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Article

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

María Concepción García, Jochen Endermann, Estefanía González-García, and María Luisa Marina

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1   **HPLC-Q-TOF-MS Identification of Antioxidant and Antihypertensive**

2   **Peptides Recovered from Cherry (*Prunus Cerasus* L.) Subproducts**

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6   MARÍA CONCEPCIÓN GARCÍA*, JOCHAN ENDERMANN, ESTEFANÍA GONZÁLEZ-GARCÍA,

7   MARÍA LUISA MARINA

8

9   Department of Analytical Chemistry, University of Alcalá, Ctra. Madrid-Barcelona,

10   Km. 33.600, E-28871 Alcalá de Henares, Madrid, Spain.

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18   * Corresponding author: concepcion.garcia@uah.es,

19   Tel: (+34) 918854915, Fax: (+34) 918854971

20

21 **ABSTRACT**

22   The processing of fruits, such as cherries, is characterized by generating a lot of

23 waste material such as fruit stones, skins, etc. In order to contribute to environmental

24 sustainability, it is necessary to recover these residues. Cherry stones contain seeds with

25 a significant amount of proteins that are underused and undervalued. The aim of this

26 work was to extract cherry seed proteins, to evaluate the presence of bioactive peptides,

27 and to identify them by mass spectrometry. The digestion of cherry seed proteins was

28 optimized and three different enzymes were employed: Alcalase, Thermolysin, and

29 Flavourzyme. Peptide extracts obtained by the digestion of the cherry seed protein

30 isolate with Alcalase and Thermolysin yielded the highest antioxidant and

31 antihypertensive capacities. Ultrafiltration of hydrolysates allowed obtaining fractions

32 with high antioxidant and antihypertensive capabilities. HPLC-Q-TOF-MS together

33 with bioinformatics tools enabled to identify peptides in these fractions.

34

35 **KEYWORDS:** cherry, seed proteins, peptide, antihypertensive, antioxidant, MS

36 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]

62   In fact, the intake of antioxidant peptides could be helpful to fight against many diseases

63   originated by reactive oxygen species[8] that, through the oxidation of lipids or proteins,

64   breakage of DNA, or modification of nucleotides, can lead to serious diseases like

65   atherosclerosis or cancer.[5] Moreover, food oxidation results in food deterioration by the

66   production of undesirable off-flavours, odours, dark colours, potentially toxic reaction

67   products, etc. Despite there are some synthetic antioxidants, the questionable safety of

68   these synthetic compounds and the negative consumer perception, make natural

69   antioxidants like bioactive peptides very valued.[6,9,10] On the other hand, hypertension

70   can lead to many serious diseases like cardiovascular and kidney diseases.[6,7]

71   Angiotensin converting enzyme (ACE) plays a central role in the development of high

72   blood pressure. Therefore, inhibition of ACE activity is the main way to defeat this

73   disease. Despite there are some ACE inhibitors that have been synthesized, they usually

74   lead to adverse side effects.[11] Again, the consumption of antihypertensive peptides could

75   be of great interest.

76   The main objective of this work was to extract cherry seed proteins, to optimize

77   proteins digestion using suitable enzymes to obtain bioactive peptides, to fractionate

78   most active hydrolysates, and to identify potential bioactive peptides by RP-HPLC-Q-

79   TOF-MS and bioinformatics tools.

80

81   **MATERIAL AND METHODS**

82   **Chemicals and Samples.** All chemicals and reagents were of analytical grade. Water

83   was daily obtained from a Milli-Q system from Millipore (Bedford, MA, USA). Sodium

84   dodecylsulfate (SDS), tris(hydroxymethyl)aminomethane (Tris) (all from Merck), n-

85   hexane (96%), acetone (both from Scharlab (Barcelona, Spain)), and dithiothreitol

86   (DTT) (from Sigma-Aldrich (St. Louis, MO, USA)) were used for the extraction of

87   cherry seed proteins. Tris/glycine/SDS running buffer, Laemmli buffer, Bio-Safe

88   Coomassie G250 stain, Mini Protean precast gels, Precision Plus Protein Standards

89   (recombinant proteins expressed by *E. Coli* with molecular masses of 10, 15, 20, 25, 37,

90   50, 75, 100, 150, and 250 kDa) (all from Bio-Rad-Laboratories (Hercules, CA, USA))

91   were employed for the separation of proteins by SDS-PAGE. Quick Start Bradford –

92   1xDye reagent from Bio-Rad-Laboratories was employed for the estimation of proteins

