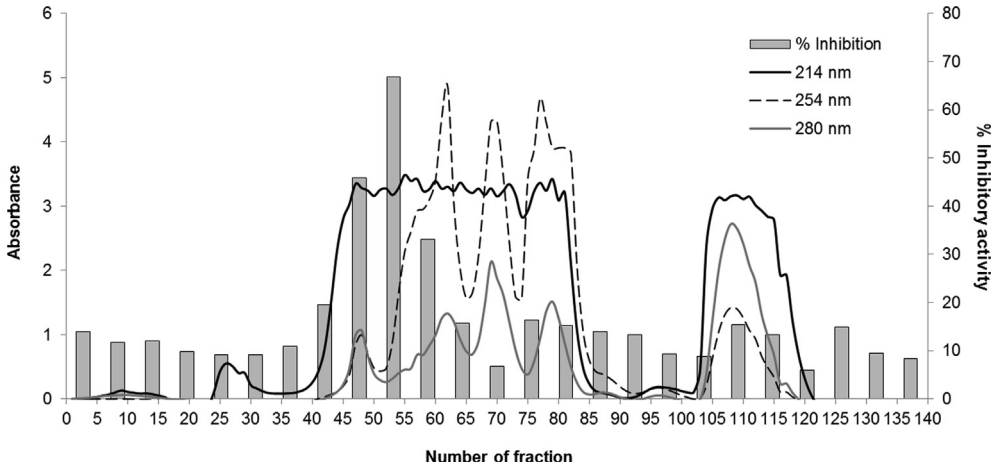

Fig. 1. Fractionation of Iberian dry-cured ham extract in a Sephadex G25 gel filtration column. Fractions were collected and assayed for their α-glucosidase inhibitory activity. Fractionation of dry-cured ham extract and bioactivity assays were done in triplicate.

