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Identification of novel antioxidant peptides from snakehead (*Channa argus*) soup generated during gastrointestinal digestion and insights into the anti-oxidation mechanisms

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29 **Abstract:** Antioxidant peptides obtained from snakehead (*Channa argus*) soup (SHS) after simulated
30 gastrointestinal (GI) digestion were separated, identified and characterized. Results showed that the
31 fraction with MW < 3 kDa had the highest antioxidant capacity. Four novel antioxidant peptides were
32 identified after RP-HPLC and UPLC-MS/MS. PGMLGGSPGLLGGSP and SDGSNIHFNP had the
33 highest DPPH radical scavenging activity (IC₅₀ = 1.39 mM) and Fe²⁺ chelating ability (IC₅₀ = 4.60
34 mM), respectively. Structures *in silico* for IVLPDEGK, PGMLGGSPGLLGGSP and
35 SDGSNIHFNP suggest that at least one β-turn and/or α-helix, are associated with antioxidant activity.
36 Moreover, our results showed that these three peptides docked with a recombinant Kelch-like
37 ECH-associated protein 1 (Keap1) with a binding score greater than TX6, a good ligand of Keap1. The
38 cell viability assay also showed significant cytoprotective effects against H₂O₂-induced cellular
39 oxidative damage. This information implies that antioxidant mechanisms of novel peptides occurred
40 via activation of cellular anti-oxidation Keap1-Nrf2 signaling pathway.

41

42 **Keywords:** antioxidant peptides; snakehead soup; gastrointestinal digestion; molecular docking;
43 UPLC-MS/MS; RP-HPLC; Kelch-like ECH-associated protein 1; cytoprotective effect

44

45 **1. Introduction**

46 Snakehead (*Channa argus*) is a traditional, high-value, freshwater fish species indigenous to
47 Asia-Pacific countries such as China and Malaysia (Wahab et al., 2015; Wu, Zhang, Huo, Xiong, &
48 Du, 2018), and its annual aquaculture production exceeded 0.48 million tons in 2018 in China (China
49 Fishery Statistical Yearbook, 2019). Snakeheads contain few bones, which means the edible portion
50 makes up 63%, and like many fish, is rich in high-quality protein and micronutrients (Wu et al., 2018).
51 Due to its high levels of essential amino acids and fatty acids, snakeheads are usually used as
52 ingredients in soup, a popular side dish in Asia-Pacific countries (Sahid et al., 2018; Zhang, Zheng,
53 Feng, Shen, Xiong, & Du, 2018). Moreover, snakehead fish has been reported to significantly promote
54 the healing of wounds and burns (Sahid et al., 2018).

55 The wound repair process could induce cellular oxidative stress, producing various types of free
56 radicals, which severely interfere with wound healing (Schäfer & Werner, 2008). Many previous
57 studies have reported that the wound healing promotion potency of fish and fish products may mainly
58 be derived from their released antioxidant peptides (Wang, Doan, Nguyen, Nguyen, & Wang, 2019;
59 Venkatesan, Anil, Kim, & Shim, 2017). Antioxidant peptides can protect the human body against
60 cellular oxidative stress by scavenging reactive oxygen species, chelating transition metal and
61 inhibiting lipid peroxidation (Delgado, Nardo, Pavlovic, Rogniaux, Añón, & Tironi, 2016; Sila &
62 Bougatef, 2016). Their antioxidant activity is closely related to the molecular weight (MW),
63 hydrophobicity, amino acid residue composition and folding pattern (Delgado et al., 2016). Our
64 previous study found an increase in antioxidant activity of SHS during gastrointestinal (GI) digestion
65 (Zhang et al., 2018). However, the antioxidant peptides from snakehead fish have rarely been studied,
66 and the antioxidant peptides generated and released from SHS during digestion have not been reported

67 yet.

68 Although the *in vivo* antioxidant mechanism of peptides is still not fully understood, various
69 reports have indicated that the Keap1-Nrf2 anti-oxidation signaling pathway is one of the most
70 probable approaches (Han et al., 2018; Huerta et al., 2016; Li et al., 2017). This pathway is an
71 important regulator of cytoprotective responses to oxidative stress (Magesh, Chen, & Hu, 2012). In
72 this pathway, the transcription factor Nrf2 (nuclear factor erythroid 2-related factor 2) is a key factor
73 involved in the cellular anti-oxidation process, while the repressor protein Keap1 (Kelch-like
74 ECH-associated protein 1) promotes the degradation of Nrf2 (Zhang, Lo, Sun, Habib, Lieberman, &
75 Hannink, 2005). Therefore, external molecules that can bind to Keap1 and inhibit the Keap1-Nrf2
76 complex formation would enhance the *in vivo* antioxidant activity (Li et al., 2017). However, it is still
77 not clear whether the antioxidant activity of peptides from SHS is associated with the Keap1-Nrf2
78 signaling pathway. Such potential interactions between peptides and Keap1 can be evaluated through
79 the use of *in silico* molecular docking and cell model.

