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# PLUM (*Prunus Domestica L.*) BY-PRODUCT AS A NEW AND CHEAP SOURCE OF BIOACTIVE PEPTIDES: EXTRACTION METHOD AND PEPTIDES CHARACTERIZATION

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**Short title: CHEAP BIOACTIVE PEPTIDES FROM PLUM (*Prunus Domestica L.*) BY-PRODUCT**

## ABSTRACT

Fruit processing produces a great amount of wastes. Stone of fruits such as plum (*Prunus Domestica L.*) are part of these residues. Plum stones contain a seed with a high content in proteins and lipids. Although the plum seed oil has attracted some attention, seed proteins are totally underused and undervalued. This work proposes, for the first time, to extract proteins from this by-product, to evaluate their potential as a source of bioactive peptides, and to identify by using RP-HPLC-MS/MS obtained peptides. A method for the extraction of plum seed proteins using high intensity focused ultrasounds has been developed. Extracted proteins have been digested using four different enzymes (Alcalase, Thermolysin, Flavourzyme, and Protease P). Digestion protocols were optimized and bioactive properties of resulting peptides were evaluated. Alcalase was the enzyme yielding the extracts with the highest ABTS radical scavenging capacity (79.8 %) and with the highest capacity to inhibit lipid peroxidation (95 %). Since this enzyme also showed a high capacity to scavenge hydroxyl radicals (52.3 %) and to inhibit ACE enzyme ($IC_{50}$= 0.4 mg/mL), it was selected for the identification of peptides. Analysis of the Alcalase extract by RP-HPLC-ESI-Q-TOF enabled the identification of short chain and highly hydrophobic peptides.



**Keywords**: fruit by-product; bioactive peptide; plum seed; antioxidant; RP-HPLC-ESI-Q-TOF.

## 1. Introduction

Food industry produces a large amount of waste whose percentage ranges from 5 to 90% depending on the processed raw material (Restrepo, 2006). Specifically, the processing of fruits and vegetables is characterized by generating most part of this waste material. These residues are used, in part, for animal feeding and the rest is discarded. Some alternative uses of these solid residues are the production of fertilizers and fuels (biomass) (Deng et al., 2012; Duda-Chodak & Tarko, 2007; Rodríguez et al., 2008). In the case of some fruits, skin and stone have been employed in the manufacture of foodstuffs and cosmetics (mango peel marmalade, peach pit oil, etc.). However, it is still small the waste fraction that is reused. In order to contribute to environmental sustainability it is necessary to recover these residues and to look for new opportunities of application. In this sense, there are initiatives in which fruit by-products have been used for the production of substances with high added value. Some examples are the use of flavonoids extracted from the lemon peel or the use of the dietary fiber and anthocyanins contained in the waste generated in the production of fruit juices (Russo, Bonaccorsi, Torre, Saró, Dugo, Mondello, 2014; Zhang et al., 2013).

Stones of fruits such as plum (*Prunus domestica L.*) consist of a hull covering the seed. This underused seed is rich in proteins and lipids, thus, they maybe a cheap source of substances that could be useful for food, cosmetic, and pharmaceutical industries. The lipid content of plum seeds has already been explored (Hassanein, 1999). In order to exploit plum seeds as cheap sources of proteins, a higher knowledge on the extraction of proteins from these matrices is required. Protein extraction from plant tissues is a major challenge since plant cells contain relatively low concentrations of soluble proteins due to the presence of vacuoles and rigid cell walls (Wang, Tai & Chen, 2008; Rose, Bashir, Giovannoni, Jahn & Saravanan, 2004). Furthermore, plant

tissues contain a large amount of proteases and other compounds such as polysaccharides, lipids, phenolic compounds, and secondary metabolites that can interfere in both extraction and subsequent separation and detection of proteins (Desfrancs, Thiellement & Devienne, 1985; Rabilloud, 1996; Gegenheimer, 1990). Consequently, protein extraction from plants usually is a tedious process that is difficult to automate and, very often, presents limited effectiveness and reproducibility. Extraction techniques such as high-intensity focused ultrasounds (HIFU) have allowed the acceleration of extraction processes. An ultrasound probe provides mechanical energy in form of acoustic energy when it is introduced into a fluid. This mechanical energy allows the acceleration of reactions and processes based on a phenomenon known as cavitation (Awad, Moharram, Shaltout, Asker & Youssef, 2012).

A potential application of extracted proteins could be the production of bioactive peptides. Indeed, bioactive peptides can be in a latent state as part of the sequence of a protein from which they may be released by digestion (García, Puchalska, Esteve & Marina, 2013). A peptide is a functional ingredient if it has been successfully demonstrated its beneficial effect on one or more functions of the body beyond its nutritional effects, so that their effect is significant for health, in general, or enables to reduce the risk to suffer a disease (Diplock, Aggett, Ashwell, Bornet, Fern & Roberfroid, 1999). Bioactive peptides can present diverse activities (antioxidant, antihypertensive, hypocholesteroalemic, immunostimulating, etc.) although antioxidant and antihypertensive activities are the most common (Sarmadi & Ismail, 2010).

The growing interest in finding new approaches for the recovery of waste material from food industry and the production of valuable substances, makes the main objective of this work was to develop a method enabling the extraction and digestion of proteins

from plum seeds (waste product), to evaluate the presence of peptides with antioxidant and potential antihypertensive capacities, and to identify these peptides by RP-HPLC-ESI-Q-TOF.

