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Peptides with potential cardioprotective actions derived from dry-cured ham by-products

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Abstract

The interest of using food by-products as a source of bioactive peptides has increased significantly in the recent years. The goal of this study was to evaluate the presence and stability of peptides showing angiotensin I-converting enzyme (ACE-I), endothelin converting enzyme (ECE), dipeptidyl peptidase-IV (DPP-IV), and platelet-activating factor-acetylhydrolase (PAF-AH) inhibitory activity derived from dry-cured ham bones, which could exert cardiovascular health benefits. ACE-I and DPP-IV inhibitory peptides were stable against heating typically used in Mediterranean household cooking methods and also to *in vitro* digestion. PAF-AH inhibitory activity significantly increased following simulated gastrointestinal digestion whereas ECE inhibitory significantly decreased. The mass spectrometry analysis revealed a notable degradation of hemoglobin-derived peptides after simulated digestion, and the release of a large number of dipeptides that may have contributed to the observed bioactivities. These results suggest that natural peptides from Spanish dry-cured ham bones could contribute to a positive impact on cardiovascular health.

Keywords: Ham bones, bioactive peptides, cooking, *in vitro* digestion, mass spectrometry.

1. Introduction

Hypertension, diabetes, obesity, alterations in lipid metabolism, and thrombotic disorders are risk factors in the development of cardiovascular diseases, which are the main cause of death globally. Some of these factors can be controlled by regulating enzymatic pathways through the ingestion of food-protein derived hydrolysates and peptides with different types of bioactivity [1,2]. Bioactive peptides are short sequences of amino acids that have a positive health impact on the human body, including antihypertensive, antioxidant, antidiabetic and antithrombotic activities, among others [3]. These peptides are inactive in the parent protein but can be released after proteolytic digestion and exert physiological effects. For this reason, they should be able to resist degradation by gastrointestinal proteases, cross the intestinal barrier and reach their target sites in an active form [4,5]. Bioactive peptides can be generated from parent proteins using chemical or enzymatic hydrolysis, microbial fermentation or gastrointestinal digestion, but also during food processing conditions such as cooking, freezing, storage, pH variations, and physical treatments [2,6].

The meat processing industry generates a large amount of by-products including bones, meat trimmings, blood, skins, and horns, which represent high economic and environmental costs [7]. Some of these by-products constitute part of the human diet since the consumption of internal organs with high nutritive value is a normal practice in certain countries. Additionally, blood is used in traditional Asian and European foods for its technological properties, and rinds and bones in Mediterranean cooking to add flavor to soups, stews, and broths. Other applications of by-products are for animal feed, fertilizers, biodiesel generation, pharmaceuticals and cosmetics [7-9]. In recent years, several works have reported the presence of peptides generated by enzymatic hydrolysis, processing conditions and gastrointestinal digestion of meat by-products. Peptides

identified to date have reported antihypertensive, antioxidant, antimicrobial, opioid and other bioactivities [2,10-12].

The aim of this work was to evaluate the presence of bioactive peptides with potential cardiovascular health benefits derived from dry-cured ham by-products as well as their stability under different acid conditions and heat treatments that simulated traditional Mediterranean household cooking methods. Furthermore, *in vitro* simulated digestion was used to mimic what happens to these peptides in the human gut and to assess if gastrointestinal digestion can assist in the generation of bioactive peptides.

2. Materials and methods

2.1 Chemicals and reagents

Enzymes used for the *in vitro* gastrointestinal digestion were purchased from Sigma-Aldrich, Co. (St. Louis, MO, USA). Regarding chemicals used for enzymatic inhibition assays, angiotensin I-converting enzyme (ACE-I) from rabbit lung, captopril, phosphoramidon disodium salt, 4-morpholineethanesulfonic acid (MES), dipeptidyl peptidase-IV (DPP-IV) from porcine kidney, diprotin A, Gly-Pro-7-amido-4-methylcoumarin hydrobromide (Gly-Pro-AMC), and DL-dithiothreitol (DTT), 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 *o*-aminobenzoylglycyl-*p*-nitro-*L*-phenylalanyl-*L*-proline (Abz-Gly-p-nitro-Phe-Pro-OH) trifluoroacetate salt was from Bachem AG. (Bubendorf, Switzerland). Recombinant human endothelin-I converting enzyme (ECE) and fluorogenic peptide substrate V (FPS V) were from R&D Systems (Minneapolis, MN, USA). The platelet-activating factor-acetylhydrolase (PAF-AH) inhibitory assay kit was supplied by

Cayman Chemical Company (Ann Arbor, MI, USA). All other chemicals and reagents used were of analytical grade.

2.2 Sample preparation and peptide extraction

Dry-cured ham bones (50 g) were minced and processed according to the method of Gallego et al. [12] in triplicate. Briefly, one sample was homogenized in 200 mL of water (control); two samples were mixed with 200 mL of 0.5 N HCl and 0.01 N HCl, respectively; and two samples were prepared in order to simulate Mediterranean household cooking: samples in 200 mL of water heated at 100ºC for 20min and 100ºC for 1h, respectively. After the extraction of peptides (8 min, 4ºC, overnight) using a stomacher (IUL, Barcelona, Spain), samples were centrifuged (12,000 *g*, 20 min, 4°C), deproteinized (3 volumes ethanol, 20 h, 4ºC), and centrifuged again (12,000 *g*, 10 min, 4°C). Finally, supernatants were dried using a rotatory evaporator and lyophilized. All procedures were prepared in triplicate.

2.3 *In vitro* gastrointestinal digestion

Samples (200 mg/mL) were subjected to *in vitro* digestion according to the methodology described by Gallego et al. [12] Briefly, 27 U/mL of salivary α-amylase was used to simulate the oral phase (3 min, 37ºC, pH 7), 2000 U/mL of porcine pepsine for the gastric phase (2 h, 37ºC, pH 3), and a mixture of 100 U/mL of trypsin, 25 U/mL of chymotrypsin, 200 U/mL of porcine pancreatic α-amylase, 2000 U/mL of porcine pancreatic lipase and 10 mM of porcine bile extract for the intestinal phase (2 h, 37ºC, pH 7). The process was finished by heating at 95ºC for 2 min, and then the mixture was deproteinized with 3 volumes ethanol (20 h, 4ºC) and centrifuged (12,000 *g*, 10 min, 4ºC). Finally, the supernatant was dried in a rotatory evaporator and lyophilized. Analysis was done in triplicate.