93   by Bradford assay. Hydrochloric acid, sodium dihydrogen phosphate, sodium hydroxide

94   (all from Merck), sodium tetraborate, and Thermolysin enzyme (both from Sigma

95   Aldrich) were used for the digestion of proteins. Alcalase 2.4 L FG and Flavourzyme

96   1000 L were kindly donated by Novozymes Spain S.A. (Madrid, Spain). Sodium

97   chloride (Merck), angiotensin converting enzyme (ACE), ethanol (EtOH), 2-[4-(2-

98   hydroxyethyl)-1-piperazinyl]-ethanesulfonic acid (HEPES), hippuryl-histidyl-leucine

99   (HHL),   1,10-phenanthroline,   2-mercaptoethanol,   6-hydroxy-2,5,7,8-

100   tetramethylchroman-2-carboxylic acid (Trolox), 2,2'-azino-bis(3-ethylbenzothiazoline-

101   6-sulphonic acid) (ABTS), albumin from bovine serum, ammonium thiocyanate,

102   hydrogen peroxide, ferrous sulfate, iron (II) sulphate, iron (II) chloride,

103   iron(III)chloride, L-gluthathion (GSH), linolenic acid, methanol, ortho-phthalaldehyde,

104   potassium ferricyanide, trichloroacetic acid, and potassium persulfate (all from Sigma-

105   Aldrich) were used to carry out *in vitro* assays. Acetonitrile (ACN) HPLC gradient

106 grade (Scharlab), trifluoroacetic acid (TFA), and acetic acid (both from Sigma-Aldrich)

107 were employed in the preparation of mobile phases in HPLC and HPLC-Q-TOF

108 experiments.

109 **Protein Extraction.** The extraction procedure was similar to the described by Esteve et

110 al.[12] with some modifications. Cherry pulp was removed and the stone was broken to

111 extract cherry seeds. Seeds were milled and fat was removed by extracting three times

112 with hexane (25 mL per 0.5 g seeds). Seeds moisture was determined keeping them at

113 $103 \pm 2\ ^0C$ until constant weight. Defatted seeds were stored at -20 ^0C until use. The

114 procedure used to extract proteins from cherry seeds consisted of mixing 30 mg of

115 defatted seeds with 5 mL of 100 mM Tris-HCl buffer (pH 7.5) containing 1 % SDS and

116 0.5 % DTT. Extraction was carried out using high intensity focused ultrasounds (HIFU)

117 for 5 min and an amplitude of 30 % followed by centrifugation at 4000g for 20 min.

118 Proteins from the supernatant were next precipitated with 10 mL of cold acetone at 4 °C

119 for 1 h. After centrifugation at 4000g for 10 min, precipitated proteins were dried over

120 night at 37 °C. Pellets were weighed and then dissolved in the previous Tris-HCl buffer

121 (without DTT) using HIFU for 5 min at 30% amplitude. The solution was filtrated

122 through 0.45 µm and stored at 4 °C until use. The protein content in this solution was

123 estimated by Bradford assay.[13]

124

125 **Protein Separation.** Proteins were separated by SDS-PAGE and RP-HPLC. SDS-

126 PAGE separation was carried out in a Mini-Protean from Bio Rad. Samples were

127 prepared by mixing 15 µL of the cherry seed protein isolate with 15 µL of Laemmli

128 buffer containing 5 % (v/v) β-mercaptoethanol and by heating for 5 min at 100 ºC.

129 Electrophoresis was carried out on commercial Ready Gel Precast Gels using

130 Tris/glycine/SDS as running buffer and applying 80 V for 5 min and 200 V for 30 min.

6

131   For the estimation of molecular weights, peptide standards (10, 15, 20, 25, 37, 50, 75,

132   100, 150, and 250 kDa) were used as ladder. After separation, proteins were fixed with

133   50 mL of 10 % (v/v) glacial acetic acid/40 % (v/v) MeOH gently shaking for 30 min

134   and stained with 50 mL of Bio-Safe Coomassie stain by slightly shaking for 1 h.

135   Finally, the gel was washed with Milli-Q water for 2 h.

136   RP-HPLC separation of the cherry seed protein isolate was performed in a

137   modular Agilent Technologies liquid chromatograph (Pittsburg, PA, USA). All samples

138   were prepared twice and measured by duplicate. A Poros R2/10 Perfusion column (4.6

139   mm D × 50 mm) at a flow-rate of 2 mL/min and a temperature of 25 °C were used. The

140   gradient was 30-40 %B in 13 min, 40-60 % B in 6 min, 60- 80 %B in 1 min, and 80-

141   30 % B in 2 min. Mobile phase A was water with 0.1 % (v/v) of TFA and mobile phase

142   B consisted of ACN with 0.1 % (v/v) TFA. Detection was carried out with a fluorescent

143   detector at λ_{exc}= 280 and λ_{em}= 360 nm.

