Novel strategy for the revalorization of olive (*Olea europaea*) residues based on the extraction of bioactive peptides

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Abstract

This work proposes a new strategy for the revalorization of residual materials from table-olive and olive oil production based on the extraction of bioactive peptides. Enzymatic hydrolysates of the olive seed protein isolate were prepared by treatment with five different proteases: Alcalase, Thermolysin, Neutrase, Flavourzyme, and PTN. Although all hydrolysates presented antioxidant properties, Alcalase was the enzyme that yielded the hydrolysate with the highest antioxidant capacity. All hydrolysates showed antihypertensive capacity, obtaining IC_{50} values from 29 to 350 µg/mL. Thermolysin was the enzyme yielding the hydrolysate with the highest ACE-inhibitory capacity. Hydrolysates were fractionated by ultrafiltration showing a high concentration of short chain peptides which exhibited significantly higher antioxidant and antihypertensive capacities than fractions with higher molecular weights. Peptides in most active fractions were identified by LC-MS/MS, observing homologies with other recognized antioxidant and antihypertensive peptides. Finally, their antioxidant and antihypertensive capacities were evaluated after *in vitro* gastrointestinal digestion.

Keywords

Olive seeds, bioactive peptides, antioxidant capacity, antihypertensive capacity, mass spectrometry

1. Introduction

The Mediterranean basin provides 97% of the total olive production in the world, constituting one of the major agro-industrial activities for countries as Spain, Italy, Greece, Turkey, and Tunisia. Despite the evolutionary changes carried out in olive oil and table-olive brine production processes, a large volume of residues is still generated in the processing of this fruit. Olive oil extraction results in two different kinds of waste materials: the solid waste, also known as olive pomace or olive oil cake, that is a combination of olive pulp and stone; and an aqueous liquor, the so-called olive mill wastewater, constituted by vegetation water, soft olive tissues, and water added during refinement. These residues result in notoriously polluting products that are phytotoxic, not easily biodegradable, and difficult to treat (Fiorentino et al., 2003; Vlyssides, Loizides, & Karlis, 2004; Khoufi, Aloui, & Sayadi, 2009). Additionally, the management of these residues is detrimental for the economic profit of producers. In order to contribute to environmental sustainability and to maximize economic return, it is necessary to recover these residues and to look for new opportunities of application. In this sense, these residues could be useful for the production of substances with high added value. Examples of similar initiatives have been the recovery of terpenes (Parra, López, & García-Granados, 2010), polyphenols (Luján, Capote, Marinas, & Luque de Castro, 2008), and polar lipids (Karantonis et al., 2008) from the oil solid waste while olive mill and table-olive brine wastewaters have been used for the extraction of phenolic compounds (Obied et al., 2005; Bouaziz et al., 2008; Hamza, Khoufi, & Sayadi, 2012) and polar lipids (Karantonis et al., 2008).

Regarding the olive stone, it has been proposed as a cheap source of protein (up to 22%) (Rodríguez et al., 2008). Despite this, it has been mainly used as an energy source (biomass) or for other purposes non focused on the recovery and use of olive seed

proteins (Rodríguez et al., 2008). Nevertheless, olive seed proteins could be of interest for the production of valuable substances such as bioactive peptides. Bioactive peptides can be defined as food components that have a positive impact on body functions or conditions and may ultimately influence health (Heisel & Fitzgerald, 2003). Bioactive peptides can demonstrate a wide variety of bioactivities being antihypertensive and antioxidant peptides the most popular.

Antioxidants intake has been inversely related with aging, cellular death, diabetes, and cancer. In fact, reactive oxygen species (ROS) cause lipid oxidation, protein oxidation, DNA strand break and base modification, and modulation of gene expression (Lee, Koo, & Min, 2004). Moreover, antioxidant compounds are very useful for the control of lipid oxidation in food products. Peroxidation of fatty acids can cause deleterious effects in foods resulting in the development of undesirable off-flavors, odors, dark colors, and potentially toxic reaction products (Rajapaks, Mendis, Jung, Je, & Kim, 2005). The demand for natural antioxidants has increased in the last years because of their safety and due to the negative consumer perception on synthetic antioxidants (Sakanaka, Tachibana, Ishihana, & Juneja, 2004; Sakanaka, Tachibana, Ishihara, & Juneja, 2005). On the other hand, hypertension is an important public-health challenge worldwide (Kearney et al., 2004). Despite the fact that there are a wide number of synthetic compounds for the treatment of hypertension, they can produce undesirable effects such as cough, taste disturbances or rashes. Because of this reason, antihypertensive peptides coming from food have become an interesting alternative.

Different studies have demonstrated the health benefits of some peptides obtained from oilseeds protein hydrolysates (García, Puchalska, Esteve, & Marina, 2013). Indeed, bioactive peptides with antihypertensive and antioxidant ability have already been

observed in sunflower seeds, rapeseed, maize, peanut, sesame, quinoa, amaranth, and soybean (Aluko, & Monu, 2003; Megias et al., 2004; Pedroche et al., 2004; Yust et al., 2004; Silva-Sánchez et al., 2008; Ren, Zheng, Liu, & Liu, 2010; Fritz, Vecchi, Rinaldi, & Añón, 2011).