2.4 ACE-I inhibitory activity

The ACE-I inhibitory activity was measured according to the method developed by Sentandreu and Toldrá [13], which is based on the ability of ACE-I enzyme to hydrolyze the internally quenched fluorescent substrate Abz-Gly-Phe(NO_2)-Pro. For that, 50 µL of each sample was mixed with 50 µL of ACE-I (3 mU/mL in 150 mM Tris-base buffer, pH 8.3). The reaction was started by the addition of 200 µL of substrate (0.45 mM in 150 mM Tris-base buffer with 1.125 mM NaCl, pH 8.3). After 45 min of incubation at 37ºC, the fluorescence was measured at 355 nm excitation and 405 nm emission wavelengths. The assay was done in triplicate and captopril (10 µM) was used as the positive control. Results were expressed as percentage inhibition of ACE-I and the IC_{50} values for each sample were also calculated.

2.5 ECE inhibitory activity

Inhibition of ECE activity was assessed using synthetic FPS V and quantifying the reaction product 7-methoxycoumarin group with fluorescence detection. The assay was done according to the procedure described previously by Fernández-Musoles et al. [14] with slight modifications. Briefly, 25 µL of each sample was mixed with 200 µL of ECE-1 (0.125 µg /mL in 100 mM MES with 150 mM NaCl, pH 6), and 125 µL of FPS V (20 mM in in 100 mM MES with 150 mM NaCl, pH 6). The fluorescence was measured after 20 min of incubation at 355 nm excitation and 405 nm emission wavelengths. The assay was done in triplicate and phosphoramidon (10 µM) was used as the positive control. Results were expressed as percentage of ECE inhibition and the IC_{50} values were also calculated.

2.6 DPP-IV inhibitory activity

The DPP-IV inhibition assay was carried out according to the methodology described by Gallego et al. [15] This assay measures the fluorescence generated due to the release of

153 7-amido-4-methylcoumarin (AMC) from the internally quenched fluorescent substrate
154 Gly-Pro-AMC by the action of the DPP IV enzyme. A total of 50 µL of each sample (5
155 mg/mL) was mixed with 50 µL of DPP-IV (4.55 mU/mL in 50 mM Tris-HCl buffer, pH
156 8.0). The reaction was initiated by the addition of 200 µL of substrate Gly-Pro-AMC
157 (0.25 mM in 50 mM Tris-HCl buffer pH 8.0, 0.5 mM DTT, 1 mM EDTA). The mixture
158 was incubated for 20 min at 37ºC, and the fluorescence was measured using excitation
159 and emission wavelengths of 355 nm and 460 nm, respectively. Samples were assayed
160 in triplicate at a concentration of 5 mg/mL and diprotin A (100 µM) was used as
161 positive control. Results were expressed as DPP-IV inhibition percentage.

162 **2.7 PAF-AH inhibitory activity**

163 The assay is based on the capacity of the enzyme PAF-AH to hydrolyze the substrate 2-
164 thio PAF, which generates free thiols that can be colorimetrically detected using 5,5'-
165 dithio-bis(2-nitrobenzoic acid) (DTNB). For that purpose, samples were assayed at a
166 concentration of 5 mg/mL using a PAF-AH inhibitor assay kit in accordance with the
167 manufacturers' instructions. The assay was done in triplicate and the results were
168 expressed as the percentage PAF-AH inhibition achieved by the peptides.

169 **2.8 Peptide identification by nLC-MS/MS analysis**

170 Samples H_2O (control) and samples cooked at 100ºC for 1h, before and after the *in vitro*
171 digestion, were prepared at a concentration of 0.3 mg/mL in 0.1% of trifluoroacetic acid
172 (TFA) and analyzed by nanoliquid chromatography-tandem mass spectrometry (nLC-
173 MS/MS) for the identification of peptides. A total of 5 µL of each sample was injected
174 into a Eksigent Nano-LC Ultra 1D Plus system (Eksigent AB Sciex, CA, USA), where
175 it was preconcentrated onto a trap column (NanoLC Column, 3µm C18-CL, 75µm x
176 15cm; Eksigent AB Sciex, CA, USA) at a flow rate of 3 µL/min for 5 min with 0.1%
177 TFA as mobile phase. The sample was then loaded into the nano-HPLC capillary

column (3µm C18-CL, 72µm x 12 cm, Nikkyo Technos Co, Ltd. Japan) where peptide elution was carried out at a flow rate of 0.30 µL/min and 30ºC, using 0.1% formic acid in water as solvent A, and 0.1% formic acid in acetonitrile as solvent B. Chromatographic conditions were a linear gradient from 5% to 35% of solvent B in 30 min. Peptides were analyzed in a quadrupole/time-of-flight (Q/TOF) TripleTOF® 5600+ system (AB Sciex Instruments, MA, USA) with a nano-electrospray ionization (nESI) system. The Q/TOF operated in positive polarity and data-dependent acquisition mode, in which a 250 ms TOF MS scan from 150 to 1250 *m/z* was performed, followed by 50 ms product ion scans from 100 to 1250 *m/z* on the 25 most intense 2 to 5 charged ions.

2.9 Data analysis

Statistical analysis of enzymatic inhibition included one-way analysis of variance (ANOVA) and Fisher's multiple range tests using XLSTAT 2011 v5.01 software (Addinsoft, Barcelona, Spain). Results were expressed as the mean of 3 replicates ± standard deviations, and differences were considered significant at $P < 0.05$.

Data were processed using ProteinPilot™ v4.5 search engine (AB Sciex, MA, USA) for the identification of peptides and protein of origin. The Paragon algorithm of ProteinPilot was used to search in Uniprot database, selecting mammalian taxonomy and no enzyme specificity. The search of sequences of bioactive peptides previously identified was done using BIOPEP database (http://www.uwm.edu.pl/biochemia/index.php/en/biopep).

