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Dipeptidyl peptidase IV inhibitory peptides generated in Spanish dry-cured ham

Marta Gallego, Maria-Concepción Aristoy, and Fidel Toldrá*

Instituto de Agroquímica y Tecnología de Alimentos (CSIC), Avd. Agustín Escardino 7, 46980, Paterna, Valencia, Spain

* Corresponding author: Tel: +34963900022 ext.2112; fax: +34963636301.
E-mail address: ftoldra@iata.csic.es

Abstract

Dipeptidyl peptidase IV (DPP-IV) inhibitors are promising new therapies for type 2 diabetes. Therefore, the aim of this study was to identify DPP-IV inhibitory peptides present in a water soluble extract of Spanish dry-cured ham. Such extract was fractionated by size-exclusion chromatography and the *in vitro* DPP-IV inhibitory activity was determined in each collected fraction. Several peptides present in the active fractions were synthesised and assayed for DPP-IV inhibition. Peptides KA and AAATP showed the strongest DPP-IV inhibitory activity, with IC₅₀ values of 6.27 mM and 6.47 mM, respectively. Dipeptides AA, GP, PL, and carnosine, as well as peptides AAAAG, ALGGA, and LVSGM also demonstrated to be DPP-IV inhibitors, although at a lower degree. These findings suggest the potential of Spanish dry-cured ham as a natural precursor of DPP-IV inhibitory peptides. These biopeptides could also be used as ingredients for functional foods or pharmaceutical products against type 2 diabetes.

Keywords: Dry-cured ham, bioactive peptides, dipeptidyl peptidase IV inhibitor, type 2 diabetes.

1. Introduction

Dipeptidyl peptidase IV (DPP-IV) is a serine protease expressed in both membrane and soluble forms in a diversity of cell types. This enzyme cleaves preferentially X-proline or X-alanine dipeptides from N-terminus of multiple substrates, including incretin hormones named glucagon-like peptide (GLP-1) and glucose-dependent insulintropic peptide (GIP) (Cunningham & O'Connor, 1997; Thoma, Löffler, Stihle, Huber, Ruf & Henning, 2003). GLP-1 and GIP are gut-derived peptides secreted in response to nutrient ingestion that potentiate glucose-stimulated insulin release by the pancreas, suppress glucagon secretion and improve plasma glucose concentrations. Nevertheless, incretin hormones are quickly degraded by the ubiquitous DPP-IV (Mudaliar & Henry, 2012).

Recent advances in the understanding of incretins have led to the development of two main classes of GLP-1-mediated therapies against type 2 diabetes: GLP-1 receptor agonists resistant to degradation by DPP-IV, and DPP-IV inhibitors that reduce the degradation of GLP-1. The choice of the therapy to be used depends on the specific needs of the diabetic patient (Fineman, Cirincione, Maggs & Diamant, 2012). GLP-1 agonists are intravenously administered and offer higher target selectivity but they present undesirable side effects. Alternatively, DPP-IV inhibitors are orally administered and cause only transient and minimal side effects. Several synthetic DPP-IV inhibitors have emerged in recent years as potent antidiabetic drugs, for example vildagliptin, sitagliptin, saxagliptin, and linagliptin (Pratley & Gilbert, 2008; Fineman et al., 2012).

DPP-IV inhibitors extend the half-life of the endogenous GLP-1 and GIP and consequently their plasma concentrations increase. Then, these incretin hormones enhance glucose-dependent insulin secretion, reduce glucagon production and hepatic

glucose production, and other glycaemic effects. Notably GLP-1 is more effective than GIP in stimulating insulin release at an equivalent level of glycaemia. Furthermore, GLP-IV and GIP are also involved in extra-glycaemic effects such as improve β -cell function, and cardiovascular and central nervous system effects (Mudaliar & Henry, 2012; Duez, Cariou & Staels, 2012).

There are some reports describing bioactive peptides with DPP-IV inhibitory activity, and a recent *in silico* approach can be used to identify potential dietary proteins as precursors of DPP-IV inhibitors (Lacroix & Li-Chan, 2012). Some of these inhibitory peptides were found in salmon, bovine meat, chicken egg, milk, wheat, rice bran, and others (Lacroix & Li-Chan, 2012; Li-Chan, Hunag, Jao, Ho & Hsu, 2012; Hatanaka et al., 2012). Most of the inhibitory peptides contain proline and/or hydrophobic amino acids within their sequence, and the third N-terminal residue of the peptide plays an important role in determining the inhibitory activity of a peptide (Hatanaka et al., 2012). Thus, the DPP-IV inhibitory activity of such peptides is determined by their composition and sequence of amino acids but not by their length (Huang, Jao, Ho & Hsu, 2012).

Spanish dry-cured ham has been studied as a natural source of bioactive peptides with antihypertensive and antioxidant activity (Escudero, Mora, Fraser, Aristoy, Arihara & Toldrá, 2013a; Escudero, Mora, Fraser, Aristoy & Toldrá, 2013b), but according to our knowledge, to date there are no studies about DPP-IV inhibitory peptides present in dry-cured ham. The purpose of the present study was to verify the existence of peptides, present in water soluble fractions of dry-cured ham, with inhibitory activity against DPP-IV.