## 2. Materials and methods

### 2.1. *Chemicals and samples*

All chemicals and reagents were of analytical grade. Water was daily obtained from a Milli-Q system from Millipore (Bedford, MA, USA). Supergradient HPLC grade acetonitrile (ACN), acetic acid, acetone, methanol (MeOH), and hexane were purchased from Scharlau Chemie (Barcelona, Spain). Tris(hydroxymethyl)aminomethane (Tris), hydrochloric acid (HCl), sodium dihydrogen phosphate, ammonium bicarbonate (ABC), and sodium dodecyl sulphate (SDS) were from Merck (Darmstadt, Germany). Sodium hydroxide, dithiothreitol (DTT), trifluoroacetic acid (TFA), bovine serum albumin (BSA), o-phthalaldehyde (OPA), sodium tetraborate (STB), β-mercaptoethanol (β-ME), glutathione (GSH), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), potassium persulphate, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox), 2,2-diphenyl-1-picrylhydrazyl (DPPH), 1,10-phenantroline, ferrous sulphate, hydrogen peroxide, potassium ferricyanide, trichloroacetic acid (TCA), ferric chloride, linoleic acid, ammonium thiocyanate, ferrous chloride, ACE from rabbit lung, hippuryl-histidyl-leucine (HHL), 2-[4-(2-hydroxyethyl)-1-piperazinyl]-ethanesulphonic acid (HEPES), sodium chloride, and Thermolysin were all purchased from Sigma-Aldrich (Saint Louis, MO, USA). Laemmli buffer, Mini-Protean precast gels, Tris/glycine/SDS running buffer, Bio-Safe Coomassie stain, Precision Plus Protein Standards (recombinant proteins expressed by *E. Coli* with molecular weights of 10, 15, 20, 25, 37, 50, 75, 100, 150, and 250 kDa), and Bradford reagent (Coomassie Blue G-250) were acquired at Bio-Rad (Hercules, CA, USA). Alcalase 2.4 L FG and Flavourzyme 1000 L were kindly donated by Novozymes Spain S.A. (Madrid, Spain). Protease P was generously donated by Amano (Amano Enzyme Inc., Nagoyo, Japan). Seeds were from plums purchased in a local market (Alcalá de Henares, Madrid, Spain).

## 2.2.*Protein extraction*

Plums were manually pitted and seeds were extracted with a nutcracker. Seeds were ground with a domestic mill and stored at -20ºC until use. Seeds moisture was determined keeping them at 103 ± 2 ºC until constant weight.

Ground seeds (0.5 g) were defatted three times with 20 mL of hexane during 30 min with shaking. Proteins were extracted by mixing defatted seeds (30 mg) with 5 mL of a 100 mM Tris-HCl buffer (pH 7.5) containing 1 % (w/w) SDS and 0.25 % (w/w) DTT. In order to accelerate the extraction, an HIFU probe (model VCX130, Sonics Vibra-Cell, Hartford, CT, USA) at 30 % of amplitude was employed for 1 min. The resulting extract was centrifuged for 10 min at 4000 x g and the supernatant was mixed with 10 mL of cold acetone and kept in the fridge for 30 min. Finally, it was centrifuged for 10 min at 4000 x g and the precipitated proteins were redissolved in 100 mM Tris-HCl buffer (pH 7.5) containing 1 % (w/w) SDS. Protein extract was filtered through regenerated cellulose filters (0.45 µm). Bradford assay (Bradford, 1976) was used to estimate the protein content of extracts.

## 2.3. *RP-HPLC separation of proteins*

Protein separation was performed using a HPLC equipment from Agilent Technologies (Pittsburg, PA, USA) model 1100, equipped with a vacuum degasser, a quaternary pump, an automatic injection system, a thermostatic column compartment, a diode array detector, and a fluorescence detector. Control of the equipment and data acquisition were performed with the HP-ChemStation software. The separation was carried out in an Aeris Widepore XB-C18 column (100 mm x 2.1 mm ID and 3.6 µm particle diameter) from Phenomenex (Torrance, CA, USA). Elution gradient was 30-40 % B in 20 min. Mobile phases were: A, water with 0.1 % (v/v) TFA and B, ACN with

0.1 % (v/v) TFA. Rest of chromatographic conditions were: flow rate, 0.5 mL/min; temperature, 60 ºC; injection volume, 20 µL; UV detection at 210, 254, and 280 nm and fluorescence detection at $\lambda_{exc}$= 280 and $\lambda_{em}$= 360 nm.

### 2.4. *SDS-PAGE separation of proteins*

Proteins were also separated using a Bio-Rad Mini-Protean system (Hercules, CA, USA). Samples were prepared by mixing 15 µL of protein extract with 15 µL Laemmli buffer containing 5 % (v/v) β-ME and heating for 5 min at 100 ºC. Electrophoresis was carried out on commercial Ready Precast Gels using Tris/glycine/SDS as running buffer and applying 80 V for 5 min and 200 V for 30 min. For the estimation of molecular weights, protein standards (10, 15, 20, 25, 37, 50, 75, 100, 150, and 250 kDa) were used. After separation, proteins were fixed with 50 mL of 10 % (v/v) glacial acetic acid/40 % (v/v) MeOH shaking for 30 min and stained with 50 mL of Bio-Safe Coomassie stain by slightly shaking for 1 h. Finally, the gel was washed with water for 2 h.

### *2.5. Protein digestion*

Protein extracts obtained from plum seeds were hydrolyzed using four different food enzymes: Alcalase, Thermolysin, Flavourzyme, and Protease P. The optimized conditions for every enzyme are grouped in **Table 1**. In every case, the extract was dissolved in the corresponding buffer at a suitable concentration. Then, the enzyme was added at the optimum ratio and the solution was incubated in a Thermomixer Compact (Eppendorf AG, Hamburg, Germany) at the optimal temperature with agitation. The digestion was stopped (100 ºC for 10 min) and centrifuged for 5 min at 7000 x g. Finally, the supernatant was collected for the determination of the degree of hydrolysis and peptide bioactivity.

### *2.6. Determination of the degree of hydrolysis (%DH)*

The degree of hydrolysis was determined by the OPA assay, following a procedure previously described by Wang et al. (2008) with some modifications. This assay was based on the formation of an adduct between α-amino groups of peptides and OPA reagent. OPA mixture (5 mL) was prepared by mixing 2.5 mL of 100 mM sodium tetraborate, 1 mL of 5 % (w/v) SDS, 100 µL of 40 mg/mL OPA in MeOH, 10 µL of β-ME, and 1.39 mL of water. The assay consisted of mixing 2.5 µL of sample with 100 µL of OPA mixture and, after 8 min at room temperature, measuring the absorbance at 340 nm in a spectrophotometer Lambda 35 (Perkin-Elmer, Waltham, MA, USA). GSH (0 – 5 mg/mL) was used to build the calibration curve and the peptide content was obtained by interpolation. The degree of hydrolysis was calculated by the following equation:

$$DH = \frac{peptide\ content}{protein\ content}\ x\ 100 \qquad (1)$$

where the protein content was determined by Bradford assay. The replicates of every sample were measured at least three times.