3. Results and discussion

Bones are the most abundant dry-cured ham by-products and they are traditionally used in Mediterranean household cooking of broths and stews. Bones are an important source

of collagen protein, which has been described to contain in its sequence bioactive peptides with potential health benefits, especially antioxidant and ACE inhibitory activities [16]. Blood is also a rich protein by-product which has been reported to show antioxidant, antihypertensive, antimicrobial and opioid activities in hemoglobin and plasma hydrolysates [17,18].

Samples from dry-cured ham bones were subjected to different treatments simulating traditional Mediterranean cooking and *in vitro* digestion in order to evaluate the presence of bioactive peptides as inhibitors of the enzymes ACE-I, ECE, DPP-IV and PAF-AH. These enzymes are involved in metabolic pathways that regulate the cardiovascular system so their inhibition can result in the reduction of high blood pressure and alleviation of disorders including type 2 diabetes, obesity, hypercholesterolemia, atherosclerosis and inflammatory diseases [19,20].

3.1 ACE-I inhibitory peptides

The ACE-I enzyme is involved in the Renin-Angiotensin--Aldosterone System (RAAS) by cleaving angiotensin-I into the vasoconstrictor angiotensin-II, and induces the release of aldosterone. ACE-I also inactivates the vasodilatory protein bradykinin. Thus, ACE-I inhibitory peptides are widely used in the treatment of hypertension and inflammation-associated cardiovascular diseases [21].

Peptide extracts obtained from dry-cured ham bones were analyzed for ACE-I inhibitory activity, showing few differences after the treatments (Figure 1). The sample cooked at 100ºC for 1h showed ACE-I IC_{50} inhibition values of 279 µg/mL and 592 µg/mL before and after digestion, respectively. Sample treated with 0.5 N HCl prior to digestion showed approximately 30% less ACE-I inhibitory activity than the rest of samples, which presented similar inhibition percentages but slightly lower IC_{50} values after digestion.

To date, some studies have identified ACE-I inhibitory peptides generated from the hydrolysis of meat by-products [22-24]. The obtained results show the stability of ACE-I inhibitory activity during cooking and following gastrointestinal digestion.

3.2 ECE inhibitory peptides

The endothelin system participates in the regulation of blood pressure as ECE releases the peptide endothelin (ET)-1 that binds with different receptors and induces vasoconstriction among other physiological effects. So, ECE inhibitors and ET receptor agonists are currently being used in treatments against hypertension and recent studies are focused on evaluating the effect of food-derived peptides on the endothelin system [25,26]

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This work showed the presence of peptides derived from dry-cured ham bones with inhibitory effect on ECE activity (Figure 2). Samples cooked at 100ºC for 20min and 1h showed the highest ECE inhibition before digestion, noting the low IC_{50} value (38 µg/mL) obtained for the sample cooked for 20min. Samples extracted under acidic conditions did not present this bioactivity. After *in vitro* digestion (Figure 2B), there was a 50% reduction in the ECE inhibitory activity of the control and cooked samples while the samples treated with HCl showed 20% inhibition of ECE. These results would suggest that breakdown of the ECE inhibitory peptides in the control and cooked samples occurred. In addition, it appears that novel peptides are generated in the acidic samples due to the action of the enzymes used for the gastrointestinal digestion.

3.3 DPP-IV inhibitory peptides

DPP-IV enzyme degrades the incretin hormones glucagon-like peptide-1 (GLP-1) and gastric inhibitory peptide (GIP), which potentiate glucose-stimulated insulin release, suppress glucagon secretion and improve plasma glucose concentrations. As a result,

252 DPP-IV inhibitors are promising new therapies for type 2 diabetes and hypertension
253 [27,28].

254 The DPP-IV inhibitory activity of peptides from dry-cured ham bones was lower than
255 30% in all samples (see Figure 3). There was a notable increase (20%) in the inhibition
256 value in the control sample after the *in vitro* digestion, whereas the activity of the acidic
257 samples after the enzymatic action increased to a lesser degree. No significant
258 differences were found between cooked samples before and after digestion, but the
259 DPP-IV activity showed an increase compared to the control sample prior to digestion.
260 Cooking of dry-cured ham bones and simulated gastrointestinal digestion may lead to
261 the formation of novel DPP-IV inhibitory peptides, which is in agreement with a
262 previous *in silico* analysis that demonstrated the potential of meat and by-products as a
263 source of these bioactive peptides [29].

264 **3.4 PAF-AH inhibitory peptides**

265 The PAF-AH enzyme catalyzes the pro-inflammatory phospholipid mediator PAF,
266 which participates in several inflammatory and vascular diseases. Recent works have
267 focused on the identification of food-derived peptides showing PAF-AH inhibition as
268 they are promising therapeutic targets for the prevention of atherosclerotic lesions [30,31].
269 As can be seen in Figure 4, the inhibitory effect of peptides on PAF-AH activity was
270 low in all the assayed samples. The control and cooked samples reached PAF-AH
271 inhibitory values between 5.2% and 10.4% before digestion, whereas acidic samples did
272 not show activity. However, the simulated digestion led to an increase of PAF-AH
273 inhibition, mainly for samples H_2O and 0.01 N HCl where approximately 20% increase
274 in PAF-AH inhibitory activities was observed compared to the other samples analyzed.
275 To the best of our knowledge, this is the first work reporting PAF-AH inhibitory
276 peptides derived from meat by-products.

3.5 Stability of peptides

Bioactive peptides must undergo processing conditions and gastrointestinal digestion to exert their potential function *in vivo* [32]. Household preparations including acidic and cooking treatments can destroy amino acids, affect physical and structural properties of proteins and peptides, and modify the digestion rate by gastrointestinal enzymes [33,34]. In fact, temperature, pH, amino acid composition and peptide sequence can affect stability, permeability through gastrointestinal barriers, and solubility of proteins and peptides, leading to a low bioavailability of the biologically active compounds. For instance, ACE-I and DPP-IV inhibitory peptides have been reported to present poor solubility due to the high proportion of hydrophobic amino acids in their sequences, which could require higher amounts of peptide to exert biological effects in the organism [35].