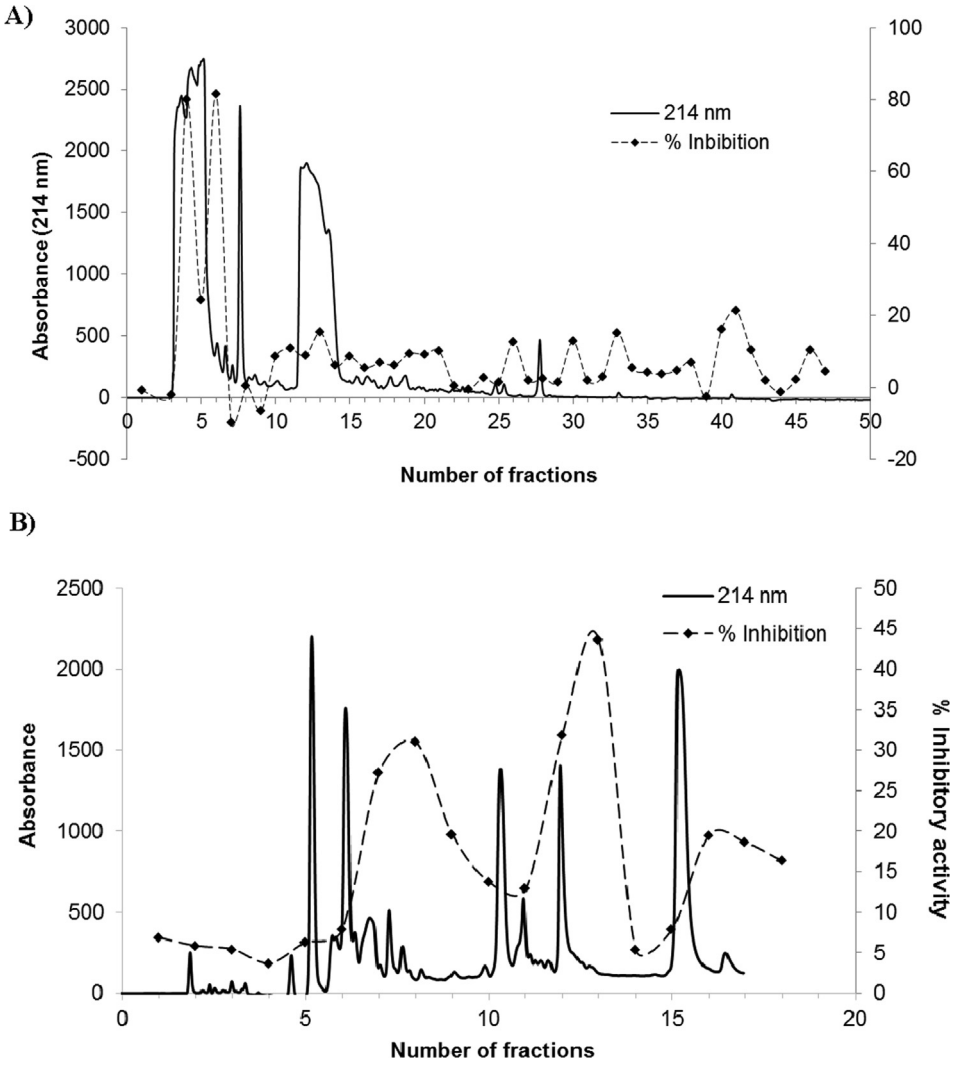

Fig. 2. HPLC separation of most active fractions observed in size-exclusion chromatography (fr 51–55). (A) Reversed-phase chromatographic separation of the selected pooled fractions obtained from size-exclusion chromatography. (B) HILIC chromatographic separation of the selected pooled fractions obtained from size-exclusion chromatography.

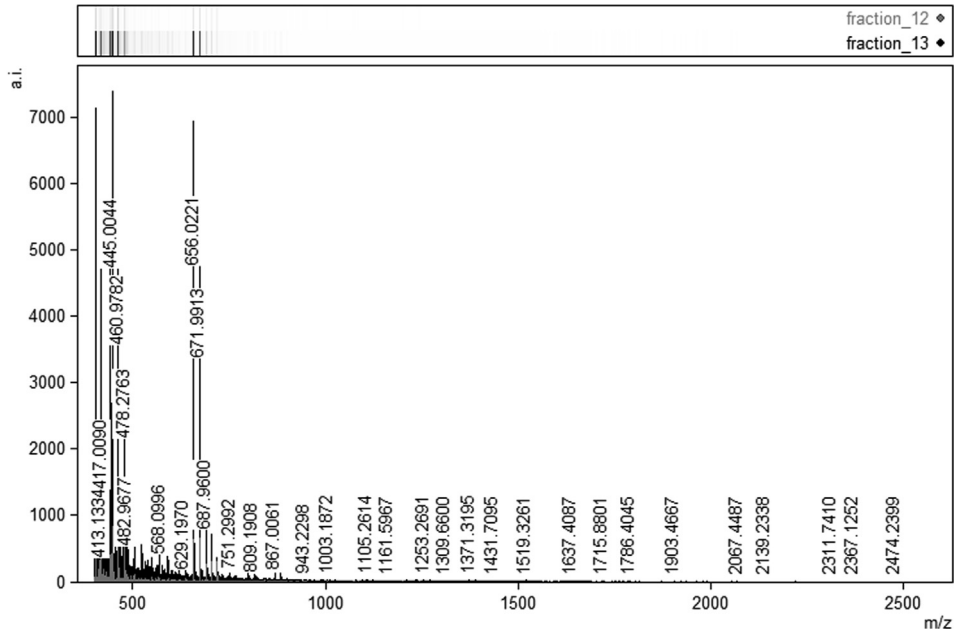

Fig. 3. MALDI-TOF LC-MS spectra of active fractions obtained after HILIC-HPLC separation. A and B in the figure corresponds to fractions 12 and 13, respectively.