### *2.7. In vitro evaluation of the antioxidant capacity of peptides*

Antioxidant capacity of hydrolyzates was measured using five different *in vitro* assays evaluating the ability to scavenge free radicals (ABTS, DPPH, and hydroxyl radicals), the ability to reduce Fe (III), and the ability to inhibit lipid peroxidation. Three replicates of each sample were measured at least three times. Solvent blanks were measured for every assay.

2.7.1. *ABTS radical scavenging assay*

Scavenging capacity against ABTS radicals was evaluated according to the method of Wiriyaphanet al. (2012) with slight modifications. In this assay, ABTS oxidation is produced by its reaction with potassium persulphate resulting in the formation of deep green $ABTS^{\bullet+}$. $ABTS^{\bullet+}$ stock solution containing 7.4 mM ABTS and 2.6 mM potassium persulphate in 10 mM phosphate buffer (PB) (pH 7.4) was prepared and kept in the dark for, at least, 16 h. The working solution was prepared daily by mixing the $ABTS^{\bullet+}$ stock solution with 10 mM PB (pH 7.4) to obtain an absorbance of 0.70 ± 0.01 at 734 nm. The assay was carried out by mixing 1 µL of sample with 100 µL of $ABTS^{\bullet+}$ working solution for 6 min at room temperature. Afterwards, the absorbance corresponding to $ABTS^{\bullet+}$ was measured at 734 nm. $ABTS^{\bullet+}$ scavenging capacity was calculated using the following equation:

$$ABTS\ radical\ scavenging\ capacity(\%) = \left(\frac{Abs_{blank} - Abs_{sample}}{Abs_{blank}}\right) x\ 100 \qquad (2)$$

where $Abs_{sample}$ is the absorbance of the sample with $ABTS^{\bullet+}$ and $Abs_{blank}$ is the absorbance of the digestion buffer with $ABTS^{\bullet+}$. Trolox (1 mM) was employed as positive control.

*2.7.2. DPPH radical scavenging assay*

DPPH radical scavenging capacity was evaluated through a method previously proposed by You et al. (2011) with some modifications. This assay was based on the reduction of DPPH radicals. The assay involved the reaction of 50 µL of sample with 50 µL of 0.1 mM DPPH solution in 95 % (v/v) EtOH in the dark and at room temperature. After 30 min reaction, the absorbance was measured at 517 nm and the DPPH radical scavenging capacity was calculated according to the following equation:

$$DPPH\ radical\ scavenging\ capacity\ (\%) = (1 - \frac{Abs_{sample} - Abs_{control}}{Abs_{blank}})\ x\ 100 \quad (3)$$

where $Abs_{sample}$ is the absorbance of the sample with DPPH, $Abs_{control}$ is the absorbance of the sample with 95 % EtOH, and $Abs_{blank}$ is the absorbance of the digestion buffer with DPPH. GSH (1 mg/mL) was used as positive control.

*2.7.3. Hydroxyl radical scavenging assay*

The capacity to scavenge hydroxyl radicals was evaluated by the method of Ajibola et al. (2011) with some modifications. On this assay, hydroxyl radicals were generated by the reaction of hydrogen peroxide with Fe (II) which was oxidized to Fe (III). The presence of Fe (II) was monitored by its reaction with 1,10-phenanthroline generating a colored compound. The presence of antioxidant compounds inhibited the oxidation of Fe (II) to Fe (III) and the formation of $^{\bullet}OH$, so it was favoured the formation of the colored compound. The assay consisted of mixing 50 µL of 3 mM 1,10-phenanthroline in 0.1 M PB (pH 7.4), 50 µL of 3 mM ferrous sulphate, 50 µL of sample, and 50 µL of 0.01 % (v/v) hydrogen peroxide. The mixture was incubated for 1 h at 37 $^{0}$C and the absorbance was measured at 536 nm. The hydroxyl radical scavenging capacity was calculated according to the following equation:

$$Hydroxyl\ Radical\ Scavenging\ Capacity\ (\%) = (\frac{Abs_{sample} - Abs_{blank}}{Abs_{control} - Abs_{blank}})\ x\ 100 \quad (4)$$

where $Abs_{sample}$ is the absorbance of the sample, $Abs_{blank}$ is the absorbance of the digestion buffer, and $Abs_{control}$ is the absorbance of the solution resulting when adding

the digestion buffer instead of the sample and water instead of hydrogen peroxide. GSH (1 mg/mL) was employed as positive control.

*2.7.4. Ferric reducing antioxidant power (FRAP)*

This assay measures the capacity to reduce Fe (III) and it was evaluated according to a method previously proposed by Ajibola et al. (2011) with modifications. The assay was based on measuring the increase in absorbance occurring when Fe (III) from the ferricyanide complex turned into Fe (II). For that purpose, 12.5 µL of hydrolyzate, 12.5 µL of 0.2 M PB (pH 6.6), and 25 µL of potassium ferricyanide were mixed and incubated for 20 min at 50 $^{0}$C. Thereafter, the reaction was stopped by adding 25 µL of 10 % (w/v) TCA. For the assay, 50 µL of that solution, 40 µL of water, and 10 µL of 2.5 % (w/v) ferric chloride were mixed and, after 3 min at room temperature, the absorbance was measured at 700 nm. The reducing power was calculated according to the following equation:

$$Reducing\ power = \frac{Abs_{\ sample}}{Peptide\ content} \quad (5)$$

where $Abs_{sample}$ is the absorbance obtained for the hydrolyzate and the peptide content was calculated by the OPA assay. The positive control was GSH (0 – 1 mg/mL). The percentage of inhibition was calculated taking as maximum reduction power the value of 1 mg/mL GSH.