Some studies about the stability of bioactive peptides derived from meat by-products have been reported to date. Fu et al. [36] described that ACE inhibitory peptides derived from bovine collagen hydrolysates retained their activity after temperature, pH treatments, and simulated digestion. This high resistance of collagen peptides could be due to post-translational hydroxylation of the proline residues that are in their sequences [37]. On the other hand, a previous work in dry-cured ham bones evidenced the generation and stability of antioxidant peptides after household preparations and gastrointestinal digestion, showing that those peptides derived from collagen protein were main responsible for differences in the activity between uncooked and cooked samples after digestion [12]. In fact, ACE-I, ECE, DPP-IV, and PAF-AH inhibitory activities found in the present work may be attributed to the previously identified collagen-derived peptides [12], although further studies are required to confirm this statement.

300 **3.6 Identification of peptides**

301 Control samples and samples cooked for 1h were analyzed by nLC-MS/MS to evaluate
302 the peptidic profile before and after digestion, focusing on those peptides derived from
303 the hydrolysis of the hemoglobin protein. Table 1 shows the sequence of the 212
304 identified peptides derived from the alpha and beta subunits of this protein, noting that
305 most of them are present in the samples prior to digestion and are later hydrolyzed by
306 the gastrointestinal enzymes. A sequential loss of amino acids from the C-terminal end
307 is also observed in many of the identified peptides, for instance in the peptides 1-11 or
308 16-24 from the hemoglobin alpha. These facts give rise to the generation of smaller
309 fragments that could maintain, decrease, or increase their bioactivity depending on their
310 characteristics as well as to the release of a large amount of tripeptides, dipeptides and
311 free amino acids [38].

312 The identified peptides were compared to previously reported ACE-I and DPP-IV
313 inhibitory peptides available in the BIOPEP database [39]. Some dipeptides such as KA
314 and NV obtained from the degradation of peptides 114 and 112 to generate peptides 112
315 and 111, respectively, have been described as DPP-IV inhibitors. Moreover, EA and
316 HG from peptides 122 and 119 to generate peptides 120 and 118, respectively, have
317 been reported to show ACE-I inhibitory activity, as well as peptides 208 and 209 have
318 similar sequences to the ACE-I inhibitor VVYPWT [40,41]. Some of the identified
319 peptides could be also multifunctional and have a synergistic effect on blood pressure
320 lowering actions [42,43], such as dipeptide KF released from peptide 24 which has been
321 reported to inhibit both ACE-I and DPP-IV enzymes [44,45]. The great number of short
322 peptides generated during the sample processing could contribute notably to the
323 bioactivities found in the present work. Moreover, di- and tripeptides are more likely to

be resistant to gastrointestinal digestion and to be absorbed directly into the circulatory system [46].

In conclusion, this work shows the potential of dry-cured ham bones as a source of bioactive peptides with *in vitro* ACE-I, ECE, DPP-IV, and PAF-AH inhibitory activity. The stability of ACE-I and DPP-IV inhibitory peptides was observed after cooking and *in vitro* digestion of samples. PAF-AH inhibitory activity increased after the action of the gastrointestinal enzymes while the amount of ECE inhibitory peptides showed a significant decrease. These results suggest that dry-cured ham bones traditionally used in the Mediterranean household cooking of broths and stews could have a positive impact on cardiovascular health and a possible reduction of high blood pressure for consumers. However, further works are needed in order to identify the peptide sequences responsible for these bioactivities as well as *in vivo* studies to assess the bioavailability and physiological effect in the organism.

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478

FIGURE CAPTIONS

Figure 1. ACE-I inhibitory activity of the samples H_2O (control), 0.5 N HCl, 0.01 N HCl, 100ºC 20min, and 100ºC 1h, A) before and B) after *in vitro* gastrointestinal digestion. The values represent means of three replicates and bars represent standard deviations. Table shows the calculated IC_{50} value for each sample.

Figure 2. ECE inhibitory activity of the samples H_2O (control), 0.5 N HCl, 0.01 N HCl, 100ºC 20min, and 100ºC 1h, A) before and B) after *in vitro* gastrointestinal digestion. The values represent means of three replicates and bars represent standard deviations. Table shows the calculated IC_{50} values for each sample.

Figure 3. DPP-IV inhibitory activity of the samples H_2O (control), 0.5 N HCl, 0.01 N HCl, 100ºC 20min, and 100ºC 1h, before and after *in vitro* gastrointestinal digestion. The values represent means of three replicates and bars represent standard deviations. Bar letters indicate significant differences among the values at $P < 0.05$.

Figure 4. PAF-AH inhibitory activity of the samples H_2O (control), 0.5 N HCl, 0.01 N HCl, 100ºC 20min, and 100ºC 1h, before and after *in vitro* gastrointestinal digestion. The values represent means of three replicates and bars represent standard deviations. Bar letters indicate significant differences among the values at $P < 0.05$.

Table 1. Peptides from hemoglobin protein identified by nLC-MS/MS in samples H2O and 100 ºC 1h before and after *in vitro* digestion.