144

145   **Protein Digestion and Fractionation.** Digestion of extracted proteins was carried out

146   using three different enzymes: Alcalase (borate buffer (pH 8.5), 0.10 AU enzyme /g

147   protein), Thermolysin (phosphate buffer (pH 8.0), 0.4 g enzyme/g protein), and

148   Flavourzyme (bicarbonate buffer (pH 6.0), 75 AU enzyme/g protein). In all cases, a

149   protein concentration of 2.5 mg/mL, a digestion temperature of 50 °C, and a digestion

150   time of 7 h were selected. Additionally, blank digestions without proteins prepared by

151   mixing buffer and enzyme were carried out. Digestions were carried out by slight

152   mixing (600 rpm) at a controlled temperature in a Thermomixer Compact (Eppendorf

153   AG, Hamburg, Gernmany). Digestions were stopped by heating to 100 °C for, at least,

154   10 min and, then, they were centrifuged (5 min at 7000 rpm).

155   Hydrolysates were fractionated by ultrafiltration using Vivaspin 500 PES

156   molecular weight (Mw) cut-off filters (5 kDa) (Sartorius Stedim biotech, Goettingen,

157   Germany) and Amicon Mw cut-off filters (3 kDa) (Millipore).

158

159   **Degree of Hydrolysis (%DH).** The degree of hydrolysis was determined by the OPA

160   assay following the method of Wang et al.[14] with modifications. OPA mixture (5 mL)

161   consisted of 2.5 mL of 100 mM sodium tetraborate, 1 mL of 5 % (w/v) SDS, 100 μL of

162   40 mg/mL OPA dissolved in MeOH, 10 μL of β-mercaptoethanol, and 1.39 mL of

163   water. The assay consisted of mixing 2.5 μL sample with 100 μL OPA mixture and

164   measuring the absorbance at 340 nm (spectrophotometer Lambda 35, Perkin-Elmer,

165   Waltham, MA) after 8 min at room temperature. The peptide content was calculated by

166   interpolation in a calibration curve obtained using GSH (0-5 mg/mL). The degree of

167   hydrolysis was calculated by the following equation:

$$DH = \frac{peptide\ content}{protein\ content}\ x\ 100 \qquad [1]$$

168   where the protein content was determined by the Bradford assay.

169

170   **Antioxidant Capacity.** Four different *in vitro* antioxidant assays were carried out for

171   the evaluation of the antioxidant capacity of hydrolysates from the cherry seed protein

172   isolate.

173   *ABTS radical scavenging assay*. The scavenging capacity against ABTS radicals

174   was evaluated according to the method of Wiriyaphan et al.[15] with modifications.

175   ABTS$^{\bullet+}$ stock solution was prepared by mixing a 7.4 mM ABTS solution with 2.6 mM

176   potassium persulfate in 10 mM phosphate buffer (pH 7.4) for, at least, 16 h kept in the

177   dark. ABTS$^{\bullet+}$ working solution was daily prepared by mixing the stock solution with 10

178   mM phosphate buffer (pH 7.4). The assay was carried out by mixing 1 μL of sample

179   with 100 µL of ABTS$^{\bullet+}$ working solution for 6 min at room temperature. The

180   absorbance corresponding to ABTS$^{\bullet+}$ was measured at 734 nm. ABTS$^{\bullet+}$ scavenging

181   capacity was calculated using the following equation:

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

182

183   where Abs_{sample} was the absorbance of 1 µL sample with 100 µL of ABTS$^{\bullet+}$ working

184   solution and Abs_{blank} was the absorbance of 1 µL digestion buffer with 100 µL of

185   ABTS$^{\bullet+}$ working solution. Trolox (1 mM) was employed as positive control.

186   *Hydroxyl radical scavenging assay*. The capacity to scavenge hydroxyl radicals

187   was evaluated by the method of Ajibola *et al.*[16] with modifications. Hydroxyl radicals

188   were obtained by the oxidation of Fe (II) with hydrogen peroxide. Antioxidant

189   compounds inhibit the oxidation of Fe (II) and the formation of hydroxyl radicals. The