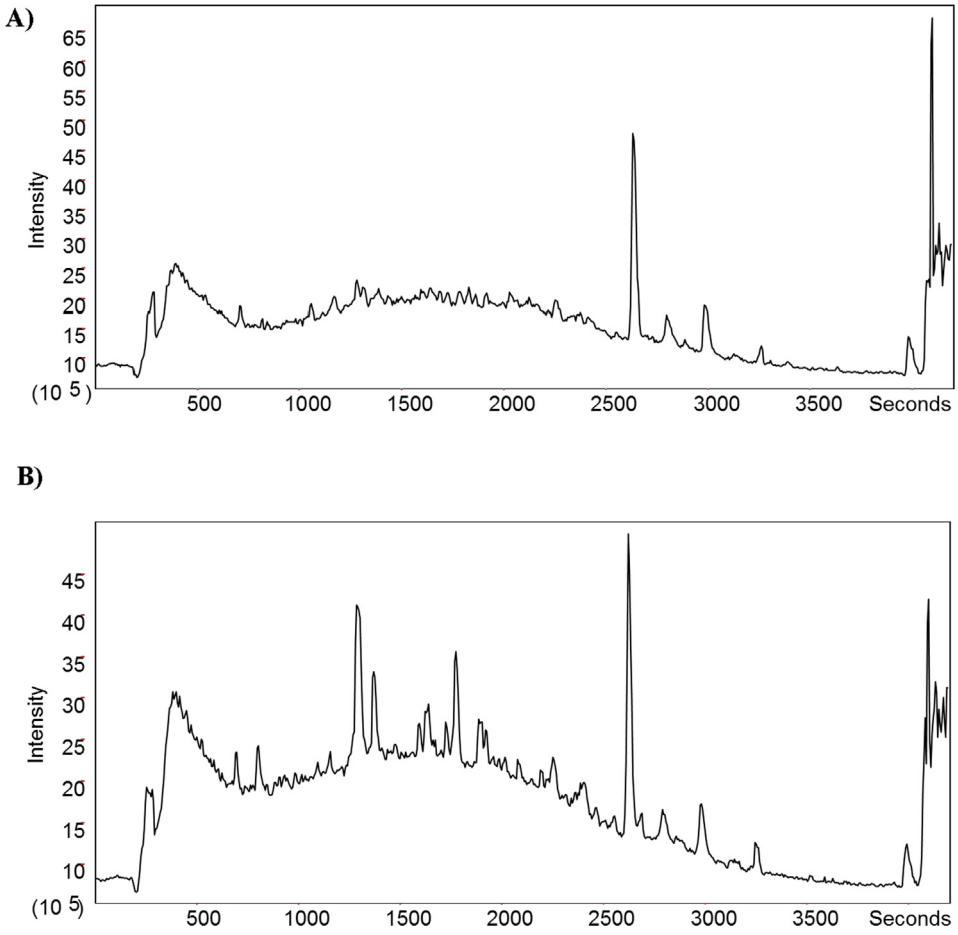

Fig. 4. Total ion chromatogram (TIC) of main fractions eluted from HILIC-HPLC. A and B in the figure corresponds to fractions 12 and 13, respectively.

3.2. Isolation of the peptides of interest using high-performance liquid chromatography

Fractions 51–55 obtained after SEC were pooled together, concentrated and injected into a reversed phased column using HPLC chromatography. The obtained results are shown in Fig. 2A, where the percentage of α-glucosidase inhibitory activity is also represented. Reversed-phase chromatography is the most common HPLC separation

Table 1
Sequences of peptides identified in fraction 12 obtained after HILIC-HPLC separation of the Iberian dry-cured ham aqueous extract. The identification was done using mass spectrometry in tandem in a Quadrupole/Time of Flight instrument.

Pi	Sequence	Pf	Exp_m/z[1]	Exp_Mr[2]	Exp_z[3]	Calc_Mr[4]	PTMs[5]	Prot_accession[6]	Prot_description
A	FPPDVGGN	V	401.69	801.36	2	801.37		MLRS_BOVIN	Myosin regulatory light chain 2
A	FPPDVGGNVD	Y	508.74	1015.46	2	1015.46		MLRS_BOVIN	Myosin regulatory light chain 3
A	FPPDVGGNVDY	K	590.27	1178.53	2	1178.52		MLRS_BOVIN	Myosin regulatory light chain 4
L	IGDEPLENYLDTEYF	G	909.41	1816.80	2	1816.80		PEPA_PIG	Pepsin A
N	VPEVGGEALGR	L	542.27	1082.53	2	1082.57		HBB_CHETO	Hemoglobin subunit beta
H	GIITNWDDM	E	540.74	1079.46	2	1079.46	Oxidation (M)	ACTA_BOVIN	Actin
K	SYELPDGQVIT	I	611.30	1220.59	2	1220.59		ACTA_BOVIN	Actin
D	DEGDQLAAL	A	466.73	931.44	2	931.41	Deamidated (NQ)	MYO7A_HUMAN	Unconventional myosin-VIIa
I	EDPFDQDDWGA	W	647.77	1293.52	2	1293.48		ENOA_HUMAN	Alpha-enolase
F	ILLNLLK	V	413.79	825.57	2	825.57		RHCG_HUMAN	Ammonium transporter Rh type C
S	ITVIAEEL	S	444.24	886.47	2	886.50		CPNE4_HUMAN	Copine-4
E	ASDDAVQGQ	D	891.38	890.38	1	890.36	Deamidated (NQ)	PACS2_MOUSE	Phosphofurin acidic cluster sorting protein 2
S	YNLNSST	Q	400.69	799.37	2	799.32	2 Deamidated (NQ)	CC50C_MOUSE	Cell cycle control protein 50C
E	TVMSLMYTMVT	P	442.21	1323.62	3	1323.58	3 Oxidation (M)	OLFI9_RAT	Olfactory receptor-like protein I9
Q	QSTQGASGQ	T	432.23	862.45	2	862.38		TBP_MESAU	TATA-box-binding protein
P	PVQQNMMNSASGP	L	460.18	1377.51	3	1377.55	Oxidation (M); 2 Deamidated (NQ)	YAP1_HUMAN	Transcriptional coactivator YAP1

