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Isolation and characterization of angiotensin converting enzyme inhibitory peptides from peach seed hydrolysates: *in vivo* assessment of antihypertensive activity

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ABSTRACT

Peptide fraction with molecular masses below 3 kDa (PSH-3kDa) from a peach seed hydrolysate demonstrated high angiotensin converting enzyme (ACE) inhibitory activity (concentration to inhibit 50% ACE (IC_{50}) = 16.4 µg/mL) in our previous work. This work proposes a further study of this highly active fraction. RP-HPLC enabled to isolate two fractions (F3 and F4) with high inhibitory activity (IC_{50} = 2.0 ± 0.5 µg/mL and 1.2 ± 0.2 µg/mL, respectively). Peptide analysis by LC-Q-TOF-MS/MS using reverse-phase and hydrophilic interaction chromatography enabled to identify 33 peptides within both fractions. Among them, peptide IYSPH showed the highest capacity. The lack of cytotoxicity of peptides was demonstrated in three different cell lines (HeLa, HT-29, and HK-2). Oral administration of PSH-3kDa fraction or peptide IYSPH caused a significant systolic blood pressure reduction (-30 mmHg) on spontaneously hypertensive rats after 3-6 h treatment.

Keywords: peach seed hydrolysate, ACE-inhibitory peptides, cytotoxic assay, *in vivo* assay, hypertension, chromatography, mass spectrometry.

16 **Introduction**

17 Hypertension is a cardiovascular risk factor that can lead to stroke, myocardial infarction, cardiac
18 failure, dementia, renal failure, and even blindness.[1, 2] According to the World Health Organization
19 (WHO), the global prevalence of raised blood pressure (defined as systolic and/or diastolic blood
20 pressure equal, or above, 140/90 mmHg) in adults was around 22% in 2014.[1]

21 Renin-angiotensin system plays an important role in the regulation of blood pressure. It is based on
22 different enzymatic reactions where angiotensin converting enzyme (ACE) is involved.[3] ACE is a
23 multifunctional zinc containing enzyme located in different tissues in mammalian species.[2, 4, 5] ACE
24 catalyzes the conversion of the decapeptide angiotensin I (ANG I), derived from angiotensinogen, to the
25 octapeptide angiotensin II (ANG II) by the release of peptide carboxyl-terminal histidyl-leucine (HL).[3,6]
26 ANG II induces vasoconstriction and stimulates the release of aldosterone from adrenal cortex.
27 Moreover, ACE also catalyzes the degradation of the vasodilator bradykinin.[4] Synthetic drugs such as
28 Captopril, Enalapril, Lisinopril have been employed to inhibit excessive ACE activity in hypertension
29 treatment. Despite their effectiveness, undesirable side effects such as dry cough, edema, and other health
30 complications usually appear after long-term administration.[7] At this regard, some peptides have
31 demonstrated potent ACE-inhibitory activity and many interesting studies are being carried out to find
32 out their usefulness to reduce moderate levels of hypertension.[8, 9]

33 Many by-products from food processing present high contents of valuable compounds such as
34 proteins.[10] Our research group has reported that fruit stones belonging to *Prunus* and *Olea europaea*,
35 contain seeds with a high protein content. Protein digestion with different enzymes has enabled to obtain
36 peptides with antioxidant, hypocholesterolemic, and antihypertensive properties from plum, cherry,
37 apricot, peach, and olive seeds.[11-16] Within hydrolysates, that obtained with thermolysin enzyme from
38 the peach seed proteins (PSH) showed the highest *in vitro* ability to inhibit ACE. Moreover, most

39 antihypertensive peptides in this hydrolysate were in the fraction passing through 3 kDa cut-off
40 ultrafiltration filters (PSH-3kDa).[13] Nevertheless, the usefulness of these studies is relative if no further
41 research is carried out to demonstrate the *in vivo* ability of "supposed" antihypertensive peptides.

42 This work aims to further study of peptides in fraction PSH-3kDa from peach seeds, which exerted a
43 potent ACE-inhibitory capacity in our previous work [13]. Peptides in fraction PSH-3kDa were fractionated
44 by RP-HPLC and their *in vitro* ACE inhibition activity was determined. Peptides in most active fractions
45 were identified by tandem mass spectrometry using two orthogonal chromatographic modes for the
46 comprehensive separation of peptides. Selected peptides were synthesized and their activity and
47 cytotoxicity were evaluated. Finally, an *in vivo* study was conducted to demonstrate the antihypertensive
48 activity of peptides when administered to spontaneously hypertensive rats.

49 Materials and methods

50 *Materials*

51 All chemicals and reagents were of analytical grade. Milli-Q water was obtained using a Milli-Q
52 system from Millipore (Bedford, MA, USA). Acetonitrile (ACN) of HPLC-and MS-grade, hexane, and
53 acetone were acquired from Scharlau Chemie (Barcelona, Spain). Thermolysin, pepsin, pancreatin,
54 angiotensin converting enzyme (ACE) from rabbit lung, ammonium bicarbonate, dithiothreitol (DTT),
55 sodium hydroxide, acetic acid (AA), N-(2-hydroxyethyl)-1-piperazinyl-N'-(2-ethanesulfonic) acid
56 (HEPES), hippuryl-L-histidyl-L-leucine (HHL), trifluorocetic acid (TFA), 3-(4,5-dimethyl-2-thiazolyl)-
57 2,5 diphenyl tetrazolium bromide (MTT), Dubelcco's modified eagle medium (DMEM), fetal bovine
58 serum (FBS), phosphate-buffered saline (PBS), dimethylsulphoxide (DMSO), and Captopril were from
59 Sigma-Aldrich (St. Louis, MO, USA). Tris(hydroxymethyl)aminomethane (Tris), hydrochloric acid
60 (HCl), sodium phosphate, and sodium dodecyl sulphate (SDS) were purchased from Merck (Darmstadt,
61 Germany). Peptides IYSPH (615.3 g/mol), IYTPH (629.3 g/mol), ILMH (512.3 g/mol), IMAPH (567.3

62 g/mol), IFSPR (618.3 g/mol), VAIP (398.2 g/mol), PILNDE (699.3 g/mol), and ALPDEV (642.3 g/mol)
63 were synthesized by GenScript Corp. (Piscataway, NJ, USA) with a purity above 95%. Peaches were
64 purchased in a local market (Alcalá de Henares, Madrid, Spain).

65 ***Cell lines***

66 Cell lines from human cervical cancer (HeLa), human colon cancer (HT-29), and human renal
67 proximal tubule epithelial (HK-2) were acquired from the American Type Culture Collection ATCC
68 (Rockwell, MD, USA).

69 ***Protein extraction***

70 Protein extraction was carried out using the method described by Vásquez-Villanueva et al.[13] Briefly,
71 a 100 mM Tris-HCl buffer (pH 7.5) containing 0.5% (w/v) SDS and 0.5% (w/v) DTT was added to 30
72 mg of dried and defatted peach seeds. The mixture was sonicated for 1 min at 30% of amplitude using a
73 high intensity focused ultrasound probe. After sonication, the extract was centrifuged at 4000 xg for 10
74 min and extracted proteins were precipitated with cold acetone by keeping in the fridge for 15 min. Then,
75 cold acetone was removed and precipitated proteins were dried and kept for enzymatic digestion.

76 ***Enzymatic protein digestion***

77 Isolated peach seed proteins were enzymatically hydrolyzed as previously reported by Vásquez-
78 Villanueva et al.[13] Isolated proteins were dissolved in 5 mM phosphate buffer (pH 8.0) and thermolysin
79 enzyme was added at an enzyme/substrate ratio of 0.1 g enzyme/g protein. The blend (5 mg/mL; total
80 volume, 5 mL) was incubated at 50 °C for 4 h. Afterwards, the enzyme was inactivated by increasing the
81 temperature up to 100 ºC following by the centrifugation, at 4500 xg for 5 min, and the supernatant was
82 collected. Molecular mass cut-off filters of 3 kDa from Merck Millipore (Tullagreen, Ireland) were
83 employed for the fractionation of the whole hydrolysate. The permeate was collected and named as PSH-
84 3kDa. Peptide concentration in this fraction was 3.5 mg/mL.

