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Article

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HPLC-Q-TOF-MS Identification of Antioxidant and Antihypertensive Peptides Recovered from Cherry (*Prunus Cerasus* L.) Subproducts

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ABSTRACT

The processing of fruits, such as cherries, is characterized by generating a lot of waste material such as fruit stones, skins, etc. In order to contribute to environmental sustainability, it is necessary to recover these residues. Cherry stones contain seeds with a significant amount of proteins that are underused and undervalued. The aim of this work was to extract cherry seed proteins, to evaluate the presence of bioactive peptides, and to identify them by mass spectrometry. The digestion of cherry seed proteins was optimized and three different enzymes were employed: Alcalase, Thermolysin, and Flavourzyme. Peptide extracts obtained by the digestion of the cherry seed protein isolate with Alcalase and Thermolysin yielded the highest antioxidant and antihypertensive capacities. Ultrafiltration of hydrolysates allowed obtaining fractions with high antioxidant and antihypertensive capabilities. HPLC-Q-TOF-MS together with bioinformatics tools enabled to identify peptides in these fractions.

KEYWORDS: cherry, seed proteins, peptide, antihypertensive, antioxidant, MS identification

INTRODUCTION

The correct management of residues is one of the main goals of the United Nations Environmental Programme.[1] In fact, United Nations, through its Resource Efficient and Cleaner Production Programme, emphasizes the need for an efficient use of natural resources, minimizing generation of wastes and emissions and fostering safe and responsible production.[1] Related to this is the fact that food industry generates a high amount of waste. These wastes constitute a clear environmental risk. As a consequence, food processing must be focused on the reuse of these residues.

In the case of fruit processing, wastes mainly consist of leaves, skins, and stones. These residues are employed, in part, for animal feeding and the rest is discarded. The use of these by-products for animal feeding supposes an additional economic cost and an adequate planning. In addition, in those areas where there is no livestock, these residues are transferred directly to waste. Some alternative uses of these solid residues are the production of fertilizers and fuels (biomass). Nevertheless, the state-of-the-art for the utilization of these wastes is in its early stages and they are not commonly implemented.[2] The absence of alternatives for reusing these wastes makes further investigations really important.[2, 3]

Fruit stones, like those present in cherries, usually are the main part of the wastes generated in fruit processing. The cherry stone contains a seed with high protein content.[3] Therefore, cherry seeds really constitute a cheap protein source that could be useful to palliate protein world deficiencies, especially in developing countries, or that could be of interest for the production of bioactive peptides.

More than 37 different kinds of bioactive peptides are listed in BioPep database.[4] Angiotensin Converting Enzyme (ACE) inhibitors (antihypertensive) and antioxidant peptides are of special interest because they could help to defeat widespread diseases.[5-7]

In fact, the intake of antioxidant peptides could be helpful to fight against many diseases originated by reactive oxygen species[8] that, through the oxidation of lipids or proteins, breakage of DNA, or modification of nucleotides, can lead to serious diseases like atherosclerosis or cancer.[5] Moreover, food oxidation results in food deterioration by the production of undesirable off-flavours, odours, dark colours, potentially toxic reaction products, etc. Despite there are some synthetic antioxidants, the questionable safety of these synthetic compounds and the negative consumer perception, make natural antioxidants like bioactive peptides very valued.[6,9,10] On the other hand, hypertension can lead to many serious diseases like cardiovascular and kidney diseases.[6,7] Angiotensin converting enzyme (ACE) plays a central role in the development of high blood pressure. Therefore, inhibition of ACE activity is the main way to defeat this disease. Despite there are some ACE inhibitors that have been synthesized, they usually lead to adverse side effects.[11] Again, the consumption of antihypertensive peptides could be of great interest.

The main objective of this work was to extract cherry seed proteins, to optimize proteins digestion using suitable enzymes to obtain bioactive peptides, to fractionate most active hydrolysates, and to identify potential bioactive peptides by RP-HPLC-Q-TOF-MS and bioinformatics tools.

MATERIAL AND METHODS

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). Sodium dodecylsulfate (SDS), tris(hydroxymethyl)aminomethane (Tris) (all from Merck), n-hexane (96%), acetone (both from Scharlab (Barcelona, Spain)), and dithiothreitol (DTT) (from Sigma-Aldrich (St. Louis, MO, USA)) were used for the extraction of cherry seed proteins. Tris/glycine/SDS running buffer, Laemmli buffer, Bio-Safe Coomassie G250 stain, Mini Protean precast gels, Precision Plus Protein Standards (recombinant proteins expressed by *E. Coli* with molecular masses of 10, 15, 20, 25, 37, 50, 75, 100, 150, and 250 kDa) (all from Bio-Rad-Laboratories (Hercules, CA, USA)) were employed for the separation of proteins by SDS-PAGE. Quick Start Bradford – 1xDye reagent from Bio-Rad-Laboratories was employed for the estimation of proteins by Bradford assay. Hydrochloric acid, sodium dihydrogen phosphate, sodium hydroxide (all from Merck), sodium tetraborate, and Thermolysin enzyme (both from Sigma Aldrich) were used for the digestion of proteins. Alcalase 2.4 L FG and Flavourzyme 1000 L were kindly donated by Novozymes Spain S.A. (Madrid, Spain). Sodium chloride (Merck), angiotensin converting enzyme (ACE), ethanol (EtOH), 2-[4-(2-hydroxyethyl)-1-piperazinyl]-ethanesulfonic acid (HEPES), hippuryl-histidyl-leucine (HHL), 1,10-phenanthroline, 2-mercaptoethanol, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), albumin from bovine serum, ammonium thiocyanate, hydrogen peroxide, ferrous sulfate, iron (II) sulphate, iron (II) chloride, iron(III)chloride, L-gluthathion (GSH), linolenic acid, methanol, ortho-phthalaldehyde, potassium ferricyanide, trichloroacetic acid, and potassium persulfate (all from Sigma-Aldrich) were used to carry out *in vitro* assays. Acetonitrile (ACN) HPLC gradient

grade (Scharlab), trifluoroacetic acid (TFA), and acetic acid (both from Sigma-Aldrich) were employed in the preparation of mobile phases in HPLC and HPLC-Q-TOF experiments.