[1] Theoretical mass/charge ratio of the identified peptide.
[2] Theoretical precursor molecular weight of the identified peptide, including modifications.
[3] Expected charge (z) of the fragmented ion as calculated by the Mascot algorithm.
[4] Molecular mass of the peptide calculated including modifications.
[5] Posttranslational modifications detected on the identified peptides and found by the search.
[6] Protein accession number obtained according to Uniprot database.

technique and is used for separating hydrophobic compounds, although less polar compounds can also be retained. According to this, the highest values of α-glucosidase inhibitory activity (80%) were obtained in the front of the chromatographic separation corresponding to the most polar peptides. Non-significant values of α-glucosidase inhibitory activity were observed in the rest of the chromatogram, indicating the polar characteristics of the most active peptides. In RP-HPLC, polar-ionic compounds are usually eluted near the dead volume so that effective analysis of these compounds is difficult. Techniques such as derivatization or ion-pairing are used in order to improve the retention of these compounds in RP. However, these methodologies are not compatible with further analysis using mass spectrometry approaches. In this sense, HILIC is a complementary technique to reversed-phase chromatography and elution occurs with increasing polarity of the analytes (Buszewski & Noga, 2012).

Due to the polar character of the peptides showing the highest values of α-glucosidase inhibitory activity, another chromatographic separation was done using HILIC in order to improve the separation of the most active peptides. The obtained results are shown in Fig. 2B. Fractions of 1 mL were collected and the α-glucosidase inhibitory activity was measured obtaining two main peaks of interest in fractions 12 and 13. According to these results, fraction 13 shows lower absorbance intensity at 214 nm but significantly higher percentage of inhibitory activity reaching more than 40%.

3.3. Mass spectrometry analysis

The fractions 12 and 13 obtained from HILIC-HPLC were analysed using MALDI-ToF mass spectrometry to determine the molecular mass of the peptides. The results are showed in Fig. 3. In this regards, the values of intensity and number of peaks observed in fraction 12 are lower than in fraction 13 probably due to the less amount of peptides and their less capacity to be ionized using MALDI ionization.

On the other hand, mass spectrometry in tandem was used to elucidate the sequences of peptides contained in fractions 12 and 13. Fig. 4 shows the Total Ion Chromatogram (TIC) obtained after the LC separation and Electrospray Ionisation (ESI). The obtained spectra were analysed using the software Mascot Distiller and the search engine Mascot for the identification of the peptides and origin proteins. In this respect, Tables 1 and 2 show a total of 16 and 47 sequences of peptides identified in fractions 12 and 13, respectively, including the expected and calculated molecular mass, charge, posttranslational modifications and protein accession numbers. The specific contribution to the total α-glucosidase inhibitory capacity of each identified peptide will be potentially studied in further studies. In Table 1, the identification of the sequences FPPDVGGN and FPPDVGGNVD elucidates the release of the dipeptide VD from the C-terminal site during proteolysis from the protein myosin regulatory light chain, probably due to the action of a dipeptidyl peptidase enzyme (Sentandreu & Toldrá, 2001). This dipeptide has been previously described as DPP IV inhibitor (Lan et al., 2015) that is a property related with antidiabetic activity (Gallego, Aristoy, & Toldrá, 2014). In Table 2, the dipeptides WK and VV could have been excised from longer β-enolase peptides, and both have also been previously described as DPP IV inhibitory peptides (Bella, Erickson, & Kim, 1982; Nongonierma, Mooney, Shields, & FitzGerald, 2014). Something similar occurs with dipeptides IE and SI from α-enolase protein, which were previously described as ACE inhibitory and DPP IV inhibitory peptides, respectively (Lan et al., 2015; van Platerink, Janssen, & Haverkamp, 2008).