80 Therefore, the objective of this study was to separate, identify and characterize antioxidant
81 peptides from SHS generated by GI digestion. Ultrafiltration and reversed-phase high-performance
82 liquid chromatography (RP-HPLC) were used to separate antioxidant peptides. Ultra-high pressure
83 liquid chromatography coupled with mass spectrometry (UPLC-MS/MS) and 3D structure *in silico*
84 prediction were utilized to identify the sequences and characterize the structures of these novel
85 antioxidant peptides. The molecular docking technology and HepG2 cell model were also adopted for
86 understanding their *in vivo* antioxidant mechanism. The information obtained from the present study
87 could help our understanding of the antioxidant mechanism of SHS and provide theoretical support for
88 the high-value utilization of freshwater fish products.

89

90 **2. Materials and methods**

91 *2.1. Materials*

92 Fresh snakeheads (*C. argus*, approximately ~750 g per fish, n = 20) were purchased from a local
93 market in Wuhan, China and taken to the laboratory in a plastic bag within 20 min.
94 1,1-diphenyl-2-picrylhydrazyl (DPPH), 1,10-phenanthroline monohydrate, ferrozine, trichloroacetic
95 acid (TCA), glutathione (GSH), lysozyme, L-tyrosine (L-Tyr), bovine serum albumin (BSA), vitamin
96 B12 (VB12), vitamin C (VC) and ethylenediaminetetraacetic acid (EDTA) were bought from China
97 National Medicines Co., Ltd. (Beijing, China). Sephadex G-25 was obtained from Sigma-Aldrich Co.,
98 Ltd. (St. Louis, MO, USA). HepG2 cells were acquired from the Beijing Institute of Biochemistry and
99 Cell Biology (Beijing, China). All other reagents used were analytical grade, including pepsin
100 (3000-3500 National Formulary Unit/mg, BIOSHARP, St Louis, MO, USA) and pancreatin (4000 U/g,
101 Yuanye Biotechnology Co., Ltd., Shanghai, China).

102

103 *2.2. Preparation and simulated gastrointestinal (GI) digestion of snakehead soup (SHS)*

104 The fresh snakehead, with scales, branchia, and offal removed, was rinsed several times before
105 use. The fish was immersed in water at a ratio of 1:4 (w/v) and then cooked using an induction cooker
106 (RT2134, Midea group, Foshan, China). The cooking power was 500 W for the first 20 min until the
107 sample soup started to boil, and then reduced to 300 W and kept boiling for 70 min. The soup was then
108 filtered through six layers of gauze to remove solids.

109 Subsequently, the simulated GI digestion of SHS was performed using a two-step enzymatic
110 process according to the method of Zheng, Ren, Su, Yang, & Zhao. (2013) and Zhu, Zhang, Zhou, &

111 Xu. (2016) with minor modifications. The soup sample was adjusted to pH 2.0 with 1 M HCl, and
112 pepsin was then added to a level of 40 g/kg of the protein content, which was determined by the
113 method of Lowry, Rosebrough, Farr, & Randall. (1951). The mixture was blended and incubated at
114 37 °C for 2 h with shaking at a speed of 100 rpm (COS-100B, Bilon Instruments Co., Ltd., China) to
115 simulate gastric digestion. Subsequently, the mixture was adjusted to pH 5.3 using 0.9 M NaHCO₃ and
116 then to pH 7.5 with 1 M NaOH. Pancreatin was then added at 40 g/kg of the protein content and the
117 reaction mixture was further incubated at 37 °C for 2.5 h to simulate intestinal digestion. Then test
118 tubes were placed in boiling water for 10 min to terminate the digestion. Samples were then cooled to
119 room temperature and centrifuged at 16,000 × g for 20 min (TGL-16GA, Xingkekeji, Instruments Co.,
120 Ltd., Changsha, China). The supernatant was filtrated and then freeze-dried (ALPHA 1-4 LD, Martin
121 Christ, Osterode, Germany) for the separation of antioxidant peptides.

122

123 *2.3. Measurement of peptide molecular weight (MW) distribution*

124 The peptide MW distribution of SHS after simulated GI digestion was purified by gel filtration
125 chromatography. The Sephadex G-25 column (1.6 cm × 70 cm) was loaded and eluted with 0.02%
126 NaN₃ for 12 h until reaching equilibration. 1 mL 10 mg/mL GI digested sample solution was then
127 added into the pre-equilibrated column and eluted at a flow rate of 16 mL/h with 0.02% NaN₃ at room
128 temperature. The elution was collected at 15 min intervals and assayed at 280 nm with a UV-1750
129 spectrophotometer (Shimadzu, Kyoto, Japan). GSH, lysozyme, L-Tyr, BSA and VB₁₂ were used as the
130 standard substances of this measurement. The relationship between lg (MW) of each substance and the
131 occurrence time of the corresponding maximum absorption peak was drawn as the standard curve.