The objective of the present study is to propose a new strategy for the recovery of waste proteins from olive seed and to evaluate their potential to produce antioxidant and antihypertensive peptides.

2. Materials and methods

2.1. Chemical and samples

Tris(hydroxymethyl)aminomethane (Tris), hydrochloric acid, sodium dodecyl sulfate (SDS) (all from Merck, Darmstadt, Germany), ammonium bicarbonate (ABC), potassium phosphate (PB), dithiothreitol (DTT) (all from Sigma-Aldrich, St. Louis, MO), and reagent grade acetone (from Scharlau) were used for the extraction and precipitation of olive seed proteins. Alcalase 2.4 L FG, Neutrase 0.8 L, Flavourzyme 1000 L, and PTN 6.0 Saltfree*N enzymes were kindly donated by Novozymes Spain S. A. (Madrid, Spain). Thermolysin, porcine pepsin, and pancreatin were obtained from Sigma-Aldrich. Sodium tetraborate, o-phthaldialdehyde (OPA), 2-mercaptoethanol, 1,1-diphenyl-2-picrylhydrazyl (DPPH), ethanol (EtOH), glutathione (GSH), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), potassium persulfate, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 1,10-phenantroline, linoleic acid, iron (II) chloride, ferrous sulphate ($FeSO_4$), ammonium thiocyanate, and hydrogen peroxide (H_2O_2), all obtained from Sigma-Aldrich, were employed for the antioxidant assays. Hippuryl-His-Leu

(HHL), hippuric acid (HA), angiotensin converting enzyme (ACE) from rabbit lung, HEPES, and sodium chloride (Sigma-Aldrich) were used for the ACE-inhibitory assay. Supergradient HPLC grade acetonitrile (ACN) (Scharlau, Barcelona, Spain), formic acid (FA), acetic acid (AA) (Sigma-Aldrich), and HPLC grade water (Milli-Q system; Millipore, Bedford, MA) were used in the preparation of mobile phases. Glycerol and off-gel ampholyte buffer 3-10 from Agilent Tecnologies (Pittsburgh, PA) were used for the off-gel IEF separation. Raw olives of 'Arbequina' variety with violet maturity index were collected in Toledo (Spain). Olive fruits were manually depulped and stones stored at -20 ºC until use.

2.2. Protein isolation

Olive stones were crashed to release the seed and seeds were ground in a domestic mill. Protein extraction from olive seeds was performed by a method previously developed (Esteve, del Río, Marina, & García, 2010). Briefly, 0.03 g of olive seeds were mixed with 5 mL of an extracting solution containing 125 mM Tris-HCl (pH 7.5), 1% (m/v) SDS, and 0.1% (m/v) DTT. The mixture was vigorously shaken for 1 min and centrifuged twice at 4000g for 10 min. Proteins in the supernatant were precipitated with 25 mL of cold acetone at -20 ºC for 1 h and collected by centrifugation for 10 min at 4 ºC. Proteins were precipitated again with 10 mL of cold acetone, centrifuged, and dried at room temperature.

2.3. Hydrolysis of the olive seed protein isolate and ultrafiltration

The olive seed protein isolate was hydrolysed with five different enzymes (Alcalase, Thermolysin, Neutrase, PTN, and Flavourzyme) using optimal hydrolysis conditions (see **Table 1**). The protein isolate was dissolved in the corresponding buffer up

126 to a final concentration of 5 mg/mL. Afterwards, the enzyme was added and the digestion
127 was carried out for 2 h. Then, the mixture was heated to 100 ºC for 10 min to deactivate
128 the enzyme and centrifuged at 7000g for 5 min. Enzyme autolysis blanks were obtained
129 for every enzyme under the same digestion conditions but in absence of sample. The
130 supernatants were taken for subsequent determination of the degree of hydrolysis (% DH)
131 and antioxidant and antihypertensive capacities.

132 Hydrolysates fractionation by ultrafiltration was performed using Vivaspin 500
133 PES molecular weight (Mw) cut-off filters (5 kDa) (Sartorius Stedim Biotech,
134 Goettingen, Germany) and AmiconMw cut-off filters (3 kDa) (Millipore).

135 *In vitro* gastrointestinal digestion was carried out using the method proposed by
136 Garret *et al.* (Garret, Failla, & Sarama, 1999). Briefly, most active fractions were
137 acidified (pH 2.0) with 1 M HCl and mixed with porcine pepsin at an enzyme-to-substrate
138 ratio of 1:35. The solution was incubated at 37 ºC by shaking at 300 rpm for 1 h. Next,
139 the pH of the resulting solution was raised to 5.0 by adding 0.1 M sodium bicarbonate and
140 to 8.0 with 0.1 M sodium hydroxide. Next, pancreatin enzyme dissolved in 0.1 M PB (pH
141 8.0) was added at an enzyme-to-substrate ratio of 1:25 and samples were incubated by
142 shaking at 37 ºC for 2 h. Finally, the mixture was heated to 100 ºC for 10 min for the
143 inactivation of enzyme.