The results of the *in silico* analysis of the identified peptides, show a total of 15 sequences with a Peptide Ranker value higher than 0.5, which indicates the predicted probability for the peptide to be bioactive. Due to the length of these peptides, all of them showed a very low probability to be cell penetrating, between 0.19 and 0.05. In fact, the *in silico* simulated gastrointestinal digestion resulted in the hydrolysis of all the sequences, generating new peptides previously described as bioactives showing ACEI inhibitory and DPPIV and DPPIII inhibitory activities (Table 3).

3.4. Bioactivity of previously identified peptides in Spanish dry-cured ham

Some α-glucosidase inhibitory peptides have been described to be multifunctional as they can exert two or more health-promoting activities with effects on other carbohydrate-mediated diseases acting as antiviral, anticancer, antihepatitis and immunoregulatory compounds (Bakherad et al., 2019; Ibrahim, Serem, Bester, Neitz, & Gaspar, 2019; Patel, 2016; Pili et al., 1995; Zitzmann et al., 1999). Due to the previously published manuscripts regarding the potential capacity of Spanish dry-cured ham decreasing basal glucose levels (Montoro-García et al., 2017), the α-glucosidase inhibitory capacity of some peptides

Table 2
Sequences of peptides identified in fraction 13 obtained after HILIC-HPLC separation of the Iberian dry-cured ham aqueous extract. The identification was done using mass spectrometry in tandem in a Quadrupole/Time of Flight instrument.