190   assay was monitored by measuring the absorbance corresponding to the colored

191   complex resulted from the reaction between Fe (II) and 1,10-phenanthroline. The assay

192   consisted of mixing 50 µL of 3 mM 1,10-phenanthroline in 0.1 M phosphate buffer (pH

193   7.4), 50 µL of 3 mM ferrous sulfate, 50 µL of sample, and 50 µL of 0.01 % (v/v)

194   hydrogen peroxide. The mixture was incubated for 1 h at 37 ^{0}C and the absorbance of

195   the Fe (II) complex was measured at 536 nm. The hydroxyl radical scavenging capacity

196   was evaluated according to the following equation:

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

197

198   where Abs_{sample} was the absorbance of the sample, Abs_{blank} was the absorbance of the

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

9

200   adding the digestion buffer instead of sample and water instead of hydrogen peroxide.

201   GSH (1 mg/mL) was employed as positive control.

202

203   *Ferric ion reducing antioxidant power (FRAP) assay.* This assay measures the

204   capacity of the sample to reduce Fe (III) from ferricyanide and it was evaluated

205   according to the method proposed by Ajibola *et al.*[16] with modifications. The assay

206   consisted of incubating 12.5 µL of sample with 12.5 µL of 0.2 M phosphate buffer

207   (pH 6.6) and 25 µL of 1% potassium ferricyanide solution for 20 min at 50 °C in the

208   Thermomixer. The reaction was stopped with 25 µL of 10% (w/v) trichloroacetic acid

209   solution. This solution (50 µL) was mixed with 60 µL of water and 15 µL of 2.5 %

210   iron (III) chloride solution and after 3 min, the absorption at 700 nm was measured. The

211   reducing power was calculated according to the following equation:

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

212   where Abs_{sample} was the absorbance obtained for the hydrolyzate and the peptide

213   content was that calculated by the OPA assay. The positive control was GSH ($0 - 1$

214   mg/mL). The percentage of inhibition was calculated considering the reducing power

215   corresponding to 1 mg/mL GSH as the maximum reduction.

216

217   *Capacity to inhibit lipid peroxidation.* This assay was carried out by the method

218   developed by Chen *et al.*[17] with modifications. The assay involved mixing 20 µL of

219   sample, 20 µL of 0.13 % (v/v) linoleic acid in EtOH, and 10 µL of water at 40 ^{0}C for

220   144 h (6 days) in the dark. The degree of oxidation was monitored by mixing 2.5 µL of

221   that solution with 175 µL of 75 % (v/v) EtOH, 2.5 µL of 30 % (w/v) ammonium

222   thiocyanate, and 2.5 µL of 20 mM Fe (II) chloride in 3.5 % (v/v) HCl for 3 min at room

223   temperature and measuring the absorbance of the resulting ferric thiocyanate at 500 nm.

224   The capacity to inhibit lipid peroxidation of linoleic acid was calculated using the

225   following equation:

Lipid peroxidation inhibition capacity

$$= \left(1 - \frac{Abs_{sample,144h} - Abs_{sample,0h}}{Abs_{blank,144h} - Abs_{blank,0h}}\right) x \; 100 \qquad [6]$$

226

227   where $Abs_{sample,144h}$ and $Abs_{blank,144h}$ were the absorbances of the sample and

228   digestion buffer for 144 h, respectively; and $Abs_{sample,0h}$ and $Abs_{blank,0h}$ were the

229   initial absorbances of the sample and digestion buffer, respectively.

230

231   **ACE Inhibition Capacity.** ACE inhibition capacity was expressed as the peptide

232   concentration needed for the 50% inhibition of ACE activity (IC_{50}). For that purpose, an

233   assay developed by Geng *et al.*[18] based on the hydrolysis of the tripeptide HHL into

234   hippuric acid (HA) by the action of ACE was employed. The assay measures the

235   capacity of hydrolyzates to inhibit ACE activity by monitoring the signal corresponding

236   to HA. In order to calculate IC_{50} value, the whole peptide extract and dilutions up to

237   1:10 ratio were measured for every hydrolysate. All measurements were made by

238   duplicate. In every case, 2.5 µL of sample were mixed with 10 µL of ACE (0.05 U/mL),

239   17.5 µL of 50 mM HEPES buffer (pH 8.3) with 300 mM NaCl, and 5 µL of HHL

240   (1.25 mg/mL). For the controls, 2.5 µL of the corresponding digestion buffer were used.