Protein Extraction. The extraction procedure was similar to the described by Esteve et al.[12] with some modifications. Cherry pulp was removed and the stone was broken to extract cherry seeds. Seeds were milled and fat was removed by extracting three times with hexane (25 mL per 0.5 g seeds). Seeds moisture was determined keeping them at 103 ± 2 ^{0}C until constant weight. Defatted seeds were stored at -20 ^{0}C until use. The procedure used to extract proteins from cherry seeds consisted of mixing 30 mg of defatted seeds with 5 mL of 100 mM Tris-HCl buffer (pH 7.5) containing 1 % SDS and 0.5 % DTT. Extraction was carried out using high intensity focused ultrasounds (HIFU) for 5 min and an amplitude of 30 % followed by centrifugation at 4000g for 20 min. Proteins from the supernatant were next precipitated with 10 mL of cold acetone at 4 °C for 1 h. After centrifugation at 4000g for 10 min, precipitated proteins were dried over night at 37 °C. Pellets were weighed and then dissolved in the previous Tris-HCl buffer (without DTT) using HIFU for 5 min at 30% amplitude. The solution was filtrated through 0.45 μm and stored at 4 °C until use. The protein content in this solution was estimated by Bradford assay.[13]

Protein Separation. Proteins were separated by SDS-PAGE and RP-HPLC. SDS-PAGE separation was carried out in a Mini-Protean from Bio Rad. Samples were prepared by mixing 15 μL of the cherry seed protein isolate with 15 μL of Laemmli buffer containing 5 % (v/v) β-mercaptoethanol and by heating for 5 min at 100 ºC. Electrophoresis was carried out on commercial Ready Gel 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, peptide standards (10, 15, 20, 25, 37, 50, 75, 100, 150, and 250 kDa) were used as ladder. After separation, proteins were fixed with 50 mL of 10 % (v/v) glacial acetic acid/40 % (v/v) MeOH gently 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 Milli-Q water for 2 h.

RP-HPLC separation of the cherry seed protein isolate was performed in a modular Agilent Technologies liquid chromatograph (Pittsburg, PA, USA). All samples were prepared twice and measured by duplicate. A Poros R2/10 Perfusion column (4.6 mm D × 50 mm) at a flow-rate of 2 mL/min and a temperature of 25 ºC were used. The gradient was 30-40 %B in 13 min, 40-60 % B in 6 min, 60- 80 %B in 1 min, and 80-30 % B in 2 min. Mobile phase A was water with 0.1 % (v/v) of TFA and mobile phase B consisted of ACN with 0.1 % (v/v) TFA. Detection was carried out with a fluorescent detector at λ_{exc}= 280 and λ_{em}= 360 nm.

Protein Digestion and Fractionation. Digestion of extracted proteins was carried out using three different enzymes: Alcalase (borate buffer (pH 8.5), 0.10 AU enzyme /g protein), Thermolysin (phosphate buffer (pH 8.0), 0.4 g enzyme/g protein), and Flavourzyme (bicarbonate buffer (pH 6.0), 75 AU enzyme/g protein). In all cases, a protein concentration of 2.5 mg/mL, a digestion temperature of 50 ºC, and a digestion time of 7 h were selected. Additionally, blank digestions without proteins prepared by mixing buffer and enzyme were carried out. Digestions were carried out by slight mixing (600 rpm) at a controlled temperature in a Thermomixer Compact (Eppendorf AG, Hamburg, Gernmany). Digestions were stopped by heating to 100 ºC for, at least, 10 min and, then, they were centrifuged (5 min at 7000 rpm).

Hydrolysates were fractionated by ultrafiltration using Vivaspin 500 PES molecular weight (Mw) cut-off filters (5 kDa) (Sartorius Stedim biotech, Goettingen, Germany) and Amicon Mw cut-off filters (3 kDa) (Millipore).

Degree of Hydrolysis (%DH). The degree of hydrolysis was determined by the OPA assay following the method of Wang *et al.*[14] with modifications. OPA mixture (5 mL) consisted of 2.5 mL of 100 mM sodium tetraborate, 1 mL of 5 % (w/v) SDS, 100 μL of 40 mg/mL OPA dissolved in MeOH, 10 μL of β-mercaptoethanol, and 1.39 mL of water. The assay consisted of mixing 2.5 μL sample with 100 μL OPA mixture and measuring the absorbance at 340 nm (spectrophotometer Lambda 35, Perkin-Elmer, Waltham, MA) after 8 min at room temperature. The peptide content was calculated by interpolation in a calibration curve obtained using GSH (0-5 mg/mL). 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 the Bradford assay.

Antioxidant Capacity. Four different *in vitro* antioxidant assays were carried out for the evaluation of the antioxidant capacity of hydrolysates from the cherry seed protein isolate.

ABTS radical scavenging assay. The scavenging capacity against ABTS radicals was evaluated according to the method of Wiriyaphan *et al.*[15] with modifications. $ABTS^{\bullet+}$ stock solution was prepared by mixing a 7.4 mM ABTS solution with 2.6 mM potassium persulfate in 10 mM phosphate buffer (pH 7.4) for, at least, 16 h kept in the dark. $ABTS^{\bullet+}$ working solution was daily prepared by mixing the stock solution with 10 mM phosphate buffer (pH 7.4). 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. 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} was the absorbance of 1 µL sample with 100 µL of $ABTS^{\bullet+}$ working solution and Abs_{blank} was the absorbance of 1 µL digestion buffer with 100 µL of $ABTS^{\bullet+}$ working solution. Trolox (1 mM) was employed as positive control.