Pi	Sequence	Pf	Exp_m/z[1]	Exp_Mr[2]	Exp_z[3]	Calc_Mr[4]	PTMs[5]	Prot_accession[6]	Prot_description
N	VGDEGGF	A	680.3	679.3	1	679.28		ENOB_PIG	Beta-enolase
D	PFDQDDWK	T	525.7	1049.4	2	1049.45		ENOB_PIG	Beta-enolase
E	DPFDQDDWK	T	583.2	1164.5	2	1164.47		ENOB_PIG	Beta-enolase
I	EDPFDQDDWK	T	647.8	1293.5	2	1293.52		ENOB_PIG	Beta-enolase
Y	PVVSIEDPFDQD	D	680.8	1359.6	2	1359.62		ENOB_PIG	Beta-enolase
P	VVSIEDPFDQDD	W	689.8	1377.6	2	1377.59		ENOB_PIG	Beta-enolase
S	IEDPFDQDDWK	T	704.3	1406.6	2	1406.60		ENOB_PIG	Beta-enolase
V	SIEDPFDQDDWK	T	747.8	1493.6	2	1493.63		ENOB_PIG	Beta-enolase
S	IEDPFDQDDWKT	W	754.8	1507.6	2	1507.65		ENOB_PIG	Beta-enolase
D	PFDQDDWGA	W	525.7	1049.4	2	1049.41		ENOA_HUMAN	Alpha-enolase
E	DPFDQDDWGA	W	583.2	1164.5	2	1164.44		ENOA_HUMAN	Alpha-enolase
I	EDPFDQDDWGA	W	647.8	1293.5	2	1293.48		ENOA_HUMAN	Alpha-enolase
S	IEDPFDQDDWGA	W	704.3	1406.6	2	1406.56		ENOA_HUMAN	Alpha-enolase
V	SIEDPFDQDDWGA	W	747.8	1493.6	2	1493.59		ENOA_HUMAN	Alpha-enolase
A	FPPDVGGN	V	401.7	801.4	2	801.37		MLRS_BOVIN	Myosin regulatory light chain 2
A	FPPDVGGNVD	Y	508.7	1015.4	2	1015.46		MLRS_BOVIN	Myosin regulatory light chain 3
A	FPPDVGGNVDY	K	590.3	1178.5	2	1178.52		MLRS_BOVIN	Myosin regulatory light chain 4
I	TNWDDMEK	I	519.7	1037.4	2	1037.41		ACTC_BOVIN	Actin
E	YDEAGPSIVH	R	544.3	1086.5	2	1086.50		ACTC_BOVIN	Actin
G	DGVTHNVPIYE	G	622.3	1242.6	2	1242.59		ACTC_BOVIN	Actin
G	DGVTHNVPIYEG	Y	650.8	1299.6	2	1299.61		ACTC_BOVIN	Actin
E	VAEKQNNVN	R	508.7	1015.5	2	1015.49	Deamidated (NQ)	EIF1A_RAT	Probable RNA-binding protein EIF1AD
S	ADNPDGGL	Q	380.2	758.3	2	758.31	Deamidated (NQ)	NCKP5_HUMAN	Nck-associated protein 5
L	EDDENGA	M	375.6	749.3	2	749.24	Deamidated (NQ)	SPRN_MOUSE	Shadow of prion protein
L	ESGQDGQP	D	410.2	818.3	2	818.29	2 Deamidated (NQ)	SPNDC_MOUSE	Spindlin interactor
E	IQDQHDE	Y	443.2	884.3	2	884.35	Deamidated (NQ)	KIF3C_BOVIN	Kinesin-like protein KIF3C
T	VDGPSGKLW	R	479.7	957.5	2	957.49		G3P_CAVPO	Glyceraldehyde-3-phosphate dehydrogenase
Y	DGQPNAH	K	740.3	739.3	1	739.28	2 Deamidated (NQ)	EXOC6_RAT	Exocyst complex component 6
N	VPEVGGEALGR	L	542.3	1082.5	2	1082.57		HBB_CHETO	Hemoglobin subunit beta
R	HPGDFGA	D	350.7	699.3	2	699.30		MYG_BALBO	Myoglobin
A	ENGFPQGNPEP	Q	594.7	1187.4	2	1187.46	3 Deamidated (NQ)	FA83G_MOUSE	Protein FAM83G
L	GEHGDSSVPVWSG	V	657.3	1312.6	2	1312.57		LDHA_MONDO	L-lactate dehydrogenase A chain
K	PGLTGPQGPQ	G	477.2	952.4	2	952.45	2 Deamidated (NQ)	COHA1_MOUSE	Collagen alpha-1(XVII) chain
E	DAELFMSLYDPH	K	719.3	1436.6	2	1436.63		DOCK2_MOUSE	Dedicator of cytokinesis protein 2
S	GSPKMSNIMQSI	A	442.2	1323.7	3	1323.62	2 Oxidation (M)	EMSY_MOUSE	BRCA2-interacting transcriptional repressor
K	SVCCMVPVRMDNI	–	741.8	1481.7	2	1481.65	Oxidation (M)	DFB50_MOUSE	Beta-defensin 50
W	DHGGRIFSCSFCH	N	489.2	1464.5	3	1464.60		ZN330_BOVIN	Zinc finger protein 330
K	ASGVCVD	S	650.2	649.2	1	649.27		SCRN1_BOVIN	Secernin-1
S	DSVMNVFKDRNFDSCC	I	633.2	1896.7	3	1896.73	Oxidation (M); 2 Deamidated (NQ)	MD13L_MOUSE	Mediator of RNA polymerase II transcription
M	MMSMQGMMGPQQNIMIPPQMRPRG	M	943.8	2828.4	3	2828.19	5 Oxidation (M); 2 Deamidated (NQ)	BCL9_HUMAN	B-cell CLL/lymphoma 9 protein
T	CQRPLPSSTPM	P	617.3	1232.6	2	1232.55	Oxidation (M); Deamidated (NQ)	CS071_MACFA	Uncharacterized protein
K	NGGCMHMKCPQPQCKLEWC	W	737.0	2208.1	3	2207.89	Oxidation (M)	PRKN_MOUSE	E3 ubiquitin-protein ligase parkin
G	SNGSVED	R	354.2	706.3	2	706.28		MAFB_RAT	Transcription factor MafB
P	FLGSGGT	I	638.3	637.3	1	637.31		NEK1_HUMAN	Serine/threonine-protein kinase Nek1
R	RQLIEFNPSHFQS	A	535.2	1602.7	3	1602.78	Deamidated (NQ)	MPP4_HUMAN	MAGUK p55 subfamily member 4
L	DPCCEGST	C	406.2	810.3	2	810.25		ADAM9_HUMAN	Disintegrin
K	KTKNKKKKNKKKKAA	T	591.1	1770.3	3	1770.17		LRRF1_MOUSE	Leucine-rich repeat flightless-interacting

[1] Theoretical mass/charge ratio of the identified peptide.
[2] Theoretical precursor molecular weight of the identified peptide, including modifications.
[3] Expected charge (z) of the fragmented ion as calculated by the Mascot algorithm.
[4] Molecular mass of the peptide calculated including modifications.
[5] Posttranslational modifications detected on the identified peptides and found by the search.
[6] Protein accession number obtained according to Uniprot database.