2. Materials and methods

2.1 Reagents

Dipeptidyl peptidase IV (from porcine kidney), Diprotin A (Ile-Pro-Ile), Gly-Pro-7-amido-4-methylcoumarin hydrobromide (Gly-Pro-AMC), DL-Dithiothreitol (DTT), trifluoroacetic acid (TFA) and the synthetic peptides Ala-Ala (AA), Lys-Ala (KA), Gly-Pro (GP), Pro-Leu (PL), L-carnosine, L-anserine nitrate salt, and L-glutathione reduced (GSH) were purchased from Sigma-Aldrich, Co. (St. Louis, Mo., USA). Ethylenediaminetetraacetic Acid Disodium salt 2-hydrate (EDTA) was from Panreac Química, S.L.U. (Barcelona, Spain) and Acetonitrile HPLC grade was from Sharlab, S.L. (Barcelona, Spain). All other chemicals and reagents used were of analytical grade.

2.2 Spanish dry-cured ham preparation

Three Spanish dry-cured hams were produced using raw hams from 6 months old pig (Landrace x Large White). Hams were bled and prepared according to traditional procedures consisting of the pre-salting stage using a mixture of salt, nitrate and nitrite, the salting state where hams were covered with solid salt, piled up and placed in a cold room (2-4 °C and 90-95% relative humidity for 12 days), post-salting where hams were kept at 4-5 °C, 75-85% relative humidity for 60 days, and finally, the ripening-drying period at 14-20 °C and relative humidity down to 70%. The total length of the curing process was 10 months.

2.3 Extraction and deproteinisation

Fifty grams of *Biceps femoris* muscle from the processed Spanish dry-cured hams were minced and homogenised with 200 mL of 0.01 N HCl for 8 minutes in a stomacher (IUL Instrument, Barcelona, Spain). The homogenate was centrifuged (12000 g for 20 min at 4 °C), filtered through glass wool and then the supernatant was deproteinised by adding 3 volumes of ethanol and maintaining the sample at 4 °C for 20 hours. Next, the sample was centrifuged again (12000 g for 10 min at 4 °C) and the supernatant was

dried in a rotatory evaporator and then lyophilised for further ultrafiltration, as described in 2.4. The same procedure was followed for the size-exclusion chromatography as described in 2.5. In this case, the extract was dissolved in 25 mL of 0.01 N HCl, filtered through a 0.45 µm nylon membrane filter (Millipore, Bedford, MA) and stored at -20 °C until use.

2.4 Ultrafiltration

An aliquot of the deproteinised extract was fractionated by ultrafiltration using a Microcon microconcentrator model 3 (Amicon, Inc., MA, USA) and a Microsep™ UF Spin Filter 1K (Pall Corporation, MI, USA) with membranes having molecular mass cut-offs of 3 kDa and 1kDa, respectively. The collected fractions contained peptides between 3-1 kDa and lower than 1kDa, which were assayed for DPP-IV inhibition.

2.5 Size-exclusion chromatography

A 5 mL aliquot of the deproteinised extract was subjected to size-exclusion chromatography to fractionate the peptides according to their molecular mass. A Sephadex G25 column (2.5 x 72 cm, Amersham Biosciences, Uppsala, Sweden) was employed, previously equilibrated with 0.01 N HCl filtered through a 0.45 µm nylon membrane filter (Millipore) and degassed. The separation was performed at 4 °C and at a flow rate of 15 mL/h of 0.01 N HCl as eluent. 5 mL fractions were collected using an automatic fraction collector and further monitored by ultraviolet (UV) absorption at 214 nm (Ultrospec 3000 UV/Visible spectrophotometer, Pharmacia Biotech, Cambridge, England). The first 105 mL were discarded and the remaining fractions were collected and grouped as follows: fractions corresponding to elution volumes from 110 mL to 185 mL (named sample 1), from 190 mL to 310 mL (sample 2), from 315 mL to 420 mL (sample 3), from 425 mL to 475 mL (sample 4) and fractions eluting from 480 mL to 625 mL (sample 5). Each fraction was lyophilised and then was redissolved in 2 mL of

50mM Tris-HCl buffer (pH 8.0). For the DPP IV inhibitory assay, each sample was tested at various concentrations.

2.6 Determination of DPP-IV inhibitory activity

The DPP-IV inhibition assay was carried out using 96-well microplates by measuring fluorescence due to the release of 7-amido-4-methylcoumarin (AMC) by the action of DPP IV from the internally quenched fluorescent substrate Gly-Pro-7-amido-4-methylcoumarin hydrobromide (Gly-Pro-AMC). Test samples (50 µL each sample and dilutions) were added 50 µL of DPP-IV (diluted with 50 mM Tris-HCl buffer pH 8.0 to 4.55 mU/mL). The reaction was initiated by the addition of 200 µL of 0.25 mM reaction buffer (containing 50 mM Tris-HCl buffer pH 8.0, 5 mM DTT, 1 mM EDTA and 20 mM Gly-Pro-AMC). The mixture was then incubated for 20 min at 37°C. The generation of fluorescence was measuring using excitation and emission wavelengths of 355 nm and 460 nm, respectively (Fluoroskan Ascent microplate fluorometer, Thermo Scientific, MA, USA). DPP-IV inhibition is expressed as percentage and the IC₅₀ value was defined as the concentration of sample or peptide required to inhibit 50% of DPP-IV activity. Diprotin A (Ile-Pro-Ile) was used as a reference inhibitor.

2.7 Selection of DPP-IV inhibitory peptides and peptide synthesis

The selection of peptides with potential DPP-IV inhibitory activity among the large number of them identified in the Spanish dry-cured ham (Sentandreu, Stoeva, Aristoy, Laib, Voelter & Toldrá, 2003; Sentandreu & Toldrá, 2007; Escudero et al., 2013a) was done according to the characteristics defined for DPP-IV inhibitory peptides, which generally are characterized by a hydrophobic nature and in particular by the presence of a proline or an alanine in position 2 of the N-terminus (Pieter, 2006). Thus, dipeptides with theoretical DPP-IV inhibitory activity AA, KA, GP, and PL were assayed as well as the natural meat peptides carnosine, anserine, and glutathione reduced.