Protein subunit	Peptide number	Peptide sequence	Modification	Observed (*m/z*)[a]	Calculated MW (Da)[b]	Charge (+)	Before digestion[c]		After digestion[d]	
							H2O	100ºC 1h	H2O	100ºC 1h
Alpha	1	AAHHPDDF		455.194	908.378	2			x	x
	2	AAHHPDDFN		512.216	1022.421	2			x	
	3	AAHHPDDFNPS		604.246	1206.505	2		x		
	4	AAHHPDDFNPSV		436.198	1305.574	3				x
	5	AAHHPDDFNPSVH		481.846	1442.633	3	x	x	x	x
	6	AAHHPDDFNPSVHA		505.530	1513.670	3		x	x	x
	7	AAHHPDDFNPSVHAS		534.547	1600.702	3	x	x		
	8	AAHHPDDFNPSVHASL		572.249	1713.786	3		x		
	9	AAHHPDDFNPSVHASLD		610.601	1828.813	3	x	x		
	10	AAHHPDDFNPSVHASLDK		490.197	1956.908	4	x	x		
	11	AAHHPDDFNPSVHASLDKF		526.972	2103.976	4	x	x		
	12	AGAHGAEALER		541.245	1080.531	2		x		
	13	AGAHGAEALERM		404.812	1211.572	3		x		
	14	AHELRVDPV		518.267	1034.551	2		x		
	15	AHGAEALER		477.202	952.473	2		x		
	16	AHHPDDFN		476.658	951.383	2		x		
	17	AHHPDDFNPS		568.719	1135.468	2		x	x	x
	18	AHHPDDFNPSV		618.264	1234.537	2		x		x
	19	AHHPDDFNPSVH		458.163	1371.596	3	x	x	x	x
	20	AHHPDDFNPSVHA		481.846	1442.633	3		x	x	x
	21	AHHPDDFNPSVHAS		510.862	1529.665	3	x	x		
	22	AHHPDDFNPSVHASLD		586.916	1757.776	3	x	x		
	23	AHHPDDFNPSVHASLDK		472.436	1885.871	4	x	x		
	24	AHHPDDFNPSVHASLDKF		509.209	2032.939	4	x	x		
	25	AVGHLDDLPGALS		632.822	1263.646	2		x		
	26	AWGKVGGQAGAHGA	Oxidation (W)	428.166	1281.621	3	x			
	27	DDFNPSVH		465.658	929.388	2	x	x	x	
	28	DDFNPSVHA		501.185	1000.425	2	x	x		x
	29	DDFNPSVHAS		544.708	1087.457	2	x	x		
	30	DDFNPSVHASLD		658.788	1315.568	2		x		
	31	DDFNPSVHASLDK		722.854	1443.663	2	x	x		
	32	DDLPGALSALSDL		643.823	1285.640	2		x		
	33	DFNPSVH		408.135	814.361	2	x	x		
	34	DFNPSVHASLDK		443.840	1328.636	3	x	x		
	35	DPVNFK		360.131	718.365	2	x			
	36	FLGFPTTKT		506.243	1010.544	2	x	x		
	37	GAHGAEALER		505.717	1009.494	2		x		
	38	GAHGAEALERM	Oxidation (M)	386.465	1156.530	3		x		
	39	GGQAGAHGAEAL		519.720	1037.489	2	x	x		

	40	GGQAGAHGAEALE		584.253	1166.532	2	x	x			
	41	GGQAGAHGAEALER		441.839	1322.633	3	x	x			
	42	GGQAGAHGAEALERM	Oxidation (M)	490.860	1469.668	3	x	x			
	43	GHLDDLPGALS		547.750	1093.540	2		x			
	44	GKVGGQAGAHGAEA		605.289	1208.590	2	x	x	x		
	45	GKVGGQAGAHGAEAL		661.843	1321.674	2	x				
	46	GKVGGQAGAHGAEALE		484.540	1450.716	3	x	x			
	47	GKVGGQAGAHGAEALER		536.586	1606.818	3	x	x			
	48	GKVGGQAGAHGAEALERM	Oxidation (M)	585.608	1753.853	3	x	x			
	49	GQAGAHGAEALE		555.742	1109.510	2	x	x			
	50	GQAGAHGAEALER		633.806	1265.611	2	x	x			
	51	GQAGAHGAEALERM	Oxidation (M)	471.848	1412.647	3	x	x			
	52	HGAEALER		441.679	881.436	2	x	x			
	53	HGAEALERM	Oxidation (M)	515.212	1028.471	2	x	x			
	54	HGQKVADALTKA		413.517	1237.678	3	x				
	55	HHPDDFNP		489.671	977.399	2	x				
	56	HHPDDFNPS		533.194	1064.431	2	x	x	x		
	57	HHPDDFNPSV		582.738	1163.500	2		x	x		x
	58	HHPDDFNPSVH		651.282	1300.558	2	x	x	x		x
	59	HHPDDFNPSVHA		458.163	1371.596	3	x	x			
	60	HHPDDFNPSVHAS		487.180	1458.628	3	x	x			
	61	HHPDDFNPSVHASLD		563.232	1686.739	3	x	x			
	62	HHPDDFNPSVHASLDK		605.937	1814.834	3	x	x			
	63	HLDDLPGA		419.158	836.403	2		x			
	64	HLDDLPGAL		475.710	949.487	2	x	x			
	65	HLDDLPGALS		519.234	1036.519	2		x			
	66	HLDDLPGALSA		554.762	1107.556	2	x				
	67	HPDDFNPS		464.652	927.372	2	x				
	68	HPDDFNPSVH		582.738	1163.500	2	x	x	x		x
	69	HPDDFNPSVHA		618.275	1234.537	2	x		x		x
	70	HPDDFNPSVHAS		661.792	1321.569	2	x				
	71	HPDDFNPSVHASLDK		560.243	1677.775	3	x	x			
	72	KLRVDPV		413.709	825.507	2		x			
	73	KVGGQAGAHGA		489.715	977.504	2		x			
	74	KVGGQAGAHGAEA		589.777	1177.584	2		x			x
	75	KVGGQAGAHGAEALE		710.871	1419.711	2	x	x			
	76	KVGGQAGAHGAEALER		517.574	1549.796	3		x			
	77	KVGGQAGAHGAEALERM		575.258	1722.847	3		x			
	78	LAAHHPDDFNPSVH		519.578	1555.717	3			x		x
	79	LANVSTVL		408.690	815.475	2		x			
	80	NLSHGSDQV	Deamidation (N)	479.178	956.420	2	x	x			
	81	NLSHGSDQVK		362.124	1083.531	3	x	x			
	82	NLSHGSDQVKA	Deamidation (N)	386.137	1155.552	3	x				
	83	NLSHGSDQVKAHGQK		535.913	1604.802	3	x	x			

