González-García, E., Puchalska, P., Marina, M.L. & García, M.C. 2015, "Fractionation and identification of antioxidant and angiotensin-converting enzyme-inhibitory peptides obtained from plum (Prunus domestica L.) stones", Journal of Functional Foods, vol. 19, pp. 376-384.

Available at http://dx.doi.org/10.1016/j.jff.2015.08.033

FRACTIONATION AND IDENTIFICATION OF ANTIOXIDANT AND ANGIOTENSIN-CONVERTING ENZYME-INHIBITORY PEPTIDES OBTAINED FROM PLUM (*PRUNUS DOMESTICA L.*) STONES

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Running title: Identification of bioactive peptides from plum stones

ABSTRACT

Peptides with antioxidant and ACE-inhibitory capacities have been obtained from a plum (*Prunus Domestica L.*) by-product by *in vitro* hydrolysis. The aim of this work was to fractionate the most active plum hydrolysates, to identify peptides in these fractions, and to evaluate their resistance to gastrointestinal enzymes. Highest antioxidant capacity was observed in the whole Alcalase hydrolysate while peptides yielding the highest ability to inhibit ACE activity were concentrated in the fraction < 3 kDa from the Thermolysin hydrolysate. RP-HPLC-ESI-MS/MS enabled the identification of 13 and 7 potential antioxidant and antihypertensive peptides, respectively, in these fractions. Nevertheless, RP-HPLC alone did not guarantee the successful separation of all peptides. The combination of RP-HPLC and hydrophilic interaction liquid chromatography (HILIC) coupled to ESI-Q-TOF-MS/MS enabled the identification of all peptides. Five potential antioxidant and three potential antihypertensive peptides could be additionally identified by HILIC. A total of 7 potential antioxidant peptides and 3 potential antihypertensive peptides resisted a simulated gastrointestinal digestion.

Keywords: plum stone, ACE-inhibitory peptides, antioxidant peptides, hydrophilic interaction chromatography, mass spectrometry.

1. Introduction

Fruit residues are one of the main constituents of food industry waste. Most of these wastes are discarded causing important environmental pollution and health risks (Deng et al., 2012). In other occasions, these by-products are processed into low market-valued products such as animal feed (Restrepo Gallego, 2006). New approaches to reuse these wastes and obtain high valuable compounds are, nowadays, important trends (Deng et al., 2012; Duda-Chodak & Tarko, 2007).

According to the Food and Agricultural Organization (FAO) of the United Nations (FAOSTAT, 2015), plum production in Spain in 2013 was about 172 thousand tons, which supposes a fiftieth of the plum world production. From this production, about 10 to 25 % are by-products generated by plum canning and beverage industries (Ros et al., 2012). Plums (*Prunus domestica L.*) seeds are by-products with a considerably high protein content (around 20%) (Alpers, 1917; Giaja, 1927). Albeit plum seeds are rich in highly valuable proteins, they are irretrievably lost since they are considered a useless waste. Nevertheless, these proteins could be a cheap source of valuable peptides.

Bioactive peptides are generally small, with around 2-20 amino acids residues, where activity depends mainly on their structure and amino acid composition (Erdmann, Cheung, & Schroeder, 2008). Antioxidant peptides are typically rich in highly hydrophobic and aromatic amino acids (Erdmann et al., 2008; Sarmadi & Ismail, 2010). while antihypertensive peptides usually contain aromatic amino acids as phenylalanine, tyrosine, and tryptophan (Erdmann et al., 2008; Jung et al., 2006; Saito, 2008) and/or hydrophobic amino acids like proline close to the C-terminal position (Erdmann et al., 2008; Jung et al., 2006; Ondetti, Sabo, & Cushman, 1980). Identification of peptides has been mainly performed using reversed-phase (RP)-HPLC coupled to mass spectrometry (Esteve, Marina, & García, 2015; González-García, Marina, & García, 2014).

Nevertheless, RP is less effective for the separation of short chain peptides. There has not been an attractive alternative to RP for many years for the separation of short chain peptides. Hydrophilic interaction liquid chromatography (HILIC) emerged as an appealing chromatographic mode for the separation of polar compounds like small size peptides (D'Attoma & Heinisch, 2013). HILIC uses hydrophilic stationary phases like those traditionally employed in normal phase (NP) (e.g., silica, amino, and cyano) (Buszewski & Noga, 2012) and also new specific ones. HILIC mobile phases consist of water miscible solvents that are highly compatible with electrospray ionization-mass spectrometry (ESI-MS) (Jandera, 2011). Separations are performed by gradient elution with increasing concentrations of the aqueous phase. Unlike RP, separation by HILIC could involve a partition between the aqueous layer imbibed on the stationary phase and the hydrophilic elution buffer. Additionally, electrostatic interactions or hydrogen bonding to the stationary phase could also be present. Buffering salts (ammonium acetate or formate) are usually added to the mobile phase in order to reduce these electrostatic interactions (Bernal, Ares, Pol, & Wiedmer, 2011).

In a previous work, proteins from plum seeds were extracted and digested using different enzymes and digestion conditions. Results showed that hydrolysates obtained with Alcalase and Thermolysin presented, respectively, interesting antioxidant and ACE-inhibitory capabilities (González-García, García, & Marina, 2014). In the present work, we will focus on the fractionation of Alcalase and Thermolysin hydrolysates, the comprehensive identification of bioactive peptides in most active fractions using HILIC and RP-HPLC coupled to ESI-Q-TOF-MS/MS, and on the evaluation of peptide resistance to gastrointestinal enzymes.

2. Materials and methods

2.1.Chemicals and samples.