Other 7 peptides like AAATP, ASGPINFT, DVITGA, SAGPN, AAAAG, ALGGA, and LVSGM previously identified in dry-cured ham (Escudero et al., 2013a; Escudero et al., 2013b) were also selected for testing DPP-IV inhibitory activity because of their hydrophobic character in N-terminal residues. These peptides were synthesised by the solid phase procedure using the Fmoc protected amino acid synthesis method in a Focus XC (AAPPTec[®] Louisville, KY, USA). The synthesised peptides were purified by HPLC (Agilent 1100 HPLC system, Agilent Tech., Palo Alto, CA, USA) and the column used was a Symmetry C18 (4.6 mm x 250 mm, 5µm) from Waters (Milford, MA, USA). Mobile phases consisted of solvent A containing 0.1% TFA in water and solvent B containing 0.085% TFA in acetonitrile:water (60:40, v/v). Both mobile phases were filtered through a 0.45 µm nylon membrane filter and degassed prior to analytical run. We used an isocratic gradient of 99% solvent A for 5 min, followed by a linear gradient from 1% to 100% of solvent B in 80 min at a flow rate of 0.8 mL/min. The separation was monitored using a diode array detector at a wavelength of 214 nm. The purity and the molecular mass of the purified peptides were determined in an Ultimate 3000 RSLC nano system from Dionex (Thermo Fisher Scientific Ltd., Leicestershire, UK) coupled to an AmaZon ETD ion-trap mass spectrometer equipped with an nanoelectrospray ionisation source (Bruker Daltonik GmbH, Bremen, Germany).

3. Results and discussion

3.1 DPP-IV inhibitory activity of ultrafiltered fractions

Fractions separated by ultrafiltration were assayed for DPP-IV inhibitory activities (Figure 1). The results showed that peptides between 3-1 kDa had the highest DPP-IV inhibition rate of 92.5 %, while peptides < 1 kDa displayed an inhibition rate of 75.2%.

Because both groups of ultrafiltered fractions were strong inhibitors, fractions obtained from gel filtration were also assayed for DPP-IV inhibitory activity.

3.2 DPP-IV inhibitory activity of fractions from gel filtration chromatography

Fractions separated using the Sephadex G-25 gel filtration column (Figure 2) were assayed for *in vitro* DPP-IV inhibitory activity at various concentrations: samples solutions (lyophilized fractions dissolved in 2 mL of 50mM Tris-HCl buffer, pH 8) and then serially diluted 1/10, 1/100, and 1/1000.

The assays showed that sample 1 (corresponding to an elution volume from 110 to 185 mL) and samples 3, 4 and 5 (corresponding to elution volumes in the ranges 315 to 625 mL) did not exert a remarkable inhibitory effect on DPP-IV activity. Moreover, their concentrations were 0.19 mg/mL for sample 1, 13.54 mg/mL for sample 3, 1.59 mg/mL for sample 4 and 7.04 mg/mL for sample 5, each concentration expressed as tyrosine equivalents measured at 280 nm.

Sample 2 corresponding to an elution volume from 190 mL to 310 mL was the only sample that showed DPP-IV inhibitory activity, thus it was diluted 1/2, 1/4, 1/10, 1/12.5, 1/20, and 1/100 to determine its IC₅₀ value. This sample corresponded to molecular mass lower than 1700 Da (Aristoy & Toldrá, 1995) and its concentration was 5.67 mg/mL expressed as tyrosine equivalents. The IC₅₀ value was determined and found to be 0.69 mg/mL (Figure 3). In addition, we tested the effect of two minerals, sodium and calcium, on DPP-IV enzyme activity. In dry-cured ham, current levels of salt added in the curing process result in sodium concentrations within the range 1100-1800 mg/100g, while calcium is usually between 12 and 35 mg/100g of ham (Jiménez-Colmenero, Ventanas & Toldrá, 2010). Moreover, former studies showed that the elution of NaCl from the gel filtration column appeared in fraction corresponding to sample 2 (Sentandreu et al., 2003). Then, CaCl₂ and NaCl were tested if affected the inhibition

results obtained for sample 2, but the results indicated that both salts were not DPP-IV inhibitors.

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3.3 DPP-IV inhibitory activity of selected peptides

Four dipeptides identified in dry-cured ham (AA, KA, GP, and PL), three natural meat peptides (carnosine, anserine and GSH) and other seven dry-cured ham peptides of greater length (AAATP, ASGPINFT, DVITGA, SAGPN, AAAAG, ALGGA, and LVSGM) were selected and evaluated for their *in vitro* activity on DPP-IV. Diprotin A (Ile-Pro-Ile), which is a well-known peptide with the greatest DPP-IV inhibitory activity, was used as a control and showed an IC₅₀ value of 11.08 µM as measured under the present study conditions (Figure 4).

The first four dipeptides were mainly generated by the action of porcine muscle dipeptidyl peptidases (Sentandreu & Toldrá, 2007) and were selected because they contain a proline or alanine in the first or second N-terminal residue. The experimental results indicated that AA and KA were DPP-IV inhibitors with IC₅₀ of 9.40 ± 0.10 mM and 6.27 ± 0.59 mM, respectively. In relation to GP, contradictory results are reported in the literature with some studies indicating DPP-IV inhibitory activity (Lacroix & Li-Chan, 2012) and other study indicating the opposite (Hatanaka et al., 2012). In our assay, GP presented inhibitory activity and its IC₅₀ value was 9.69 ± 0.49 mM. PL peptide showed the lowest inhibitory activity and its IC₅₀ value was found to be greater than 10 mM (Table 1).