	84	NLSHGSDQVKAHGQKV		568.943	1703.870	3	x	x		
	85	NLSHGSDQVKAHGQKVA		592.624	1774.907	3	x	x		
	86	NLSHGSDQVKAHGQKVAD		630.977	1889.934	3	x	x		
	87	NLSHGSDQVKAHGQKVADA		491.213	1960.972	4		x		
	88	NVSTVLTSKY	Deamidation (N)	556.768	1111.576	2	x			
	89	PDDFNPSVH		514.194	1026.441	2		x		
	90	PTTKTYFPH		364.463	1090.545	3		x		
	91	QAGAHGAEALER		596.774	1191.563	2		x		
	92	QYGAEALER		518.741	1035.499	2		x		
	93	QYGAEALERM	Oxidation (M)	592.276	1182.534	2		x		
	94	SAADKANVK		452.203	902.482	2	x	x		
	95	SAADKANVKAA		523.255	1044.556	2	x			
	96	SHGSDQVKAHGQK		460.190	1377.675	3	x	x		
	97	SHGSDQVKAHGQKV		493.219	1476.743	3	x	x		
	98	SHGSDQVKAHGQKVAD		555.255	1662.807	3		x		
	99	TKAVGHLDDLPG		408.166	1221.635	3		x		
	100	VADALTKA		394.675	787.444	2		x		
	101	VGGQAGAHGAEA		512.712	1023.473	2	x	x	x	x
	102	VGGQAGAHGAEAL		569.264	1136.557	2	x	x	x	x
	103	VGGQAGAHGAEALE		633.798	1265.600	2	x	x		
	104	VGGQAGAHGAEALER		474.867	1421.701	3	x	x		
	105	VGGQAGAHGAEALERM		518.557	1552.742	3	x	x		
	106	VGHLDDLPG		461.693	921.456	2	x			
	107	VGHLDDLPGA		497.253	992.493	2	x			x
	108	VGHLDDLPGAL		553.794	1105.577	2	x			x
	109	VGHLDDLPGALS		597.296	1192.609	2	x	x		
	110	VGHLDDLPGALSA		632.822	1263.646	2	x	x		
	111	VLSAADKA		387.666	773.428	2	x	x		
	112	VLSAADKANV	Carbamylation	515.746	1029.546	2	x	x		
	113	VLSAADKANVK		558.300	1114.635	2	x	x		
	114	VLSAADKANVKA		396.176	1185.672	3	x	x		
	115	VLSAADKANVKAA	Carbamylation	650.857	1299.715	2	x	x		
	116	VLSAADKANVKAAWGK		543.615	1627.905	3	x			
	117	VLSAADKANVKAAWGKVGGQAGA		543.024	2168.170	4		x		
	118	WGKVGGQAGA		465.699	929.472	2		x		
	119	WGKVGGQAGAHG		375.468	1123.552	3	x			
	120	WGKVGGQAGAHGA		399.150	1194.589	3	x	x		
	121	WGKVGGQAGAHGAE		442.173	1323.632	3	x			
	122	WGKVGGQAGAHGAEA		465.857	1394.669	3	x			
	123	WGKVGGQAGAHGAEALE		546.581	1636.796	3	x			
	124	WGKVGGQAGAHGAEALER		449.190	1792.897	4	x	x		
	125	WGKVGGQAGAHGAEALERM	Oxidation (M)	647.649	1939.932	3	x			
Beta	126	AEEKEAVLG		473.209	944.482	2	x			
	127	ARRLGHDFNPDVQAA		556.261	1665.833	3		x		

	128	DAVMGNPKVKAHGK	Oxidation (M)	489.896	1466.766	3	x				
	129	DEVGGEAL		395.131	788.355	2	x				
	130	DEVGGEALG		423.647	845.377	2	x				
	131	DEVGGEALGR		501.711	1001.478	2	x	x			
	132	DEVGGEALGRL		558.264	1114.562	2	x	x			
	133	DEVGGEALGRLL		614.819	1227.646	2		x			
	134	DGLKHLDN		456.188	910.451	2	x	x			
	135	DGLKHLDNL		512.742	1023.535	2	x				
	136	DGLKHLDNLK		384.827	1151.630	3	x	x			
	137	DGLKHLDNLKG		403.839	1208.651	3	x				
	138	DGLKHLDNLKGTFA		510.241	1527.805	3	x	x			
	139	DLSNADAV		402.637	803.366	2	x	x			
	140	EEKEAVLGL		494.236	986.528	2	x				
	141	ESFGDLSNA		470.169	938.398	2	x				
	142	ESFGDLSNADA		563.214	1124.462	2	x	x			
	143	ESFGDLSNADAV		612.760	1223.531	2	x	x			
	144	EVGGEALGR		444.185	886.451	2		x			
	145	EVGGEALGRL		500.740	999.535	2	x				
	146	FESFGDLSNA		543.713	1085.467	2		x			
	147	FESFGDLSNAD		601.240	1200.494	2		x			
	148	FESFGDLSNADA		636.765	1271.531	2	x	x			
	149	FESFGDLSNADAV		686.311	1370.599	2	x	x			
	150	FGDLSNADAV		504.700	1007.456	2	x	x			
	151	FGDLSNADAVMGNPKVK	Oxidation (M)	593.622	1777.867	3	x				
	152	GDLSNADAV		431.151	860.388	2	x	x			
	153	GGEALGRL		386.663	771.424	2		x			
	154	GGEALGRLL		443.215	884.508	2		x			
	155	GHDFNPDVQ		514.692	1027.436	2	x	x			x
	156	GHDFNPDVQAA		585.746	1169.510	2	x	x			
	157	GKVNVDEV		430.180	858.445	2	x	x			
	158	GKVNVDEVGGE		551.772	1101.530	2			x		x
	159	GKVNVDEVGGEA		587.273	1172.567	2	x	x	x		
	160	GKVNVDEVGGEAL		643.825	1285.651	2	x	x			
	161	GKVNVDEVGGEALG		672.344	1342.673	2	x	x			
	162	GKVNVDEVGGEALGRL		538.267	1611.858	3	x				
	163	GLWGKVNVDEV		608.308	1214.630	2	x	x			
	164	HDFNPDVQAA		557.228	1112.489	2		x			
	165	HLSAEEKE		471.687	941.445	2	x	x			
	166	HLSAEEKEA		507.214	1012.483	2	x	x			
	167	HLSAEEKEAV		556.759	1111.551	2	x	x			
	168	HLSAEEKEAVL		613.315	1224.635	2	x	x			
	169	HLSAEEKEAVLG		641.829	1281.656	2	x				
	170	HLSAEEKEAVLGL		465.878	1394.741	3	x	x			
	171	HVDPENF		429.144	856.372	2	x	x			