144 *2.4. Peptide separation by off-gel IEF*

145 Fractionation of hydrolysates by their isoelectric point (pI) was performed using a
146 3100 OFFGEL Kit pH 3-10 (Agilent Technologies) with a 24-well setup. Prior to sample
147 loading, IPG gel strips (General Electric, supplied by VWR, Barcelona, Spain), with a
148 linear pH gradient ranging from 3 to 10, were rehydrated by adding 40 µL of peptide

focusing buffer (12% (v/v) glycerol and ampholytes) in every well. Sample (0.72 mL) was mixed with focusing buffer (2.88 mL) and 150 µL of this mixture were loaded to every well. The sample was, then, focused at a maximum current of 50 µA and voltages ranging from 500 to 4000 V until 50 kV/h were reached. Peptides fractionated by their pI were recovered and subjected to analysis.

2.5. Determination of the degree of hydrolysis (DH, %)

The % DH was determined using OPA (*o*-phthalaldehyde) method with some modifications (Want et al., 2008). OPA mixture (5 mL) was obtained by mixing 2.5 mL of 100 mM sodium tetraborate, 1 mL of 5% (m/v) SDS, 100 µL of 40 mg/mL of OPA dissolved in MeOH, 10 µL of 2-mercaptoethanol, and 1.39 mL of water. The OPA mixture (100 µL) was incubated with the sample (2.5 µL) for 8 min at room temperature. The absorbance at 340 nm of the resulting solution was then measured using a Lambda 35 spectrometer (Perkin-Elmer, Walthman, MA). Peptide concentration was calculated by interpolation in a calibration curve obtained using GSH (0-5 mg/mL) as standard.

2.6. Measurement of antioxidant capacity

Antioxidant capacity was evaluated by measuring the capability to scavenge different radicals (DPPH, ABTS, and hydroxyl radicals) and by evaluating the capacity to inhibit the peroxidation of linoleic acid. Appropriate solvent blanks were run in every assay. Three individual replicates were measured by triplicate in every determination.

2.6.1. DPPH radical-scavenging assay

Scavenging capacity of olive seed protein hydrolysates against DPPH radicals was evaluated according to You *et al.* (You, Zhao, Regenstein, & Ren, 2011) with some modifications. Briefly, 50 µL of hydrolysates were mixed with 50 µL of 0.1 mM DPPH

in 95% EtOH. The mixture was kept at room temperature for 30 min and the absorbance of DPPH radicals was measured at 517 nm. GSH (0-5 mg/mL) was used as positive control. Scavenging capacity was calculated using the following equation:

$$\text{DPPH radical scavenging capacity (\%)} = \left(1 - \frac{Abs_{sample} - Abs_{sample\_control}}{Abs_{blank}}\right) \times 100$$

were Abs_{sample} is the absorbance corresponding to the sample mixed with DPPH solution; $Abs_{sample\ control}$ is the absorbance corresponding to the sample mixed with 95% EtOH; and Abs_{blank} is the absorbance obtained with a solution containing digestion buffer mixed with DPPH solution.

2.6.2. ABTS radical-scavenging assay

ABTS radical scavenging capacity assay was performed according to Wiriyaphan *et al.* (Wiriyaphan, Chitsomboon, & Yongsawadigul, 2012) with some modifications. An $ABTS^{0+}$ stock solution was prepared by mixing 7.4 mM ABTS solution and 2.6 mM potassium persulfate solution in 10 mM phosphate buffer (PB) (pH 7.4) and kept in the dark for 16 h. Fresh $ABTS^{0+}$ working solution was prepared by diluting $ABTS^{0+}$ stock solution in 10 mM PB (pH 7.4) to attain an absorbance of 0.7 ± 0.01 at 734 nm. 1 µL of hydrolysate was mixed with 100 µL of fresh $ABTS^{0+}$ working solution. The reaction mixture was kept in the dark for 6 min and absorbance corresponding to ~~the~~ $ABTS^{0+}$ radicals was measured at 734 nm. ABTS radical scavenging capacity was calculated according to the following equation:

$$\text{ABTS radical scavenging capacity (\%)} = \left(\frac{Abs_{blank} - Abs_{sample}}{Abs_{blank}}\right) \times 100$$

were Abs_{sample} is the absorbance of 1 µL of sample with 100 µL of $ABTS^{0+}$ working solution; and Abs_{blank} is the absorbance of 1 µL of buffer with 100 µL of $ABTS^{0+}$ working solution. Furthermore, Trolox equivalent antioxidant capacity (TEAC, concentration of sample giving the same % of inhibition as 1 mM Trolox) was also determined. For that purpose, ABTS radical scavenging capacities of hydrolysates and Trolox at five different concentrations (3-35 µg/mL and 0-15 µM, respectively) were determined and plotted against their corresponding concentrations. TEAC values were obtained by the comparison of the slopes corresponding to these plots.