132

133 *2.4. Separation of antioxidant peptides*

134 *2.4.1. Ultrafiltration*

135 SHS samples collected from simulated GI digestion were ultrafiltered sequentially using Amicon
136 Ultra-15 centrifugal filters with 10 kDa and 3 kDa MWCO (Millipore, Bedford, MA, USA). All
137 recovered fractions (SHS-I, MW > 10 kDa; SHS-II, 3-10 kDa; SHS-III, MW < 3 kDa) were
138 freeze-dried and stored at -80 °C for use. Their antioxidant activities were determined as described in
139 Section 2.5 and compared with the original simulated GI digested SHS sample and its different
140 fractions.

141

142 *2.4.2. Reversed-phase high performance liquid chromatography (RP-HPLC)*

143 According to the method of Shen, Chahal, Majumder, You, & Wu. (2010) with some
144 modifications, the fractions with the highest antioxidant activity were investigated using an Agilent
145 1260 semi-preparative HPLC instrument (Agilent Technologies Inc., Santa Clara, CA, USA) equipped
146 with a reversed-phase Techmate ST C18 analytical column (5 µm, 150 mm × 4.6 mm) (Techmate
147 Technology Co. Ltd., Beijing, China). The gradient elution was conducted at a flow rate of 0.2
148 mL/min with 20 mM ammonium formate in deionized water (pH = 10, adjusted with ammonium
149 hydroxide) as elution A and 20 mM ammonium formate in 80% acetonitrile solution added with
150 equivalent 25% ammonium hydroxide as elution B. The column was maintained at room temperature.
151 The elution was collected at 3 min intervals and assayed at 216 nm. Ultimately, 10 fractions (F1-F10)
152 were collected and their DPPH radical scavenging activities were detected as described later. Then
153 fractions were freeze-dried for further studies.

154

155 *2.5. Evaluation of antioxidant activity of peptides*

156 *2.5.1. DPPH radical scavenging activity*

157 The DPPH radical scavenging activity was determined according to the method of Zhang, Li,
158 Miao, & Jiang. (2011) with minor modifications. Specifically, a 4 mL sample was mixed with 1 mL
159 0.1 mM DPPH solution (in 99.7% ethanol); the control group comprised of 4 mL sample solution
160 mixed with 1 mL 99.7% ethanol; and the blank group contained 4 mL deionized water mixed with 1
161 mL 0.1 mM DPPH (in 99.7% ethanol). The mixture was blended and kept in the dark for 30 min at
162 room temperature. Subsequently, the absorbance of the reaction mixture was detected at 517 nm. The
163 DPPH radical scavenging activity was calculated according to the following equation:

164
$$\text{DPPH radical scavenging activity (\%)} = [1 - (A_s - A_b) / A_c] \times 100\% \quad (\text{Eq. 1})$$

165 where A_s , A_b and A_c represent the absorbance of the sample group, blank group and control group,
166 respectively.

167

168 *2.5.2. Hydroxyl radical scavenging activity*

169 The hydroxyl radical scavenging activity was measured using the method of Li, Jiang, Zhang, Mu,
170 & Liu. (2008) with some modifications. Briefly, a reaction mixture solution was composed of 2 mL
171 sample mixed with 1 mL 0.75 mM 1,10-phenanthroline, 1 mL 0.75 mM FeSO₄ and 2 mL 0.2 M
172 phosphate buffer (pH = 7.4). Then 1 mL 0.12% H₂O₂ solution was added to the mixture and incubated
173 at 37 °C for 1 h, and the absorbance was detected at 536 nm. The control group comprised of the same
174 solutions as the sample group, except that equivalent deionized water was used instead of the sample
175 solution. The composition of the blank group was the same as that of the sample group, except that
176 equivalent deionized water was used instead of the 1,10-phenanthroline monohydrate and FeSO₄

177 solutions. The hydroxyl radical scavenging activity was determined using the equation shown as
178 follows:

179
$$\text{OH radical scavenging activity (\%)} = (A_s - A_c) / (A_b - A_c) \times 100\% \quad (\text{Eq. 2})$$

180 where A_s , A_b and A_c represent the absorbance of the sample group, blank group and control group,
181 respectively.

182

183 2.5.3. Fe^{2+} chelating ability

184 The Fe^{2+} chelating ability was measured as described by Decker & Welch (1990) with slight
185 modifications. Specifically, the sample group comprised of 1 mL sample solution mixed with 3.7 mL
186 deionized water, 0.1 mL 2 mM FeCl_2 and 0.2 mL 5 mM ferrozine. The mixture was blended and kept
187 at room temperature for 20 min, and then the absorbance was detected at 562 nm. The deionized water
188 was used as the control, while the deionized water instead of FeCl_2 and ferrozine was used for the
189 blank. The chelating ability of Fe^{2+} was determined by the following equation:

190
$$\text{Fe}^{2+} \text{ chelating ability (\%)} = [1 - (A_s - A_b) / A_c] \times 100\% \quad (\text{Eq. 3})$$

191 where A_s , A_b and A_c represent the absorbance of the sample group, blank group and control group,
192 respectively.