Hydroxyl radical scavenging assay. The capacity to scavenge hydroxyl radicals was evaluated by the method of Ajibola *et al.*[16] with modifications. Hydroxyl radicals were obtained by the oxidation of Fe (II) with hydrogen peroxide. Antioxidant compounds inhibit the oxidation of Fe (II) and the formation of hydroxyl radicals. The assay was monitored by measuring the absorbance corresponding to the colored complex resulted from the reaction between Fe (II) and 1,10-phenanthroline. The assay consisted of mixing 50 µL of 3 mM 1,10-phenanthroline in 0.1 M phosphate buffer (pH 7.4), 50 µL of 3 mM ferrous sulfate, 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 of the Fe (II) complex was measured at 536 nm. The hydroxyl radical scavenging capacity was evaluated according to the following equation:

$$Hydroxyl\ Radical\ Scavenging\ Capacity\ (\%) = \left(\frac{Abs_{sample} - Abs_{blank}}{Abs_{control} - Abs_{blank}}\right) x\ 100 \qquad [4]$$

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

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

Ferric ion reducing antioxidant power (FRAP) assay. This assay measures the capacity of the sample to reduce Fe (III) from ferricyanide and it was evaluated according to the method proposed by Ajibola *et al.*[16] with modifications. The assay consisted of incubating 12.5 µL of sample with 12.5 µL of 0.2 M phosphate buffer (pH 6.6) and 25 µL of 1% potassium ferricyanide solution for 20 min at 50 °C in the Thermomixer. The reaction was stopped with 25 µL of 10% (w/v) trichloroacetic acid solution. This solution (50 µL) was mixed with 60 µL of water and 15 µL of 2.5 % iron (III) chloride solution and after 3 min, the absorption at 700 nm was measured. The reducing power was calculated according to the following equation:

$$Reducing\ power = \frac{Abs_{\ sample}}{Peptide\ content} \quad [5]$$

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

Capacity to inhibit lipid peroxidation. This assay was carried out by the method developed by Chen *et al.*[17] with modifications. The assay involved mixing 20 µL of sample, 20 µL of 0.13 % (v/v) linoleic acid in EtOH, and 10 µL of water at 40 ^{0}C for 144 h (6 days) in the dark. The degree of oxidation was monitored by mixing 2.5 µL of that solution with 175 µL of 75 % (v/v) EtOH, 2.5 µL of 30 % (w/v) ammonium thiocyanate, and 2.5 µL of 20 mM Fe (II) chloride in 3.5 % (v/v) HCl for 3 min at room temperature and measuring the absorbance of the resulting ferric thiocyanate at 500 nm.

The capacity to inhibit lipid peroxidation of linoleic acid was calculated 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 $Abs_{blank,144h}$ were the absorbances of the sample and digestion buffer for 144 h, respectively; and $Abs_{sample,0h}$ and $Abs_{blank,0h}$ were the initial absorbances of the sample and digestion buffer, respectively.

ACE Inhibition Capacity. ACE inhibition capacity was expressed as the peptide concentration needed for the 50% inhibition of ACE activity (IC_{50}). For that purpose, an assay developed by Geng *et al.*[18] based on the hydrolysis of the tripeptide HHL into hippuric acid (HA) by the action of ACE was employed. The assay measures the capacity of hydrolyzates to inhibit ACE activity by monitoring the signal corresponding to HA. In order to calculate IC_{50} value, the whole peptide extract and dilutions up to 1:10 ratio were measured for every hydrolysate. All measurements were made by duplicate. In every case, 2.5 µL of sample were mixed with 10 µL of ACE (0.05 U/mL), 17.5 µL of 50 mM HEPES buffer (pH 8.3) with 300 mM NaCl, and 5 µL of HHL (1.25 mg/mL). For the controls, 2.5 µL of the corresponding digestion buffer were used. The mixture was kept at 37 °C (in the Thermomixer) and the reaction was stopped by the addition of 50 µL of cold ACN (- 20 °C). Separation and detection of HA was performed by HPLC using a Chromolith Performance RP-18e column (100 mm x 4.6 mm I D) from Merck. Separation was carried out at 25 °C and mobile phases consisted of water with 0.025 % (v/v) TFA (mobile phase A) and ACN with 0.025 % (v/v) TFA (mobile phase B). The injection volume was 10 µL. A linear gradient from 5-

95 % B in 10 min followed by 95-5% B in 2 min was used. Detection was made at 228 nm. Percentage of ACE inhibition was calculated using the following expression where $A_{control}$ was the area under the peak of HA in the control sample and A_{sample} was the area under the peak of HA in the sample.

$$\%\, ACEInhibition = \frac{A_{control} - A_{sample}}{A_{control}} \qquad [7]$$

For every hydrolysate, the % of ACE inhibition of the undiluted and diluted sample were plotted against the peptide concentration (determined by OPA assay) and the IC_{50} value was determined from the signal corresponding to an inhibition of 50 % of ACE.

HPLC-Q-TOF-MS Identification of Bioactive Peptides. Identification of peptides was carried out by MS/MS detection using a Quadrupole Time-of-Flight (Q-TOF) series 6530 coupled to a HPLC (model 1100) both from Agilent Technologies. An Ascentis Express peptide ES-C18 (100 mm x 2.1 mm, 2.7 μm particle size, 160 Å pore size) analytical column with a guard column Ascentis Express peptide ES-C18 (5 mm x 2.1 mm), both from Sigma Aldrich, were employed for the separation of peptides. Elution gradient employed for the separation of peptides in fraction > 5 kDa was: 3% B for 6 min, followed by 3-43% B in 20 min, 43-55% B in 2 min, 55-74 %B in 9 min, 74-95% B in 2 min, 1 min at 95% B, and 95- 3% B in 2 min. Separation of peptides in the fraction < 3 kDa was carried out with an elution gradient at 3% B for 7 min, 3-60% B in 28 min, 60- 95% B in 3 min, 95% B for 2 min, and 95- 3% B in 2 min. Rest of parameters were: separation temperature, 25 °C; injection volume, 15 μL; and flow rate, 0.3 mL/min. Mobile phases A and B consisted of water and ACN, both containing 0.3 % (v/v) acetic acid. Electrospray ionisation in the positive ion mode at a capillary voltage of 3500 V and a MS-range from m/z 200 to 2200 were employed. Other MS

conditions were: dry gas flow, 12 L/min; dry gas temperature, 350 °C; nebulizer pressure, 50 psig; sheath gas flow, 12 L/min; and sheath gas temperature, 400 °C. The fragmentation energy was calculated taking into account 5 V per 100 Da molecular weight.