previously identified as bioactive peptides in Spanish dry-cured ham has been determined. In this sense, peptides GGLGP and LGVGG were previously identified in Spanish dry-cured ham derived from elastin protein and were evaluated as antioxidant peptides (Mora et al., 2014). Also the peptide AEEEYPDL was identified in the same study as a potent antioxidant peptide derived from creatine kinase protein and later quantified at a concentration of 0.148 fg per g of dry-cured ham using multiple reaction monitoring methodology (Gallego, Mora, & Toldrá, 2018). The evaluated dipeptides (EA, PP, VE, PE, and AD) could come from different origin proteins as they are not unique sequences but all of them were identified in Spanish dry-cured ham. As it is described in Table 4, dipeptides EA, PP and VE were previously described as ACEI-inhibitory peptides, whereas dipeptides PP, VE, PE, and AD were described as DPP IV inhibitory peptides. The IC_{50} values obtained for all these dipeptides in relation of the α-glucosidase activity is also described in Table 4, that shows the lowest values for AEEEYPDL, LGVGG

Table 3
In silico study of the identified peptides in Iberian dry-cured ham.

Peptide Ranker[a]	Peptide Sequence	CPPpred[b]	Results for enzyme action[c]	Active fragments[d]	Bioactivities
0.91	NGGCMHMKCPQPQCKLEWC	0.19	N – GGCM – H – M – K – CPQPQCK – L – EW – C	EW	ACE and DPPIV inhibitory
0.79	DHGGRIFSCSFCH	0.05	DH – GGR – IF – SCSF – CH	IF	ACE inhibitory
0.77	PFDQDDWGA	0.06	PF – DQDDW – GA	GA	ACE and DPPIV inhibitory
				PF	DPP III and DPPIV inhibitory
0.75	PFDQDDWK	0.08	PF – DQDDW – K	PF	DPP III and DPPIV inhibitory
0.71	VDGPSGKLW	0.18	VDGPSGK – L- W	–	
0.71	DPFDQDDWGA	0.06	DPF – DQDDW – GA	GA	ACE and DPPIV inhibitory
0.70	FPPDVGGN	0.06	F – PPDVGGN	–	
0.70	HPGDFGA	0.05	H – PGDF – GA	GA	ACE and DPPIV inhibitory
0.70	ADNPDGGL	0.08	ADN – PDGGL	–	
0.68	DPFDQDDWK	0.07	DPF – DQDDW – K	–	
0.67	CQRPLPSSTPM	0.18	CQR – PL – PSSTPM	PL	ACE and DPPIV inhibitory
0.60	SVCCMVPVRMDNI	0.12	SVCCM – VPVR – M – DN – I	DN	DPPIV inhibitory
0.53	EDPFDQDDWGA	0.06	EDPF – DQDDW – GA	GA	ACE and DPPIV inhibitory
0.52	DSVMNVFKDRNFDSCC	0.09	DSVM – N – VF – K – DR – N – F – DSCC	VF	ACE and DPPIV inhibitory
				DR	DPPIV inhibitory
0.51	EDPFDQDDWK	0.07	EDPF – DQDDW – K		

[a] Peptide Ranker: ranks peptide(s) by the predicted probability that the peptide will be bioactive.
[b] CPPpred: ranks peptide(s) by the probability that the peptide will be cell penetrating.
[c] Results after simulated action of chymotrypsin (EC 3.4.21.1), trypsin (EC 3.4.21.4), and pepsin (EC 3.4.23.1).
[d] Active fragments identified in BIOPEP (http://www.uwm.edu.pl/biochemia/index.php/en/biopep).

Table 4
Multifunctional peptides previously identified in Spanish dry-cured ham with IC_{50} values obtained in this study for α-glucosidase inhibitory activity and other types of bioactivity reported in the literature.