193

194 2.5.4. Reducing power

195 The reducing power was determined by the method of Oyaizu (1986) with some modifications. 2
196 mL sample solution and 2 mL 0.2 M phosphate buffer (pH = 6.6) were mixed with 2 mL 1% K_3Fe
197 $(\text{CN})_6$. The mixture was blended and incubated at 50 °C for 20 min and then mixed with 2 mL 10%
198 TCA, followed by centrifugation at $1750 \times g$ for 10 min. Then 2 mL supernatant was added with 2 mL

199 deionized water and 0.4 mL 0.1% FeCl₃. Subsequently, the reaction mixture was blended thoroughly
200 and kept at room temperature for 10 min before the absorbance detection of the resulting Prussian blue
201 at 700 nm.

202

203 2.6. UPLC-MS/MS-based peptide identification

204 The collected fractions with the highest DPPH radical scavenging activity were then sequenced
205 using a UPLC-MS/MS system. All experiments were performed on a triple time-of-flight (Triple TOF)
206 System (5600 plus, AB SCIEX, Foster City, CA, USA) coupled with a splitless ultra-high pressure
207 liquid chromatography (UPLC) system (Ultra 1D Plus, Eksigent, Dublin, CA, USA). After desalting
208 with Sephadex G-25, the peptides were dissolved in 0.1% formic acid mixed with 2% acetonitrile and
209 98% deionized water, and then loaded into a C18 trap column (5 μm, 5 × 0.3 mm, Agilent
210 Technologies, Inc., Santa Clara, CA, USA) at a flow rate of 5 μL/min. Subsequently, it was eluted
211 from the trap column over the C18 analytic column (75 μm × 150 mm, 3 μm particle size, 100-Å pore
212 size, Eksigent, Dublin, CA, USA) at a flow rate of 300 nL/min in a 100 min gradient. The
213 information-dependent acquisition mode was used to acquire MS/MS spectra. Survey scans were
214 acquired in 250 ms and 40 product ion scans were collected in 50 ms/scan. The precursor ion range
215 was set from m/z 350 to m/z 1500, and the product ion range was set from m/z 100 to m/z 1500.

216 Raw data from the MS/MS system were analyzed using the ProteinPilot 4.5 software. The protein
217 accession and sequence location were achieved by comparing mass data against the UniProt database
218 (<http://www.uniprot.org/>) at the conditions of “identified sample type, no cysteine alkylation, and
219 thorough search effort”. Meanwhile, according to the method outlined by Sheng et al. (2019) and
220 Zhang et al. (2020), sequences with abundance in the MS/MS spectra above 1.00×10^7 were selected

221 for further analysis because they were easy to detect. Moreover, the peptides with sequencing
222 confidence $\geq 85\%$ were further selected and considered as the finally identified antioxidant peptides.

223

224 *2.7. Synthesis and antioxidant activity of identified peptides*

225 Identified antioxidant peptides were synthesized by the solid-phase procedure using the
226 Fmoc-protected amino acid synthesis method. The peptide synthesis was conducted by Dechi
227 Biosciences Co., Ltd. (Shanghai, China). The obtained peptides showed a purity higher than 95% (w/w)
228 detected by RP-HPLC on conditions of mobile phase A, 0.1% TFA in water; mobile phase B, 0.1%
229 TFA in acetonitrile; flow rate, 1 mL/min; column, Kromasil-C18, 5 μm particle size, 250 mm \times 4.6
230 mm (Eka Nobel, Bohus, Sweden). The molecular masses of synthesized peptides were determined
231 with the MS system. The DPPH radical scavenging activity and Fe^{2+} chelating ability of synthesized
232 peptides at different concentrations (0-2 mg/mL) were assayed as described above. The half-maximal
233 inhibitory concentration (IC_{50}) of the peptide was obtained by non-linear regression analysis fitted to a
234 sigmoidal dose-response equation.

235

236 *2.8. Three-dimensional (3D) structure in silico prediction of identified peptides*

237 The 3D structure models of identified peptides were predicted using the PEP-FOLD tool V3.5
238 (<http://bioserv.rpbs.univ-paris-diderot.fr/services/PEP-FOLD3/>), a *de novo* resource aimed at
239 predicting peptide structures from amino acid sequences (Lamiable, Thévenet, Rey, Vavrusa,
240 Derreumaux, & Tufféry, 2016). The folding pattern calculated with the lowest energy was selected as
241 the conformation of peptides generated by the PEP-FOLD tool V3.5. Images and analysis of the 3D
242 structures were generated using the Discovery Studio software 2016 (Accelrys Software Inc., San