The *in vitro* gastrointestinal digestion (GID) was carried out following the method used by Vásquez-Villanueva et al.[13] Briefly, samples were adjusted at pH 2.0 with 1 M HCl and digestion with pepsin enzyme (1:35, enzyme: substrate ratio) was carried out at 37 °C for 1 h. Then, samples were adjusted to pH 5.0 with 0.1 M $NaHCO_3$ and to pH 7.0-8.0 with 0.1 M NaOH and digestion with pancreatin enzyme (1:25, enzyme: substrate ratio) was carried out at 37 °C for 3 h. The reaction was stopped by heating at 95 °C for 15 min.

RP-HPLC separation

PSH-3 kDa fraction, with a peptide concentration of 3.5 mg/mL, was further fractionated by using an HPLC system (from Agilent Technologies, Waldbron, Germany) equipped with a quaternary pump, a fraction collector (both 1100 Series), an automatic injector, and a variable wavelength detector (both 1265 Series). A reverse phase column Jupiter 4 µm Proteo 90 Å (250 mm x 10 mm) from Phenomenex (Torrance, CA, USA) was employed. Mobile phases were: 0.1% TFA/water (v/v) (mobile phase A) and 0.1% TFA/ACN (v/v) (mobile phase B). Elution gradient was: 20% B for 5 min, 25-30 % B in 15 min, and 30-40% B in 15 min at a flow rate of 1 mL/min. The injected volume~~,~~ was 600 µL and the separation temperature was set at 25 °C. Detection wavelengths were set at 210, 254, and 280 nm (for UV detection), and λ_{exc} 280 and λ_{em} 360 nm (for fluorescence detection). Every sample was injected four times and fractions were collected every 5 min from minute 10. Eight fractions were collected, dried in a centrifugal evaporator (Concentrator Plus, Eppendorf, Hamburg, Germany), and stored at -20 °C until their use. Before ACE-inhibitory capacity evaluation, every fraction was dissolved in the initial volume of buffer (2400 µL = 4 x 600 µL). Afterwards, peptide concentration were determined following the method described in a previous work by Vásquez-Villanueva et al.[13]

ACE-inhibitory capacity assay

107 ACE-inhibitory capacity was measured as previously described in Vásquez-Villanueva et al.[17]
108 Briefly, 2.5 µL of sample was mixed with 17.5 µL of 50 mM HEPES, 5 µL of 1.3 mg/mL HHL (in 50
109 mM HEPES at a pH 8.3 and containing 300 mM NaCl), and 10 µL of an ACE solution (0.05 U/mL). The
110 reaction mixture was incubated at 37 °C. After 4 h the reaction was stopped with cold ACN (50 µL). The
111 hippuric acid formed was determined by RP-HPLC using a Zorbax SB-C18 column (0.5 x 150 mm, 5
112 µm) from Agilent. Mobile phases were: 0.05% TFA/water (v/v) (mobile phase A) and in 0.05%
113 TFA/ACN (v/v) (mobile phase B). The linear gradient was 5-95% B in 12 min at a flow rate of 20
114 µL/min. The injected volume was 1 µL and temperature was set at 25 °C. Detection wavelength was set
115 at 228 nm. ACE inhibition was expressed as IC_{50} (peptide concentration required to inhibit a 50% of
116 ACE activity).

117 ***Cytotoxicity studies***

118 Cytotoxic effect of peptide fractions and synthesized peptides was evaluated using the MTT assay.[18]
119 Two cancer cell lines (HeLa and HT-29) and healthy cell line (HK-2) were employed. Cells were cultured
120 (1.0×10^4 viable cells/well) in DMEM containing penicillin-streptomycin, and 10% FBS, and incubated
121 at 37 °C in a humidified atmosphere (5% CO_2). Samples were added to cells at increasing concentrations
122 and incubated for 24 h. Afterwards, 5 mg/mL MTT solution was added to each well in a 1:10 ratio (cell
123 volume: MTT stock solution volume). After 4 h, the MTT solution was removed, cells were washed
124 carefully with PBS, and 500 µL of DMSO was added to dissolve formazan crystals formed by viable
125 mitochondria. Optical density was measured at 570 nm using an automated microplate reader (Model
126 ELX 800, Bio-tek instruments, Winooski, VT, USA). Cytotoxicity was expressed as the percentage of
127 cell viability, considering controls (untreated cells) as 100% viable.

128 ***In vivo antihypertensive effect***

129 *In vivo* experiments were performed using 25-40-week-old (382 ± 43 g) male spontaneously
130 hypertensive rats (SHRs) obtained from Envigo (NJ, USA). All experiments were approved by the ethical
131 committee of the University of Alcalá following European Directive 2010/63/UE and Royal Decree
132 53/2013 of the Spanish Ministry of Economy and Competitiveness. In all experiments, the number of
133 animals was six. The animals were kept at 23 °C under 12 h light/dark cycles. Animals could drink tap
134 water and were fed with a standard diet (Global diet 2014, Envigo, NJ, USA) *ad libitum* during the
135 experiments. Peptides dissolved in water up to a final volume of 1 mL were orally administered to the
136 animals by gastric intubation, between 9 and 10 a.m. The antihypertensive effect was evaluated for the
137 peptide fraction PSH-3kDa (10 mg/kg) and for the peptide IYSPH (1.5 mg/kg). Tap water was used as
138 negative control and captopril (10 mg/ kg), as positive control. The systolic blood pressure (SBP) of
139 SHRs was measured by the non-invasive tail-cuff method using a blood pressure monitor (Pressure Meter
140 LE 5001, PanLab Harvard Apparatus, Harvard, UK). Before blood pressure measurements, the rats were
141 kept at around 30 °C for 15 min. At least, five measurements were taken, and the average of all of them
142 was obtained. All measurements were taken by the same person, under the same conditions, and in the
143 same environment to minimize stress-induced variations in blood pressure.

144 ***Peptide identification by RP-HPLC***

145 Peptides in most active fractions were identified by RP-HPLC-MS/MS and HILIC-MS/MS following
146 the method reported by Vásquez-Villanueva et al. [17] with some modifications. An HPLC system
147 equipped with a quaternary pump (1100 Series), an automated injector, and a variable wavelength (both
148 1265 Series) and coupled to a Quadrupole-Time-of-Flight (Q-TOF) mass spectrometer (6350 Series), all
149 from Agilent Technologies, were used. RP-HPLC column was an Ascentis Express Peptide ES-C18 (100
150 mm x 2.1 mm I.D., 2.7 µm particle size) with a guard column (5 mm x 2.1 mm, 2.7 µm particle size)
151 from Supelco (Bellefonte, PA, USA). Mobile phases were: 0.3% AA/water (v/v) (mobile phase A) and

152 0.3% AA/ ACN (v/v) (mobile phase B). Elution gradient was: 3% B for 3 min and 3-25 % B in 30 min.
153 HILIC column was an Ascentis Express column (100 mm x 2.1 mm I.D., 2.7 µm particle size) with a
154 guard column (5 mm x 2.1 mm, 2.7 µm particle size), also from Supelco. Mobile phases were: 65 mM
155 ammonium acetate in ACN (mobile phase A) and 65 mM ammonium acetate in water (mobile phase B).
156 Elution gradient was: 5-20 % B in 20 min and 20-30% B in 15 min at a flow rate of 0.3 mL/min. The
157 injected volume was 10 µL and temperature was set at 25 °C. The UV absorbances were recorded at 210,
158 280, and 254 nm.

159 Mass spectrometry operated in the positive ion mode using a scanning range from 100 to 1500 m/z.
160 Auto MS/MS was employed and spectra were analyzed by the *de novo* sequencing tool using PEAKS
161 Studio 7 from Bioinformatic Solutions Inc. (Waterloo, ON, Canada). Only peptides with an average local
162 confidence (ALC, expected percentage of correct amino acid in the peptide sequence) equal or above
163 90% and a good precursor pattern were considered valid. Identifications were carried out using three
164 independent samples injected by duplicate.

165 ***Statistical analysis***

166 Three independent replicates, injected by duplicate, were used in all experiments. All results were
167 expressed as mean ± standard deviation. In *in vivo* experiments, the decrease in SBP was expressed as
168 mm Hg ± standard deviation for 5-6 measurements. To compare different treatments and to evaluate the
169 effect of time-course within experiments, data were analyzed by one-way ANOVA using Statgraphics
170 Centurion version XVII (Statpoint Technologies, Inc., Warrenton, VA).