Marušić, Aristoy & Toldrá, 2013, studied and determined the concentrations of natural meat peptides carnosine, anserine and GSH in *Biceps femoris* muscle during the processing of dry-cured ham. At 10 months of ham curing process, concentrations of carnosine and anserine were 1269.6 mg/100g of ham dry matter (equivalent to 56 mM)

and 70.9 mg/100g of ham dry matter (equivalent to 2.95 mM), respectively. GSH was present until 6.5 months of processing but it disappeared at the end of the process (9 and 10 months). This tripeptide was not present in our samples but some commercial hams with 5-7 months of curing process still contain GSH (approximately 5-10 μ M). So, these natural meat peptides were tested as inhibitors and results indicated that anserine and GSH did not inhibit the activity of DPP-IV at 10 mM or lower concentrations. However, carnosine exerted inhibitory activity and its IC₅₀ was 493 ± 4.74 mM (Table 2). Considering the high concentration of carnosine (56 mM) present in ham after 10 months of processing, this value would represent an inhibition rate of 13%.

Regarding to the seven synthesised peptides, all of them were identified previously in dry-cured ham. AAATP, ASGPINFT, and DVITGA were described as antihypertensive peptides, whereas SAGPN did not show ACE inhibitory activity (Escudero et al., 2013a), and AAAAG, ALGGA, and LVSGM were described as antioxidant peptides (Escudero et al., 2013b). More peptides were also identified but only those having a length between 2 to 8 amino acids and a hydrophobic nature within the peptide sequence, particularly a proline or an alanine in the first or second N-terminal residue, were selected. As can be seen in Table 1, AAATP was the strongest DPP-IV inhibitor with an IC₅₀ value of 6.47 ± 0.20 mM, while AAAAG showed an IC₅₀ value of 8.13 ± 0.48 mM. Peptides ALGGA and LVSGM were also DPP-IV inhibitors but their IC₅₀ were greater than 10 mM, and ASGPINFT, DVITGA, and SAGPN did not exert inhibitory activity up to 20 mM.

DPP-IV inhibitory peptides AAAAG, ALGGA, and LVSGM were identified in fractions, from size-exclusion chromatography, corresponding to our sample 2 (Escudero et al., 2013b), which is in agreement with the high DPP-IV inhibitory activity showed previously in this sample. However, AAATP was identified in fraction

corresponding to our sample 3 and it was originated from *Sus scrofa* allantoicase protein (Escudero et al., 2013a). No inhibitory activity was detected in sample 3, probably due to the low amount of AAATP originated from a minor protein (allantoicase) in ham. These findings are of interest because peptides generated in dry-cured ham may act as a natural therapeutic approach for the management of type 2 diabetes, although these are not as potent as the synthetic drugs. In addition, these biopeptides could be isolated from dry-cured ham and then be added as ingredients in products marketed as ‘Functional Foods’ or ‘Natraceuticals’, or they could be used as ingredients of pharmaceutical products against type 2 diabetes (Pihlanto & Korhonen, 2003; Hartmann & Meisel, 2007; Ryan, Ross, Bolton, Fitzgerald & Stanton, 2011).

4. Conclusions

This study reports, for the first time in Spanish dry-cured ham, the synthesis and assay for DPP-IV inhibitory activity of previously reported peptides that have been either identified in dry-cured ham or known to be products of DPP IV action. Nine peptides were shown to inhibit DPP-IV enzyme of which KA and AAATP were the most potent inhibitors, with IC₅₀ values of 6.27 mM and 6.47 mM, respectively. Therefore, dry-cured ham could serve as natural precursor of peptides with inhibitory effect on DPP-IV, and these biopeptides could be used as a natural complementary treatment in the prevention and management of type 2 diabetes. However, further research is needed to identify novel peptides responsible for DPP-IV inhibition in dry-cured ham or test a possible synergistic effect among various inhibitory peptides identified. Moreover, future *in vivo* studies will need to be performed to validate the *in vitro* DPP-IV inhibitory activity and confirm the bioavailability and efficacy of these bioactive peptides.

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FIGURES CAPTIONS

Figure 1. DPP-IV inhibitory activity of dry-cured ham extract fractionated by ultrafiltration.

Figure 2. Fractionation of dry-cured ham extract in a Sephadex G-25 gel filtration column. Fractions were collected and tested for DPP-IV inhibitory activity. Fractions corresponding to elution volumes from 110 to 185 mL were pooled and named sample 1 (S1). The same procedure was followed for fractions corresponding to elution volumes from 190 to 310 mL (S2), fractions from 315 to 420 mL (S3), fractions from 425 to 475 mL (S4) and fractions from 480 to 625 mL (S5).

Figure 3. DPP-IV inhibition rate and IC_{50} value of sample 2 at various concentrations. Bars represent standard deviations from triplicate determinations. DPP IV inhibition is regressed on $\ln(x)$ of concentration.

Figure 4. DPP-IV inhibition rate and IC_{50} value of diprotin A at various concentrations. Bars represent standard deviations from triplicate determinations. DPP IV inhibition is regressed on $\ln(x)$ of concentration.

Figure 1

Figure 2

Figure 3

Figure 4

Table 1. IC₅₀ (mM) values of diprotin A (reference inhibitor) and synthetic peptides against DPP-IV activity.