2.7.5.*Inhibition of lipid peroxidation*

This assay was carried out using a linoleic acid emulsion system and following the method developed by Chen et al.(1996) with some modifications. For that purpose, 20 µL of sample, 20 µL of 0.13 % (v/v) linoleic acid in EtOH, and 10 µL of water were

mixed and incubated at 40 $^0$C for 144 h (6 days). The degree of oxidation was measured in a solution prepared by mixing 2.5 µL of that solution with 175 µL of 75 % (v/v) EtOH, 2.5 µL of 30 % (m/v) ammonium thiocyanate, and 2.5 µL of 20 mM ferrous chloride in 3.5 % (v/v) HCl for 3 min at room temperature. Afterwards, the absorbance corresponding to ferric thiocyanate was measured at 500 nm and the capacity to inhibit the lipid peroxidation of the linoleic acid was obtained using the following equation:

$$Lipid\ peroxidation\ inhibition\ capacity = \left(1 - \frac{Abs_{sample,144h} - Abs_{sample,0h}}{Abs_{blank,144h} - Abs_{blank,0h}}\right)\ x\ 100 \quad (6)$$

where $Abs_{sample,144h}$ and $Abs_{blank,144h}$ are the absorbances of the sample and the digestion buffer after144 h, respectively; and $Abs_{sample,0h}$ and $Abs_{blank,0h}$ are the initial absorbances of the sample and the digestion buffer, respectively.

### *2.8. In vitro evaluation of the ACE inhibitor capacity*

ACE inhibitor capacity of plum seed hydrolyzates was evaluated following the method described by Geng et al. (2010) with slight modifications. This method is based on the reaction of the tripeptide HHL into hippuric acid (HA) by the action of ACE and measures the capacity of hydrolyzates to inhibit ACE activity.

Six different dilutions of two independent hydrolyzates were prepared in every case and all measurements were performed, at least, three times. The procedure involved mixing 2.5 µL of sample (or digestion buffer for the control) with 10 µL of ACE (0.05 U/mL in water), 17.5 µL of 50 mM HEPES (pH 8.3) buffer that contained 300 mM NaCl, and 5 µL of HHL (1.3 mg/mL in HEPES with NaCl). Then, the mixture was incubated at 37 $^0$C and the reaction was stopped by adding 50 µL ACN at -20 $^0$C. The separation of HHL and HA was performed in an HPLC equipment with a

Chromolith ® Performance RP-18 endcapped column (100 x 4.6 mm) (Merck, Darmsdat, Germany). The chromatographic conditions were: gradient, 5-95 % B in 10 min; flow rate, 1 mL/min; temperature, 25 $^{0}$C; injection volume, 10 µL; and UV detection at 228 nm. Mobile phases were: A, water with 0.025 % (v/v) TFA and B, ACN with 0.025 % (v/v) TFA. Inhibition of ACE was calculated using the following equation:

$$ACE\ inhibition\ \% = \frac{HA_{blank} - HA_{sample}}{HA_{blank}}\ x\ 100 \qquad (7)$$

where $HA_{blank}$ is the HA peak area without ACE inhibition and $HA_{sample}$ is the HA peak area in the sample. The inhibition (%) was plotted against peptide concentration and the peptide concentration inhibiting 50% of ACE ($IC_{50}$) was calculated by interpolation.

### 2.9. *Identification of bioactive peptides by RP-HPLC-MS/MS*

Alcalase hydrolyzate was analyzed using a 6530 series ESI Quadrupole-Time-of-Flight (Q-TOF) mass spectrometer from Agilent Technologies (Palo Alto, CA, USA) coupled to an Agilent 1100 series binary HPLC system. The separation was carried 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). Chromatographic conditions were: 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 °C; injection volume, 15 µL. The mass spectrometer was operated in the positive ion mode and the mass range was from *m/z* 100 to 1500. ESI conditions were: fragmentator voltage, 200 V; nozzle

voltage, 0 V; nebulizer pressure, 50 psig; capillary voltage, 3500 V; gas temperature, 350 ºC; gas flow, 12 L/min, and skimmer voltage, 60 V. The Jet Stream sheath gas temperature and flow were 400 ºC 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 for every 100 Da.

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 by the *do novo* sequencing tool. Only those peptides identified with an ALC (expected percentage of correct amino acids in the peptide sequence) above to 90% and with a good precursor fragmentation pattern, were considered. Moreover, only those peptides appearing in all six injections (three identical injections of the two replicates) were taken into account. Only isoforms with L are presented in our results, although peptide sequences containing I amino acid instead of L are also possible since it is not possible to differentiate I from L by MS.

## 3. Results and discussion

### 3.1. *Development of a method for the extraction of plum seed proteins*

Extraction of proteins from plum seeds has never been performed. In order to optimize a method enabling this extraction, a methodology previously developed by our research group to extract proteins from olive seeds was firstly employed (Esteve, del

Rio, Marina & García, 2010). These starting conditions briefly consisted of the extraction of proteins with a 125 mM Tris-HCl (pH 7.5) buffer containing 1 % (w/v) SDS and 0.5 % (w/v) DTT, for 5 min using an HIFU probe (30 % amplitude) followed by 1 h precipitation with acetone. The protein content when plum seed proteins were extracted using this method was 13.3 ± 1.6 % (as is basis). In order to assure that all proteins from plum seeds could be extracted, different steps of the extraction procedure were optimized: extracting buffer (pH, concentration, and presence of additives), extraction time, HIFU probe amplitude, and precipitation time. The optimization procedure was monitored by the separation of extracted proteins by RP-HPLC and SDS-PAGE.

Firstly, and in order to avoid inconveniences due the presence of an upper lipidic layer in the extracts, samples were defatted three times with hexane by shaking for 30 min. This step allowed the removal of this layer and obtaining a clearer extract and a higher protein content.