171 **Results and discussion**

172 Previous works reported by our research group revealed that plum, cherry, apricot, peach (*Prunus*
173 genus), and olive (*Olea europaea*) seeds proteins could release peptides with high *in vitro* ACE-

174 inhibitory capacity when they were enzymatically digested with thermolysin.[11-13] Among them, a
175 significant ACE-inhibitory capacity was shown by peptides in fraction PSH-3kDa, obtained from the
176 peach seed. Indeed, this fraction showed the lowest value of IC_{50} (16.4 μg/mL).[13-16] Moreover, it was
177 possible to observe that peptides IYSPH, IYTPH, and IFSRP were within this PSH-3kDa. These peptides
178 have been observed in most *Prunus* genus seeds and they were even quantified recently.[19] Nevertheless,
179 there is not a deep knowledge on which peptides are really involved in the observed ACE-inhibition
180 activity, on their innocuous use in living organisms, and on the *in vivo* activity of these peptides.

181 ***Fractionation of PSH-3kDa***

182 With the purpose to isolate those peptides showing the highest capability to inhibit ACE, and,
183 therefore, with the highest potential to prevent high blood pressure, PSH-3kDa was further fractionated
184 by RP-HPLC. After the optimization of the chromatographic method, eight fractions were collected.
185 Figure 1 depicts the chromatogram obtained for PSH-3kDa fraction and the subfractions collected every
186 5 min from the minute 10, named as F1 up to F8 (Figure 1A). It is interesting to highlight the presence
187 of a big peak at 20 min which mainly corresponded to the enzyme autolysis blank (see Figure 1A and
188 Figure 1B). Nevertheless, the size of this peak in Figure 1A is bigger than the peak in Figure 1B which
189 could indicate the co-elution of peach peptides at the same time. Figure 1A also depicts the percentage
190 of ACE-inhibition obtained with every collected fraction. Fractions from F1 to F5 exhibited a percentage
191 of ACE-inhibition higher than 50% (73.9 ± 0.6%, 56.6 ± 1.5%, 78 ± 3%, 94 ± 2%, 82 ± 9%, respectively)
192 highlighting the ACE-inhibitory capacity of fraction F4. The IC_{50} values corresponding to these five
193 fractions were also evaluated and results are included in Figure 1. The lowest values of IC_{50} were
194 observed in F3 and F4 reaching 2.0 ± 0.5 and 1.2 ± 0.2 μg/mL, respectively. These fractions also showed
195 the lowest peptide concentrations (see Figure 1). Fractions F3 and F4 exhibited higher ACE-inhibitory
196 capacity than the starting fraction, PSH-3kDa (IC_{50}= 16.4 μg/mL). The lack of relationship between the

197 measured IC_{50} value and the percentage of ACE-inhibition reached by fractions can be explained taking
198 into account the peptide concentration and peptide composition of fractions. For instance, fraction F1
199 reached 73.9 ± 0.6% of ACE-inhibition but its IC_{50} was 54 ± 4 μg/mL. This means that this fraction can
200 show a high inhibition percentage because peptides are present in a high concentration and, thus, it needs
201 a high peptide concentration for the inhibition of ACE to 50%. On the other hand, F3 fraction reached
202 78 ± 3% of ACE-inhibition but its IC_{50} was 2.0 ± 0.2 μg/mL. Thus, F3 can reach a high percentage of
203 ACE-inhibition despite the peptides, in this fraction, are in a lower concentration. Due to the lowest IC_{50}
204 values showed by F3 and F4, these fractions were selected for further studies.

205 ***Peptide identification in F3 and F4 subfractions***

206 Subfractions F3 and F4 from PSH-3 kDa were analyzed by HPLC-MS/MS using RP-HPLC and
207 HILIC modes and *de novo* tool for the identification of the amino acid sequences. RP is the
208 chromatographic mode most widely employed in the identification of peptide sequences. Nevertheless,
209 RP-HPLC is limited to peptides with low polarity while polar peptides are eluted in the dead volume.
210 Thus, the combined use of RP-HPLC and HILIC enabled the comprehensive separation and identification
211 of all peptides. Table 1 summarizes the sequence of all identified peptides in fractions F3 and F4, along
212 with their molecular masses, ALC, retention time, and mass accuracy.

213 Several peptides were identified in F3 and F4, even more than those already identified when
214 analyzing the whole PSH-3 kDa, in a previous work.[13] This can be explained considering the further
215 separation of peptides in F3 and F4 which enables the detection of additional peptides, whose ionization
216 was probably suppressed when analyzing the whole PSH-3 kDa. The separation by RP-HPLC allowed
217 the identification of 11 peptides in F3, and 11 in F4, meanwhile, by using HILIC mode, 14 peptides were
218 identified in F3 and 11 in F4. Since some peptides were simultaneously observed by RP-HPLC and
219 HILIC, an overall of 19 peptides were the peptides identified in fraction F3 and 15 in fraction F4. Among

220 the identified peptides, LYSPH, LYTPH, and LFSPR had been previously identified in peach, plum, and
221 cherry seeds hydrolysates that showed ACE-inhibition capacity.[11-13] The remaining peptides were newly
222 sequenced.

223 A recent proteomic study reported by González-García et al.,[20] enabled the identification of proteins
224 in peach and plum seeds. The comparison of peptides sequences with identified proteins enabled to find
225 out the correct sequences in cases where I/L amino acids were present since both have the same molecular
226 mass. Thus, our peptide sequences are IMAPH, ILMH, IYTPH, IFSPR (in F3), IYSPH (in F3/F4) and
227 PILNDE (in F4) instead of LMAPH, LLMH, LYTPH, IFSPR, LYSPH and PILNDE. Moreover, some
228 peptides were identified within sequences of 11S globulins. Globulins are storage proteins present in
229 seeds that are required for the development and growth of seeds. For instance, IYTPH was found in a
230 fragment of Prunin 2 and Pru 2 proteins, IYSPH, IFSPR, ALPDEV, and VAIP in fragments of Prunin 1,
231 and PILNDE in Pru 2. Other peptides such as IMAPH and ILMH were within sequences of proteins that
232 were identified by homology to other plants species such as *Sesamum indicum* (sesame) and *Oryza sativa*
233 (rice).[20]

234 These peptide sequences were searched within BIOPEP and AHTPDB databases (which are
235 databases focused on bioactive and antihypertensive peptides, respectively) but non-coincidences of
236 bioactive peptides were found.[21, 22]

237 Regarding their characteristics, all peptides were short size and showed molecular masses lower than
238 1 kDa. A significant presence of hydrophobic amino acid residues such as A, F, I, L, M, P, and V, and
239 aromatic amino acids such as F and Y in the first three C-terminal positions, was observed. These are
240 typical features for antihypertensive peptides. [23-25] ACE has E, K, R, and Y amino acid residues at the
241 active site and three hydrophobic binding subsites. Zhou et al. determined that peptides with the above
242 mentioned characteristics, theoretically, had high affinity for these active sites which favored the
243 blockade of ACE.[26]

244 Unlike expected, the hydrophobicity of peptides observed by RP-HPLC was similar to those
245 separated by HILIC. Indeed, hydrophobicity plus aromatic residues observed by RP-HPLC in F3 and F4
246 were 67% and 60%, respectively, while by HILIC, the contribution of hydrophobic plus aromatic amino
247 acids was 76% and 64%, respectively. This fact can be explained by the amino acid position in peptide
248 sequences, and by the presence of basic amino acids such as H, K, and R, which are positively charged
249 within peptide sequences separated by HILIC.[27] Finally, Figure 2 shows, as an example, the total ion
250 chromatogram (TIC) corresponding to fraction F3 obtained by RP-HPLC and HILIC modes, and the
251 fragmentation spectrum of IYSPH peptide. As observed, TIC obtained by RP-HPLC resulted in a more
252 crowded chromatogram than to the observed by HILIC. Moreover, IYSPH spectrum shows all y-ions
253 obtained after MS/MS fractionation.

254 ***Characterization of synthesized peptides***

255 Based on the hydrophobic and aromatic amino acid content, the differentiation of I/L, and their
256 potential as antihypertensive peptides, eight peptides from Table 1 were selected for their synthesis and
257 further study. Synthesized peptides were: IYSPH, IMAPH, ILMH, IYTPH, IFSPR, VAIP, PILNDE, and
258 ALPDEV. The percentage of ACE-inhibition and their IC_{50} were evaluated and results are grouped in
259 Table 2.