	Molecular weight	IC_{50} value		Other previously reported bioactivity	
Sequence	(g/mol)	(mM)	Peptide Ranker	Type	Reference
AEEEYPDL	964.98	5.58	0.19	Antioxidant	Gallego et al. (2018)
LGVGG	401.46	6.36	0.32	–	Mora et al. (2014)
GGLGP	399.45	8.71	0.73	–	Mora et al. (2014)
EA	218.21	17	0.04	ACE-inhibitor	Cheung, Wang, Ondetti, Sabo, and Cushman (1980)
PP	212.25	18.03	0.88	ACE-inhibitor	van Platerink et al. (2008)
				DPP IV inhibitor	Hatanaka, Inoue, Arima, Kumagai, Usuki, Kawakami, Kimura, and Mukaihara (2012)
VE	246.26	22.17	0.02	ACE-inhibitor	van Platerink et al. (2008)
				DPP IV inhibitor	Lan et al. (2015)
PE	244.25	25.05	0.14	DPP III inhibitor	Dhanda, Singh, and Singh (2008)
AD	204.18	25.66	0.13	DPP IV inhibitor	Lan et al. (2015)

Table 5
In silico study of the multifunctional peptides previously identified in Spanish dry-cured ham tested for α-glucosidase inhibitory activity.

Peptide Sequence	Allergenicity Prediction	Toxicity Prediction	Steric Hindrance	Amphipathicity	Hydrophobicity	pI
AEEEYPDL	Non-allergen	Non-toxin	0.61	0.48	−0.23	pH 3.5
LGVGG	Probable allergen	Non-toxin	0.65	0	0.31	pH 5.9
GGLGP	Non-allergen	Non-toxin	0.59	0	0.19	pH 3.8
EA	Non-allergen	Non-toxin	0.6	0.64	−0.85	pH 4
PP	Non-allergen	Non-toxin	0.36	0	−0.55	pH 5.9
VE	Non-allergen	Non-toxin	0.69	0.64	−0.8	pH 4.0
PE	Non-allergen	Non-toxin	0.52	0.64	−0.34	pH 4.0
AD	Non-allergen	Non-toxin	0.64	0	−0.23	pH 3.8

and GGLGP peptides, as well as values between 17 and 26 mM for the dipeptides. The obtained values are similar to those previously identified in an hydrolysate of sardine muscle using Alcalase enzyme, which identified and tested the α-glucosidase activity of the peptides VW (IC_{50} = 22.6 mM) and YYPL (IC_{50} = 3.7 mM) (Matsui et al., 1999). Regarding the potential multifunctionality, previous studies show dual ACEI and DPP IV inhibitory activity as the most common in di and tripeptides (Gallego et al., 2019b), mainly coming from proteins containing a high proportion of hydrophobic and positively charged residues (Rao et al., 2012).

In complex food matrices such as dry-cured ham, the usual methodology for the discovery of bioactive peptides is based on empirical strategies. However, its combination with *in silico* analysis permits to obtain a large amount of data regarding their physico-chemical properties, chemical structure, potential toxicity, and their bioactivity. Thus, *in silico* approaches are economical and time-saving strategies that include quantitative structure-activity relationship models (QSAR), molecular docking simulations and chemometrics. Table 5 shows the *in silico* study of the synthesized peptides, including data about allergenicity and toxicity prediction. The predicted steric hindrance of the peptides was relatively low whereas the amphipathicity was high in AEEEYPDL, EA, VE and PE peptides. The most hydrophobic peptides were LGVGG and GGLGP. Peptides showing low steric hindrance and high amphipathicity are more likely to exert their function as bioactives as stabilizes the enzyme conformation (Falciani et al., 2007; Manzo et al., 2015). These results are in agreement with those observed after

the empirical analysis of bioactivity in the synthesized peptides.

4. Conclusions

Iberian dry-cured ham is an already proved meat product known as a source of different bioactive peptides due to its characteristics of high protein content and intense hydrolysis occurred during its processing. This study reports for the first time the α-glucosidase inhibitory potential of an extract of peptides obtained from dry-cured ham, supporting the beneficial effects previously observed in clinical trials in relation to the observed decrease in basal glucose levels after the ingestion of dry-cured ham. A total of 16 and 47 sequences of peptides were identified in two most active HILIC-HPLC chromatographic fractions. Additionally, the assay of previously identified bioactive peptides such as AEEEYPDL and LGVGG in dry-cured ham revealed that these peptides can exert several bioactivities acting as multifunctional compounds.

5. Ethics statement

The authors declare that the research did not include any human subjects and animal experiments.

CRediT authorship contribution statement

Leticia Mora: Writing - review & editing, Software, Validation, Supervision. **Diego González-Rogel:** Investigation, Methodology. **Alejandro Heres:** Investigation, Methodology, Writing - original draft. **Fidel Toldrá:** Writing - review & editing, Supervision.

Declaration of Competing Interest

The authors declared that there is no conflict of interest.

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