In all cases, two independent extracts were analysed by duplicate. Peaks software (Bioinformatics Solutions Inc.; Waterloo, ON, Canada) was employed for the treatment of MS/MS data and *do novo* peptide sequencing using an error tolerance of 50.0 ppm for the precursor and 0.5 kDa for the fragment. Peptide identification was accepted if peptides appeared in the two independent samples analysed by duplicate and presented an average local confidence (ALC (%), expected percentage of correct amino acids in the peptide sequence) above 90%. Moreover, since the equipment is not able to distinguish between I and L amino acids due to their equal molecular masses, both amino acids must be considered.

Statistical analysis. Statistical analysis was performed using Statgraphics Software Plus 5.1 (Statpoint Technologies, Inc., Warrenton, VA).

RESULTS AND DISCUSSION

In order to obtain peptides from cherry seeds, it is necessary to extract firstly cherry seed proteins. Despite there is no method previously developed for the extraction of cherry seeds proteins, the group ~~previous~~ experience on the extraction of proteins from other seeds like olive[12] and plum[19] was very useful. Using extraction conditions described in Materials and Methods, the protein content, estimated by Bradford assay, was 39.2 ± 5.8 g protein/100 g in defatted and dried seeds. This content is similar to the observed previously for plum seeds[19]. Extracted proteins were next separated by SDS-PAGE (see Figure 1) observing numerous bands below 75 kDa. It was important to

highlight the bands appearing around 10, 15, and 20 kDa. Additionally, the RP-HPLC separation of the extract using fluorescent detection at wavelengths corresponding to tryptophan and tyrosine amino acids also showed many signals that could correspond to proteins (results not shown). All these results demonstrated that the procedure enabled the extraction of proteins from cherry seeds.

In order to obtain peptides from this cherry seed protein isolate, extracted proteins have to be digested with suitable enzymes.

Optimization of the Digestion of the Cherry Seed Protein Isolate. Taking into account bibliographic data and research group experience, Alcalase, Thermolysin, and Flavourzyme were the enzymes chosen for the hydrolysis of the cherry seed protein isolate. In order to obtain the highest digestion efficiency, different parameters were optimized with every enzyme: pH, protein concentration, enzyme:protein ratio, and digestion time. Digestion temperature was always 50º C, corresponding to the highest enzymes activity. The optimization of the digestion was monitored by the determination of the degree of hydrolysis. Table 1 groups the conditions tested in every case.

There was not any significant variation ($P > 0.05$) in the hydrolysis degree when using Alcalase enzyme at pHs 8.0 and 8.5 while Flavourzyme showed a higher hydrolysis degree at pH 6.0 than at higher pHs. In the case of Thermolysin, a pH 8.0 was selected taking into account previous results from our research group[19,20]. Regarding the substrate concentration, as expected, the degree of hydrolysis decreases when increasing the substrate concentration and a concentration of 2.5 mg/mL was selected for the three enzymes. Moreover, different enzyme:substrate ratios were tried. There was no significant variation ($P > 0.05$) in the hydrolysis degree when using Alcalase enzyme at different enzyme:substrate ratios and the lowest value tried (0.10 AU/g proteins) was chosen. In the case of Flavourzyme, the enzyme:substrate ratio

yielding the highest hydrolysis degree was 75 AU/g protein. Finally, the hydrolysis degree when using Thermolysin varied from 34 (at 0.03 g enzyme/g protein) to 49% (at 0.4 g enzyme/g protein) being this the enzyme:substrate ratio firstly chosen. Nevertheless, further investigations revealed that a high enzyme:substrate ratio resulted in enzyme autolysis and, thus, a lower enzyme:substrate ratio (0.05 g enzyme/g protein) was finally selected. In all cases, hydrolysis degree increased rapidly in the first hours of digestions and then, it showed a slow increase up to 24 h. Nevertheless, black digestions performed at 24 h also showed ~~the~~ enzyme autolysis and in order to prevent it, a digestion time of 7 h was chosen for the three enzymes. Finally, Table 1 shows the hydrolysis degree obtained under optimal conditions for the three enzymes. In comparison with olive[20] and plum[19] seed proteins, these hydrolysis degrees were slightly lower. There was no significant difference among the hydrolysis degrees observed with the three enzymes ($P > 0.05$).

Evaluation of the *In Vitro* Antioxidant Capacity of Hydrolysates Obtained from Cherry Seed Protein Isolate. Antioxidant properties were evaluated in hydrolysates obtained using the three enzymes under optimal conditions. For that purpose, four different assays evaluating different antioxidant capabilities were applied: radical scavenging capacity, reduction capacity, and capacity to inhibit lipid peroxidation. Figure 2 shows the antioxidant capacity obtained by the two radical scavenging assays (ABTS and hydroxyl radical scavenging assays), by the assay measuring the reduction capacity of peptides (FRAP assay), and by the assay evaluating the capacity to inhibit lipid peroxidation. In all cases, Flavourzyme was the enzyme yielding the peptides with the lowest antioxidant capacity while Alcalase and Thermolysin resulted in the peptides with the highest antioxidant capacities. These results were similar to the observed with peptides obtained from plum[19] and olive[20] seed proteins. Nevertheless, in comparison