241   The mixture was kept at 37 °C (in the Thermomixer) and the reaction was stopped by

242   the addition of 50 µL of cold ACN (- 20 °C). Separation and detection of HA was

243   performed by HPLC using a Chromolith Performance RP-18e column (100 mm x

244   4.6 mm I D) from Merck. Separation was carried out at 25 °C and mobile phases

245   consisted of water with 0.025 % (v/v) TFA (mobile phase A) and ACN with 0.025 %

246   (v/v) TFA (mobile phase B). The injection volume was 10 µL. A linear gradient from 5-

247   95 % B in 10 min followed by 95-5% B in 2 min was used. Detection was made at

248   228 nm. Percentage of ACE inhibition was calculated using the following expression

249   where $A_{control}$ was the area under the peak of HA in the control sample and A_{sample} was

250   the area under the peak of HA in the sample.

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

251   For every hydrolysate, the % of ACE inhibition of the undiluted and diluted

252   sample were plotted against the peptide concentration (determined by OPA assay) and

253   the IC_{50} value was determined from the signal corresponding to an inhibition of 50 % of

254   ACE.

255

256   **HPLC-Q-TOF-MS Identification of Bioactive Peptides.** Identification of peptides

257   was carried out by MS/MS detection using a Quadrupole Time-of-Flight (Q-TOF) series

258   6530 coupled to a HPLC (model 1100) both from Agilent Technologies. An Ascentis

259   Express peptide ES-C18 (100 mm x 2.1 mm, 2.7 μm particle size, 160 Å pore size)

260   analytical column with a guard column Ascentis Express peptide ES-C18 (5 mm x 2.1

261   mm), both from Sigma Aldrich, were employed for the separation of peptides. Elution

262   gradient employed for the separation of peptides in fraction > 5 kDa was: 3% B for

263   6 min, followed by 3-43% B in 20 min, 43-55% B in 2 min, 55-74 %B in 9 min, 74-

264   95% B in 2 min, 1 min at 95% B, and 95- 3% B in 2 min. Separation of peptides in the

265   fraction < 3 kDa was carried out with an elution gradient at 3% B for 7 min, 3-60% B in

266   28 min, 60- 95% B in 3 min, 95% B for 2 min, and 95- 3% B in 2 min. Rest of

267   parameters were: separation temperature, 25 °C; injection volume, 15 μL; and flow rate,

268   0.3 mL/min. Mobile phases A and B consisted of water and ACN, both containing

269   0.3 % (v/v) acetic acid. Electrospray ionisation in the positive ion mode at a capillary

270   voltage of 3500 V and a MS-range from m/z 200 to 2200 were employed. Other MS

12

271   conditions were: dry gas flow, 12 L/min; dry gas temperature, 350 °C; nebulizer

272   pressure, 50 psig; sheath gas flow, 12 L/min; and sheath gas temperature, 400 °C. The

273   fragmentation energy was calculated taking into account 5 V per 100 Da molecular

274   weight.

275   In all cases, two independent extracts were analysed by duplicate. Peaks

276   software (Bioinformatics Solutions Inc.; Waterloo, ON, Canada) was employed for the

277   treatment of MS/MS data and *do novo* peptide sequencing using an error tolerance of

278   50.0 ppm for the precursor and 0.5 kDa for the fragment. Peptide identification was

279   accepted if peptides appeared in the two independent samples analysed by duplicate and

280   presented an average local confidence (ALC (%), expected percentage of correct amino

281   acids in the peptide sequence) above 90%. Moreover, since the equipment is not able to

282   distinguish between I and L amino acids due to their equal molecular masses, both

283   amino acids must be considered.

284   **Statistical analysis.** Statistical analysis was performed using Statgraphics Software

285   Plus 5.1 (Statpoint Technologies, Inc., Warrenton, VA).

286   **RESULTS AND DISCUSSION**

287   In order to obtain peptides from cherry seeds, it is necessary to extract firstly

288   cherry seed proteins. Despite there is no method previously developed for the extraction

289   of cherry seeds proteins, the group ~~previous~~ experience on the extraction of proteins

290   from other seeds like olive[12] and plum[19] was very useful. Using extraction conditions

291   described in Materials and Methods, the protein content, estimated by Bradford assay,

292   was 39.2 ± 5.8 g protein/100 g in defatted and dried seeds. This content is similar to the

293   observed previously for plum seeds[19]. Extracted proteins were next separated by SDS-

294   PAGE (see Figure 1) observing numerous bands below 75 kDa. It was important to

295 highlight the bands appearing around 10, 15, and 20 kDa. Additionally, the RP-HPLC

296 separation of the extract using fluorescent detection at wavelengths corresponding to

297 tryptophan and tyrosine amino acids also showed many signals that could correspond to

298 proteins (results not shown). All these results demonstrated that the procedure enabled

299 the extraction of proteins from cherry seeds.