Two different buffers at pHs from 6.5 to 9.0 were tested: phosphate buffer (pH 6.5, 7.0, and 7.5) and Tris-HCl buffer (pH 7.5, 8.0, 8.5, and 9.0). Results showed that pH and buffer did not significantly affect the amount of extracted proteins and a Tris-HCl buffer at pH 7.5 was selected. Next, different Tris-HCl buffer (pH 7.5) concentrations (20, 50, 75, and 125 mM) were tested. Again, buffer concentration did not seem to significantly increase the amount of extracted proteins and a 100 mM concentration was selected for further experiments. Moreover, the effect of the addition of two different additives (SDS and DTT) in the extracting buffer was studied. Buffers containing SDS at 0, 0.5, 1.0, 1.5, and 2.0 % (w/v) and DTT at 0, 0.25, 0.50, 0.75, and 1.00 % (w/v) were tested. The highest percentage of extracted protein was observed with 1 % (w/v) SDS and 0.25 % (w/v) DTT. Extraction time using HIFU was also

studied in the range from 0.5 to 15 min and with probe amplitudes of 20, 30, 50, 75, and 100 %. Extraction times longer than 1 min and ultrasound amplitudes higher than 30 % did not result in an improved extraction of proteins. Precipitation time of proteins with acetone after their extraction was also optimized in the range from 0.25 to 24 h. Precipitation times higher than 30 min did not increase protein recovery.

The protein content, estimated by Bardford assay, when proteins were extracted using the optimized conditions was 16.9 ± 1.3%, as is basis, and 38.6 ± 2.7 % in dried and defatted plum seeds. The optimization of the extraction procedure enabled a 20% increase in the amount of extracted proteins in relation to the amount extracted when using initial conditions. **Fig.1 and 2** show electrophoretic profiles and chromatographic separation of the extracts obtained using the starting and the optimized extraction conditions. The number of electrophorestic bands and their intensity increased when proteins were extracted using the optimized procedure. The separation obtained by SDS-PAGE showed numerous bands below 75 kDa highlighting two bands between 37 and 50 kDa and three bands at molecular weights below 25 kDa. Chromatographic separations also showed a higher amount of peaks when using optimized extracting protocol.

Antioxidant and potential antihypertensive capacities of intact proteins in the extract were next evaluated. Both antioxidant and ACE-inhibitory capacity were negligible. Since intact proteins did not show bioactivity, next step was to obtain peptides from these proteins and to investigate their antioxidant and ACE-inhibitory capacities.

### 3.2. *Optimization of the digestion of extracted protein using different enzymes*

In order to evaluate the presence of bioactive peptides in plum seeds, extracted proteins were next digested using four different enzymes: Alcalase, Thermolysin, Flavourzyme, and Protease P. These enzymes were chosen taking into account bibliographic data on enzymes used to produce bioactive peptides (Korhonen & Pihlanto, 2006; Pedroche, Yust, Girón-Calle, Alaiz, Millan, & Vioque, 2002). In order to reach a high efficiency in the digestions, the following digestion parameters were optimized for every enzyme: substrate concentration, enzyme/substrate ratio, buffer and pH, and digestion time. Digestion temperature was established at 50 $^{0}$C for Alcalase, Thermolysin, and Flavourzyme, and at 40 $^{0}$C for Protease P since they were the temperatures enabling enzyme maximum activity. Digestion efficiency during optimization was monitored by the estimation of the hydrolysis degree.

Substrate concentrations ranging between 2.5-10 mg/mL were tested observing that the degree of hydrolysis slightly decreased when decreasing substrate concentration. The higher degree of hydrolysis was found at a substrate concentration of 2.5 mg/mL for Thermolysin and 5 mg/mL for Flavourzyme, Alcalase, and Protease P. Digestion time was varied between 15 min and 24 h for all enzymes and % DH was determined in every case (see **Fig.3**). In general, the hydrolysis degree increased sharply when increasing the digestion time up to reach a plateau. The selected digestion times for Alcalase, Thermolysin, Flavourzyme, and Protease P were 3, 4, 7, and 24 h, respectively. Furthermore, different enzyme/substrate ratios were also tried with every enzyme (see **Table 1**). In general, the degree of hydrolysis increased with the amount of enzyme used in the digestion. Nevertheless, the degree of enzyme autolysis also increased, so the chosen enzyme/substrate ratio for every enzyme was that providing the highest degree of hydrolysis at a low enzyme autolysis. Another optimized parameter was the digestion buffer and pH. pHs 7-9 were tried in the case of Alcalase,

Thermolysin, and Protease P while pHs 5.5to8.0 were tried in the case of Flavouzyme. In the case of Alcalase, there was not a significant variation in the DH in the studied pH range and the selected buffer was a 5 mM borate buffer (pH 8.5). Thermolysin showed a higher DH at pHs 8-9 and a 5 mM phosphate buffer (pH 8.0) was chosen. Flavourzyme yielded a similar DH when using buffers at pHs 5.5-7.5 and a 5 mM phosphate buffer (pH 7.0) was selected. Finally, Protease P showed the highest DH with a 5 mM phosphate buffer at pH 7.5.

Using these optimal digestion conditions, Thermolysin was the enzyme yielding the highest hydrolysis degree (91.4 ± 1.2 %) followed by Flavourzyme (74.1 ± 6.7 %), Protease P (68.5 ± 6.1 %), and Alcalase (61.7 ± 6.1 %).

### 3.3. *Evaluation of the in vitro antioxidant capacity of hydrolyzates*

The antioxidant capacity of a food or biological sample depends on many factors being advisable to use more than one assay for its comprehensive evaluation. Therefore, five different antioxidant assays that measured the capacity to scavenge different free radicals, the capacity to reduce Fe (III), and the capacity to inhibit lipid peroxidation were used in this work. Antioxidant capacities observed for every enzyme in every assay and the hydrolysis degree determined for every extract are presented in **Fig.4**.

In general, all extracts showed radical scavenging capacity despite values greatly depended on the assay. DPPH radical scavenging assay could not be used to measure the antioxidant capacity when digesting with Alcalase, Flavourzyme, and Protease P due to the formation of a precipitate. This precipitate could be from the precipitation of remaining non hydrolyzed proteins and/or enzymes with the EtOH used in this antioxidant assay. There was not a unique enzyme yielding the highest radical

scavenging capacity in all assays. Alcalase and Thermolysin seemed to be the enzymes showing the highest antioxidant capacities.

The assay showing the highest variability among hydrolysates was that evaluating the capacity to inhibit lipid peroxidation. Indeed, it ranged from 95% of inhibition, corresponding to the Alcalase hydrolysate, to 20% of inhibition, corresponding to the Protease P hydrolysate. Again, Alcalase hydrolyzate followed by Thermolysin hydrolysate yielded the highest antioxidant capacity.