243 Diego, CA, USA).

244

245 *2.9. Molecular docking analysis*

246 The molecular docking of identified peptides with Keap1 was conducted following the methods
247 provided in Han et al. (2018) and Li et al. (2017) with minor modifications. The semi-flexible program
248 CDOCKER in the Discovery Studio software 2016 was used for this procedure. Before the docking,
249 the crystal structure of human Keap1 (PDB ID: 2FUL) was downloaded from the PDB database and
250 pretreated with the Prepare Program in Discovery Studio to build loops, minimize the energy, remove
251 water molecules, remove Nrf2 16-mer peptide and protonize. The docking pocket was defined based
252 on the active sites of Keap1 as x: 5, y: 9, z: 1 and radius: 15 Å (Li et al., 2017). Furthermore, the TX6
253 (PubChem Compound ID: 121488089) was used as the reference ligand since it showed strong
254 binding capacity with Keap1 and activated the Nrf2 pathway (Huerta et al., 2016). Meanwhile, the
255 identified antioxidant peptides were also minimized in energy. The docking program was performed
256 with the partial flexibility CDOCKER protocol, specifically, the receptor (Keap1) was set as rigid and
257 the ligands (the identified peptides or TX6) were set as flexible. The molecular docking results were
258 evaluated based on the –CDOCKER interaction energy (–CIE) score, interaction site, and interaction
259 fore types with Keap1.

260

261 *2.10. Cytoprotective effects against cell damage induced by H₂O₂*

262 The identified antioxidant peptides with successful molecular docking performance were selected
263 and their cytoprotective effects were evaluated using cell viability assay. Briefly, HepG2 cells were
264 cultivated in a humidified atmosphere (37 °C, 5% CO₂) with DEME medium (containing 10% fetal
265 bovine serum and 1% penicillin-streptomycin). To determine the cytotoxicity of the synthetic peptides

266 (125-1000 μM) on HepG2 cells, the cells were seeded in a 96-well plate at a concentration of 1.0×10^4
267 cells/well. After 24 hours, cells were incubated with different concentrations of the synthetic peptides
268 for another 24 hours. Then, cell survival was determined by the MTT
269 (3-(4,5-dimethyl-2thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide) assay. The protective effects of
270 the synthetic peptides (125-1000 μM) on HepG2 cells were appraised by establishing a H_2O_2 -induced
271 (350 $\mu\text{g}/\text{mL}$) oxidative stress model. Briefly, HepG2 cells (1.0×10^4 cells/mL) were seeded in culture
272 plates at 37 °C for 24 h. The experiment was divided into five different groups, including the blank
273 group (100 μL cell suspension), oxidative damage group (100 μL H_2O_2 solution), low-concentration
274 peptide-protected group (50 μL H_2O_2 and 50 μL peptide solution), medium-concentration
275 peptide-protected group (50 μL H_2O_2 and 50 μL peptide solution) and high-concentration
276 peptide-protected group (50 μL H_2O_2 and 50 μL peptide solution). The cell viability was determined
277 by absorbance at 570 nm using a microplate reader.

278

279 2.11. Statistical analysis

280 All experiments were performed in triplicate. Tables were made by Microsoft Excel 2016 while
281 figures were drawn using Origin V8.0 and Microsoft PowerPoint 2016. Analysis of variance and
282 regression were conducted using the SAS program V8 (SAS Institute Inc., Carry, NC, USA).
283 Differences among mean values were established using the Duncan multiple range test. The significant
284 difference was confirmed when $P < 0.05$.

285

286 **3. Results and Discussion**

287 *3.1. MW distribution of peptides from simulated GI digested SHS*

288 The peptide MW distribution of simulated GI digested SHS is shown in Fig. 1a and the fitted
289 linear equation between lg (MW) (y) and elution time (x) were calculated by the method of least
290 square as $y = -0.0106 x + 6.2445$ ($R^2=0.9733$) (Fig. 1b). It can be seen from Fig. 1a that four main
291 fractions (F-I to F-IV) differed in MW range derived from simulated GI digested SHS. F-I was
292 approximately 20-kDa or larger and accounted for about 7.36% of the whole GI digesta. It was
293 considered as less completely digested if macromolecular proteins consisted of several hundreds of
294 amino acid residues. F-II contained the least component (14.68% of the whole digesta) with MW of
295 3.5-21.7 kDa, indicating that it had mainly peptides containing several tens of amino acid residues. At
296 last, the rest (about 77.96%) of the whole digesta was distributed in two fractions with MW < 3 kDa.
297 F-III had MW of 400-2,400 Da, demonstrating that this fraction was mainly short-chain peptides with
298 3-20 amino acid residues. F-IV accounted for the maximal proportion and possessed MW < 400 Da
299 with a sharp peak. This fraction is probably comprised of dipeptides and free amino acids. Tyrosine
300 (Y), one of the main free amino acids in SHS (Zhang et al., 2018), has a strong absorbance peak at 280
301 nm due to its conjugated double bond. This may be the main contributor to the prominent sharp peak
302 of F-IV with a corresponding MW of 186 Da, close to that of Y (181.19 Da).