Peptides	IC ₅₀ (mM)
Diprotin A	0.011 ± 0.001
KA	6.27 ± 0.59
AA	9.40 ± 0.10
GP	9.69 ± 0.49
PL	> 10
AAATP	6.47 ± 0.20
AAAAG	8.13 ± 0.48
ALGGA	> 10
LVSGM	> 10
ASGPINFT	No inhibition *
DVITGA	No inhibition *
SAGPN	No inhibition *

* Tested up to 20 mM of peptide.

Table 2. IC₅₀ (mM) value of natural meat peptides against DPP-IV activity.

Natural meat peptides	IC ₅₀ (mM)
Carnosine	493 ± 4.74 [#]
Anserine	No inhibition *
Glutathione reduced	No inhibition *

[#] Carnosine concentration after 10 months of ham curing process is very high (56 mM).

* Tested up to 10 mM of peptide.

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**Dipeptidyl peptidase IV inhibitory peptides generated in Spanish Dry-
Cured ham**

Marta Gallego, Maria-Concepción Aristoy, and Fidel Toldrá*

Instituto de Agroquímica y Tecnología de Alimentos (CSIC), Avd. Agustín Escardino 7, 46980, Paterna, Valencia, Spain

* Corresponding author: Tel: +34963900022 ext.2112; fax: +34963636301.
E-mail address: ftoldra@iata.csic.es

Abstract

Dipeptidyl peptidase IV (DPP-IV) inhibitors are promising new therapies for type 2 diabetes. Therefore, the aim of this study was to identify DPP-IV inhibitory peptides present in a water soluble extract of Spanish dry-cured ham. Such extract was fractionated by size-exclusion chromatography and the *in vitro* DPP-IV inhibitory activity was determined in each collected fraction. Several peptides present in the active fractions were identified, synthesised and assayed for DPP-IV inhibition. Peptides KA and AAATP showed the strongest DPP-IV inhibitory activity, with IC₅₀ values of 6.27 mM and 6.47 mM, respectively. Dipeptides AA, GP, PL, and carnosine, as well as peptides AAAAG, ALGGA, and LVSGM also demonstrated to be DPP-IV inhibitors, although at a lower degree. These findings suggest the potential of Spanish dry-cured ham as a natural precursor of DPP-IV inhibitory peptides. These biopeptides could also be used as ingredients for functional foods or pharmaceutical products against type 2 diabetes.

Keywords: Dry-cured ham, bioactive peptides, dipeptidyl peptidase IV inhibitor, type 2 diabetes.

1. Introduction

Dipeptidyl peptidase IV (DPP-IV) is a serine protease expressed in both membrane and soluble forms in a diversity of cell types. This enzyme cleaves preferentially X-proline or X-alanine dipeptides from N-terminus of multiple substrates, including incretin hormones named glucagon-like peptide (GLP-1) and glucose-dependent insulintropic peptide (GIP) (Cunningham & O'Connor, 1997; Thoma, Löffler, Stihle, Huber, Ruf & Henning, 2003). GLP-1 and GIP are gut-derived peptides secreted in response to nutrient ingestion that potentiate glucose-stimulated insulin release by the pancreas, suppress glucagon secretion and improve plasma glucose concentrations. Nevertheless, incretin hormones are quickly degraded by the ubiquitous DPP-IV (Mudaliar & Henry, 2012).

Recent advances in the understanding of incretins have led to the development of two main classes of GLP-1-mediated therapies against type 2 diabetes: GLP-1 receptor agonists resistant to degradation by DPP-IV, and DPP-IV inhibitors that reduce the degradation of GLP-1. The choice of the therapy to be used depends on the specific needs of the diabetic patient (Fineman, Cirincione, Maggs & Diamant, 2012). GLP-1 agonists are intravenously administered and offer higher target selectivity but they present undesirable side effects. Alternatively, DPP-IV inhibitors are orally administered and cause only transient and minimal side effects. Several synthetic DPP-IV inhibitors have emerged in recent years as potent antidiabetic drugs, for example vildagliptin, sitagliptin, saxagliptin, and linagliptin (Pratley & Gilbert, 2008; Fineman et al., 2012).

DPP-IV inhibitors extend the half-life of the endogenous GLP-1 and GIP and consequently their plasma concentrations increase. Then, these incretin hormones enhance glucose-dependent insulin secretion, reduce glucagon production and hepatic

glucose production, and other glycaemic effects. Notably GLP-1 is more effective than GIP in stimulating insulin release at an equivalent level of glycaemia. Furthermore, GLP-IV and GIP are also involved in extra-glycaemic effects such as improve β -cell function, and cardiovascular and central nervous system effects (Mudaliar et al., 2012; Duez, Cariou & Staels, 2012).

There are some reports describing bioactive peptides with DPP-IV inhibitory activity, and a recent *in silico* approach can be used to identify potential dietary proteins as precursors of DPP-IV inhibitors (Lacroix et al., 2012). Some of these inhibitory peptides were found in salmon, bovine meat, chicken egg, milk, wheat, rice bran, and others (Lacroix et al., 2012; Li-Chan, Hunag, Jao, Ho & Hsu, 2012; Hatanaka et al., 2012). Most of the inhibitory peptides contain proline and/or hydrophobic amino acids within their sequence, and the third N-terminal residue of the peptide plays an important role in determining the inhibitory activity of a peptide (Hatanaka et al., 2012). Thus, the DPP-IV inhibitory activity of such peptides is determined by their composition and sequence of amino acids but not by their length (Huang, Jao, Ho & Hsu, 2012).

Spanish dry-cured ham has been studied as a natural source of bioactive peptides with antihypertensive and antioxidant activity (Escudero, Mora, Fraser, Aristoy, Arihara & Toldrá, 2013a; Escudero, Mora, Fraser, Aristoy & Toldrá, 2013b), but according to our knowledge, to date there are no studies about DPP-IV inhibitory peptides present in dry-cured ham. The purpose of the present study was to verify the existence of peptides, present in water soluble fractions of dry-cured ham, with inhibitory activity against DPP-IV.