In comparison with the capacity to scavenge free radicals or to inhibit lipid peroxidation, the capacity of hydrolysates to reduce iron (III) was always very low. The highest reducing power was found for the Thermolysin hydrolyzate followed by the Alcalase hydrolysate.

Comparing antioxidant capacities observed by all assays and all enzymes with the hydrolysis degree (see Figure 3), it was observed that only the profiles obtained by the hydroxyl radical scavenging assay and by the FRAP assay could show some correlation with the hydrolysis degree, while the other three assays did not show any correlation. Regarding antioxidant capacity, the highest values were obtained with Alcalase and Thermolysin hydrolysates being the hydrolysate obtained with Protease P that yielding the lowest antioxidant capacity.

In order to compare antioxidant capacity observed with the plum seed extracts with other foods or peptides, the trolox (a water soluble vitamin E analog) equivalent antioxidant capacity (TEAC) was calculated. TEAC values for Alcalase and Thermolysin were 460 ± 22 and 772 ± 27 µmol trolox/g sample, respectively. These values, as example, are in the range of red wine (570-710 µmol trolox/g sample) (Paixao, Perestrelo, Marques & Camara, 2007).

### 3.4.*Evaluation of the in vitro ACE inhibitory capacity of hydrolyzates*

In order to evaluate the potential antihypertensive of hydrolyzates, the ACE inhibition assay was employed and $IC_{50}$ values (see **Fig.4**) were calculated. Like the antioxidant capacity, the highest ACE inhibitory capacity was observed when using Thermolysin and Alcalase enzymes while Flavourzyme and Protease P resulted in very high $IC_{50}$ values and, thus, very low potential antihypertensive capacity. In comparison with other food sources such as blackberries ($IC_{50}$ = 169 µg/mL) (Ivanov, Garbuz, Malfanov & Ptitsyn, 2013), oat ($IC_{50}$ = 30 µg/mL) (Cheung, Nakayama, Hsu, Samaranayaka & Li-Chan, 2009) or potato ($IC_{50}$ = 50 µg/mL) (Pihlanto, Akkanen & Korhonen, 2008), it is possible to affirm that plum seed protein isolate could be an attractive source of peptides from which it could be possible to isolate potential antihypertensive peptides like those from garlic ($IC_{50}$ ranging from 3.7 to 280 µg/mL) (Suetsuna, 1998) or famous VPP ($IC_{50}$ = 2.8 µg/mL) and IPP ($IC_{50}$ = 1.7 µg/mL) peptides from milk (Lopez-Fandino, Otte, & van Camp, 2006).

### 3.5.*Identification of peptides in the Alcalase hydrolysate*

Since Alcalase seemed to be the enzyme that yielded the highest antioxidant and ACE inhibitory capacity, it was chosen for the identification of peptides by RP-HPLC-ESI-Q-TOF-MS/MS. A list of identified peptides, retention time, ALC, and molecular masses are presented in **Table 2**. As an example, **Fig. 5** shows the mass spectrum of the chromatographic peak at 23.73 min and the MS/MS spectrum of ion at 757.444 m/z. After sequence interpretation, this spectrum was matched to VKPVAPF peptide. All peptides showed a molecular mass below 1 kDa. These results are rational taking into account the low specificity of Alcalase enzyme. It was possible the identification of 13 different peptides with 4 to 7 amino acids. The analysis of amino acid composition of

identified peptides showed high amount of hydrophobic (L/I, P, and V) and aromatic (H, F, W, and Y) amino acids within their sequences. Indeed, they accounted for the 70% of total amino acids (57% L/I/P/V and 13% H/F/Y). The presence of these amino acids is important since they are common features within antioxidant and ACE inhibitor peptides (Sarmadi & Ismail, 2010).

In conclusion, the present work has developed a method for the extraction of proteins from a residual material from plum. The method involved the use of high intensity focused ultrasounds which enabled plum seed protein isolate preparation with a protein content of about 40 % (in dry and defatted basis) in less than 1 h. Moreover, the optimization of digestion conditions with four different enzymes resulted in hydrolysis degrees ranging from 90% in the case of Thermolysin to 60% in the case of Alcalase. Under the optimal digestion conditions, Alcalase enzyme seemed to be the enzyme showing the most promising extract for the isolation of both antioxidant and potential antihypertensive peptides. Analysis of Alcalase hydrolysate by RP-HPLC-ESI-Q-TOF enabled the identification of 13 peptides with typical features of antioxidant and antihypertensive peptides. This method could be a strategy for the recovery and revalorization of this plum by-product.

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**Figure captions**

**Fig.1.** Electrophoretic profiles obtained by SDS-PAGE corresponding to intact proteins extracted from plum seeds using initial (In) and optimized (Op) conditions.

**Fig.2.** Chromatographic separation by RP-HPLC of plum seed proteins extracted using initial (In) and optimized (Op) conditions.

**Fig.3.** Variation of the degree of hydrolysis (% DH) with the time of incubation during the digestion of plum seed proteins with four different enzymes using hydrolysis optimized conditions.

**Fig.4.** Antioxidant and ACE inhibitory capacities of the hydrolyzates obtained from plum seed proteins using four different enzymes. Non compatible assay means that it was not possible to use this assay due to the formation of a precipitate.

**Fig. 5.** Mass spectrum of the peak at 23.73 min and MS/MS spectrum corresponding to the signal at 757.444 m/z.

**Table 1.** Digestion conditions studied and selected for the digestion of proteins extracted from plum seeds.

| Enzyme | T ($^0$C) | Substrate concentration (mg/mL) | Enzyme/substrate ratio | Buffer and pH | Digestion time (h) |
|---|---|---|---|---|---|
| **Alcalase** | 50 | 2.5 **5.0** 7.5 10 | 0.15 **0.30** 0.60 0.90 1.20 1.50 **AU/g protein** | 5 mM PB pH 7 7.5 8 **5 mM Borate pH 8.5** 9 | 0.25 0.5 1 2 **3** 4 7 24 |
| **Thermolysin** | 50 | **2.5** 5.0 7.5 10 | 0.05 **0.10** 0.30 0.50 **g enzyme/g protein** | **5 mM PB pH** 7 7.5 **8** 5mM Borate pH 8.5 9 | 0.25 0.5 1 2 3 **4** 7 24 |
| **Flavourzyme** | 50 | 2.5 **5.0** 7.5 10 | 25 50 **75** 100 **AU/g protein** | 5 mM ABC pH 5.5 6 6.5 **5 mM PB pH 7** 7.5 8 | 0.25 0.5 1 2 3 4 **7** 24 |
| **Protease P** | 40 | 2.5 **5.0** 7.5 10 | 0.30 0.50 **1** 2 **% (w/w)** | **5 mM PB pH** 7 **7.5** 8 5 mM Borate pH 8.5 9 | 0.25 0.5 1 2 3 4 7 **24** |

*Optimized conditions in bold. PB: Phosphate buffer. ABC: Ammonium bicarbonate.