260 The highest ACE-inhibitory effect was obtained for IFSPR and IYSPH while peptides IYTPH and
261 VAIP reached a moderate percentage of ACE-inhibition. Concerning IC_{50} values, peptide IYSPH exerted
262 the highest ACE-inhibitory capacity (IC_{50} = 24 ± 3 μg/mL (39 ± 5 μM)), while IFSPR (IC_{50} = 31 ± 2
263 μg/mL (50 ± 4 μM)) and VAIP exhibited moderate IC_{50} (142 ± 22 μg/mL (358 ± 55 μM)). The IC_{50}
264 values of synthesized peptides from peach seeds are higher than those obtained for F3 and F4 fractions
265 (2.0 ± 0.5 μg/mL and 1.2 ± 0.2 μg/mL, respectively). Similar results were obtained by Ben Henda et al.

266 with synthesized peptides from Tilapia (hydrolysates with molecular masses < 1 kDa).[28] This behavior
267 could be due to the presence of synergistic effects among peptides with F3 and F4 fractions.

268 In order to evaluate the resistance to gastrointestinal enzymes, peptides were subjected to *in vitro*
269 gastrointestinal digestion (GID). IC_{50} values after GID are also shown in Table 2. After simulated GID,
270 peptides IFSPR and IYSPH kept their activity above 50% of ACE-inhibition although IC_{50} value
271 corresponding to peptide IFSPR was doubled (from 31 to 67 µg/mL) and the corresponding to the IYSPH
272 peptide slightly increased (from 24 to 35 µg/mL). The presence of proline amino acid residue within
273 three C-terminal position probably makes peptide IYSPH to be less susceptible to intestinal peptidases.[29]
274 The IC_{50} for peptide VAIP after GID could not be evaluated since the percentage of ACE inhibition was
275 below 50 %.

276 ***Cytotoxicity of peptides***

277 Cytotoxicity of peptides in PSH-3kDa, F3 and F4 fractions, and IYSPH peptide was evaluated by the
278 MTT assay at different concentrations. This method measures the mitochondrial dehydrogenase activity
279 in living cells.[18] Since each cell line has their own sensitivity, it is recommended the use of different cell
280 lines.[30] Cells were treated with increasing concentrations of samples several times above IC_{50} value.
281 Figure 3, shows the viability of three different kinds of cells (HeLa, HT-29, and HK-2) in presence of
282 peptides from PSH-3kDa, F3 and F4 fractions, and IYSPH peptide. All tested peptides exerted similar
283 tendency, even at high concentrations, reaching a constant value around 80% of cell viability, (always
284 above IC_{50}). Results demonstrated that studied samples did not show cytotoxic effects after 24 h of
285 incubation and that their use in *in vivo* future assays was safe.

286 ***In vivo antihypertensive effects in SHRs***

287 *In vitro* results are often not well correlated with results obtained by *in vivo* experiments. Thus, *in*
288 *vivo* studies are required to assure the antihypertensive effect which greatly depends on the capacity of
289 compounds to reach target organs. *In vivo* assays were carried out with fraction PSH-3kDa that
290 significantly inhibited ACE and that could be easy prepared from peach seed. Moreover, synthetic
291 peptide IYSPH, that exerted a high ACE inhibition, was also employed in *in vivo* assays. Figure 4 depicts
292 the decrease in SBP after the oral administration of the above-mentioned peptides in comparison to
293 captopril (a potent ACE inhibitor), used as positive control. Oral administration of PSH-3kDa and peptide
294 IYSPH at doses of 10 mg/kg and 1.5 mg/kg, respectively, induced a significant reduction of SBP, from
295 187 mmHg (before treatment) to 156 mmHg (after treatment) (p-value < 0.05). Maximum
296 antihypertensive effect was observed between 3-6 h post administration. Captopril at 10 mg/kg showed
297 its maximum hypotensive effect at 3 h after administration, which is statistically higher than the observed
298 for peptide IYSPH and fraction PSH-3kDa (p-value < 0.05) at same time-course. Moreover, there were
299 no statistical differences between hypotensive effects of captopril and peptides at 6 h after administration
300 (p-value > 0.05). The antihypertensive effect of these samples was almost reverted 24 h after
301 administration. These results are comparable to those obtained for lactopeptides VPP and IPP that have
302 also proven their antihypertensive activity in SHR. While peptide IYSPH and fraction PSH-3kDa enabled
303 a SBP reduction around -30 mmHg, the reduction in SBP caused by VPP and IPP peptides is around -29
304 mmHg and -24 mmHg, at 6 h after administration, respectively. [31] Recently, Liu et al. reported
305 antihypertensive peptides from hazelnut protein hydrolysate. [32] Within peptides identified, a peptide with
306 the sequence amino acid YLVR showed a significant reduction in SBP around -40 mmHg at 8 h after
307 administration. [32] Similarly, Dang et al. found a peptide with sequence LVLPGE in broccoli which
308 decreased SBP around -40 mmHg at 8 h after administration. [33] Despite greater hypotensive effect was
309 observed, higher doses (10 mg/kg), in both cases, than for peptide IYSPH (1.5 mg/kg) were employed.

310 Moreover, the required concentration of peach seed hydrolysate to observe the reduction of SBP was
311 low. Indeed, Wang et al. prepared a rice bran protein hydrolysate that showed a suppressive effect by the
312 administration of 50 mg hydrolysate/kg rat,[34] 5 times higher than the required in our case. Other example
313 is the antihypertensive effect of palm kernel cake protein hydrolysate reported by Zarei et al., which
314 significantly inhibited hypertension at a dose of 75 mg/kg.[25]

315 Taking into account the PSH-3 kDa and IYSPH concentrations required to decrease SBP in SHR,
316 and following the Food and Drug Administration draft guidelines,[35] it was possible to calculate the
317 corresponding human equivalent dose that was 0.243 mg/kg, in the case of peptide IYSPH, and 1.62
318 mg/kg, for PSH-3kDa fraction. These results demonstrate the significant potential of using peach seed
319 by-product for the production of bioactive peptides with high hypotensive properties. Nevertheless,
320 additional evaluations are required to elucidate the mechanism of PSH-3kDa and peptide IYSPH for their
321 antihypertensive potency in SHRs.

322 In summary, peptides isolated from a peach by-product have demonstrated potent antihypertensive
323 activity during *in vivo* experiments carried out with hypertensive rats. A high ACE-inhibitory activity
324 was detected in two fractions (F3 and F4) obtained after fractionation by ultrafiltration and RP-HPLC of
325 the starting peach seed hydrolysate. Sequencing by HPLC-MS/MS using two orthogonal
326 chromatographic modes, RP-HPLC and HILIC, guaranteed the comprehensive detection of peptides in
327 these fractions. Most peptides displayed typical features of antihypertensive peptides such as short size
328 and a high content in hydrophobic and aromatic amino acids residues.[36, 37] Moreover, some of them also
329 resisted gastrointestinal digestion. Additionally, a cytotoxic study using three different cell lines (HeLa,
330 HK-2, and HT-29) revealed that these peptides did not result in a significant reduction in cell viability
331 and could be administered to living organisms. The oral administration of PSH-3kDa peptide fraction or
332 peptide IYSPH produced a 16% reduction of systolic blood pressure (around 30 mm Hg) after 3-6 h

333 treatment. Results suggest that peach seed, which is an underused by-product, is useful for the production
334 of peptides with effective antihypertensive activity that can be employed for the development of
335 nutraceuticals and functional foods.