All chemicals and reagents were of analytical grade. Water was taken daily from a Millipore Milli-Q system (Bedford, MA, USA). Acetonitrile (ACN), acetic acid (AA), acetone, methanol (MeOH), and hexane were from Scharlau Chemie (Barcelona, Spain). Tris(hydroxymethyl)aminomethane (Tris), hydrochloric acid (HCl), sodium dodecyl sulfate (SDS), sodium bicarbonate, phosphate buffer, and ammonium acetate were from Merck (Darmstadt, Germany). Sodium hydroxide, dithiothreitol (DTT),o-phthalaldehyde (OPA), sodium tetraborate, β-mercaptoethanol, glutathione, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), potassium persulfate, (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) (Trolox), 1,10-phenantroline, ferrous sulfate, hydrogen peroxide, potassium ferricyanide, trichloroacetic acid, ferric chloride, linoleic acid, ammonium thiocyanate, ferrous chloride, angiotensin converting enzyme (ACE) from rabbit lung, hippuryl-histidyl-leucine, 2-[4-(2-hydroxyethyl)-1-piperazinyl]-ethanesulfonicacid, sodiumchloride, Thermolysin, pepsin from porcine gastric mucosa (P-7012), and pancreatin from porcine pancreas (P-7545) were all purchased at Sigma-Aldrich (Saint Louis, MO, USA). Alcalase 2.4 L FG was kindly donated by Novozymes Spain S.A. (Madrid, Spain). Plums were acquired in a local supermarket in Alcalá de Henares (Madrid).

2.2. Protein extraction, digestion, and ultrafiltration.

Plum seed proteins were extracted using previously optimized conditions González-García et al. (2014). Briefly, 0.5 g of ground seeds were defatted three times with 20 mL of hexane. Proteins were extracted with 100 mM Tris-HCl buffer (pH 7.5) containing 1 % (w/w) SDS and 0.25 % (w/w) DTT using a high intensity focused ultrasound probe

(model VCX130 from Sonics Vibra-Cell, Hartford, CT, USA) for 1 min at 30% of amplitude. After centrifugation, extracted proteins were precipitated with 10 mL of cold acetone for 30 min in the fridge. Precipitated proteins were centrifuged (10 min, 4000 x g) and redissolved in the appropriate buffer for its further digestion.

Digestion of proteins was performed with Alcalase or Thermolysin which provided, in a previous work González-García et al. (2014), the most antioxidant and ACE-inhibitory hydrolysates, respectively. Optimized conditions were employed. In the case of Alcalase, precipitated proteins were dissolved up to a concentration of 5.0 mg/mL in 5 mM borate buffer (pH = 8.5) and incubated with the enzyme at an enzyme:substrate ratio of 0.30 AU/g protein for 3 h at 50 ºC in a Thermomixer Compact from Eppendorf AG (Hamburg, Germany). In the case of Thermolysin, a 5 mM phosphate buffer (pH = 8.0) was employed to dissolve proteins at a concentration of 2.5 mg/mL and the solution was incubated with the enzyme at a 0.10 g enzyme/g protein for 4 h at 50 ºC. In both cases, the digestion was stopped by raising the temperature to 100 ^{0}C for 10 min followed by centrifugation (5 min, 7000 x g).

Hydrolysates were fractionated by ultrafiltration (60 min, 7000 x g). For that purpose, Vivaspin 500 PES filters with 5 kDa molecular weight cut-off (MWCO) (Sartorious Stedim Biotech, Goettingen, Germany) and Amicon filters with 3 kDa MWCO (Millipore) were employed. Obtained fractions (> 5 kDa, 3-5 kDa, and < 3 kDa) were dissolved to the initial volume with the digestion buffer in order to compare antioxidant and ACE-inhibitory capacities with those of the whole hydrolysate. Fractions were stored at -20 ^{0}C until use.

2.3. O-phthalaldehyde (OPA) assay.

Peptide content was measured using the OPA assay, following the procedure described by González-García et al. (2014). Replicates of every sample were measured at least three times.

2.4. *In vitro* bioactivity assays.

In vitro antioxidant capacity of hydrolysates and fractions were measured using four different assays evaluating: the ability to scavenge free radicals (ABTS and hydroxyl radicals), the ability to reduce Fe (III) (Ferric reducing antioxidant power (FRAP) assay), and the ability to inhibit lipid peroxidation. Every sample was prepared by triplicate and every measurement was done at least three times. Solvent blanks were measured for every assay. All assays were carried out following the procedures described by González-García et al. (2014). Antioxidant capacity was expressed as percentage of inhibition.

In vitro antihypertensive capacity was evaluated by measuring the ability to inhibit ACE, one of the main enzymes responsible for regulating blood pressure. ACE inhibitory capacity of hydrolysates and fractions was measured according to the procedure described by González-García et al. (2014). Potential antihypertensive capacity was expressed as IC_{50} which is the peptide concentration needed to inhibit a 50% ACE.

2.5. Simulated gastrointestinal (GI) digestion.

Most active peptide fractions were next digested following the method described by Puchalska, García, & Marina (2014). Briefly, sample pH was fixed to 2 with 1 M HCl, pepsin was added at an enzyme:substrate ratio 1:35, and the mixture was incubated for 1 h at 37 ^{0}C and shaking. After pepsin digestion, sample was adjusted to pH 5 with 0.1 M $NaHCO_3$ and to pH 7-8 with 0.1 M NaOH. Pancreatin digestion was carried out in 0.1 M phosphate buffer (pH 8) by the addition of the enzyme at an enzyme:substrate ratio 1:25 for 2 h at 37 ^{0}C and shaking. Reaction was stopped by boiling for 10 min.