**Table 2.** Peptide sequence, retention time, ALC, and experimental molecular masses of the peptides identified in the whole Alcalase hydrolysate.

| Peptide sequence | Retention time (min) | ALC (%) | Molecular mass |
|---|---|---|---|
| **MLPSLPK** | 23.19 | 95 | 784.4517 |
| **HLPLL** | 26.27 | 95 | 591.3744 |
| **NLPLL** | 28.17 | 95 | 568.3585 |
| **HNLPLL** | 26.02 | 95 | 705.4174 |
| **KGVL** | 13.54 | 94 | 415.2794 |
| **HLPLLR** | 27.68 | 94 | 747.4755 |
| **HGVLQ** | 8.76 | 93 | 552.3020 |
| **GLYSPH** | 16.12 | 92 | 672.3231 |
| **LVRVQ** | 13.58 | 92 | 613.3911 |
| **YLSF** | 25.19 | 92 | 528.2584 |
| **DQVPR** | 6.07 | 92 | 613.3184 |
| **LPLLR** | 22.70 | 91 | 610.4166 |
| **VKPVAPF** | 23.73 | 91 | 756.4534 |

L was used to represent leucine or isoleucine.

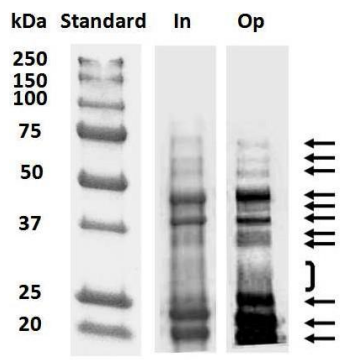
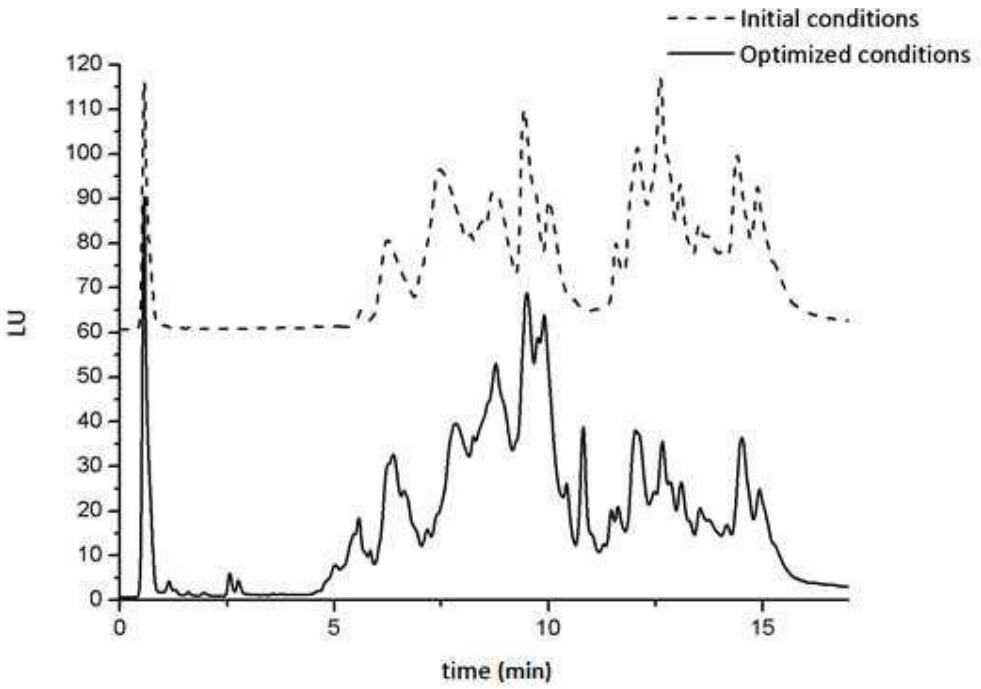



Fig.1


Initial conditions
Optimized conditions
LU
time (min)
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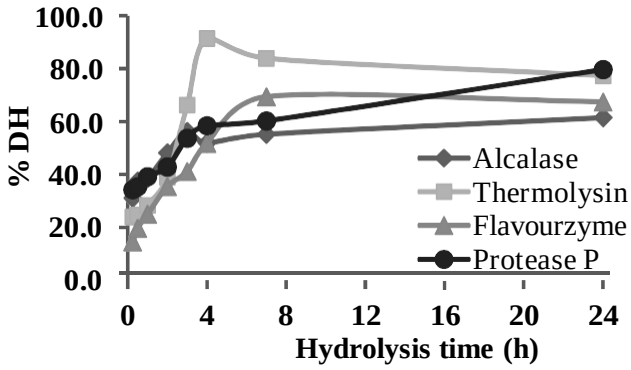
40
50
60
70
80
90
100
110
120
0
5
10
15