2. Materials and methods

2.1 Reagents

Dipeptidyl peptidase IV (from porcine kidney), Diprotin A (Ile-Pro-Ile), Gly-Pro-7-amido-4-methylcoumarin hydrobromide (Gly-Pro-AMC), DL-Dithiothreitol (DTT), trifluoroacetic acid (TFA) and the synthetic peptides Ala-Ala (AA), Lys-Ala (KA), Gly-Pro (GP), Pro-Leu (PL), L-carnosine, L-anserine nitrate salt, and L-glutathione reduced (GSH) were purchased from Sigma-Aldrich, Co. (St. Louis, Mo., USA). Ethylenediaminetetraacetic Acid Disodium salt 2-hydrate (EDTA) was from Panreac Química, S.L.U. (Barcelona, Spain) and Acetonitrile HPLC grade was from Sharlab, S.L. (Barcelona, Spain). All other chemicals and reagents used were of analytical grade.

2.2 Spanish dry-cured ham preparation

Three Spanish dry-cured hams were produced using raw hams from 6 months old pig (Landrace x Large White). Hams were bled and prepared according to traditional procedures consisting of the pre-salting stage using a mixture of salt, nitrate and nitrite, the salting state where hams were covered with solid salt, piled up and placed in a cold room (2-4 °C and 90-95% relative humidity for 12 days), post-salting where hams were kept at 4-5 °C, 75-85% relative humidity for 60 days, and finally, the ripening-drying period at 14-20 °C and relative humidity down to 70%. The total length of the curing process was 10 months.

2.3 Extraction and deproteinisation

Fifty grams of *Biceps femoris* muscle from the processed Spanish dry-cured hams were minced and homogenised with 200 mL of 0.01 N HCl for 8 minutes in a stomacher (IUL Instrument, Barcelona, Spain). The homogenate was centrifuged (12000 g for 20 min at 4 °C), filtered through glass wool and then the supernatant was deproteinised by adding 3 volumes of ethanol and maintaining the sample at 4 °C for 20 hours. Next, the sample was centrifuged again (12000 g for 10 min at 4 °C) and the supernatant was dried in a rotatory evaporator and then lyophilised for further ultrafiltration, as

described in 2.4. The same procedure was followed for the size-exclusion chromatography as described in 2.5. In this case, the extract was dissolved in 25 mL of 0.01 N HCl, filtered through a 0.45 µm nylon membrane filter (Millipore, Bedford, MA) and stored at -20 °C until use.

2.4 Ultrafiltration

An aliquot of the deproteinised extract was fractionated by ultrafiltration using a Microcon microconcentrator model 3 (Amicon, Inc., MA, USA) and a Microsep™ UF Spin Filter 1K (Pall Corporation, MI, USA) with membranes having molecular mass cut-offs of 3 kDa and 1kDa, respectively. The collected fractions contained peptides between 3-1 kDa and lower than 1kDa, which were assayed for DPP-IV inhibition.

2.5 Size-exclusion chromatography

A 5 mL aliquot of the deproteinised extract was subjected to size-exclusion chromatography to fractionate the peptides according to their molecular mass. A Sephadex G25 column (2.5 x 72 cm, Amersham Biosciences, Uppsala, Sweden) was employed, previously equilibrated with 0.01 N HCl filtered through a 0.45 µm nylon membrane filter (Millipore) and degassed. The separation was performed at 4 °C and at a flow rate of 15 mL/h of 0.01 N HCl as eluent. 5 mL fractions were collected using an automatic fraction collector and further monitored by ultraviolet (UV) absorption at 214 nm (Ultrospec 3000 UV/Visible spectrophotometer, Pharmacia Biotech, Cambridge, England). The first 105 mL were discarded and the remaining fractions were collected and grouped as follows: fractions corresponding to elution volumes from 110 mL to 185 mL (named sample 1), from 190 mL to 310 mL (sample 2), from 315 mL to 420 mL (sample 3), from 425 mL to 475 mL (sample 4) and fractions eluting from 480 mL to 625 mL (sample 5). Each fraction was lyophilised and then was redissolved in 2 mL of

50mM Tris-HCl buffer (pH 8.0). For the DPP IV inhibitory assay, each sample was tested at various concentrations.

2.6 Determination of DPP-IV inhibitory activity

The DPP-IV inhibition assay was carried out using 96-well microplates by measuring fluorescence due to the release of 7-amido-4-methylcoumarin (AMC) by the action of DPP IV from the internally quenched fluorescent substrate Gly-Pro-7-amido-4-methylcoumarin hydrobromide (Gly-Pro-AMC). Test samples (50 μ L each sample and dilutions) were added 50 μ L of DPP-IV (diluted with 50 mM Tris-HCl buffer pH 8.0 to 4.55 mU/mL). The reaction was initiated by the addition of 200 μ L of 0.25 mM reaction buffer (containing 50 mM Tris-HCl buffer pH 8.0, 5 mM DTT, 1 mM EDTA and 20 mM Gly-Pro-AMC). The mixture was then incubated for 20 min at 37°C. The generation of fluorescence was measuring using excitation and emission wavelengths of 355 nm and 460 nm, respectively (Fluoroskan Ascent microplate fluorometer, Thermo Scientific, MA, USA). DPP-IV inhibition is expressed as percentage and the IC₅₀ value was defined as the concentration of sample or peptide required to inhibit 50% of DPP-IV activity. Diprotin A (Ile-Pro-Ile) was used as a reference inhibitor.