300 In order to obtain peptides from this cherry seed protein isolate, extracted

301 proteins have to be digested with suitable enzymes.

302 **Optimization of the Digestion of the Cherry Seed Protein Isolate.** Taking into

303 account bibliographic data and research group experience, Alcalase, Thermolysin, and

304 Flavourzyme were the enzymes chosen for the hydrolysis of the cherry seed protein

305 isolate. In order to obtain the highest digestion efficiency, different parameters were

306 optimized with every enzyme: pH, protein concentration, enzyme:protein ratio, and

307 digestion time. Digestion temperature was always 50º C, corresponding to the highest

308 enzymes activity. The optimization of the digestion was monitored by the determination

309 of the degree of hydrolysis. Table 1 groups the conditions tested in every case.

310 There was not any significant variation ($P > 0.05$) in the hydrolysis degree when

311 using Alcalase enzyme at pHs 8.0 and 8.5 while Flavourzyme showed a higher

312 hydrolysis degree at pH 6.0 than at higher pHs. In the case of Thermolysin, a pH 8.0

313 was selected taking into account previous results from our research group[19,20].

314 Regarding the substrate concentration, as expected, the degree of hydrolysis decreases

315 when increasing the substrate concentration and a concentration of 2.5 mg/mL was

316 selected for the three enzymes. Moreover, different enzyme:substrate ratios were tried.

317 There was no significant variation ($P > 0.05$) in the hydrolysis degree when using

318 Alcalase enzyme at different enzyme:substrate ratios and the lowest value tried (0.10

319 AU/g proteins) was chosen. In the case of Flavourzyme, the enzyme:substrate ratio

14

320   yielding the highest hydrolysis degree was 75 AU/g protein. Finally, the hydrolysis

321   degree when using Thermolysin varied from 34 (at 0.03 g enzyme/g protein) to 49% (at

322   0.4 g enzyme/g protein) being this the enzyme:substrate ratio firstly chosen.

323   Nevertheless, further investigations revealed that a high enzyme:substrate ratio resulted

324   in enzyme autolysis and, thus, a lower enzyme:substrate ratio (0.05 g enzyme/g protein)

325   was finally selected. In all cases, hydrolysis degree increased rapidly in the first hours of

326   digestions and then, it showed a slow increase up to 24 h. Nevertheless, black digestions

327   performed at 24 h also showed ~~the~~ enzyme autolysis and in order to prevent it, a

328   digestion time of 7 h was chosen for the three enzymes. Finally, Table 1 shows the

329   hydrolysis degree obtained under optimal conditions for the three enzymes. In

330   comparison with olive[20] and plum[19] seed proteins, these hydrolysis degrees were

331   slightly lower. There was no significant difference among the hydrolysis degrees

332   observed with the three enzymes ($P > 0.05$).

333

334   **Evaluation of the *In Vitro* Antioxidant Capacity of Hydrolysates Obtained from**

335   **Cherry Seed Protein Isolate.** Antioxidant properties were evaluated in hydrolysates

336   obtained using the three enzymes under optimal conditions. For that purpose, four

337   different assays evaluating different antioxidant capabilities were applied: radical

338   scavenging capacity, reduction capacity, and capacity to inhibit lipid peroxidation.

339   Figure 2 shows the antioxidant capacity obtained by the two radical scavenging assays

340   (ABTS and hydroxyl radical scavenging assays), by the assay measuring the reduction

341   capacity of peptides (FRAP assay), and by the assay evaluating the capacity to inhibit

342   lipid peroxidation. In all cases, Flavourzyme was the enzyme yielding the peptides with

343   the lowest antioxidant capacity while Alcalase and Thermolysin resulted in the peptides

344   with the highest antioxidant capacities. These results were similar to the observed with

345   peptides obtained from plum[19] and olive[20] seed proteins. Nevertheless, in comparison

15

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.

16

372   Thermolysin fractions were next obtained by ultrafiltration and ACE inhibition capacity

373   was evaluated in every fraction (see Figure 4B). The fraction below 3 kDa yielded the

374   highest capacity to inhibit ACE activity while the fraction from 3 to 5 kDa showed no

375   ACE inhibition.

376

377   **Peptide Identification in most Bioactive Fractions by RP-HPLC-ESI-Q-TOF.**

378   Peptide identification was carried out in the fractions yielding the highest antioxidant

379   capacity (fractions above 5 kDa obtained with Alcalase and Thermolysin enzymes) and

380   antihypertensive capacity (fraction below 3 kDa obtained with the Thermolysin

381   enzyme).