346 with olive and plum seeds, antioxidant capacity of cherry seed peptides resulted lower
347 when using the ABTS and the hydroxyl radical assays. In order to obtain a fraction
348 containing most antioxidant peptides, whole peptide extracts obtained with Alcalase and
349 Thermolysin enzymes were fractionated according to their molecular weights within
350 fractions above 5 kDa, from 5 to 3 kDa, and below 3 kDa. Figure 3 shows the
351 antioxidant capacity of the whole peptide extracts and fractions. In all cases, the whole
352 peptide extract showed a higher antioxidant capacity than the fractions, being the
353 fraction from 3 to 5 kDa that always yielding the lowest antioxidant capacity while
354 fractions above 5 kDa and below 3 kDa showed higher values. These results were
355 compared with the amount of peptides determined by OPA assay in the whole peptide
356 extract and in every fraction. As expected, the fraction showing the highest peptide
357 amount was the fraction below 3 kDa while the fraction above 5 kDa presented a much
358 lower peptide content. This situation was also observed in olive seed peptide fractions[20].
359 Taking into account this data, the fraction containing peptides with the highest
360 antioxidant capacities seemed to be the fraction above 5 kDa for both Alcalase and
361 Thermolysin enzymes. Therefore, these fractions were selected for the identification of
362 peptides by MS.
363
364 **Evaluation of the antihypertensive capacity of hydrolysates obtained from the**
365 **cherry seed protein isolate.** Antihypertensive capacity of whole peptide extracts was
366 evaluated by measuring the capacity to inhibit ACE activity. Figure 4A shows no ACE
367 inhibition in the case of the Flavourzyme peptide extract while the Thermolysin peptide
368 extract showed the lowest IC_{50} value (0.31 ± 0.07 mg/mL) and, thus, the highest
369 inhibition capacity. Results obtained were again compared with those from plum[19] and
370 olive[20] seed proteins. ACE inhibition activity of cherry seed peptides was similar to the
371 observed in plum seed peptides but lower to the observed in olive seeds peptides.

Thermolysin fractions were next obtained by ultrafiltration and ACE inhibition capacity was evaluated in every fraction (see Figure 4B). The fraction below 3 kDa yielded the highest capacity to inhibit ACE activity while the fraction from 3 to 5 kDa showed no ACE inhibition.

Peptide Identification in most Bioactive Fractions by RP-HPLC-ESI-Q-TOF. Peptide identification was carried out in the fractions yielding the highest antioxidant capacity (fractions above 5 kDa obtained with Alcalase and Thermolysin enzymes) and antihypertensive capacity (fraction below 3 kDa obtained with the Thermolysin enzyme).

Peptide identification in the fraction above 5 kDa from the Alcalase peptide extract. A total of 51 different peptides were identified in the two independent samples of cherry seed peptides injected by duplicate. Nevertheless, only 6 common peptides were observed in the four analysis. In order to increase the number of identified peptides, more concentrated peptide extracts were next analysed (10-fold more concentrated). Using these concentrated samples, 8 additional peptides were observed although one peptide (TELAL), originally observed in the diluted sample, was not identified in the concentrated one. Ionization of this peptide could have been suppressed by other peptides in the concentrated sample. Identified peptides have been grouped in Table 2. Identified peptides showed molecular weights below 5 kDa in all cases (Mw < 1.3 kDa). These results could suggest a very low selectivity in the ultrafiltration process as it was observed previously[21]. No common peptide was observed with peptides identified in the olive seed[20] peptide extract while one common peptide (NLPLL) was identified when comparing with peptides in plum seed[19] peptide extract.

397 *Peptide identification in the fraction above 5 kDa from the Thermolysin peptide extract.*
398 In this case, 50 different peptides were identified in the two independent samples of
399 cherry seed peptides injected by duplicate. Among them, 14 common peptides were
400 observed. When more concentrated samples were analysed, 3 new peptides were
401 identified but there were 6 peptides, observed in the diluted samples, that could not be
402 detected in the concentrated ones probably due to ionization suppression. All identified
403 peptides are listed in the Table 2. Again, the low efficiency of the ultrafiltration
404 procedure could be the reason of the low molecular weight of identified peptides. All
405 peptides observed in the fractions above 5 kDa, showing antioxidant ability (see Table
406 2), presented common features. Indeed, all peptides showed a high percentage of
407 hydrophobic (V, I, L) and aromatic amino acids (H, F, W, Y), common features within
408 antioxidant peptides[22].

409 *Peptide identification in the fraction below 3 kDa from the Thermolysin peptide extract.*
410 A total of 21 different peptides were identified in the two independent samples of
411 cherry seed peptides injected by duplicate, being just 5 the number of common peptides.
412 Some of these peptides presented proline (P) in its sequence that is a characteristic
413 feature within antihypertensive peptides[11]. All these peptides were previously observed
414 in the fraction above 5 kDa confirming the low selectivity of ultrafiltration filters. Many
415 of these peptides were also observed in plum seed[19] peptide extract (LYSPH, LYTPH,
416 LLAQA, LAGNPENE, LLNDE, LLMQ) but no common peptide was detected when
417 comparing with olive seed peptides[20].

418

419 In conclusion, it has been possible to extract proteins from a waste material
420 derived from the processing of cherry. Protein content of cherry seeds was close to 39%
421 of the dried and defatted seed. The digestion of extracted proteins with different

422 enzymes enabled to obtain peptides showing antioxidant and antihypertensive
423 properties. Flavourzyme was the enzyme yielding the peptides with the lowest
424 antihypertensive and antioxidant capacity while Thermolysin was the enzyme showing
425 the peptides with the highest antihypertensive capacity. Fractionation of peptides by
426 ultrafiltration allowed obtaining fractions with high antioxidant or antihypertensive
427 capacities. HPLC-ESI-Q-TOF enabled to identify the peptides in most active fractions
428 which are potentially responsible for the observed bioactivities. Identified peptides in
429 antioxidant fractions showed less than 10 amino acids and a high number of
430 hydrophobic and aromatic amino acids, which are characteristic features for antioxidant
431 peptides. Some identified peptides in antihypertensive fraction contained proline which
432 is a characteristic amino acid within antihypertensive peptides. These results open a new
433 pathway for the recovery of this waste material, its revalorization, and reuse.
434

435

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443

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FIGURE CAPTIONS

Figure 1. SDS-PAGE separation of proteins from the cherry seed protein isolate.