2.6. Analysis by HPLC-MS/MS.

Samples were analyzed using a Quadrupole-Time-of-Flight (Q-TOF) mass spectrometer (instrument series 6530) from Agilent Technologies (Palo Alto, CA, USA) coupled to an HPLC system 1100 series also from Agilent Technologies. Separation of peptides was carried out through two different modes: Reversed Phase (RP) and Hydrophilic Interaction Liquid Chromatography (HILIC). Both columns consisted of fused core superficially porous particles.

The separation by RP mode was performed on an Ascentis Express Peptide ES-C18 column (100 mm x 2.1 mm I.D., 2.7 µm particle size) with an Ascentis Express guard column (5 mm x 2.1 mm I.D., 2.7 µm particle size), both from Supelco (Bellefonte, PA, USA). Following chromatographic conditions were used: mobile phase A, water with 0.3 % (v/v) AA; mobile phase B, ACN with 0.3 % (v/v) AA; elution gradient, 3 % B for 5 min, 3–25 % B in 30 min, 25–95 % B in 5 min, and 95–3 % B in 2 min; flow rate, 0.3 mL/min; temperature, 25 ^{0}C; injection volume, 15 µL. The separation by HILIC was carried out on an Ascentis Express HILIC (100 mm x 2.1 mm, I.D., 2.7 µm particle size) column with an Ascentis Express guard column (5 mm x 2.1 mm I.D., 2.7 µm particle size), both from Supelco. The chromatographic conditions were: mobile phase A, ACN; mobile phase B, water with 50 mM ammonium acetate (pH 7); elution gradient,10–40 % B in 20 min, 40 % B for 10 min, and 40–10 % B in 5 min; flow rate, 0.3 mL/min; temperature, 25 ^{0}C; injection volume, 15 µL. In both modes, UV (210, 254, and 280 nm) and MS/MS signals were simultaneously registered.

The mass spectrometer was operated in the positive ion mode and the mass range was from *m/z* 100 to 1500 Da. ESI conditions were: fragmentator voltage, 200 V; nozzle voltage, 0 V; nebulizer pressure, 50 psig; capillary voltage, 3500 V; gas temperature, 350

^{0}C; gas flow, 12 L/min, and skimmer voltage, 60 V. The Jet Stream sheath gas temperature and flow were 400 ^{0}C and 12 L/min, respectively. MS/MS was performed employing the auto mode and the following optimized conditions: 1 precursor per cycle, dynamic exclusion after two spectra (released after 1 min), and collision energy of 5 V per 100 Da.

2.7. Identification of bioactive peptides.

Tandem MS/MS spectra were obtained for the molecular ion with the highest abundance. Every sample was prepared in duplicate and injected in triplicate into the MS system. In order to assure that identified peptides came from plum seed proteins, blank samples containing everything except plum seed proteins were also analyzed.

MS/MS spectra were analyzed using PEAKS Studio Version 7 (Bioinformatics Solutions Inc., Waterloo, Canada). Analysis of data was performed with the *de novo* sequencing tool. All results were refined applying a certain average local confidence (ALC), which is the expected percentage of correct amino acids in the peptide sequence. Peptides identified above ALC equal or superior to 90% threshold and with good precursor fragmentation pattern, have been treated as a true identification and used in further data analysis. Only those peptides appearing in all six injections were taken into account. It must be noted that it is not possible to differentiate I from L by MS due to their equal molecular masses. Only isoforms with L are presented in our results, although peptide sequences containing I amino acid instead of L are also possible.

Venn diagrams were created employing the GenoTool bioinformatics facility.

2.8. Statistical analysis

Statistical analysis was carried out employing Statgraphics Software Plus 5.1 (Statpoint Technologies, Inc., Warrenton, VA, USA). In order to find statistically significant differences among obtained results, analysis of variance (ANOVA) and t-test were applied.

3. Results and discussion

Results obtained in a previous study showed that plum seeds were a relevant source of antioxidant and ACE-inhibitory peptides (González-García et al., 2014). In that work, the extraction of plum seed proteins and their digestion using different enzymes were optimized. Alcalase was the enzyme yielding the peptides with the highest antioxidant capacity while Thermolysin was the enzyme producing the most potent ACE-inhibitory peptides. The aim of this work was to fractionate most active hydrolysates, to comprehensively identify the peptides responsible for these abilities using HILIC and RP-HPLC-ESI-MS/MS, and to evaluate the resistance of most active peptides in a simulated gastrointestinal digestion.

3.1.Selection of most active peptide fractions.

Alcalase and Thermolysin hydrolysates were fractionated according to their molecular masses by ultrafiltration obtaining fractions < 3 kDa, from 3-5 kDa, and > 5 kDa. The peptide content and antioxidant capacities of the whole Alcalase hydrolysate and its fractions were measured and results are presented in **Figure 1**. Antioxidant capacity of extracts was estimated using four different assays covering different mechanisms of oxidation inhibition. The highest peptide content was obtained for the peptide fraction below 3 kDa, while the lowest was observed for the peptide fraction between 3 and 5 kDa. In general, antioxidant capacity was not concentrated in a particular peptide fraction observing the highest antioxidant capacity in the whole extract for all

assays, with the exception of FRAP one. This behavior is usual (Ghiselli, Serafini, Natella, & Scaccini, 2000) within antioxidant compounds since their cooperation is responsible of the superior defense against oxidants attack. In fact, antioxidant radicals are formed during the inhibition of oxidation and, in order to reduce their reactivity and to recover reduced antioxidants, antioxidant systems work in a synergistic way and regenerate each other (Vertuani, Angusti, & Manfredini, 2004). The deficiency of a component in an antioxidant system can, therefore, affect the efficiency of other. This explanation may explain why the peptide fractions showed lower antioxidant capacity than that observed for the whole Alcalase hydrolysate. Therefore, identification of peptides was carried out in the whole Alcalase hydrolysate.