2.6.3. Hydroxyl radical scavenging assay

Hydroxyl radical scavenging capacity was assayed according to the method of Ajibola *et al.* (Ajibola, Fashakin, Fagbemi, & Aluko, 2011) with some modifications. 3 mM 1,10-phenanthroline was dissolved in 0.1 M PB (pH 7.4) while $FeSO_4$ (3 mM) and 0.01% (v/v) H_2O_2 were separately dissolved in distilled water. 25 µL of 1, 10-phenanthroline and 25 µL of $FeSO_4$ were added to 25 µL of sample. To initiate reaction in wells, 25 µL of H_2O_2 solution was added to the mixture and incubated at 37 ºC for 1 h. Thereafter, the absorbance of the mixtures was measured at 536 nm. GSH at 0-5 mg/mL was used as positive control. Hydroxyl radical scavenging capacity was calculated by using the following equation:

$$\text{Hydroxyl radical scavenging capacity (\%)} = \left(\frac{Abs_{sampe} - Abs_{blank}}{Abs_{control} - Abs_{blank}} \right) \times 100$$

where Abs_{sample} is the absorbance of the sample; Abs_{blank} is the absorbance of the blank solution using distilled water instead of sample; and $Abs_{control}$ is the absorbance of a control solution in absence of H_2O_2.

2.6.4. Lipid peroxidation inhibition assay

The antioxidant capacity of olive seed protein hydrolysates was also evaluated following ~~a~~ linoleic acid as model according to the method of Chen *et al.* (Chen, Muramoto, Yamauchi, & Nokihara, 1996) with some modifications. 20 µL of sample was mixed with 20 µL of a solution of 0.13% (v/v) of linoleic acid in EtOH and 10 µL of water. The mixture was incubated for 144 h (6 days) at 40 ºC in the dark and the degree of oxidation was evaluated at different times by measuring ~~the~~ ferric thyocianate absorbance (Chen, Muramoto, & Yamauchi, 1995). For that purpose, 2.5 µL of reaction solution were mixed with 175 µL of 75% (v/v) EtOH, 2.5 µL of 30% (m/v) ammonium thyocianate, and 2.5 µL of 20 mM ferrous chloride in 3.5% (v/v) HCl. After standing for 3 min, the thyocianate absorbance was measured at 500 nm. Blank digestion was carried out in absence of sample for every enzyme. GSH at 1 mg/mL was used as positive control. Lipid peroxidation inhibition capacity was calculated by using the following equation:

$$\text{Lipid peroxidation inhibition activity (\%)} = \left[1 - \frac{\left(Abs_{sample,144h} - Abs_{sample,0h}\right)}{Abs_{blank,144h} - Abs_{blank,0h}}\right] \times 100$$

where $Abs_{sample,144h}$ and $Abs_{sample,0h}$ are the absorbances for the sample at 144 h and 0 h, respectively; and $Abs_{blank,144h}$ and $Abs_{blank,0h}$ are the absorbances for the blank at 144 h and 0 h, respectively.

2.7. Measurement of antihypertensive capacity

ACE (0.05 U/mL) was dissolved in water and HHL (1.25 mg/mL) was dissolved in 50 mM HEPES buffer (pH 8.3) containing 300 mM NaCl. The resulting solution was made up of 20 µL ACE, 10 µL HHL, 35 µL HEPES with NaCl, and 5 µL sample

solution. A control reaction mixture containing 5 µL of every digestion buffer was also employed. After incubation at 37 ºC in a water bath, the reaction was stopped by the addition of 100 µL ACN at -20 ºC. Finally, HHL and reaction product HA were measured by HPLC-MS.

HPLC-MS experiments were performed in a modular capillary chromatographic system (Agilent Technologies) consisting of a micro vacuum degasser (model 1100), a capillary LC pump (model 1100), a thermostated autosampler (model 1100), a thermostated column compartment (model 1200), and a multiple wavelength detector (model 1200). A C18 HPLC column Zorbax 300 SB (150 mm x 0.5 mm ID, 5 µm particle size, and 80 Å pore size) from Agilent Technologies was employed. The chromatographic conditions were: flow-rate, 20 µL/min; gradient, 5-100% B in 7 min; mobile phases, 0.5% (v/v) AA in water (phase A) and 0.5% (v/v) AA in ACN (phase B); injection volume, 1 µL; separation temperature, 25 ºC; and UV detection wavelength, 228 nm.

An ion trap mass spectrometer model amaZon SL (BrukerDaltonics, Bremen, Germany), equipped with an ESI source was used for quantitation of ACE inhibition as proposed by Geng*et al*. (Geng, He, Yang, & Wang, 2010). Nebulizer and drying gas conditions were 6.0 psi and 3.0 L/min at 200 ºC. ESI source operated in the negative ion mode (+ 3500 V) with an end plate offset of – 500 V. During the analysis, two reference masses: 121.0509 ($C_5H_4N_4$) and 922.0098 ($C_{18}H_{18}O_6N_3P_3F_{24}$) were continuously measured to allow constant mass correction and obtain accurate masses. $[M-H]^-$ ions at 178 *m/z* (generated from HA) and 428 *m/z* (generated from HHL) were measured. Data acquisition and analysis were performed using the DataAnalysis software. ACE inhibition was calculated as follows:

$$\% \text{ ACE Inhibition} = \frac{c_0 - c}{c_0} \times 100$$

where c_0 is the HA concentration without ACE inhibition and c is the HA concentration with inhibitor. IC_{50} values, defined as the concentration of peptide in µg/mL required to produce 50% inhibition of ACE, were also calculated. For that purpose, different solutions containing hydrolysate at different final concentrations (0.02-0.3 mg/mL) were mixed with the reaction mixture and the % of ACE inhibition was calculated. For every hydrolysate, the % ACE inhibition was plotted against hydrolysate concentration and the IC_{50} value was determined from the signal corresponding to a 50% ACE inhibition.