336 **Abbreviations Used**

337 ACE	Angiotensin converting enzyme
338 ALPDEV	Alanine- Leucine- Proline- Aspartic Acid- Glutamic Acid- Valine
339 ANG I	Angiotensin I
340 ANG II	Angiotensin II
341 HeLa	Cervical cancer cells
342 HK-2	Human renal proximal tubule epithelial cells
343 HT-29	Human colon cancer cells
344 IFSPR	Isoleucine- Phenylalanine- Serine- Proline- Arginine
345 ILMH	Isoleucine- Leucine- Methionine- Histidine
346 IMAPH	Isoleucine- Methionine- Alanine- Proline- Histidine
347 IYSPH	Isoleucine-Tyrosine-Serine-Proline-Histidine
348 IYTPH	Isoleucine-Tyrosine-Threonine-Proline-Histidine
349 PILNDE	Proline- Isoleucine- Leucine- Asparagine- Aspartic Acid- Glutamic Acid
350 PSH	Peach seed hydrolysates

351 PSH-3kDa Peach seed hydrolysate containing peptides with molecular weights

352 below 3 kDa

353 SBP Systolic blood pressure

354 SHRs Spontaneously hypertensive rats

355 VAIP Valine- Alanine- Isoleucine- Proline

356

357 **References**

358 (1) World Health Organization (WHO). Global Status Report on noncommunicable diseases **2014** URL:
359 (http://apps.who.int/iris/bitstream/10665/148114/1/9789241564854_eng.pdf). Accessed March 2019.

360 (2) Das, U. N. Is angiotensin-II an endogenous pro-inflammatory molecule? *Med. Sci. Monit.* **2005**, 11,
361 155-162.

362 (3) Struthers, A. D.; McDonald, T. M. Review of aldosterone and angiotensin II-induced target organ
363 damage and prevention. *Cardiovasc. Res.* **2004**, 21, 663-670.

364 (4) Paul, M.; Poyan Mehr, A.; Kreutz, R. Physiology of local renin-angiotensin systems. *Physiol. Rev.*
365 **2006**, 86, 747-803.

366 (5) Mehta, P. K.; Griendling, K. K. Angiotensin II cell signaling: physiological and pathological effects
367 in the cardiovascular system. *Am. J. Physiol. Cell Physiol.* **2007**, 292, 82-97.

368 (6) Curtiss, C.; Chon, J. N.; Vrobel, T.; Francious, J. A. Role of the rennin-angiotensin system in the
369 systemic vasoconstriction of chronic congestive heart failure. *Circulation,* **1978**, 58,763–70.

370 (7) Sweeting, M. J.; Thompson, S. G.; Brown, L. C.; Greenhalgh, R. M.; Powell, J. T. Use of angiotensin
371 converting enzyme inhibitors is associated with increased growth rate of abdominal aortic aneurysms. *J.*
372 *Vasc. Surg.* **2012**, 52, 1-4.

373 (8) Puchalska, P.; Marina, M. L.; García, M. C. Isolation and characterization of peptides with
374 antihypertensive activity in foodstuff. *Crit. Rev. Food Sci. Nutr.* **2015**, 55, 521-551.

375 (9) Rizzello, C. G.; Tagliazucchi, D.; Babini, E.; Rutella, G. S.; Taneyo, Saa D. L.; Gianotti, A. Bioactive
376 peptides from vegetable food matrices: Research trends and novel biotechnologies for synthesis and
377 recovery. *J. Funct. Foods,* **2016**, 27, 549-569.

378 (10) Varzakas, T.; Zakynthinos, G.; Verpoort, F. Plant food residues as a source of nutraceuticals and
379 functional foods. *Foods,* **2016**, 5, 88.

380 (11) González-García, E.; Puchalska, P.; Marina, M. L.; García, M. C. Fractionation and identification
381 of antioxidant and angiotensin-converting enzyme-inhibitory peptides obtained from plum (*Prunus*
382 *domestica L.*) stones. *J. Funct. Foods,* **2014**, 19, 376-384.

383 (12) García, M. C.; Endermann, J.; González-García, E.; Marina, M. L. Identification of antioxidant and
384 antihypertensive peptides in cherry (*Prunus cereasus L.*) kernels by HPLC-Q-TOF-MS. *J. Agric. Food*
385 *Chem.* **2015**, 63, 1514-1520.

386 (13) Vásquez-Villanueva, R.; Marina, M. L.; García, M. C. Revalorization of a peach (*Prunus persica*
387 (*L.*) Batsch) byproduct: Extraction and characterization of ACE-inhibitory peptides from peach stones.
388 *J. Funct. Foods,* **2015**, 18, 137-146.

389 (14) García, M. C.; González-García, E.; Vásquez-Villanueva, R.; Marina, M. L. Apricot and other seed
390 stones: amygdalin content and the potential to obtain antioxidant, angiotensin I converting enzyme
391 inhibitor and hypocholesterolemic peptides. *Food Funct.* **2016**, 7, 4693-4701.

392 (15) Vásquez-Villanueva, R.; Marina, M. L.; García, M. C. Identification by hydrophilic interaction and
393 reversed-phase liquid chromatography-tandem mass spectrometry of peptides with antioxidant capacity
394 in food residues. *J. Chromatogr. A* **2016**, 1428, 185-192.

395 (16) Esteve, C.; Marina, M. L.; García, M. C. Novel strategy for the revalorization of olive (*Olea*
396 *europaea*) residues based on the extraction of bioactive peptides. *Food Chem.* **2015**, 167, 272-280.

397 (17) Vásquez-Villanueva, R.; Muñoz-Moreno, L.; Carmena, M. J.; Marina, M. L.; García M. C. *In vitro*
398 antitumor and hypotensive activity of peptides from olive seeds. *J. Funct. Foods*, **2018**, 42, 177-184.

399 (18) Mosmann, T. Rapid colorimetric assay for cellular growth and survival: application to proliferation
400 and cytotoxicity assays. J. *Immuno.l Methods.***1983**, 65, 55-63.

401 (19) González-García, E.; García, M. C.; Marina, M. L. Capillary liquid chromatography-ion trap-mass
402 spectrometry methodology for the simultaneous quantification of four angiotensin-converting enzyme-
403 inhibitory peptides in *Prunus* seed hydrolysates. *J. Chromatogr. A* **2018**, 1540, 47-54.

404 (20) González-García, E.; Marina, M. L.; García, M. C.; Righetti, P. G.; Fasoli, E. Identification of plum
405 and peach seed proteins by nLC-MS/MS via combinatorial peptide ligand libraries. *J. Proteomics,* **2016**,
406 48, 105-112.

407 (21) Minkiewicz, P.; Dziuba, J.; Iwaniak, A.; Dziuba, M.; Darewicz, M. BIOPEP database and other
408 programs for processing bioactive peptide sequences. *J. AOAC Int.* **2008**, 91, 965-980.

409 (22) Kumar, R.; Chaudhary, K.; Sharma, M.; Nagpal, G.; Chauhan, J. S.; Singh, S.; Gautam, A.; Raghava,
410 G. P. S. AHTPDB: a comprehensive platform for analysis and presentation of antihypertensive peptides.
411 *Nucleic Acids Res.* **2015**, 43, 956-962.

412 (23) Vermeirssen, V.; Van Camp, J.; Verstraete, W. Bioavailability of angiotensin I converting enzyme
413 inhibitory peptides. *Br. J. Nutr.* **2004**, 92, 357-366.

414 (24) Yamamoto, N.; Akino, A.; Takano, T. Antihypertensive effect of the peptides derived from casein
415 by an extracellular proteinase from *lactobacillus helveticus* cp 790. *J. Dairy Sci.* **1994**, 77, 917-922.

416 (25) Zarei, M.; Forghani, B.; Ebrahimpour, A.; Abdul-Hamid, A.; Anwar, F.; Saari, N. *In vitro* and *in*
417 *vivo* antihypertensive activity of palm kernel protein hydrolysates: sequencing and characterization of
418 potent bioactive peptides. *Ind. Crops Prod.* **2015**, 76, 112-120.

419 (26) Zhou, Z.; Cheng, C.; Li, Y. Structure-based design and optimization of antihypertensive peptides to
420 obtain high inhibitory potency against both renin and angiotensin I-converting enzyme. *SAR*
421 *QSAR Environ. Res.* **2015**, 26, 1001-1016.

422 (27) Le Maux, S.; Nongonierma, A. B.; FitzGerald, R. J. Improved short peptide identification using
423 HILIC-MS/MS: Retention time prediction model based on the impact of amino acid position in peptide
424 sequence. *Food Chem.* **2015**, 173, 847-854.

425 (28) Ben Henda, Y.; Bonnet, A.; Nunes González da Silva, O. L.; Boscolo Wilson, R.; Arnaudin, I.;
426 Bridiau, N.; Maugard, T.; Piot, J.-M.; Sannier, F.; Bordenave-Juchereau, S. Identification of ace
427 inhibitory cryptides in Tilapia protein hydrolysate by UPLC-MS/MS coupled to database analysis. *J.*
428 *Chromatogr. B Analyt. Technol. Biomed. Life Sci.* **2017**, 1052, 43-50.