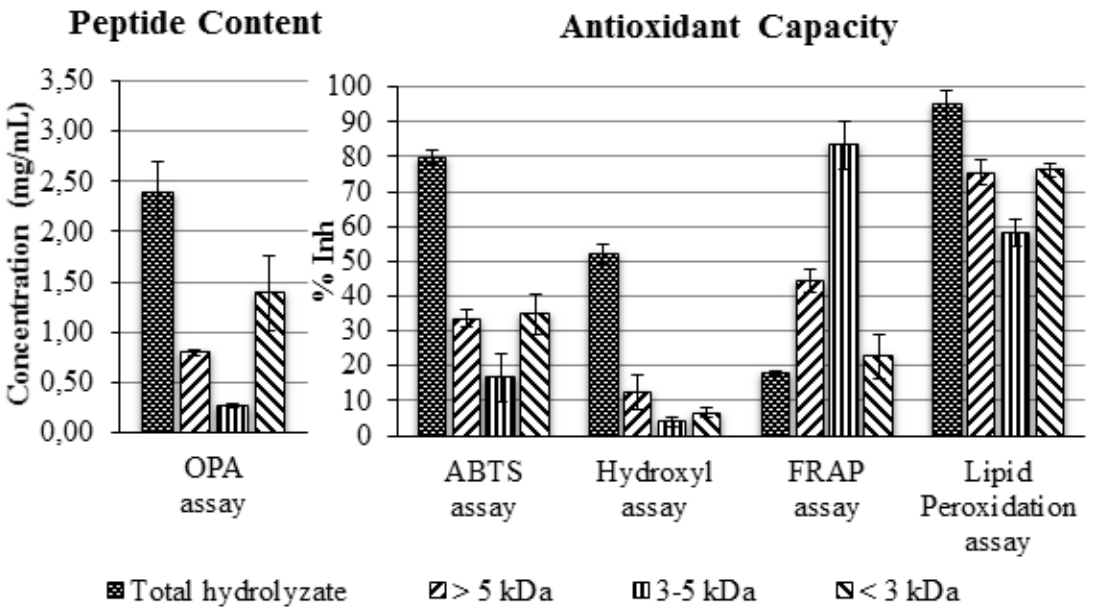

Figure 1. Peptide content and antioxidant capacity of the whole hydrolysate obtained with Alcalase enzyme from plum seed proteins and its fractions (> 5 kDa, 3 - 5 kDa, < 3 kDa) evaluated by four different antioxidant assays.

The peptide content and IC_{50} values against ACE of the whole Thermolysin hydrolysate and its fractions are depicted in **Figure 2**. Results revealed that most peptides in the Thermolysin hydrolysate had a molecular mass < 3 kDa. Moreover, almost all capacity observed in the whole Thermolysin hydrolysate proceeded from this peptide fraction (t-test, $p < 0.05$). The IC_{50} value of peptides concentrated in fraction < 3 kDa was 22.8 µg/mL. If we compare this value with the obtained with known milk peptides VPP and IPP (IC_{50} values of 2.8 µg/mL and 1.7 µg/mL, respectively), we can conclude that the ACE inhibiting capacity of peptides in this fraction is high and it could be useful to isolate single peptides as IPP and VPP (De Leo, Panarese, Gallerani, & Ceci, 2009). The peptide fractions > 5 kDa and 3-5 kDa did not exhibit ACE inhibitor capacity. This behavior was expected since ACE inhibiting peptides show short sequences (Puchalska, Marina, & García, 2015). Therefore, identification of peptides was carried out in the fraction < 3 kDa from the hydrolysate obtained with Thermolysin.

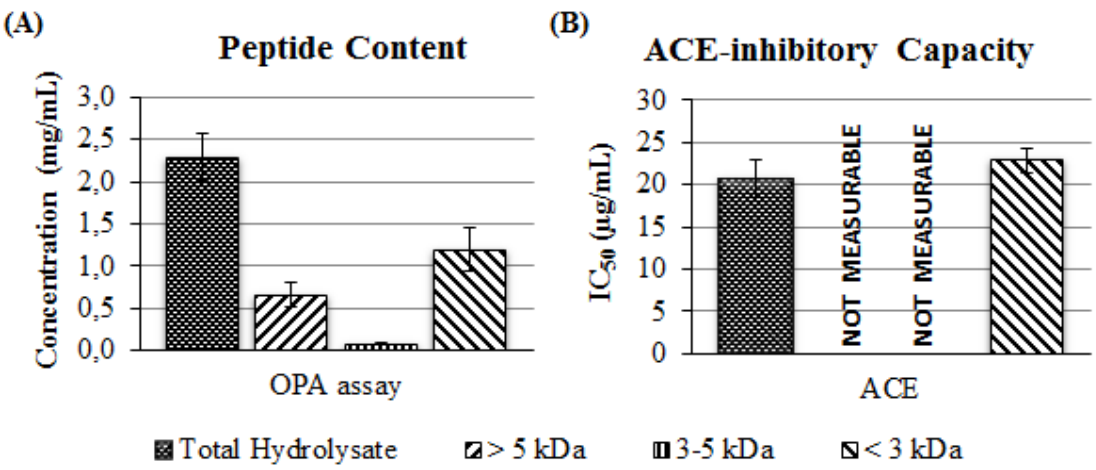

Figure 2. Peptide content and Angiotensin-converting enzyme-inhibitory capacity of the whole hydrolysate obtained with Thermolysin enzyme from plum seed proteins and its fractions (> 5 kDa, 3 - 5 kDa, and < 3 kDa).