303

304 *3.2. Separation of antioxidant peptides by ultrafiltration and RP-HPLC*

305 The antioxidant peptides in simulated GI digested SHS were separated using ultrafiltration and
306 RP-HPLC. Fig. 1c-1f shows antioxidant activities of the ultrafiltration fractions of simulated GI
307 digested SHS (SHS-I, SHS-II and SHS-III). The MW of those three fractions were > 10 kDa, 3-10

308 kDa and < 3 kDa, respectively. As shown in Fig. 1c-1f, all parameters representing antioxidant
309 activities of the sample and its fractions, including DPPH radical scavenging activity, OH radical
310 scavenging activity, Fe²⁺ chelating ability and reducing power, remarkably followed the order of
311 SHS-III > SHS-II > SHS-I ($P < 0.05$) at the concentration of 5 mg/mL. These results indicate that the
312 ultrafiltration fractions with lower MW had significantly higher antioxidant activity than the
313 higher-MW fractions. Li, Wang, Chi, Gong, Luo, & Ding (2013) found that fractions with an average
314 lower MW of fish collagen hydrolysate demonstrated significantly higher DPPH radical scavenging
315 activity and reducing power. Centenaro, Salas-Mellado, Pires, Batista, Nunes, & Prentice (2014) also
316 reported enhanced DPPH radical scavenging activity, OH radical scavenging capacity and reducing
317 power from the ultrafiltration fractions of croaker hydrolysate with relatively low MW. Our result are
318 in agreement with these previous reports. Therefore, the SHS-III was selected for further study due to
319 its potent antioxidant activity.

320 SHS-III was further separated into ten fractions (F1-F10) using RP-HPLC and the result is
321 illustrated in Fig. 1g. The DPPH radical scavenging activity of each fraction at the weight
322 concentration of 2 mg/mL is exhibited in Fig. 1h. It can be seen that F9 showed the highest DPPH
323 radical scavenging activity among all the fractions ($P < 0.05$). The DPPH radical scavenging activity
324 of F9 was $59.99 \pm 1.51\%$, about twice the lowest value (shown by F1) and 1/5 higher than the
325 second-highest level (shown by F6 and F7) ($P < 0.05$). Hence, F9 derived from SHS-III was selected
326 for further analysis.

327

328 3.3. Identification of antioxidant peptides by UPLC-MS/MS

329 The F9 fraction was then *de novo* sequenced using the UPLC-MS/MS system and 36 sequences

330 with abundance in MS/MS spectra above 1.00×10^7 were found. All sequences were obtained using
331 the ProteinPilot 4.5 software and searched in the UniProt protein database. The sequencing results are
332 exhibited in Table 1. It is shown that most of the searched sequences were from cytochrome, forkhead
333 box P2, myocyte enhancer factor 2D, NADH-ubiquinone oxidoreductase and recombination activating
334 protein. Eventually, four peptides (P1, P13, P17 and P31) were found with high sequencing confidence
335 (> 85%) (Table 1) and considered as the finally identified antioxidant peptides. Their MS/MS spectra
336 are exhibited in Fig. 2a-2d, and the corresponding sequence searching results are also shown in Table
337 1.

338 Typical antioxidative peptides are commonly small molecules containing 2-20 amino acid
339 residues (Chen, Muramoto, Yamauchi, Fujimoto, & Nokihara, 1998). Previous studies demonstrated
340 that MWs of antioxidant peptides derived from fish are commonly 500-1,500 Da (Centenaro et al.,
341 2014; Nalinanon, Benjakul, Kishimura, & Shahidi, 2011). In this study, the identified antioxidant
342 peptides had 8-17 amino acid residues with MW of approximately 0.8-1.7 kDa (Table 1 and Fig. 2),
343 which is similar to previous reports (Centenaro et al., 2014; Chen et al., 1998; Nalinanon et al., 2011).
344 Additionally, the high content of hydrophobic amino acid residues is another typical characteristic of
345 high-antioxidant peptides (Ahmed, El-Bassiony, Elmalt, & Ibrahim, 2015). The percentage of highly
346 hydrophobic residues in these identified peptides ranged from 40% (P17) to 88.24% (P13) with a
347 chain length-weighted mean higher than 70%, which is consistent with published results about the
348 antioxidant peptides (Puchalska, Marina, & García, 2014).

349

350 *3.4. Synthesis and antioxidant activity evaluation of identified peptides*

351 The identified peptides from the simulated GI digested SHS were synthesized and their

352 antioxidant properties were then determined. The DPPH radical scavenging activities and Fe²⁺
353 chelating abilities of these peptides are revealed in Fig. 2e and Fig. 2f, respectively, with the GSH, VC
354 and/or EDTA as the control.