429 (29) Manoharan S, Shuib AS, Abdullah N. Structural characteristics and antihypertensive effects of
430 angiotensin-I-converting enzyme inhibitory peptides in the renin-angiotensin and kallikrein-kinin
431 systems. *Afr. J. Tradit. Complement. Altern. Med.* **2017,** 14, 383–406.

432 (30) Phelan, M.; Aherne, A.; FitzGerald, R. J.; O'Brien N. M. Casein- derived bioactive peptides:
433 biological effects, industrial uses, safety aspects and regulatory status. *Int. Dairy J.* **2009**, 19, 643–654.

434 (31) Nakamura, Y.; Yamamoto, N.; Sakai, K.; Takano, T. Antihypertensive effect of sour milk and
435 peptides isolated from it that are inhibitors to Angiotensin I-Converting Enzyme. *J. Dairy Sci.* **1995**, 78,
436 1253-1257.

437 (32) Liu, C.; Fang, L.; Min, W.; Liu, J.; Li, H. Exploration of the molecular interactions between
438 angiotensin-I-converting enzyme (ACE) and the inhibitory peptides derived from hazelnut (*Corylus*
439 *heterophylla Fisch*). *Food Chem.* **2018**, 245, 471-480.

440 (33) Dan, Y.; Zhou, T.; Hao, L.; Cao, J.; Sun, Y.; Pan, D. In vitro and in vivo studies on the angiotensin-
441 converting enzyme inhibitory activity peptides isolated from broccoli protein hydrolysate. *J. Agric. Food*
442 *Chem.* **2019**, 67, 6757-6764.

443 (34) Shobako, N.; Ogawa, Y.; Ishikado, A.; Harada, K.; Kobayashi, E.; Suido, H.; Kusakari, T.; Maeda,
444 M.; Suwa, M.; Matsumoto, M.; Kanamoto, R.; Ohinata, K. A novel antihypertensive peptide identified
445 in thermolysin-digested rice bran. *Mol. Nutr. Food Res.* **2018**, 62, 1700732.

446 (35) USFDA. Guidance for industry estimating the maximum safe starting dose in initial clinical trials
447 for therapeutics in adult healthy volunteers. Rockville, MD: US Food and Drug Administration; 2005

448 (36) García-Mora, P.; Martín-Martínez, M.; Bonache, M.A.; González-Múniz, R.; Peñas, E.; Frias, J.;
449 Martínez-Villaluenga, C. Identification, functional gastrointestinal stability and molecular docking
450 studies of lentil peptides with dual antioxidant and angiotensin I converting enzyme inhibitory activities.
451 *Food Chem.* **2017**, 221, 464-472.

452 (37) Erdmann, K., Cheung, B.W.Y.; Schroeder, H. The possible roles of food-derived bioactive peptides
453 in reducing the risk of cardiovascular disease. J. Nutr. Biochem. **2008**, 19, 643-654.

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458 **Notes**

459 The authors declare no competing financial interest.

460

461 **Figure captions**

462 **Figure 1.** Purification of PSH-3kDa by RP-HPLC. Chromatogram (A) shows the percentage of ACE-
463 inhibition and the IC_{50} (μg/mL), for fractions exceeding 50% of ACE-inhibition, vs. time. Next to
464 chromatogram, the peptide concentration for every fraction exceeding 50% of ACE-inhibition.
465 Chromatogram (B) shows the hydrolysis blank corresponding to the autolysis of thermolysin enzyme.

466 **Figure 2.** Total ion chromatograms (TIC) obtained by RP-HPLC and HILIC coupled to ESI-Q-TOF for
467 fraction F3 and fragmentation spectrum corresponding to IYSPH peptide.

468 **Figure 3**. Percentage of cell viability obtained when treating three different cell lines (HeLa, HT-29, and
469 HK-2) with fractions PSH-3kDa (A), F3 (B), F4 (C) and with synthesized peptide IYSPH (D).

470 **Figure 4.** Time-course of systolic blood pressure (SBP) after oral administration of Captopril (10 mg/kg,
471 ●), PSH-3kDa (10 mg/kg, ■), and peptide IYSPH (1.5 mg/kg, ▲) to SHRs. Changes of SBP from baseline
472 (time 0) are expressed by the mean ± standard deviation.

Table 1. Peptide Sequence, Average Local Confidence (ALC), and Molecular Mass of Peptides Identified in Fractions F3 and F4 by RP-HPLC- and HILIC-ESI-Q-TOF-MS/MS.

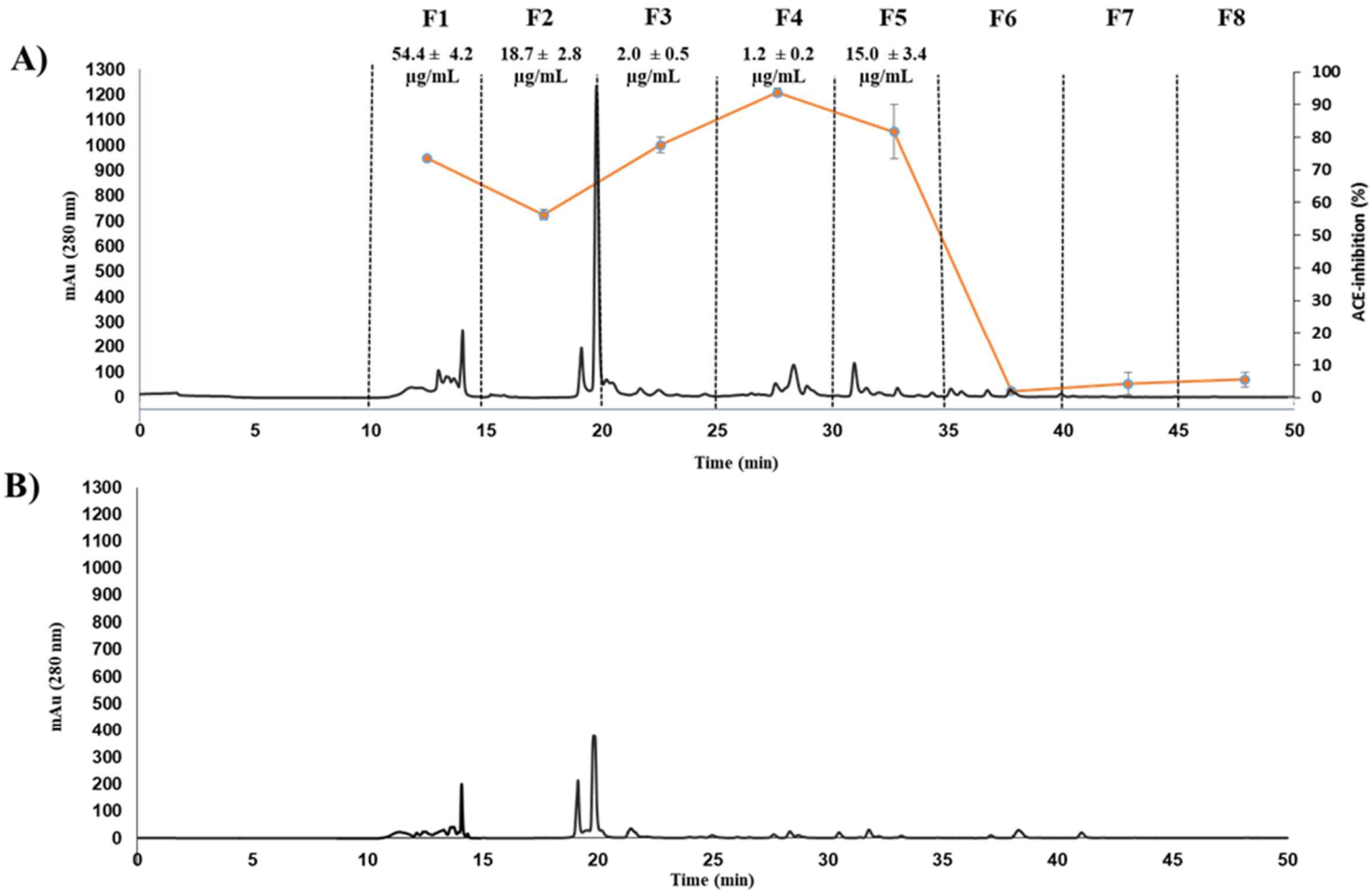
	Peptide Sequence	ALC (%)	Mass (Da)	Retention time (min)	Mass Accuracy (ppm)
F3					
RP-HPLC	LVLTE	96	573.3373	21.49	2.0
	FEET	94	524.2118	6.49	0.6
	LDDLRPR	93	883.4875	15.28	0.4
	LLVE	92	472.2892	18.77	3.1
	VEPET	90	573.2646	3.97	2.4
HILIC	LLYTPH	97	742.4014	22.62	5.9
	LLYSPH	97	728.3857	22.23	4.7
	VVVVPHN	95	762.4388	29.41	5.0
	LLVR	94	499.3482	23.93	5.4
	LVTPH	93	661.3547	29.9	5.2
	LYNPR	93	661.3547	29.71	5.7
	FEPR	93	547.2754	30.92	6.7
	VLGA	90	358.2216	9.21	4.6
RP-HPLC and HILIC	IYSPH	96[a], 97[b]	615.3016	9.74[a], 23.64[b]	1.4[a],3.6[b]
	IMAPH	92[a], 96[b]	567.2839	10.33[a], 29.37[b]	1.2[a],6.0[b]
	ILMH	94[a], 96[b]	512.2781	9.89[a],20.28[b]	2.4[a],5.0[b]
	LMSPH	93[a], 96[b]	583.2788	6.59[a],27.67[b]	0.5[a],5.5[b]
	IYTPH	94[a], 95[b]	629.3173	12.7[a],24.46[b]	1.6[a],4.1[b]
	IFSPR	90[a], 94[b]	618.3489	13.38[a],27.18[b]	1.2[a],6.7[b]
F4					
RP-HPLC	EPFE	97	520.2169	18.24	3.8
	IYSPH	96	615.3016	9.69	1.7
	MLPSLPK	95	784.4517	26.06	5.3
	VAVNL	94	514.3115	17.41	2.2
	VAIP	94	398.2529	16.39	1.7
	VAVDL	94	515.2955	24.12	3.5
	VVDEDGD	93	747.2922	2.95	4.8
	EVLED	91	603.2751	11.98	6.1
HILIC	PLLDDE	98	700.3279	24.65	2.7
	PDEV	96	458.2013	25.92	1.9
	LLLR	91	513.3638	16.3	1.5
	VLFSPR	91	717.4173	20.38	3.4
RP-HPLC and HILIC	PLLNDE	97[a], 98[b]	699.3439	18.24[a], 24.07[b]	3.2[a], 3.2[b]
	LVAY	92[a], 91[b]	464.2635	18.78[a], 6.1[b]	6.9[a], 1.3[b]
	ALPDEV	90[a], 93[b]	642.3224	16.78[a], 25.57[b]	0.2[a], 1.2[b]