2.8. RP-HPLC separation of hydrolysates

Separations were carried out in an Agilent Technologies 1100 series liquid chromatograph (Agilent Technologies) equipped with a diode array detector, an automatic injector, a degasser system, a quaternary pump, and a thermostated column compartment. HP Chemstation software was used for instrument control and data acquisition. Three different reversed-phase HPLC columns were employed: two different POROS R2/H perfusion columns (50 x 4.6 mm and 100 x 2.1 mm) from Perseptive Biosystems (Framingham, MA) and one monolithic silica column, Chromolith Performance RP-18e (100 x 4.6 mm) from Merck. Mobile phases consisted of 0.1% (v/v) FA in Milli-Q water (mobile phase A) and 0.1% (v/v) FA in ACN (mobile phase B). Separations were performed using a gradient: 3-30% B in 10 min and 30-95% B in 4 min. The flow-rate was 1 mL/min, column temperature was 25 ºC, and sample volume was 100 µL.

2.9. LC-MS/MS analysis of hydrolysates

MS/MS detection was carried out in a Quadrupole Time-of-Flight (Q-TOF) series 6530 mass spectrometer coupled to a liquid chromatograph (model 1100), both from Agilent Technologies. A guard column Ascentis Express Peptide ES-C18 (5 mm x 2.1 mm, 2.7 µm particle size) and an analytical column Ascentis Express Peptide ES-C18 (100 mm x 2.1 mm, 2.7 µm particle size, 160 Å pore size) were employed for peptide separation. Mobile phases A and B consisted of 0.1% (v/v) FA in water and 0.1% (v/v) FA in ACN, respectively. The flow-rate was set to 0.4 mL/min and the gradient was 3% B during 5 min, 3-20% B in 25 min, and 20-95% B in 5 min. The column temperature was set at 55 ºC and the injection volume was 20 µL. The mass spectrometer operated with an ESI Jet Stream source in the positive ion mode and the analyzer in mode MS/MS scanned in the range 50-3000 *m/z*. The dry gas conditions were 10 L/min and 300 ºC, the nebulizer pressure was 50 psig, and the sheath gas flow and temperature were 5.5 L/min and 250 ºC, respectively. Other MS conditions were: capillary voltage, 3500 V; fragmentator voltage, 200 V. Collision-induced dissociation (CID) at 35% energy was used for MS^2 experiments. Peaks Studio 6 (Bioinformatics Solutions Inc., Waterloo, ON, Canada) program was used for the treatment of MS/MS data and *do novo* peptide sequencing using an average local confidence (ALC% indicates the expected percentage of correct amino acids in the peptide sequence) above 90%. Peptide identifications were accepted if they appeared in, at least, two independent samples.

3. Results and discussion

Proteins extracted from olive seeds using a previously optimized method (Esteve, del Río, Marina, & García, 2010) were hydrolysed with five different enzymes: Alcalase, Thermolysin, Neutrase, Flavourzyme, and PTN. These enzymes were chosen taking into

account bibliographic data on enzymes used to produce bioactive peptides (Aluko, & Monu, 2003; Klompong, Benjakul, Kantahote, Hayes, & Shahidi, 2008; Wang, Tan, Chen, & Yan, 2009; Aijoba, Fashakin, Fagbemi, & Aluko, 2011; Valdez-Ortíz, Fuentes-Gutiérrez, Germán-Báez, Gutiérrez-Dorado, & Medina-Godoy, 2012; Wiriyaphan, Chitsomboon, & Yongsawadigul, 2012).

3.1. Optimization of enzymatic hydrolysis

Table 1 groups the optimal conditions used in the digestion of olive seed proteins with the selected enzymes. Digestion conditions were chosen after bibliographic revision. When the % DH obtained using these conditions were not satisfactory, the following enzymatic parameters were optimized: substrate concentration, reaction time, enzyme concentration, pH, and digestion buffer.

The substrate concentration was optimized using concentrations ranging from 2.5 to 10.0 mg seed protein isolate/mL and digesting with Alcalase dissolved in PB 5 mM (pH 8.0) at a temperature of 55 ºC and a digestion time of 2 h. Reaction was monitored by determining the % DH. The optimum substrate concentration for hydrolysis was 5 mg/mL since higher concentrations resulted in lower % DH and lower concentrations resulted in a similar % DH. This protein concentration was employed for all enzymes in order to be able to compare antioxidant and antihypertensive capacities. Next, the influence of reaction time, enzyme concentration, pH, and buffer on the % DH was investigated. Reaction times between 1 and 24 h were assayed with all enzymes. An initial rapid increase in % DH was observed during the first 2 h followed by a plateau or a very slow increase in % DH. Consequently, optimum digestion time was established in 2 h for all enzymes. For some enzymes, different enzyme:substrate ratios were next tried: for Alcalase, 0.15-1.5 AU/g protein; for Neutrase, 300-3000 U/g protein; for PTN, 0.001-

0.01 g enzyme/g protein. The optimum enzyme:substrate ratio, defined as that yielding maximum % DH, is indicated in Table 1. Higher enzyme concentrations did not result in an increase in % DH. The influence of pH and buffer on the hydrolysis of the seed protein isolate was studied for Alcalase, Thermolysin (both with PB (5 mM at pH 8.0) and Tris-HCl buffer (5 mM at pH 8.0 and 9.0)), and Neutrase (PB (5 mM at pH 7.0) and Tris-HCl buffer (5 mM at pH 7.0)). Results showed the highest % DH when PB was employed, choosing as optimal conditions PB 5 mM at pH 8.0 for Alcalase and Thermolysin and PB at pH 7.0 for Neutrase.