Figure 2. Antioxidant capacity of the cherry seed protein isolate digested with Alcalase, Flavourzyme, and Thermolysin and evaluated using different assays.

Figure 3. Comparison of hydrolysis degree and antioxidant capacity of fractions obtained from the cherry seed peptide extracts using Alcalase and Thermolysin with those corresponding to the whole extract.

Figure 4. IC_{50} values (mg/mL) of the cherry seed protein isolate digested with Alcalase, Flavourzyme, and Thermolysin (A) under optimal conditions and of the peptide fractions obtained from the Thermolysin peptide extract (B).

Table 1. Parameters optimized in the digestion of the cherry seed protein isolate with Alcalase, Flavourzyme, and Thermolysin enzymes[a].

Enzyme	pH	Protein concentration (mg/mL)	Enzyme:protein ratio	Digestion time (h)	Hydrolysis degree (%)[b]
Alcalase	8.0, **8.5**	**2.5**, 5.0, 7.5, 10	**0.10**, 0.15, 0.30, 0.50, 0.90 AU enzyme/g protein	0.5, 1, 1.5, 2, 3, 4, **7**, 24	51 ± 2
Flavourzyme	**6.0**, 6.5, 7.0	**2.5**, 5.0, 7.5, 10	25, 50, **75**, 100 AU enzyme/g protein	0.5, 1, 1.5, 2, 3, 4, **7**, 24	49 ± 4
Thermolysin	**8.0**	**2.5**, 5.0, 7.5, 10	0.03, **0.05**, 0.10, 0.40 mg enzyme/g protein	0.5, 1, 1.5, 2, 3, 4, **7**, 24	55 ± 5

[a] Selected conditions are in bold. [b] Hydrolysis degrees obtained under optimal digestion conditions.

Table 2. Peptides identified by RP-HPLC-Q-TOF in fractions above 5 kDa from the Alcalase or Thermolysin peptide extracts (fractions showing the highest antioxidant capacity) and in the fraction below 3 kDa from the Thermolysin digestion extract (fraction showing the highest antihypertensive capacity).

Fraction	Peptide sequence	ALC (%)	Mass
Fraction > 5 kDa obtained from the Alcalase peptide extract	DGDPLLDQ	96	871.392
	NLPLL	95	568.359
	NGDPLLDQ	95	870.408
	ESGAVTE	94	691.302
	QLPLL	93	582.374
	QLPEPDNRLQ	94	1208.615
	AFGPE	93	519.233
	DEVPR	94	614.302
	NLGNPE	91	642.297
	NLGDPS	92	601.271
	FVLGL	93	547.337
	QLNEPDNRLQ	91	1225.605
	VVNE	91	459.233
	TELAL*	92	545.306
Fraction > 5 kDa obtained from the Thermolysin peptide extract	LYSPH	97	615.302
	LYTPH	96	629.317
	FDAVGVK	96	734.396
	LLPGANH	96	720.392
	LAGNPENE	94	842.377
	LLNDE	95	602.291
	LLNDEVKEGQ	95	1143.577
	LAGNPQDE	95	842.377
	LLES	94	460.253
	LLAQA	93	514.312
	YGPQQQE	91	848.366
	VTYDYYKN*	96	1061.481
	LTPTSN*	96	631.318
	LQAE*	93	459.233
	FGPEMEQ*	95	836.338
	LLMQ*	94	503.278
	LFSPR*	91	618.349
Fraction < 3 kDa obtained from the Thermolysin peptide extract	LYSPH	96	615.302
	LYTPH	94	629.317
	LTPTSN	94	631.318
	LLPGANH	94	720.392
	LLMQ	92	503.278

* Peptides only observed in the diluted sample.