3.2. Identification of peptides in most active fractions by RP-HPLC- and HILIC-ESI-Q-TOF-MS/MS.

In order to identify antioxidant and ACE-inhibitory peptides, most active extracts (whole Alcalase hydrolysate and Thermolysin fraction < 3 kDa) were analyzed by HPLC-ESI-Q-TOF.

3.2.1. Identification of peptides in the whole Alcalase hydrolysate showing the highest antioxidant capacity

Peptides in the whole Alcalase hydrolysate were identified in a previous work using RP-HPLC-ESI-Q-TOF (González-García et al., 2014). **Figure 3A** shows the total ion chromatogram obtained by RP-HPLC. There were 13 different peptides that could be identified in the time range from 6 to 28 min. Nevertheless, less hydrophobic peptides, eluting at the beginning of the gradient, could not be efficiently separated and were not identified. In order to obtain a comprehensive identification of peptides, the whole Alcalase hydrolysate was also analyzed by HILIC-ESI-Q-TOF. When HILIC separation was carried out, a less crowded chromatogram was observed (see **Figure 3B**). Sequence, ALC, molecular mass, retention time, and mass accuracy of peptides were grouped in **Table 1**. In order to compare these results with those previously obtained by RP-HPLC-ESI-Q-TOF (González-García et al., 2014) they have also been included in **Table 1**. Peptides in **Table 1** are supposed to be those present in a higher concentration and best ionized by ESI. A total of 11 peptides with a number of amino acids ranging from 4 to 9 were identified when using HILIC. Comparison of these peptides with the 13 peptides detected by RP-HPLC-MS enabled to observe that 6 peptides (marked by *) were simultaneously detected when using both separation modes, that 7 peptides were just found when RP-HPLC was used, and that 5 peptides were observed when using just

HILIC. **Figure 3C** also shows the MS/MS spectrum of one of the peptides simultaneously observed by RP-HPLC and HILIC. Peptides simultaneously observed by both modes eluted within the first half of the HILIC chromatogram while peptides only observed by HILIC eluted very late and, thus, they were very hydrophilic.

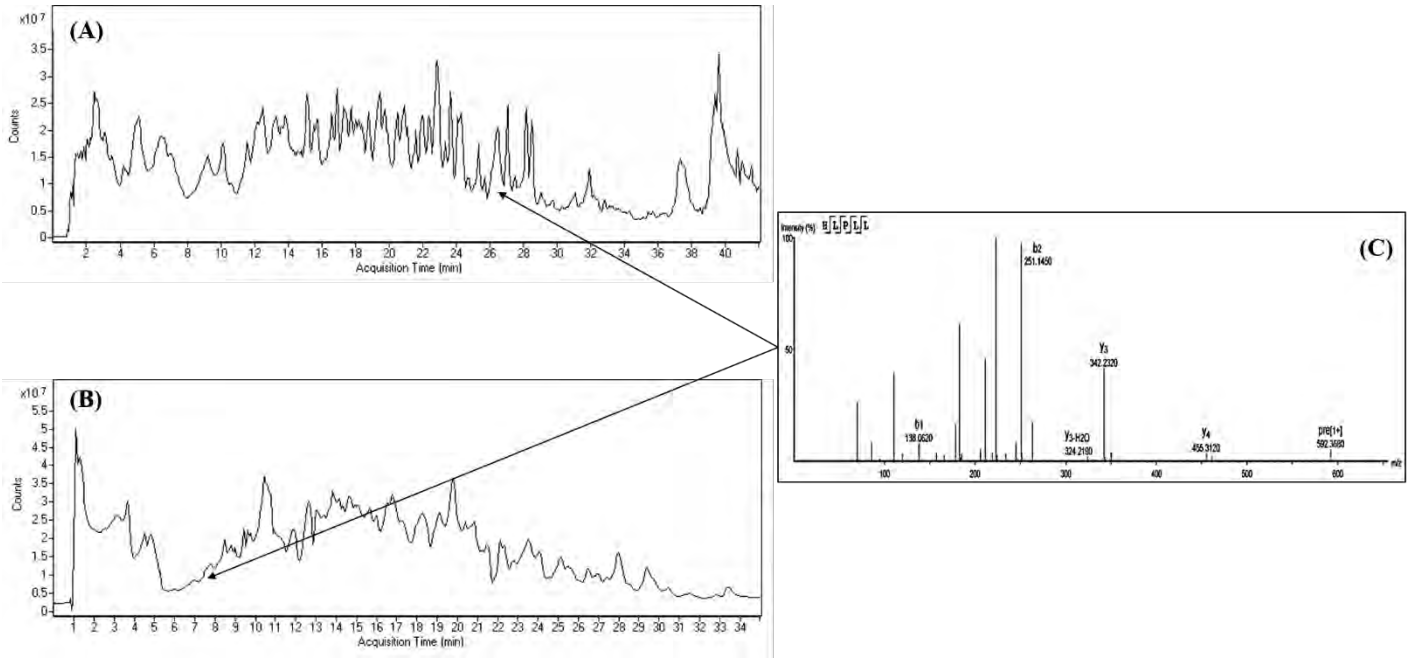

Figure 3. Total ion chromatograms corresponding to the whole Alcalase hydrolysate obtained by RP-HPLC (A) and HILIC (B) and MS/MS spectrum of a peptide simultaneously observed in both chromatographic modes (C).

Most sequenced peptides showed molecular masses below 1 kDa. These results are rational taking into account the low specificity of Alcalase enzyme. Nevertheless, these results contrast with the fact that it was possible to obtain fractions > 5 kDa and from 3 to 5 kDa. This would suggest that the ultrafiltration was not efficient in the fractionation of so small peptides.