Peptide concentrations obtained with every enzyme under optimized conditions were determined according to the OPA method. Results, summarized in **Table 2**, showed that Alcalase was the enzyme yielding the highest hydrolytic activity (% DH of 70.4). This fact had already been observed with this enzyme (Valdez-Ortíz, Fuentes-Gutiérrez, Germán-Báez, Gutiérrez-Dorado, & Medina-Godoy, 2012), being the reason why it is one of the most employed enzymes for the hydrolysis of food proteins. Intermediate hydrolysis rates were obtained when using Thermolysin, Flavourzyme, and Neutrase, with 64.6, 61.2, and 59.0% DH, respectively. PTN showed the poorest hydrolysis rate with 49.6% DH. These results could be explained taking into account that PTN has tryptic activity and, consequently, a relatively high selectivity while the other enzymes are less specific.

3.2. Antioxidant capacity of olive seed protein hydrolysates

3.2.1. Radical scavenging capacity

Since the radical system used for the evaluation of antioxidant capacity may significantly influence results, it is widely suggested the use of different radical systems

352 to assess this capacity (Fuglsang, Rattray, Nilsson & Nyborg, 2003). The antioxidant
353 capacity of protein hydrolysates was determined by the evaluation of their capacity to
354 scavenge DPPH, ABTS, and hydroxyl radicals, and results are summarized in Table 2.

355 All obtained hydrolysates were capable of scavenging DPPH radicals. DPPH
356 radical-scavenging capacities ranged from 42.8 to 68.6 %. The highest antioxidant
357 capacities were obtained with Alcalase (68.6%) and Neutrase (61.2%) enzymes, while the
358 lowest capacities were obtained for Flavourzyme (52.5%) and PTN (42.8%).

359 All protein hydrolysates exhibited good antioxidant capacity when using the
360 ABTS radical scavenging assay. In fact, antioxidant capacities ranging from 28.9 to
361 72.0% (TEAC values from 178.6-363.3) were obtained. These TEAC values are in the
362 range of other protein hydrolysates with recognized antioxidant activity such as that of
363 Douchi, a traditional Chinese salt-fermented soybean food (Esteve, del Río, Marina, &
364 García, 2010). Alcalase was again the enzyme showing the highest antioxidant capacity.

365 All hydrolysates showed capacity to scavenge hydroxyl radicals, being Alcalase
366 and Neutrase hydrolysates the most active ones. Taking into account the biological
367 significance of this radical, these results are of great interest from a physiological point of
368 view.

369 *3.2.2. Lipid peroxidation inhibition capacity*

370 The inhibitory effect of olive seed protein hydrolysates on the peroxidation of
371 lipids was evaluated using linoleic acid as model. Results obtained are grouped in Table
372 2. Autooxidation of linoleic acid in absence of antioxidant quickly increased from day 1
373 to day 6. However, in presence of GSH and every hydrolysate, that linoleic acid

peroxidation resulted inhibited. As shown in Table 2, the percentage of linoleic acid autooxidation inhibition was very high for all hydrolysates, ranging from 79.5 to 91.2%.

Overall, olive seed protein hydrolysates seem to contain antioxidative peptides. Alcalase hydrolysate, yielding the highest % DH, showed also the highest antioxidant capacity in all assays. Nevertheless, % DH was not always related with the antioxidant capacity, as it is observed in the Thermolysin digestion. Therefore, Alcalase hydrolysate was chosen for its further fractionation.

3.3. Antihypertensive capacity of olive seed protein hydrolysates

The hydrolysates obtained with Alcalase, Thermolysin, Neutrase, Flavourzyme, and PTN were next screened using the ACE inhibitory assay to evaluate their antihypertensive capacity. As shown in Table 2, Thermolysin hydrolysate showed the highest ACE inhibition capacity (29 µg/mL). Thermolysin specially catalyzes the hydrolysis of peptide bonds containing hydrophobic amino acids and it has been demonstrated that most powerful antihypertensive peptides contain hydrophobic C-terminal amino acids, as proline, or positive charged ions as lysine or arginine (Fuglsang, Rattray, Nilsson, & Nyborg). In fact, this enzyme has been widely employed for the release of hydrophobic peptides from food sources (Puchalska, Marina, & García, 2012). Alcalase, Neutrase, and PTN showed moderate inhibition capacities, while Flavourzyme displayed the lowest one. IC_{50} values were similar to those reported for other seed protein hydrolysates as quinoa (Megías et al, 2004) and amaranth (Aluko, & Monu, 2003), with recognized antihypertensive activity.