382   *Peptide identification in the fraction above 5 kDa from the Alcalase peptide extract.* A

383   total of 51 different peptides were identified in the two independent samples of cherry

384   seed peptides injected by duplicate. Nevertheless, only 6 common peptides were

385   observed in the four analysis. In order to increase the number of identified peptides,

386   more concentrated peptide extracts were next analysed (10-fold more concentrated).

387   Using these concentrated samples, 8 additional peptides were observed although one

388   peptide (TELAL), originally observed in the diluted sample, was not identified in the

389   concentrated one. Ionization of this peptide could have been suppressed by other

390   peptides in the concentrated sample. Identified peptides have been grouped in Table 2.

391   Identified peptides showed molecular weights below 5 kDa in all cases (Mw < 1.3 kDa).

392   These results could suggest a very low selectivity in the ultrafiltration process as it was

393   observed previously[21]. No common peptide was observed with peptides identified in the

394   olive seed[20] peptide extract while one common peptide (NLPLL) was identified when

395   comparing with peptides in plum seed[19] peptide extract.

396

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

18

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

443

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507

508 **FIGURE CAPTIONS**

509

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

511 **Figure 2**. Antioxidant capacity of the cherry seed protein isolate digested with Alcalase,

512 Flavourzyme, and Thermolysin and evaluated using different assays.

513 **Figure 3**. Comparison of hydrolysis degree and antioxidant capacity of fractions

514 obtained from the cherry seed peptide extracts using Alcalase and Thermolysin with

515 those corresponding to the whole extract.

516 **Figure 4**. IC_{50} values (mg/mL) of the cherry seed protein isolate digested with Alcalase,

517 Flavourzyme, and Thermolysin (A) under optimal conditions and of the peptide

518 fractions obtained from the Thermolysin peptide extract (B).

24

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).