[a] Parameters obtained by RP-HPLC.
[b] Parameters obtained by HILIC.

Table 2. ACE-Inhibition Activity (%) of Synthesized Peptides and IC_{50} (µg/mL) of Peptides exceeding 50 % of ACE-Inhibition, before and after Simulated Gastrointestinal Digestion (GID).

Peptide Sequence	Fraction	ACE-inhibition (%)*	IC_{50} (µg/mL)	GID ACE-inhibition (%)	GID IC_{50} (µg/mL)
IMAPH	3	23.8			
IYTPH	3	47.5			
ILMH	3	38.0			
IFSPR	3	75.7	31 ± 2	66 ± 1	67± 5
IYSPH	3/4	64.8	24 ±3	74 ± 2	35 ± 3
VAIP	4	51.7	142 ± 22	35 ± 4	
ALPDEV	4	6.8			
PILNDE	4	10.8			

*Peptide concentration used to determine the percentage of ACE-inhibition was 0.5 mg/mL.



Fraction	Concentration (mg/mL)
F1	2.35 ± 0.09
F2	0.37 ± 0.03
F3	0.16 ± 0.01
F4	0.10 ± 0.01
F5	0.41 ± 0.02

Figure 1.

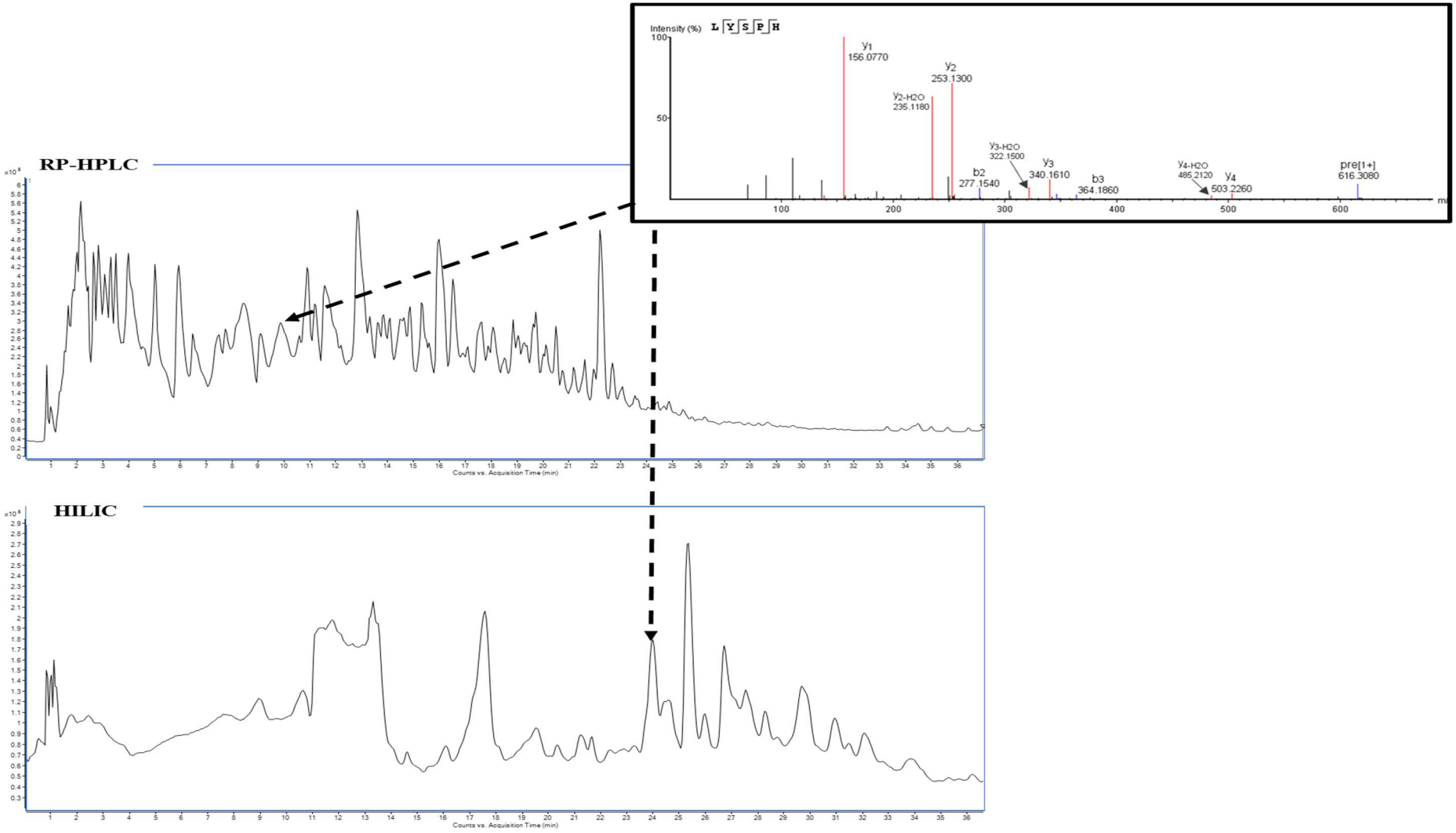

RP-HPLC
HILIC
Intensity (%)
L Y S P H
y1
156.0770
y2
253.1300
y2-H2O
235.1180
y3-H2O
322.1500
y3
340.1610
b2
277.1540
b3
364.1860
y4-H2O
485.2120
y4
503.2260
pre[1+]
616.3080
Counts vs. Acquisition Time (min)

Figure 2.