3.4. Fractionation of hydrolysates and evaluation of antioxidant and antihypertensive capacities of fractions

Most antioxidant (Alcalase hydrolysate) and antihypertensive (Thermolysin hydrolysate) hydrolysates were next fractionated by ultrafiltration. Fractions with molecular masses > 5 kDa, 3-5 kDa, and < 3 kDa were obtained and peptide concentration and antioxidant and antihypertensive capacities were evaluated in every fraction. **Figure 1** shows the results obtained for every fraction from the Alcalase hydrolysate (with the highest antioxidant capacity) in comparison with the whole Alcalase hydrolysate. The OPA assay showed the highest percentage of peptides in the fraction with molecular masses below 3 kDa. This fraction also resulted in the greatest antioxidant capacity being selected for its further fractionation. Similarly, **Figure 2** summarizes the peptide concentration and antihypertensive capacity of the fractions obtained from the Thermolysin hydrolysate. Again, the Thermolysin hydrolysate was made up mainly of short-chain peptides, with molecular masses below 3 kDa. Furthermore, IC_{50} values indicated that the antihypertensive capacity was concentrated on those small peptides while peptides from 3 to 5 kDa showed the lowest antihypertensive capacity. These results are in agreement with the knowledge that a lot of ACE inhibitors are peptides that present short sequences, with a number of amino acids ranging from 2 to 12 (Natesh, Schwager, Sturrok, & Acharya, 2003).

A further attempt to fractionate was next tried with the most antioxidant and antihypertensive fractions (Alcalase and Thermolysin fractions with peptides below 3 kDa) according to their pI by IEF. Unfortunately, ampholytes employed for the establishment of the pH gradient highly interfered on the assays used for the evaluation of the peptide concentration and antioxidant and antihypertensive capacities. In order to separate ampholytes and peptides, several clean up steps were tried (C_{18} SPE and RP-HPLC using perfusion and monolithic columns) but in no case, they resulted successful.

As a consequence, further fractionation by IEF was finally rejected and peptides in the ultrafiltration fractions were next identified.

3.5. Identification of peptides by HPLC-MS/MS

The fractions with molecular masses below 3 kDa, obtained by both the Alcalase and the Thermolysin hydrolysis, were injected into the HPLC-Q-TOF system for the identification of peptides. A high percentage of peptides presented a very high hydrophilic nature, small masses, and +1 charge, which made very difficult their identification. **Figure 3** shows, as examples, the MS/MS spectra obtained for two of the identified peptides: VLDTGLAGA in the Alcalase hydrolysate and LVVDGEGY in the Thermolysin hydrolysate.

Table 3 shows the sequences of the identified peptides on each fraction by *de novo* sequencing using PEAKS software. Peptide identifications were accepted if they appeared in, at least, two individual samples, and presented an average local confidence (ALC%, it indicates the expected percentage of correct amino acids in the peptide sequence) above 90%. The sequence of every peptide was searched on the BIOPEP database, but no correspondence with any already reported bioactive peptide was found, even though this fact does not exclude them as possible potential bioactive peptides.

The antioxidant peptides described in bibliography usually contain between 2 and 15 amino acids and present a high amount of histidine and hydrophobic amino acids (Erdman, Cheung, & Schroeder, 2008). If we take a look at **Table 3.a**, we observe a poor presence of histidine, but a very significant presence of hydrophobic peptides such as alanine, valine, leucine/isoleucine, proline, methionine, phenylalanine, and tryptophan. It has been demonstrated that most active antihypertensive peptides contain hydrophobic

444 amino acids as proline or positive charged amino acids as lysine or arginine within the
445 three amino acids closest to the C-terminal position (Natesh, Schwager, Sturrok, &
446 Acharya, 2003). Among the identified peptides in the olive seed hydrolysate, the presence
447 of proline within the last three amino acids was observed in peptides LLPSY, ALMSPH,
448 and LYSPH.

449 *3.6. Evaluation of antioxidant and antihypertensive capacity of most active fractions after*
450 *in vitro gastrointestinal digestion*

451 Most antioxidant and antihypertensive fractions (fractions < 3 kDa for Alcalase
452 and Thermolysin hydrolysates) were next subjected to *in vitro* gastrointestinal digestion
453 in order to evaluate their resistance to this process. Results obtained revealed a slight
454 increase in the antioxidant capacity after *in vitro* gastrointestinal digestion of fraction < 3
455 kDa from the Alcalase hydrolysate, showing a TEAC value of 382 ± 24. Similarly, the
456 IC_{50} value corresponding to the fraction < 3 kDa from the Thermolysin hydrolysate was
457 slightly higher (36 µg/mL) after gastrointestinal digestion. Regarding % DH, as it was
458 expected, it increased after the gastrointestinal digestion, being 82.0 ± 5.1 and 69.6 ± 3.6
459 for Alcalase and Theromolysin hydrolysates, respectively. These data revealed that
460 antioxidant and antihypertensive peptides extracted from olive seeds seemed to keep their
461 bioactivities after *in vitro* gastrointestinal digestion.