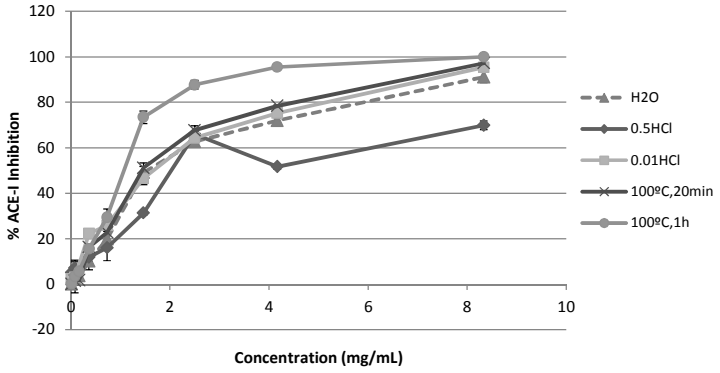
	172	HVDPENFR		507.210	1012.473	2		x		
	173	KVNVDEVGGEA		558.756	1115.546	2	x	x	x	x
	174	KVNVDEVGGEAL		615.313	1228.630	2	x	x		
	175	KVNVDEVGGEALG		643.827	1285.651	2	x	x		
	176	LGHDFNPDVQ		571.246	1140.520	2		x		
	177	LGHDFNPDVQAA		642.299	1282.594	2	x	x		
	178	LSAEEKEAVLG		573.287	1144.598	2	x			
	179	LSAEEKEAVLGL		629.840	1257.682	2	x			
	180	NVDEVGGEA		445.152	888.383	2	x			
	181	NVDEVGGEAL	Deamidation (N)	502.195	1002.451	2	x	x		
	182	NVDEVGGEALG		530.222	1058.488	2	x	x		
	183	NVDEVGGEALGR		608.290	1214.589	2	x	x		
	184	NVDEVGGEALGRL		664.843	1327.673	2	x	x		
	185	SAEEKEAVLGL		573.285	1144.598	2	x			
	186	SDGLKHLDN		499.713	997.483	2		x		
	187	SDGLKHLDNL		556.264	1110.567	2	x	x		
	188	SDGLKHLDNLK	Dehydration (S)	611.319	1220.651	2		x		
	189	SDGLKHLDNLKG		432.853	1295.683	3	x	x		
	190	SDGLKHLDNLKGTFA		539.258	1614.837	3		x		
	191	SFGDLSNADAV		548.225	1094.488	2	x	x		
	192	VDEVGGEALGRL		607.808	1213.630	2	x	x		
	193	VDPENF		360.604	719.313	2	x			
	194	VDPENFR		438.667	875.414	2		x		
	195	VGGEALGRL		436.205	870.492	2	x	x		
	196	VHLSAEEK		456.699	911.471	2		x		
	197	VHLSAEEKE		521.234	1040.514	2		x		
	198	VHLSAEEKEAV		606.303	1210.619	2	x	x		
	199	VHLSAEEKEAVL		442.197	1323.703	3		x		
	200	VHLSAEEKEAVLG		461.205	1380.725	3		x		
	201	VHLSAEEKEAVLGL		498.909	1493.809	3		x		
	202	VNVDEVGGE		459.212	916.414	2			x	
	203	VNVDEVGGEA		494.697	987.451	2	x	x	x	
	204	VNVDEVGGEAL		551.250	1100.535	2	x	x		
	205	VNVDEVGGEALG		579.767	1157.556	2	x	x		
	206	VNVDEVGGEALGRL		714.387	1426.742	2		x		
	207	VVAGVANALAH	Deamidation (N)	511.783	1021.556	2			x	
	208	VVYPWTQR		524.752	1047.550	2		x		
	209	VVYPWTQRF		598.301	1194.619	2		x		
	210	WGKVNVDE		473.693	945.456	2		x		
	211	WGKVNVDEV		523.238	1044.524	2		x		
	212	WGKVNVDEVGGEA		680.329	1358.647	2	x	x		

[a]Molecular ion mass observed in the nESI-LC-MS/MS analysis.

[b]Calculated molecular mass in Da of the matched peptide.

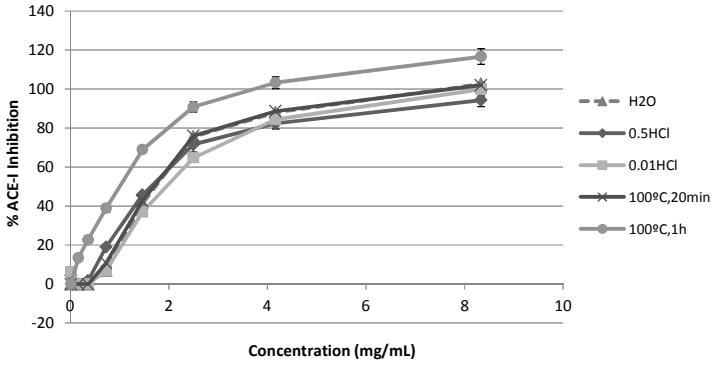
[c,d]Peptides identified at each sample.