355 As displayed in Fig. 2e, P13 showed the highest DPPH radical scavenging activity among the
356 identified peptides (IC₅₀ = 1.39 ± 0.16 mM) (*P* < 0.05), which is comparable to that of the control
357 GSH (IC₅₀ = 1.24 ± 0.06 mM) (*P* > 0.05). The high content of hydrophobic residues (A, P, V, I, L, W, F,
358 M and G) could be the main reason for its highest scavenging activity against DPPH radical among
359 these peptides, since the DPPH radical is lipid-soluble and thus the high-hydrophobicity allows
360 peptides to more readily react with lipid-soluble radicals (Pouzo, Descalzo, Zaritzky, Rossetti, &
361 Pavan, 2016). Additionally, P17 exhibited the second highest DPPH radical scavenging activity (IC₅₀ =
362 3.38 ± 1.67 mM) (*P* < 0.05), followed by P1 (IC₅₀ = 8.06 ± 2.76 mM) (*P* < 0.05). However, P31 had
363 the lowest DPPH radical scavenging activity among all these peptides (IC₅₀ = 63.96 ± 6.47 and 89.87
364 ± 29.90 mM, respectively) (*P* < 0.05). The low scavenging activity against the DPPH radical may
365 partially result from their relatively large MW and long molecular chain, since lower-MW and shorter
366 peptides are more active as they act as electron donors and react with free radicals, rendering them
367 more stable substances that stop chain reactions (Chi, Cao, Wang, Hu, Li, & Zhang, 2014; Halim,
368 Yusof, & Sarbon, 2016).

369 As revealed in Fig. 2f, all the identified peptides showed significantly higher Fe²⁺ chelating
370 ability than GSH (larger than 1 M) (*P* < 0.05). P17 exhibited the highest Fe²⁺ chelating activity among
371 the identified peptides (IC₅₀ = 4.60 ± 0.05 mM) (*P* < 0.05), which is comparable to that shown by the
372 control EDTA (IC₅₀ = 1.67 ± 0.05 mM) (*P* > 0.05). P1 and P31, closely followed P17, possessing the
373 second-highest Fe²⁺ chelating activity with IC₅₀ of about 7-26 mM (*P* < 0.05). However, P13 had the

374 lowest Fe²⁺ chelating ability among all the identified peptides (IC₅₀ > 100 mM) (*P* < 0.05). Saiga,
375 Tanabe, & Nishimura (2003) reported that the net-charged residues, including those negatively
376 charged (acidic residues, D and E) and positively charged (basic residues, H, K and R), play a crucial
377 role in Fe²⁺ and Cu²⁺ chelating abilities of peptides through electrostatic interactions. Therefore, P13,
378 containing none net-charged residues, possessed the lowest Fe²⁺ chelating ability.

379

380 3.5. 3D structures and folding patterns of identified peptides

381 The most probable conformations and 3D structures of the identified peptides *de novo* are
382 exhibited in Fig. S1, which were predicted by the PEP-FOLD tool V3.5. Results show that peptides
383 possessed different folding patterns and spatial structures. It has been found that the predominant
384 folding patterns of bioactive peptides mainly consist of β -turn (around 75% of total peptides) and
385 α -helix (about 60%) rather than β -sheet and random coil (Kaur, Garg, & Raghava, 2007). Also, the
386 α -helix plays a key role in the antioxidant capacity of peptides due to its contextual constraints (Jia,
387 Natarajan, Forte, & Bielicki, 2002). Generally, all identified peptides had well-organized folding
388 patterns including β -turn, β -sheet and/or α -helix except P31. Individually, P13 and P17 possessed 1-2
389 β -turns, while P1 and P13 had partial or intact α -helix whirls. However, the 3D structure of P31 was
390 only displayed by the random coil, which may be another important reason for its relatively low
391 scavenging activity against the DPPH radical (Fig. 2e).

392

393 3.6. Molecular docking of identified peptides with Keap1

394 The molecular docking analysis between identified antioxidant peptides and Keap1 was
395 performed using the CDOCKER program of the Discovery Studio software 2016. The molecular
396 docking models and results are shown in Fig. 3 and Table 2, respectively. The extent of binding

397 between identified antioxidant peptides and Keap1 should mainly be determined by the receptor active
398 site, the -CDOCKER interaction energy (-CIE) score and the number of interacting amino acid
399 residues (Wu, Du, Jia, & Kuang, 2016) (Table 2). Huerta et al. (2016) reported that the TX6 can
400 effectively bind to Keap1, activate the Nrf2 and thus promote the backward ARE (anti-oxidation
401 response element) expression. Hence, the TX6 was used as the reference to evaluate the binding
402 degree between identified antioxidant peptides and Keap1. The molecular docking results show that
403 almost all the identified peptide molecules successfully docked onto the active site of Keap1 except
404 P31, which failed during the docking process (Table 2). The -CIE scores of P1, P13 and P17 were
405 72.04, 63.27 and 72.85 kJ/mol, respectively, whereas the -CIE score of TX6 was just 29.55 kJ/mol
406 (Table 2). These three peptides had similar docking pockets with the TX6, which are located on the
407 same active site of Keap1 (Fig. 3-A1, B1, C1 & D1).