462

463 **4. Conclusions**

464 Olive seed hydrolysates obtained with five different enzymes demonstrated to
465 possess antioxidant properties being Alcalase the enzyme yielding the highest capacity.
466 Moreover, the hydrolysate obtained when using Thermolysin enzyme showed an

important antihypertensive capacity. In both cases, the highest capacities were observed in the fraction containing the smallest peptides (< 3 kDa). HPLC-MS/MS analysis enabled the identification and *do novo* sequencing of peptides in these fractions. These peptides presented common characteristics with other antioxidant and antihypertensive peptides already described in bibliography. Furthermore, these fractions also maintained their bioactivities after being subjected to *in vitro* gastrointestinal digestion. This shows that olive seed proteins constitute a cheap and valuable source of antioxidant and antihypertensive peptides. These results could be useful for the proposal of a strategy for the recovery of this waste material, which in turn could decrease the environmental impact of these residues and the cost related with their elimination.

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611

612

613 **Figure captions**

614 **Figure 1.** Peptide concentration and ABTS and hydroxyl radical scavenging capacities of
615 whole Alcalase hydrolysate and fractions with Mw higher than 5 kDa, between 3 and 5
616 kDa, and lower than 3 kDa.

617 **Figure 2.** Peptide concentration and ACE-inhibitory capacity (expressed as IC_{50}) of
618 whole Thermolysin hydrolysate and fractions with Mw higher than 5 kDa, between 3 and
619 5 kDa, and lower than 3 kDa.

620 **Figure 3.** Fragmentation spectra of VLDTGLAGA (a) and LVVDGEGY (b) peptides
621 obtained from the fractions with Mw below 3 kDa from the Alcalase and Thermolysin
622 hydrolysates, respectively.

Table 1. *Optimal conditions used for the hydrolysis of the olive seed protein isolate with different enzymes.*

Enzyme	Temperature (ºC)	Hydrolysis time (h)	Enzyme:substrate ratio	pH (buffer)
Alcalase	50	2	0.15 AU/g protein	8.0 (PB 5 mM)
Thermolysin	50	2	0.05 g enzyme/g protein	8.0 (PB 5 mM)
Neutrase	50	2	300 U/g protein	7.0 (PB 5 mM)
Flavourzyme	50	2	50 U/g protein	6.0 (ABC 5 mM)
PTN	50	2	0.001g enzyme/g protein	9.0 (Tris-HCl 5 mM)

Table 2. *Hydrolysis degree (% DH), DPPH, ABTS, and hydroxyl radical scavenging capacities, lipid peroxidation inhibition capacity, and ACE-inhibition capacity of olive seed protein hydrolysates.*

Protease	% DH	DPPH radical scavenging capacity (%)	ABTS radical scavenging capacity (%)	TEAC value[a]	Hydroxyl radical scavenging activity (%)	Lipid peroxidation inhibition (%)	ACE-Inh assay, IC_{50} (mg/mL)
Alcalase	70.4 ± 6.2	68.6 ± 5.6	72.0 ± 4.3	363 ± 28	54.5 ± 3.3	91.2 ± 6.9	0.20 ± 0.04
Thermolysin	64.6 ± 5.7	57.9 ± 4.3	55.2 ± 3.8	178 ± 22	37.3 ± 4.9	83.7 ± 5.9	0.029 ± 0.009
Neutrase	59.0 ± 4.4	61.2 ± 5.8	46.5 ± 4.9	272 ± 23	54.7 ± 5.0	79.5 ± 8.2	0.21 ± 0.06
Flavourzyme	61.2 ± 6.4	52.5 ± 4.5	50.8 ± 3.1	222 ± 14	43.6 ± 5.2	85.8 ± 5.8	0.35 ± 0.11
PTN	49.6 ± 4.9	42.8 ± 4.9	28.9 ± 4.6	233 ± 22	31.9 ± 3.6	85.0 ± 6.9	0.22 ± 0.06
Control		54.3 ± 4.5[b]	40.4 ± 3.9[c]		66.1 ± 6.7[b]	89.1 ± 7.2[c]	

[a] Trolox equivalent antioxidant capacity (TEAC) values expressed as micromoles of Trolox equivalents per gram of sample.

[b] Using GSH (1 mg/mL) as positive control.

[c] Using Trolox (1 mM) as positive control.

Table 3. *Sequence, average local confidence (ALC, %), and mass of peptides identified in the Alcalase fraction showing the highest antioxidant capacity (a) and in the Theromolysin fraction with the highest antihypertensive capacity (b).*

a)

Peptide sequence	ALC (%)	Mass
VLDTGLAGA	96	815.439
VPLSPT	94	612.348
FDGEVK	93	693.333
VLSPPFTGE	93	945.481
MDGAP	92	489.189
APGAGVY	92	633.312
VGPLSPT	91	669.370
VVVVPH	91	648.396
DDLPR	90	614.302
HVAGTVA	90	653.350
MGSPT	90	491.205
VGAPSVS	90	615,323

b)

Peptide sequence	ALC (%)	Mass
LTPTSN	98	631.318
LVVDGEGY	97	850.407
FDAVGVK	96	734.396
AFDAVGVK	94	805.433
VGVPGGV	93	583.333
LLPSY	92	591.327
ALMSPH	92	654.316
LFSGGES	91	695.313
LMSPH	90	583.279
LPAGA	90	724.4464