Table 1. Peptide sequence, retention time, average local confidence (ALC), experimental molecular mass, and mass accuracy of the peptides identified in the whole Alcalase hydrolysate (showing antioxidant capacity) and in the < 3 kDa Thermolysin fraction (showing Angiotensin-converting enzyme-inhibitory capacity) using RP-HPLC- and HILIC-MS/MS.

Capacity	RP-HPLC[a]					HILIC				
	Peptide sequence	Retention time (min)	ALC (%)	Molecular Mass (Da)	Mass accuracy (ppm)	Peptide sequence	Retention time (min)	ALC (%)	Molecular mass (Da)	Mass accuracy (ppm)
Antioxidant	MLPSLPK*	23.2	95	784.4517	0.3	MLPSLPK*	16.2	95	784.4517	1.6
	HLPLL*	26.3	95	591.3744	0.4	KGVL*	19.5	95	415.2794	0.9
	NLPLL*	28.2	95	568.3585	1.2	NLPLL*	2.8	94	568.3585	1.9
	HNLPLL	26.0	95	705.4174	3.1	RALVH	25.3	94	594.3602	1.1
	KGVL*	13.5	94	415.2794	3.8	HLPLL*	8.9	93	591.3744	1.6
	HLPLLR	27.7	94	747.4755	5.8	GFPPK	23.6	93	544.3009	0.3
	HGVLQ*	8.8	93	552.3020	1.7	KGVLY	16.4	93	578.3428	0.4
	GLYSPH	16.1	92	672.3231	3.1	HGVLQ*	14.8	92	552.3020	3.2
	LVRVQ	13.6	92	613.3911	1.6	DQVPR*	20.6	92	613.3184	1.2
	YLSF	25.2	92	528.2584	0.9	NGGK	26.1	91	374.1914	0.4
	DQVPR*	6.1	92	613.3184	3.6	RLPYPLGPN	18.9	91	1025.5658	1.7
	LPLLR	22.7	91	610.4166	4.7					
	VKPVAPF	23.7	91	756.4534	3.0					
ACE inhibitory	LYSPH	12.7	97	615.3016	0.7	HLLP	14.3	96	478.2903	2.1
	LYTPH	15.0	97	629.3173	1.3	VVYV*	2.0	95	478.2791	0.5
	VVYV*	21.4	97	478.2791	0.7	LAGNPENE	19.2	93	842.3770	1.0
	LLAQA	17.2	94	514.3115	1.1	LFSPR	12.8	92	618.3489	1.4
	FGEGPY	23.2	93	668.2806	0.9	VLAQ*	4.1	91	429.2587	2.6
	VLAQ*	3.3	91	429.2587	3.1					
	VAVNL	24.4	91	514.3115	2.0					

[a] Data of antioxidant peptides identified by RP-HPLC-MS/MS were taken from González-García et al. (2014). *Peptides simultaneously observed by RP-HPLC and HILIC.

Regardless of the separation mode, the analysis of the amino acid composition of identified peptides showed a high amount of hydrophobic and aromatic amino acid residues. These are very important and common features among antioxidant peptides (Erdmann et al., 2008; Sarmadi & Ismail, 2010). Moreover, the number of hydrophobic amino acid residues of peptides detected by HILIC-MS (55%) was lower than the observed in the peptides detected by RP-HPLC-MS (66%).

Sequenced peptides were checked against BIOPEP (2015) and PeptideDB (Bioinformatics Data Resource Catalogue) (2015) bioactive peptide databases. Despite some peptides could be found within longer peptides, such as KGVL peptide that was previously reported within three longer peptides exerting antibacterial activity (Morikawa, Hagiwara, & Nakajima, 1992; Simmaco, Mignogna, Barra, & Bossa, 1994; Suzuki, Ohe, Okubo, Kakegawa, & Tatemoto, 1995), most of the peptides have not been previously reported.

3.2.2. Identification of peptides in the Thermolysin fraction < 3 kDa showing the highest ACE-inhibitory capacity

Table 1 also summarizes all ACE-inhibitory peptides identified by RP-HPLC- and HILIC-MS/MS. All identified peptides showed molecular masses < 0.9 kDa. A total of 10 different peptides were observed, 5 of them were present just when using RP-HPLC and 3 when employing HILIC. Moreover, 2 peptides were simultaneously detected by both separation modes (marked by *). Most peptides contained hydrophobic amino acids and aromatic amino acids, which are typical features of ACE inhibitor peptides (Erdmann et al., 2008; Jung et al., 2006; Saito, 2008).

Identified peptide sequences were searched in BIOPEP (2015) and PeptideDB (2015) databases. Peptide VLAQ was found previously within a longer sequence (*VLAQ*YK) of an ACE inhibitor peptide (Arihara, 2006) and LLAQA was observed in the sequence of different angiotensin-like peptides and defensins.

3.3.Identification of peptides after gastrointestinal (GI) digestion by RP-HPLC- and HILIC-ESI-Q-TOF-MS/MS.

The potential of bioactive peptides depends on their capacity to reach target organs in an intact conformation. GI digestion is one of the barriers that peptides have to overcome for that purpose. In order to evaluate the resistance of peptides to GI digestion, extracts were submitted to a hydrolysis procedure using conditions simulating *in vivo* gastrointestinal digestion. Therefore, whole Alcalase hydrolysate and Thermolysin fraction < 3 kDa, that yielded the highest antioxidant and ACE-inhibitory capacities, respectively, were sequentially hydrolysed using pepsin (endopeptidase acting at stomach level) and pancreatin (mixture of trypsin, α-chymotrypsin, elastase, and carboxipeptidase A and B, acting at intestinal level) enzymes.