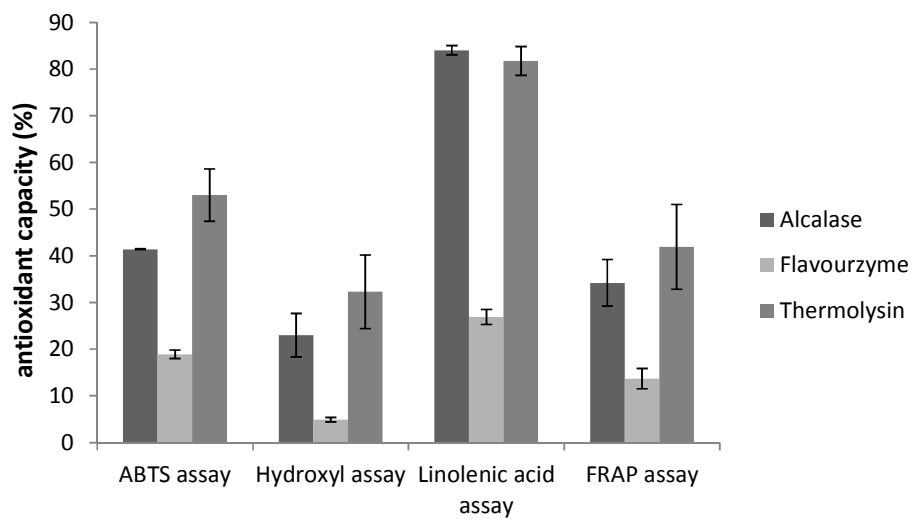| Fraction	Peptide sequence	ALC (%)	Mass
Fraction > 5 kDa obtained from the Alcalase peptide extract	DGDPLLDQ	96	871.392
	NLPLL	95	568.359
	NGDPLLDQ	95	870.408
	ESGAVTE	94	691.302
	QLPLL	93	582.374
	QLPEPDNRLQ	94	1208.615
	AFGPE	93	519.233
	DEVPR	94	614.302
	NLGNPE	91	642.297
	NLGDPS	92	601.271
	FVLGL	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.
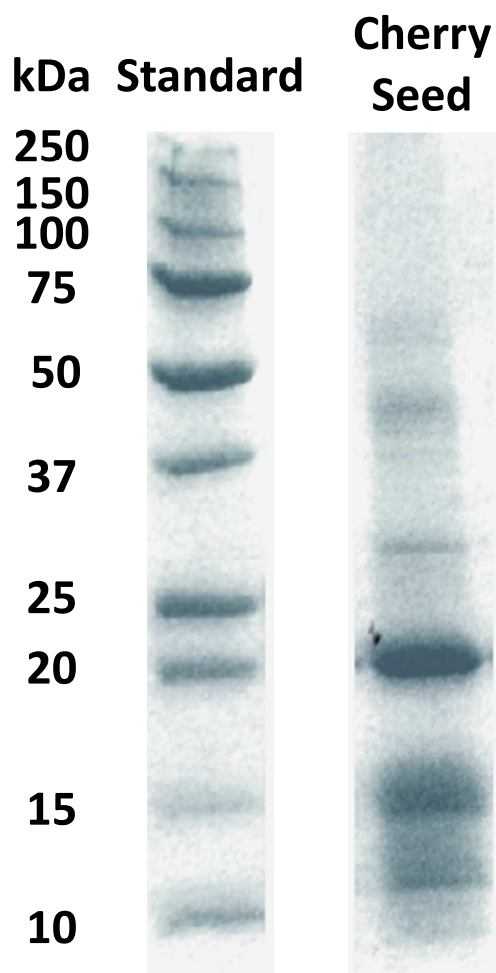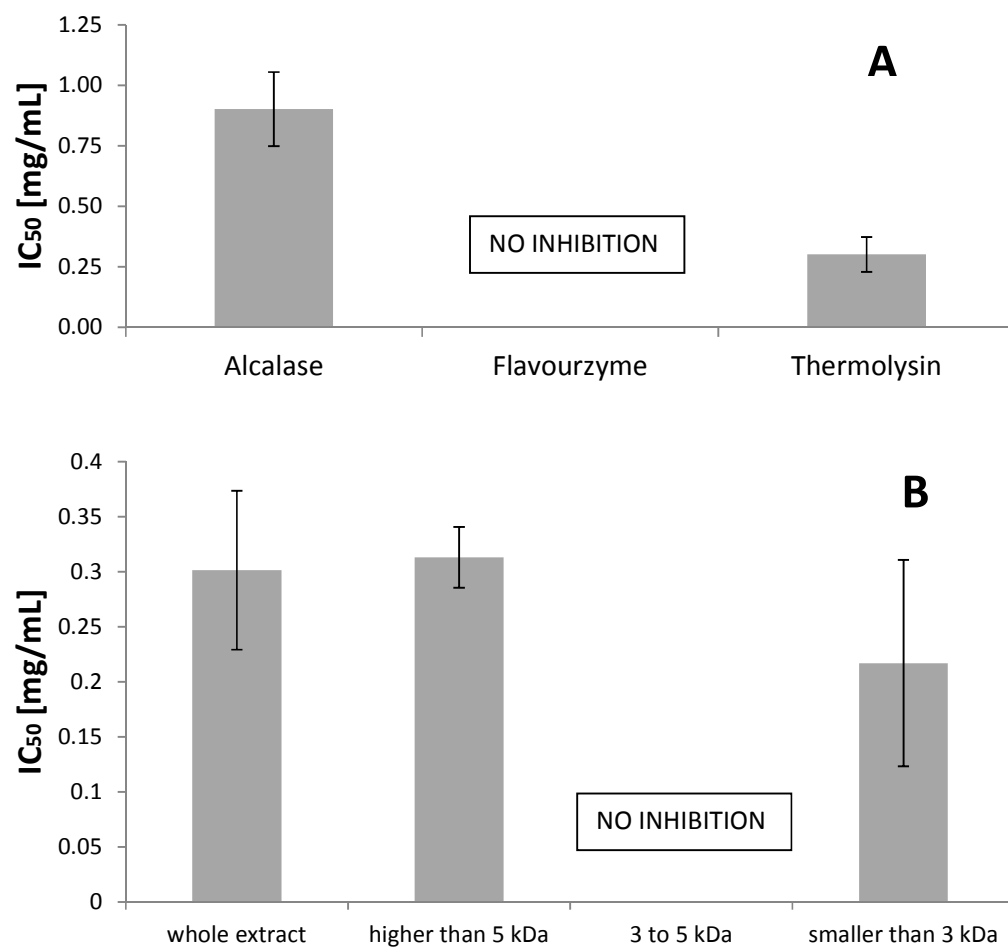
Figure 1

Figure 2

Figure 3

29

Figure 4