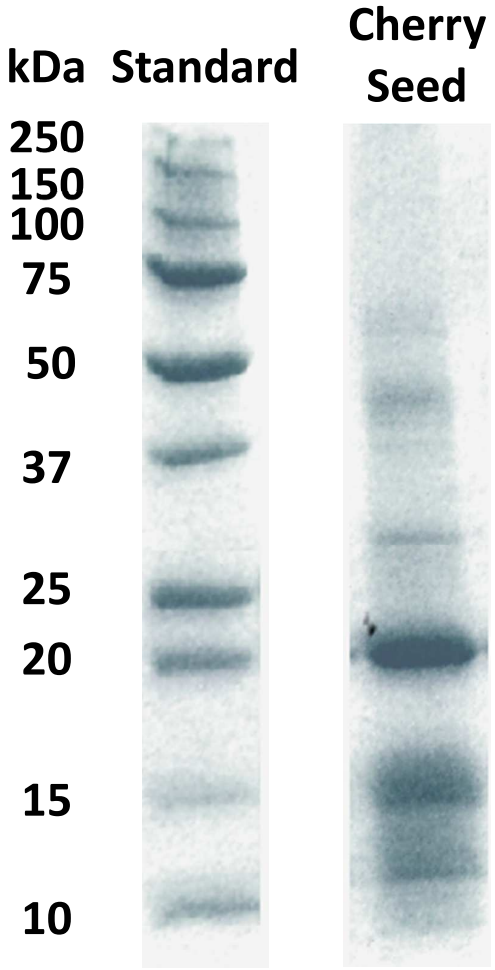


Figure 1

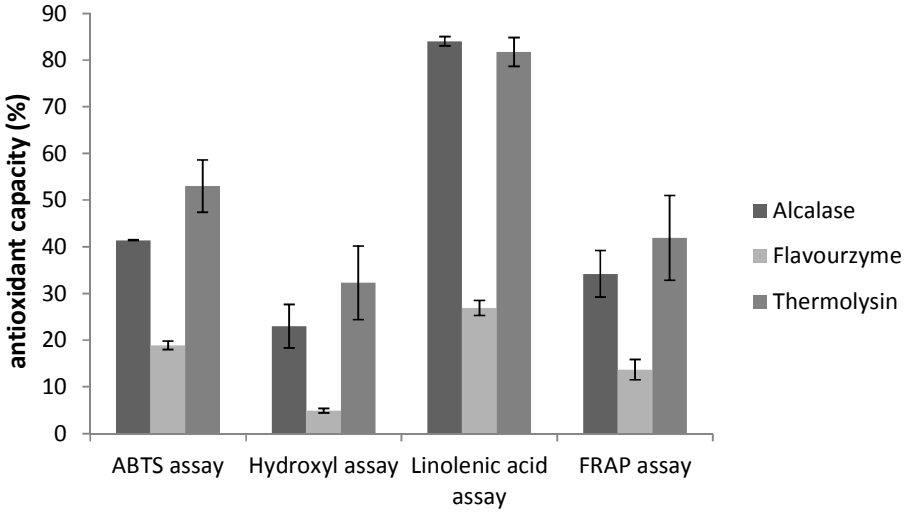

antioxidant capacity (%)
90
80
70
60
50
40
30
20
10
0
ABTS assay
Hydroxyl assay
Linolenic acid assay
FRAP assay
Alcalase
Flavourzyme
Thermolysin

Figure 2

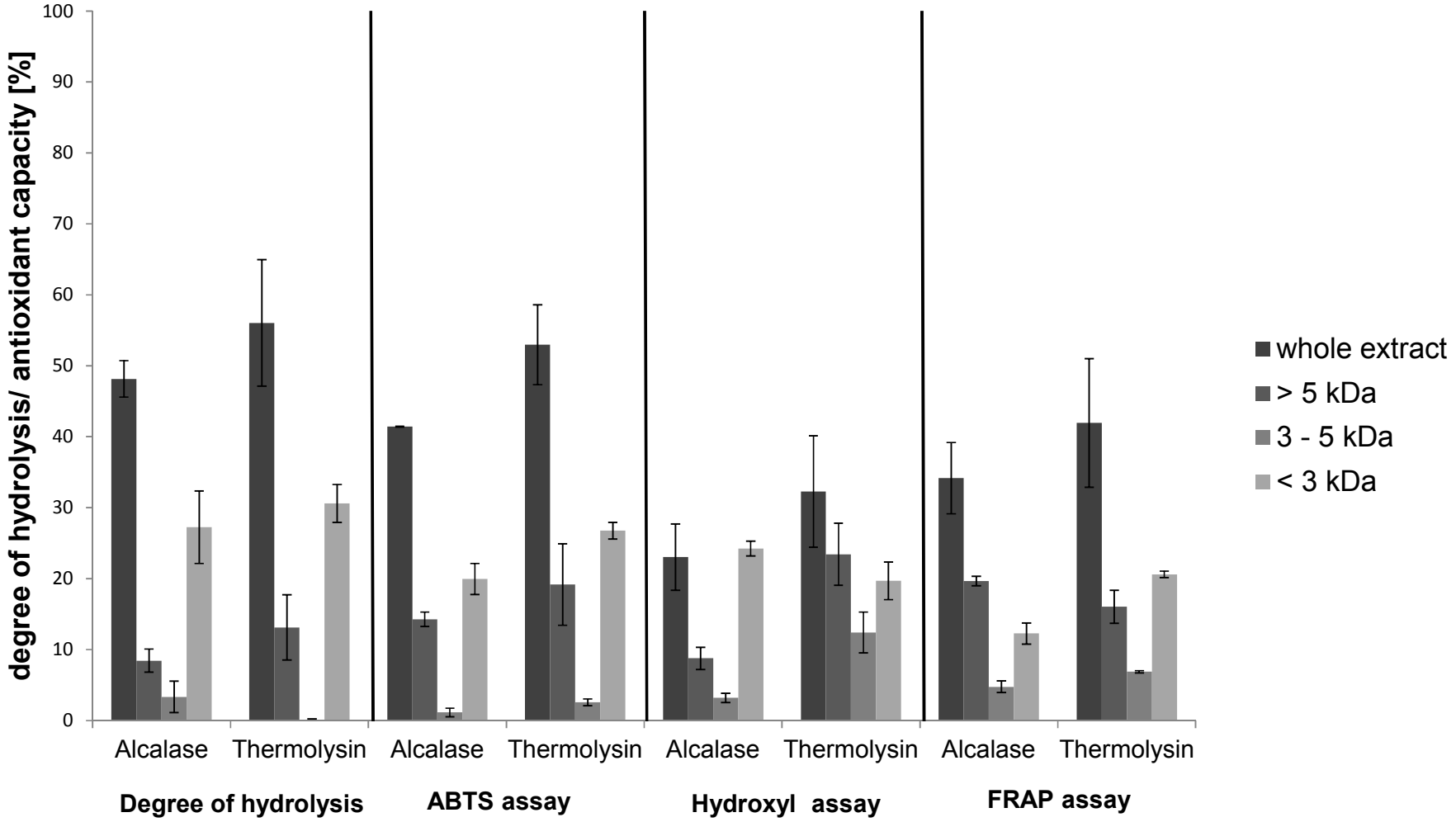

degree of hydrolysis/ antioxidant capacity [%]
100
90
80
70
60
50
40
30
20
10
0
Alcalase
Thermolysin
Alcalase
Thermolysin
Alcalase
Thermolysin
Alcalase
Thermolysin
Degree of hydrolysis
ABTS assay
Hydroxyl assay
FRAP assay
whole extract
> 5 kDa
3 - 5 kDa
< 3 kDa