2.7 Selection of DPP-IV inhibitory peptides and peptide synthesis

The selection of peptides with potential DPP-IV inhibitory activity among the large number of them identified in the Spanish dry-cured ham (Sentandreu, Stoeva, Aristoy, Laib, Voelter & Toldrá, 2003; Sentandreu & Toldrá, 2007; Escudero et al., 2013a) was done according to the characteristics defined for DPP-IV inhibitory peptides, which generally are characterized by a hydrophobic nature and in particular by the presence of a proline or an alanine in position 2 of the N-terminus (Pieter, 2006). Thus, dipeptides with theoretical DPP-IV inhibitory activity AA, KA, GP, and PL were assayed as well as the natural meat peptides carnosine, anserine, and glutathione reduced.

Other 7 peptides like AAATP, ASGPINFT, DVITGA, SAGPN, AAAAG, ALGGA, and LVSGM previously identified in dry-cured ham (Escudero et al., 2013a; Escudero et al., 2013b) were also selected for testing DPP-IV inhibitory activity because of their hydrophobic character in N-terminal residues. These peptides were synthesised by the solid phase procedure using the Fmoc protected amino acid synthesis method in a Focus XC (AAPPTec[®] Louisville, KY, USA). The synthesised peptides were purified by HPLC (Agilent 1100 HPLC system, Agilent Tech., Palo Alto, CA, USA) and the column used was a Symmetry C18 (4.6 mm x 250 mm, 5µm) from Waters (Milford, MA, USA). Mobile phases consisted of solvent A containing 0.1% TFA in water and solvent B containing 0.085% TFA in acetonitrile:water (60:40, v/v). Both mobile phases were filtered through a 0.45 µm nylon membrane filter and degassed prior to analytical run. We used an isocratic gradient of 99% solvent A for 5 min, followed by a linear gradient from 1% to 100% of solvent B in 80 min at a flow rate of 0.8 mL/min. The separation was monitored using a diode array detector at a wavelength of 214 nm. The purity and the molecular mass of the purified peptides were determined in an Ultimate 3000 RSLC nano system from Dionex (Thermo Fisher Scientific Ltd., Leicestershire, UK) coupled to an AmaZon ETD ion-trap mass spectrometer equipped with an nanoelectrospray ionisation source (Bruker Daltonik GmbH, Bremen, Germany).

3. Results and discussion

3.1 DPP-IV inhibitory activity of ultrafiltered fractions

Fractions separated by ultrafiltration were assayed for DPP-IV inhibitory activities (Figure 1). The results showed that peptides between 3-1 kDa had the highest DPP-IV inhibition rate of 92.5 %, while peptides < 1 kDa displayed an inhibition rate of 75.2%.

Because both groups of ultrafiltered fractions were strong inhibitors, fractions obtained from gel filtration were also assayed for DPP-IV inhibitory activity.

3.2 DPP-IV inhibitory activity of fractions from gel filtration chromatography

Fractions separated using the Sephadex G-25 gel filtration column (Figure 2) were assayed for *in vitro* DPP-IV inhibitory activity at various concentrations each. The assays showed that sample 1 (corresponding to an elution volume from 110 to 185 mL) and samples 3, 4 and 5 (corresponding to elution volumes in the ranges 315 to 625 mL) did not exert a remarkable inhibitory effect on DPP-IV activity. Moreover, their concentrations were 0.19 mg/mL for sample 1, 13.54 mg/mL for sample 3, 1.59 mg/mL for sample 4 and 7.04 mg/mL for sample 5, each concentration expressed as tyrosine equivalents measured at 280 nm.

Sample 2 corresponding to an elution volume from 190 mL to 310 mL was the only sample that showed DPP-IV inhibitory activity. This sample corresponded to molecular mass lower than 1700 Da (Aristoy & Toldrá, 1995) and its concentration was 5.67 mg/mL expressed as tyrosine equivalents. The IC₅₀ value was determined and found to be 0.69 mg/mL (Figure 3). In addition, we tested the effect of two minerals, sodium and calcium, on DPP-IV enzyme activity. In dry-cured ham, current levels of salt added in the curing process result in sodium concentrations within the range 1100-1800 mg/100g, while calcium is usually between 12 and 35 mg/100g of ham (Jiménez-Colmenero, Ventanas & Toldrá, 2010). Moreover, former studies showed that the elution of NaCl from the gel filtration column appeared in fraction corresponding to sample 2 (Sentandreu et al., 2003). Then, CaCl₂ and NaCl were tested if affected the inhibition results obtained for sample 2, but the results indicated that both salts were not DPP-IV inhibitors.

3.3 DPP-IV inhibitory activity of selected peptides

Four dipeptides identified in dry-cured ham (AA, KA, GP, and PL), three natural meat peptides (carnosine, anserine and GSH) and other seven dry-cured ham peptides of greater length (AAATP, ASGPINFT, DVITGA, SAGPN, AAAAG, ALGGA, and LVSGM) were selected and evaluated for their *in vitro* activity on DPP-IV. Diprotin A (Ile-Pro-Ile), which is a well-known peptide with the greatest DPP-IV inhibitory activity, was used as a control and showed an IC₅₀ value of 11.08 µM as measured under the present study conditions (Figure 4).