The antioxidant capacity and peptide content of the whole Alcalase hydrolysate after simulated GI digestion were evaluated. As expected, the peptide content increased (around 25%) due to further digestion with pepsin and pancreatin. Nevertheless, the antioxidant capacity did not change significantly ($p > 0.05$), from 57.6 ± 4.3 to 52.7 ± 4.2 % inhibition of ABTS radical. Similarly, the peptide content of the Thermolysin fraction < 3 kDa also yielded an increase (around 32%) after GI digestion. Although GI digestion did significantly decrease ACE inhibitory capacity ($p < 0.05$), it was still kept at a high level observing an $IC_{50} = 30 \pm 3$ μg/mL.

In order to identify antioxidant and ACE-inhibitory peptides resisting GI digestion, obtained extracts were again analyzed by HPLC-ESI-Q-TOF system under exactly the same conditions used previously.

3.3.1. Identification of peptides in the whole Alcalase hydrolysate after GI digestion

The analysis of the whole Alcalase hydrolysate after the GI digestion revealed the presence of 14 different peptides: 9 were separated by RP-HPLC, 3 were separated by HILIC, and 2 were observed by both modes (NLPLL and HGVLQ). Summary of identified peptides is presented in **Table 2**. Peptides showed molecular masses below 0.8 kDa.

All identified peptides were checked against BIOPEP (2015) and PeptideDB (2015) databases. Peptide LPLL, only observed by RP-HPLC, was found within various longer sequences of antibacterial (Amiche, Seon, Pierre, & Nicolas, 1999; Park, Jung, & Lee, 1994; Saberwal & Nagaraj, 1994) peptides.

Peptides identified in the whole Alcalase hydrolysate of plum seed proteins, before and after the GI digestion were compared. Results (see **Figure 4A**) highlighted 7 peptides resisting GI digestion: 3 peptides only observed by RP-HPLC, 2 just detected when using HILIC, and 2 peptides observed by both modes. Most of these resisting peptides contained proline in their sequences which makes them less susceptible to further proteolysis (Escudero, Angel Sentandreu, & Toldra, 2010). Additionally, 8 peptides, not observed before GI digestion, were also identified. Peptide LPLL appeared in the Alcalase enzyme sequence although it was not observed among peptides identified in the Alcalase digestion autolysis blank. Therefore, this peptide was not discarded since it could proceed from peptides detected previously to the gastrointestinal digestion (e.g., HN*LPLL*, H*LPLL*R,

H*LPLL*, N*LPLL* or *LPLL*R*)*. Indeed, this peptide could probably be formed by pepsin digestion since this enzyme generates peptides containing L at N-terminal position (Savoie, Gauthier, Marin, & Pouliot, 2005). Another example is the peptide NLPL that could come from H*NLPL*L. In other cases, original peptides are not included in the list of identified peptides but they could come from other less abundant or non-ionized peptides. For example, pepsin that often generates peptides with Y, F, and L at N-terminal position (Savoie et al., 2005) and /or M, F, and L at C-terminal position (Escudero et al., 2010) could have produced LSVVN, DGDPL, LPAGV, and LYTPH peptides; trypsin that generates peptides with K or R at C-terminal position could have produced NGGGK peptide, and elastase, that specifically cleavages peptide bonds at C-terminal position of G, A, and V, could have resulted in LPAGV peptide.

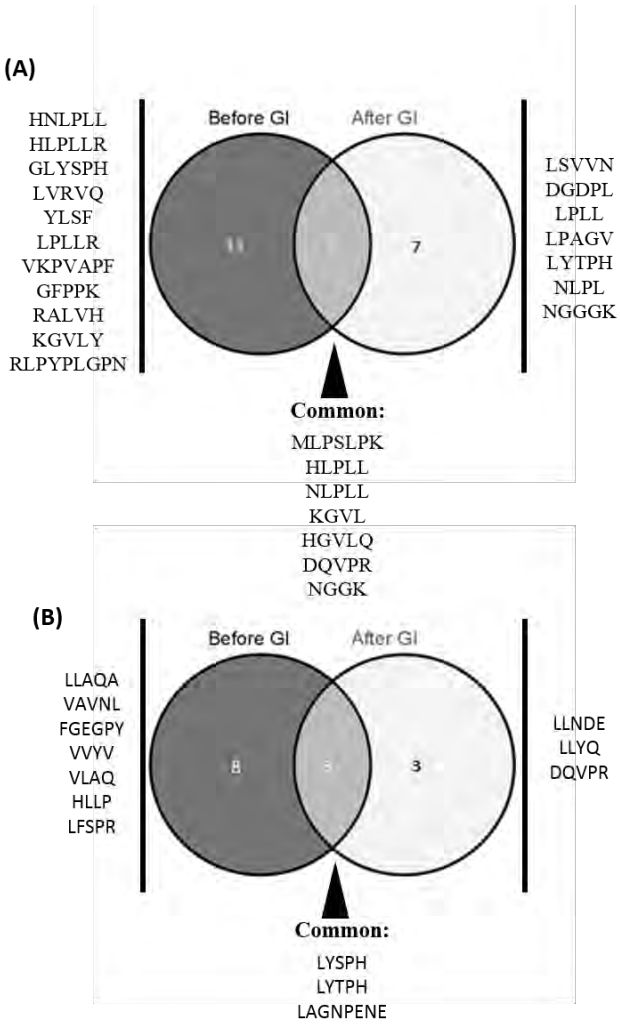

Figure 4. Venn diagrams comparing the peptides identified before and after simulated gastrointestinal digestion (GI) digestion by HPLC-Q-TOF-MS/MS in (A) the whole Alcalase hydrolysate and (B) the fraction < 3 kDa of the Thermolysin hydrolysate.