603 Fig. 2

604

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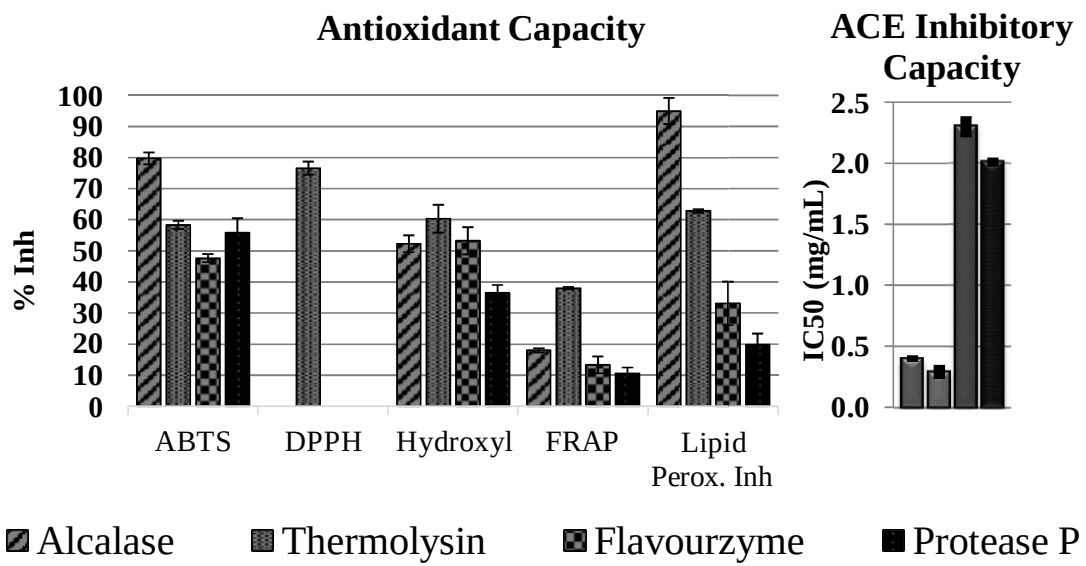
606 Fig.3

607


Antioxidant Capacity
% Inh
100
90
80
70
60
50
40
30
20
10
0
ABTS
DPPH
Hydroxyl
FRAP
Lipid Perox. Inh
ACE Inhibitory Capacity
IC50 (mg/mL)
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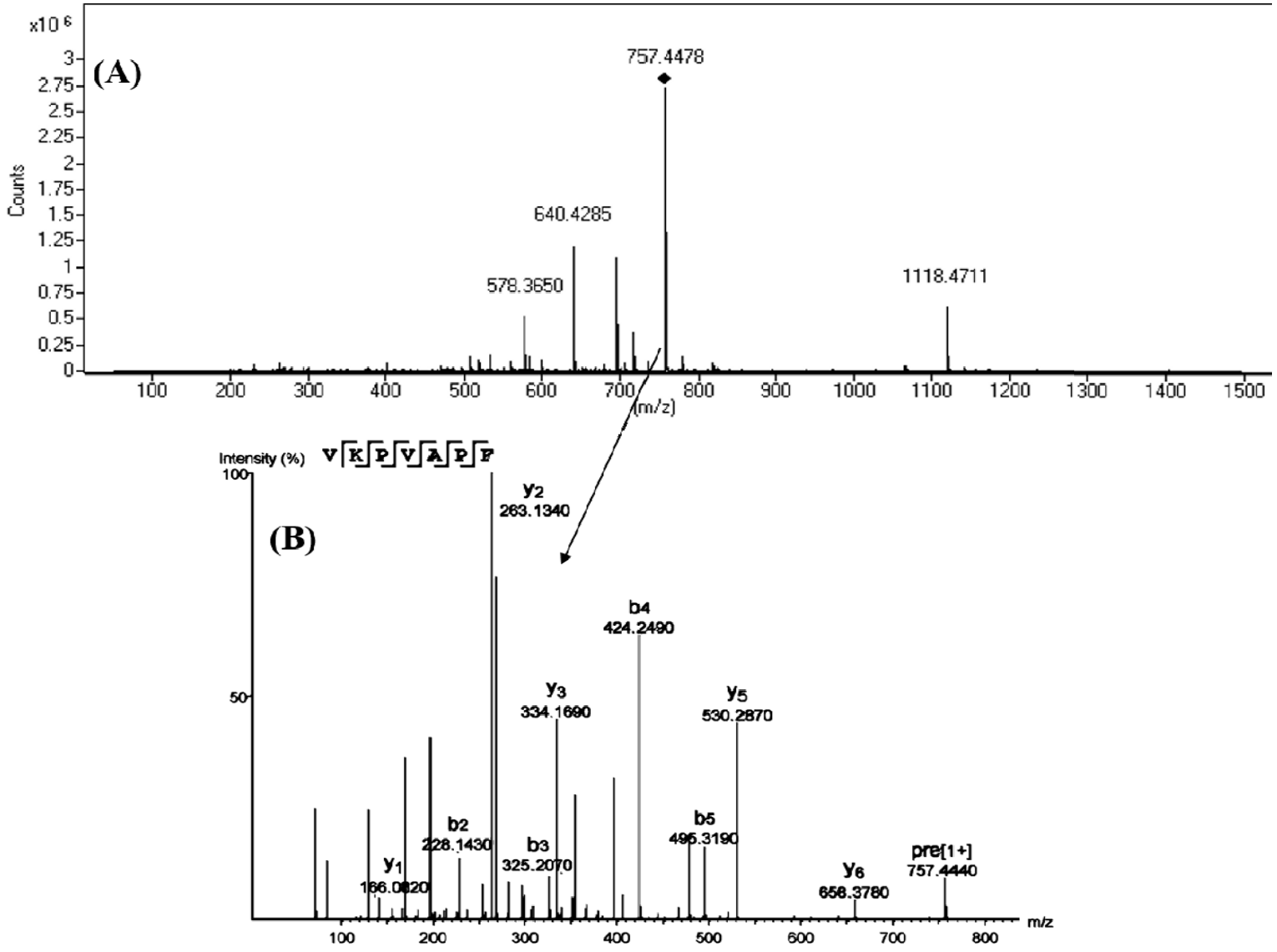
2.0
1.5
1.0
0.5
0.0
Alcalase
Thermolysin
Flavourzyme
Protease P

Fig. 4


(A)
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578.3650
1118.4711
(m/z)
(B)
Intensity (%)
V K P V A P F
y2
263.1340
b4
424.2490
y3
334.1690
y5
530.2870
b2
228.1430
b3
325.2070
b5
496.3190
y1
166.0620
y6
658.3780
pre[1+]
757.4440
m/z

Fig. 5