A)



Sample	IC_{50} (mg/mL)
H_2O	1.530
0.5N HCl	2.599
0.01 N HCl	1.585
100ºC,20min	1.499
100ºC,1h	0.279

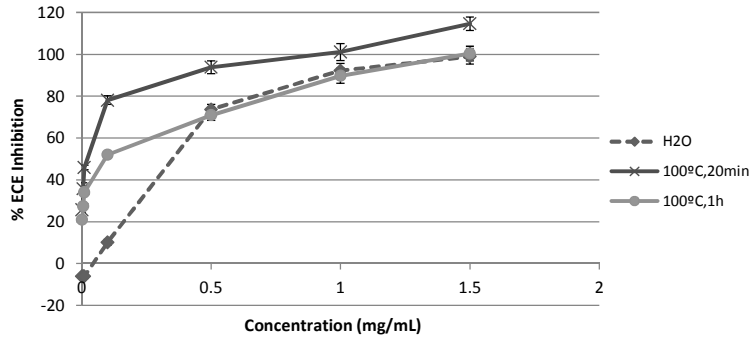
B)



Digested sample	IC_{50} (mg/mL)
H_2O	0.897
0.5N HCl	0.787
0.01 N HCl	1.060
100ºC,20min	0.866
100ºC,1h	0.592

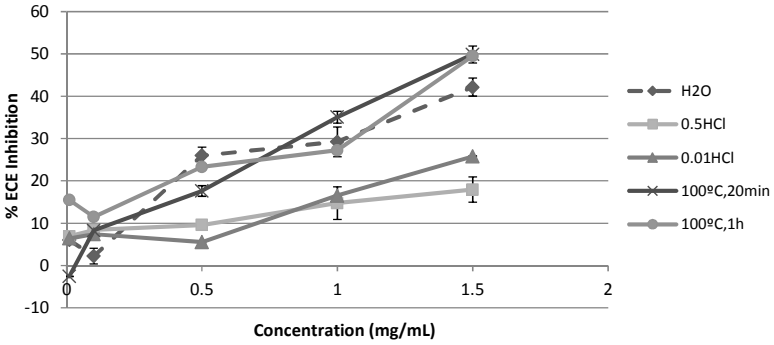
Figure 1.

A)



Sample	IC_{50} (mg/mL)
H_2O	0.805
0.5N HCl	n.d.
0.01 N HCl	n.d.
100ºC,20min	0.038
100ºC,1h	0.191

B)



Digested sample	IC_{50} (mg/mL)
H_2O	0.429
0.5N HCl	0.729
0.01 N HCl	0.895
100ºC,20min	0.660
100ºC,1h	0.665

Figure 2.

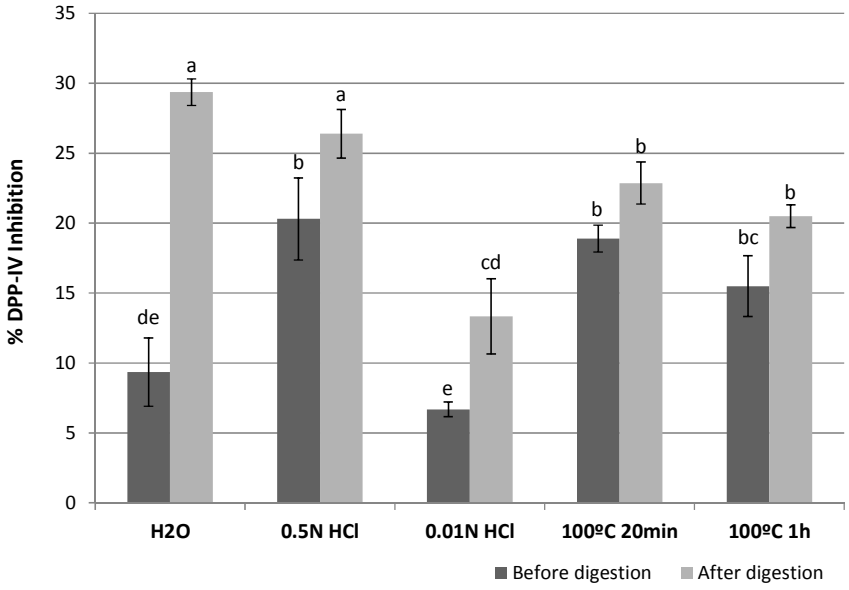

35
30
25
20
15
10
5
0
% DPP-IV Inhibition
a
a
b
b
b
b
bc
b
de
cd
e
H2O
0.5N HCl
0.01N HCl
100ºC 20min
100ºC 1h
Before digestion
After digestion

Figure 3.

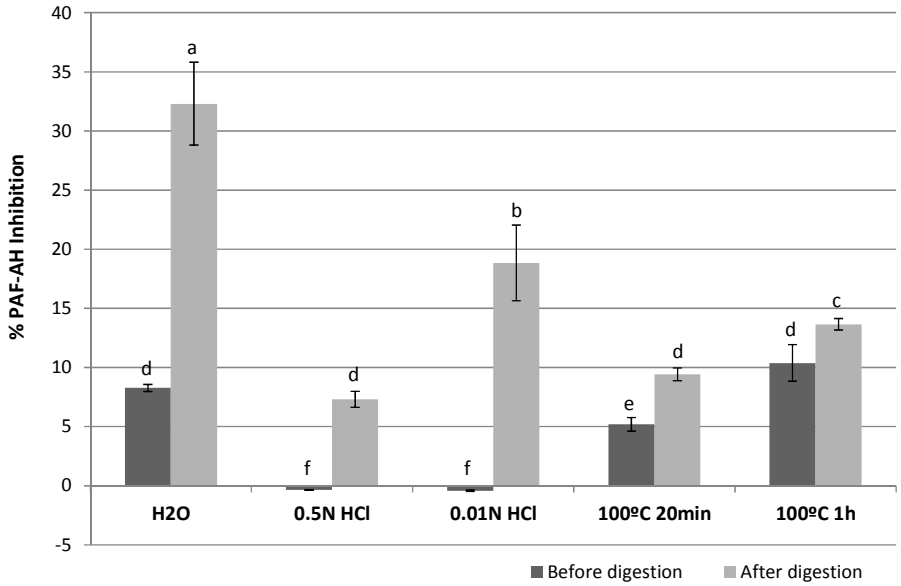

40
35
30
25
20
15
10
5
0
-5
% PAF-AH Inhibition
a
d
d
f
b
f
d
e
d
d
c
H2O
0.5N HCl
0.01N HCl
100ºC 20min
100ºC 1h
Before digestion
After digestion

Figure 4.