Table 2. Peptide sequence, retention time, average local confidence (ALC), experimental molecular mass, and mass accuracy of the peptides identified in the whole Alcalase hydrolysate (showing antioxidant capacity) and in the < 3 kDa Thermolysin fraction (showing Angiotensin-converting enzyme-inhibitory capacity) after simulated gastrointestinal digestion digestion using RP-HPLC- and HILIC-MS/MS.

Capacity	RP-HPLC					HILIC				
	Peptide sequence	**Retention time (min)**	**ALC (%)**	**Molecular mass (Da)**	**Mass accuracy (ppm)**	**Peptide sequence**	**Retention time (min)**	**ALC (%)**	**Molecular mass (Da)**	**Mass accuracy (ppm)**
Antioxidant	LSVVN	18.9	94	530.3064	1.0	NLPLL*	3.3	95	568.3585	2.7
	MLPSLPK	26.2	94	784.4517	1.2	NGGGK	27.1	95	431.2128	2.9
	LPLL	30.2	94	454.3155	2.3	APGAGV	17.1	94	470.2489	1.0
	HLPLL	35.6	93	591.3744	1.3	NGGK	26.5	93	374.1914	3.3
	KGVL	5.1	93	415.2794	0.6	HGVLQ*	14.3	92	552.3020	2.0
	DGDPL	16.7	93	515.2227	2.5	DQVPR	20.2	92	613.3184	1.6
	LPAGV	20.5	93	455.2744	1.7					
	NLPLL*	31.4	93	568.3585	3.6					
	LYTPH	16.9	92	629.3173	1.2					
	HGVLQ*	3.6	91	552.3020	0.8					
	NLPL	25.9	91	455.2744	2.3					
ACE inhibitory	LYSPH	10.3	97	615.3016	1.6	LAGNPENE	17.9	94	842.3770	2.0
	LLNDE	11.1	97	602.2911	1.8					
	LYTPH	17.2	95	629.3173	3.0					
	FDAVGVK	21.8	95	734.3962	2.6					
	LLYQ	21.6	92	535.3006	1.6					
	DQVPR	17.1	91	613.3184	1.1					

*Peptides simultaneously observed by RP-HPLC-MS/MS and HILIC-MS/MS.

3.3.2. Identification of peptides in the Thermolysin fraction < 3 kDa after GI digestion

Peptides identified in the Thermolysin fraction < 3 kDa after GI digestion were grouped in **Table 2**. Results showed seven peptides when using RP-HPLC and one peptide only observed by HILIC. No peptide was simultaneously observed by both chromatographic modes. Search against BIOPEP (2015) and PeptideDB (2015) databases showed that the peptide LLYQ had been previously reported within the sequence of an ACE inhibitor (*LLYQ*QPV) (Meisel, Walsh, Murray, & Fitzgerald, 2006) peptide and an immunomodulating (*LLYQ*EPVLGPVRGPFPIIV) (Hayes, Stanton, Fitzgerald, & Ross, 2007) peptide. Again, peptides identified before and after GI digestion were compared (see **Figure 4B**). Three peptides could resist GI digestion: LYSPH and LYTPH, observed by RP-HPLC, and LAGNPENE, showed when using HILIC. All three peptides contained P within their sequences which could justify their resistance to GI digestion. Moreover, three additional peptides, not observed before GI digestion, were now sequenced. Peptide DQVPR was also identified in the whole Alcalasa hydrolysate. This peptide could have resulted from the digestion with trypsin. Other peptides, such as LLNDE or LLYQ, could result from the digestion with pepsin (Savoie et al., 2005). Since ACE-inhibitory capacity decreased after GI digestion, additional peptides appearing after GI digestion probably showed a lower ACE-inhibitory capacity or were in a lower concentration.

4. Conclusions

Peptides fractions showing highly antioxidant and ACE inhibiting peptides have been obtained from a plum by-product by *in vitro* digestion with Alcalase and Thermolysin, respectively. No fraction obtained by ultrafiltration showed an antioxidant capacity as high as the observed value for the whole hydrolysate obtained with Alcalase

enzyme. Most ACE inhibiting peptides obtained by digestion with Thermolysin were concentrated in the fraction containing peptides below < 3 kDa. The use of both RP-HPLC-MS/MS and HILIC-MS/MS guaranteed a comprehensive identification of all peptides in both bioactive fractions. The number of identified peptides by RP-HPLC-MS/MS increased from 13 to 18, in the antioxidant fraction, and from 7 to 10, in the ACE inhibiting fraction, when both RP-HPLC and HILIC were employed. When most active fractions were submitted to a simulated gastrointestinal digestion, antioxidant capacity was maintained while ACE-inhibitory capacity slightly decreased. There were seven peptides in the antioxidant extract and three peptides in the ACE inhibiting fraction that resisted GI digestion. This work demonstrates that plum seeds are cheap sources of highly antioxidant and ACE inhibiting peptides and that this can be an attractive approach to reuse this by-product.

AKNOWLEDGEMENTS

This work was supported by the Ministry of Economy and Competitiveness (ref. AGL2012-36362), the University of Alcalá (ref. CCG2013/EXP-028) and the Comunidad Autónoma de Madrid and European funding from FEDER program (ref. S2013/ABI-3028, AVANSECAL). E.G.-G. thanks the University of Alcalá for her pre-doctoral grant. Authors thankfully acknowledge Novozymes Spain S.A. for the kind donation of Alcalase enzyme.

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