Figure 3

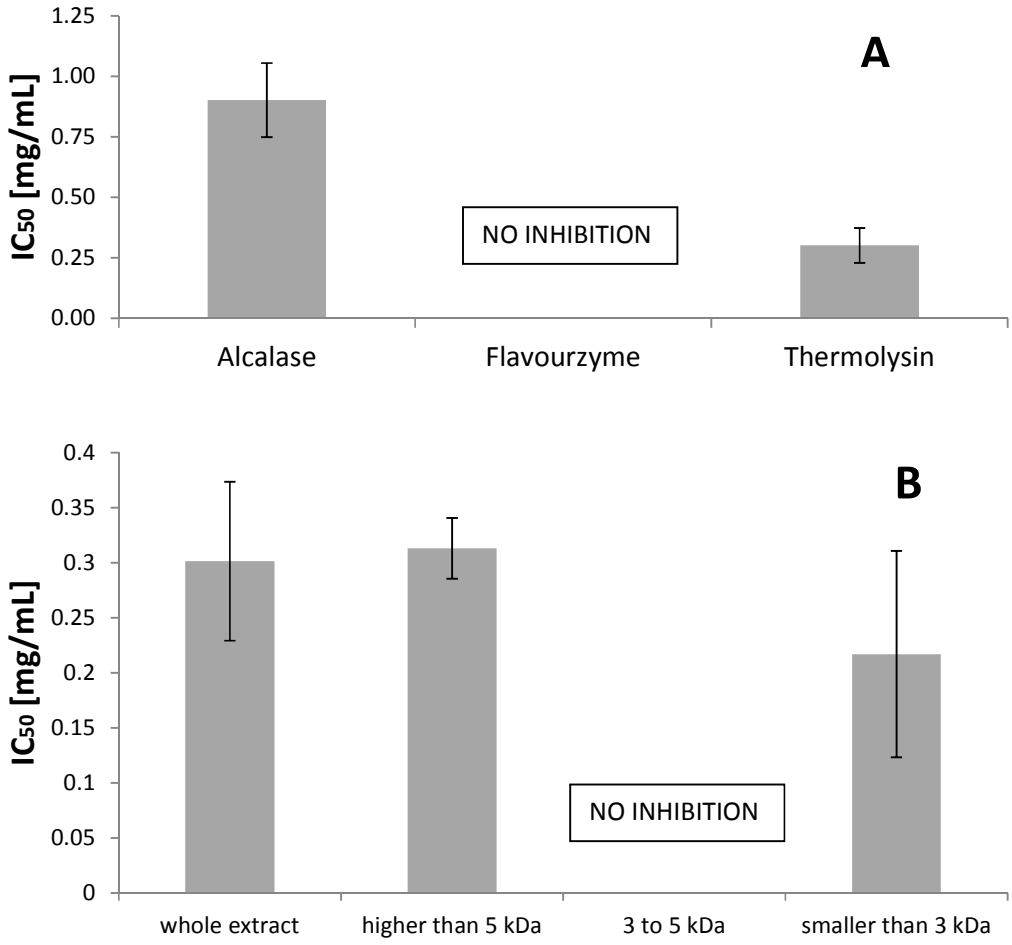
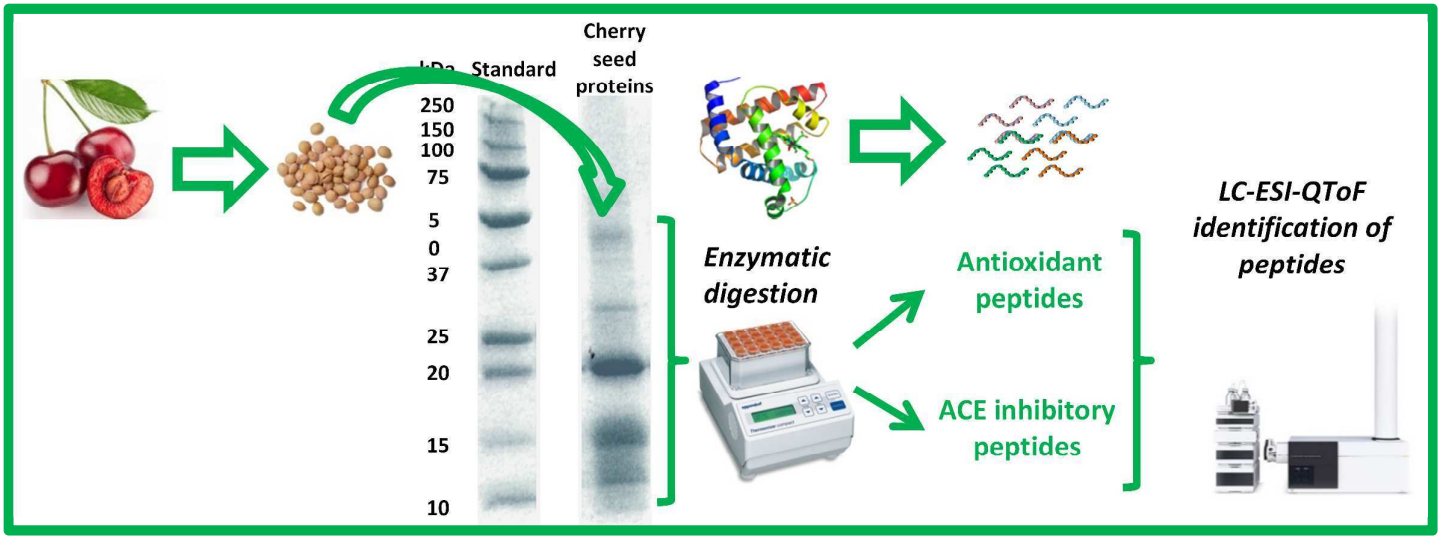

A
IC50 [mg/mL]
1.25
1.00
0.75
0.50
0.25
0.00
NO INHIBITION
Alcalase
Flavourzyme
Thermolysin
B
IC50 [mg/mL]
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
NO INHIBITION
whole extract
higher than 5 kDa
3 to 5 kDa
smaller than 3 kDa

Figure 4


Standard
Cherry seed proteins
250
150
100
75
5
0
37
25
20
15
10
Enzymatic digestion
Antioxidant peptides
ACE inhibitory peptides
LC-ESI-QToF identification of peptides