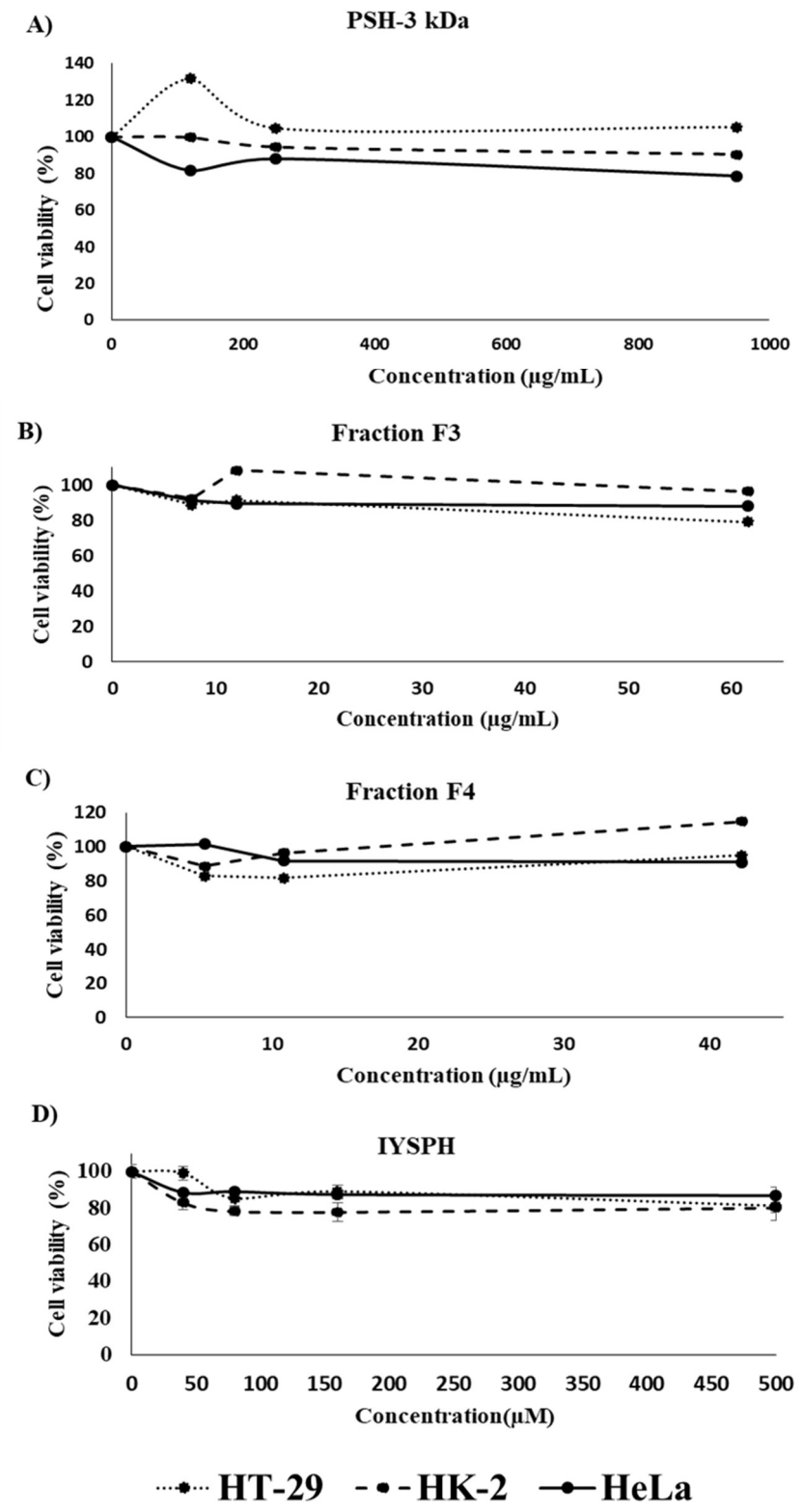

A)
PSH-3 kDa
Cell viability (%)
Concentration (µg/mL)
B)
Fraction F3
Cell viability (%)
Concentration (µg/mL)
C)
Fraction F4
Cell viability (%)
Concentration (µg/mL)
D)
IYSPH
Cell viability (%)
Concentration(µM)
HT-29
HK-2
HeLa

Figure 3.

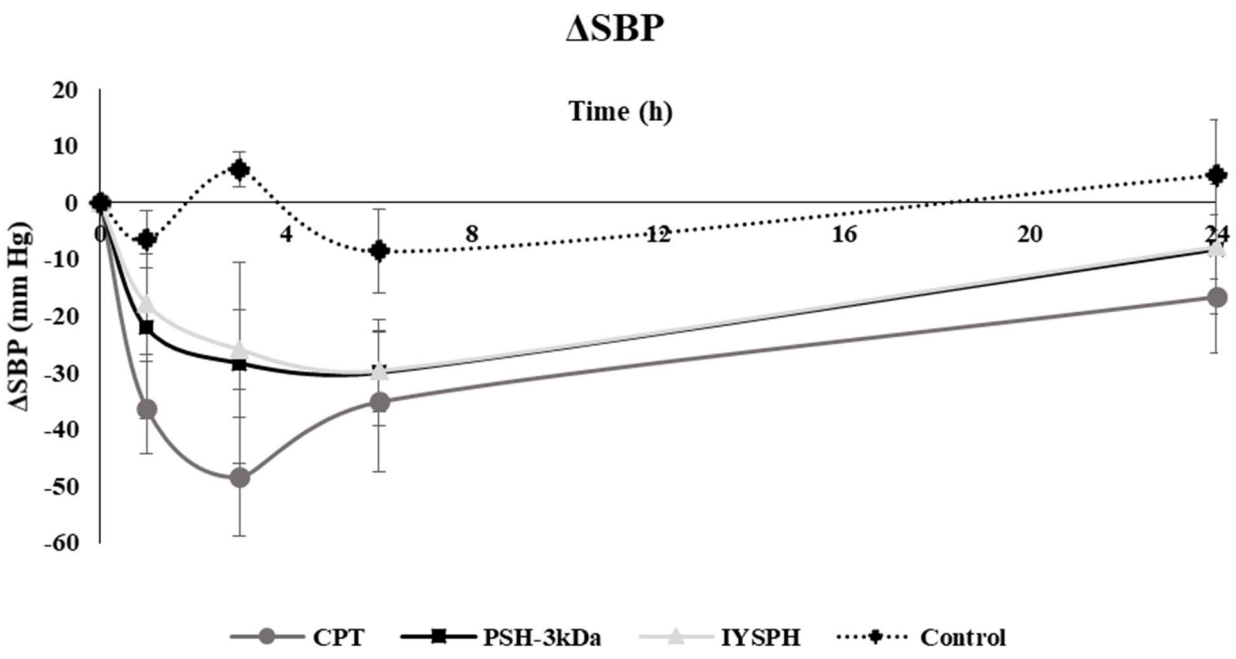
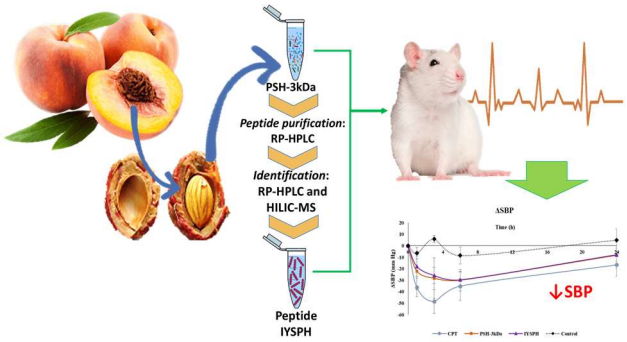

ΔSBP
Time (h)
ΔSBP (mm Hg)
20
10
0
-10
-20
-30
-40
-50
-60
0
4
8
12
16
20
24
CPT
PSH-3kDa
IYSPH
Control

Figure 4.


PSH-3kDa
Peptide purification:
RP-HPLC
Identification:
RP-HPLC and
HILIC-MS
Peptide
IYSPH
ΔSBP
Time (h)
↓SBP
CPT
PSH-3kDa
IYSPH
Control