The first four dipeptides were mainly generated by the action of porcine muscle dipeptidyl peptidases (Sentandreu et al., 2007) and were selected because they contain a proline or alanine in the first or second N-terminal residue. The experimental results indicated that AA and KA were DPP-IV inhibitors with IC₅₀ of 9.40 ± 0.10 mM and 6.27 ± 0.59 mM, respectively. In relation to GP, contradictory results are reported in the literature with some studies indicating DPP-IV inhibitory activity (Lacroix et al., 2012) and other study indicating the opposite (Hatanaka et al., 2012). In our assay, GP presented inhibitory activity and its IC₅₀ value was 9.69 ± 0.49 mM. PL peptide showed the lowest inhibitory activity and its IC₅₀ value was found to be greater than 10 mM (Table 1).

Marušić, Aristoy & Toldrá, 2013, studied and determined the concentrations of natural meat peptides carnosine, anserine and GSH in *Biceps femoris* muscle during the processing of dry-cured ham. At 10 months of ham curing process, concentrations of carnosine and anserine were 1269.6 mg/100g of ham dry matter (equivalent to 56 mM) and 70.9 mg/100g of ham dry matter (equivalent to 2.95 mM), respectively. GSH was present until 6.5 months of processing but it disappeared at the end of the process (9 and 10 months). This tripeptide was not present in our samples but some commercial hams

with 5-7 months of curing process still contain GSH (approximately 5-10 μ M). So, these natural meat peptides were tested as inhibitors and results indicated that anserine and GSH did not inhibit the activity of DPP-IV at 10 mM or lower concentrations. However, carnosine exerted inhibitory activity and its IC_{50} was 493 ± 4.74 mM (Table 2). Considering the high concentration of carnosine (56 mM) present in ham after 10 months of processing, this value would represent an inhibition rate of 13%.

Regarding to the seven synthesised peptides, all of them were identified previously in dry-cured ham. AAATP, ASGPINFT, and DVITGA were described as antihypertensive peptides, whereas SAGPN did not show ACE inhibitory activity (Escudero et al., 2013a), and AAAAG, ALGGA, and LVSGM were described as antioxidant peptides (Escudero et al., 2013b). More peptides were also identified but only those having a length between 2 to 8 amino acids and a hydrophobic nature within the peptide sequence, particularly a proline or an alanine in the first or second N-terminal residue, were selected. As can be seen in Table 1, AAATP was the strongest DPP-IV inhibitor with an IC_{50} value of 6.47 ± 0.20 mM, while AAAAG showed an IC_{50} value of 8.13 ± 0.48 mM. Peptides ALGGA and LVSGM were also DPP-IV inhibitors but their IC_{50} were greater than 10 mM, and ASGPINFT, DVITGA, and SAGPN did not exert inhibitory activity up to 20 mM.

DPP-IV inhibitory peptides AAAAG, ALGGA, and LVSGM were identified in fractions, from size-exclusion chromatography, corresponding to our sample 2 (Escudero et al., 2013b), which is in agreement with the high DPP-IV inhibitory activity showed previously in this sample. However, AAATP was identified in fraction corresponding to our sample 3 and it was originated from *Sus scrofa* allantoicase protein (Escudero et al., 2013a). No inhibitory activity was detected in sample 3, probably due to the low amount of AAATP originated from a minor protein (allantoicase) in ham.

These findings are of interest because peptides generated in dry-cured ham may act as a natural therapeutic approach for the management of type 2 diabetes, although these are not as potent as the synthetic drugs. In addition, these biopeptides could be isolated from dry-cured ham and then be added as ingredients in products marketed as ‘Functional Foods’ or ‘Natraceuticals’, or they could be used as ingredients of pharmaceutical products against type 2 diabetes (Pihlanto & Korhonen, 2003; Hartmann & Meisel, 2007; Ryan, Ross, Bolton, Fitzgerald & Stanton, 2011).

4. Conclusions

This study reports, for the first time in Spanish dry-cured ham, the identification of peptides that exhibit *in vitro* DPP-IV inhibitory activity. Nine peptides were shown to inhibit DPP-IV enzyme of which KA and AAATP were the most potent inhibitors, with IC₅₀ values of 6.27 mM and 6.47 mM, respectively. Therefore, dry-cured ham could serve as natural precursor of peptides with inhibitory effect on DPP-IV, and these biopeptides could be used as a natural complementary treatment in the prevention and management of type 2 diabetes. However, further research is needed to identify novel peptides responsible for DPP-IV inhibition in dry-cured ham or test a possible synergistic effect among various inhibitory peptides identified. Moreover, future *in vivo* studies will need to be performed to validate the *in vitro* DPP-IV inhibitory activity and confirm the bioavailability and efficacy of these bioactive peptides.

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FIGURES CAPTIONS

Figure 1. DPP-IV inhibitory activity of dry-cured ham extract fractionated by ultrafiltration.

Figure 2. Fractionation of dry-cured ham extract in a Sephadex G-25 gel filtration column. Fractions were collected and tested for DPP-IV inhibitory activity. Fractions corresponding to elution volumes from 110 to 185 mL were pooled and named sample 1 (S1). The same procedure was followed for fractions corresponding to elution volumes from 190 to 310 mL (S2), fractions from 315 to 420 mL (S3), fractions from 425 to 475 mL (S4) and fractions from 480 to 625 mL (S5).

Figure 3. DPP-IV inhibition rate and IC_{50} value of sample 2 at various concentrations. Bars represent standard deviations from triplicate determinations.

Figure 4. DPP-IV inhibition rate and IC_{50} value of diprotin A at various concentrations. Bars represent standard deviations from triplicate determinations.

Highlights

DPP IV inhibitory peptides extracted, purified and identified in Spanish dry-cured ham

Ham peptides assayed for in vitro DPP IV inhibitory activity

Spanish dry-cured ham as a source of antidiabetic peptides naturally generated during the dry-curing process.