408 Additionally, the TX6 established one hydrogen bond with GLY364 of Keap1 and six π bonds
409 with TYR334, TYR525, ALA556 and TYR572 of Keap1 (Fig. 3-A2). However, there were more than
410 eight amino acid residues involved in the interaction between P1, P13, P17 and Keap1 with larger
411 numbers of hydrogen bonds and π bonds established compared with TX6 (Table 2 and Fig. 4).
412 Individually, P1, P13 and P17 interacted with eight, sixteen and eleven amino acid residues of the
413 Keap1 active site, respectively (Table 2). Moreover, in contrast with the TX6-Keap1 interaction, the
414 interactions between these peptides and Keap1 were mainly hydrogen bonds rather than π bonds
415 (Table 2 and Fig. 3). It has been reported that ligands interact with receptors via different
416 intermolecular forces, such as hydrophobic, van der Waals's force, hydrogen bonds, π bonds and
417 electrostatic interaction, among which hydrogen bonding interactions are probably the strongest
418 (Miazaei, Mirdamadi, Ehsani, & Aminlari, 2018). All these results indicate that P1, P13 and P17 could

419 well bind to Keap1 with a binding complex even more stable than that of reference TX6.

420

421 **3.7. Cytoprotective effects on cell damage induced by H₂O₂**

422 The identified antioxidant peptides with successful molecular docking performance were further
423 evaluated by a H₂O₂-induced oxidative stress cell model and the results are shown in Fig. 4. Fig. 4a,
424 demonstrates that these peptides (125-1000 μM) had no significant toxicity effect on HepG2 cells
425 compared with the control group ($P > 0.05$). Hydrogen peroxide penetrated cell membranes and
426 caused cell damage. To evaluate the protective effect of identified peptides (P1, P13 and P17) against
427 H₂O₂-induced cell damage, cells were pretreated with different concentrations of peptides (125-1000
428 μM) before being exposed to H₂O₂ (350 μg/mL). As illustrated in Fig. 4b, the cell viability of the
429 damaged group significantly decreased to $43.40 \pm 2.18\%$ compared with the control group ($P < 0.05$).
430 However, pretreatment of the identified peptides significantly increased cell viability to 60-82% ($P <$
431 0.05), and cell survival showed a remarkable dose-effect relationship with these peptides ($P < 0.05$).
432 The results suggest that these synthesized peptides can significantly protect cells from H₂O₂-induced
433 oxidative damage.

434

435 **4. Conclusions**

436 This work has achieved, for the first time, the identification and characterization of some
437 antioxidant peptides from SHS generated from GI digestion. Specifically, four novel antioxidant
438 peptides were separated, identified and characterized using ultrafiltration, RP-HPLC, UPLC-MS/MS,
439 3D structure *in silico* prediction, molecular docking analysis and H₂O₂-induced oxidative stress cell
440 model. Results showed that the ultrafiltrated fraction with MW < 3 kDa (SHS-III), accounting for

441 about 77.96% (w/w) of the whole GI digested SHS, had the highest antioxidant capacity ($P < 0.05$).
442 The antioxidant peptides in SHS-III were further separated into ten fractions by RP-HPLC and the F9
443 was selected for peptide identification due to its highest DPPH radical scavenging activity ($P < 0.05$).
444 Among the identified peptides, PGMLGGSPGLLGGSP and SDGSNIHFNP showed high
445 antioxidant activity which may be related to the contribution of 3D structure characteristics including
446 β -turn and/or α -helix. The molecular docking study suggests that IVLPDEGK,
447 PGMLGGSPGLLGGSP and SDGSNIHFNP can bind to the active site of Keap1 with the binding
448 energy ($-CIE > 60$ kJ/mol) even higher than that of TX6 ($-CIE = 29.55$ kJ/mol). The cell viability assay
449 also indicates that these three peptides had no cytotoxicity and could significantly protect cells from
450 H_2O_2 -induced oxidative damage. Thus, it can be concluded that the peptides with strong antioxidant
451 activity generated from gastrointestinal digestion of SHS can promote *in vivo* antioxidant activity by
452 participating in cellular anti-oxidation signaling pathways.

453

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458

459 **Conflict of Interest**

460 The authors have no conflict of interest.

461

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Table 1 Sequencing results of antioxidant peptides in F9 from the simulated GI digested SHS

Protein source ^a	Peptide No.	Calculated mass (Da)	Observed mass (Da)	Sequence	Abundance in spectra	Confidence of sequencing (%)
A1-antitrypsin	P1	869.4922	869.4858	IVLPDEGK	1.01×10⁸	94.55
Cystic fibrosis transmembrane conductance regulator	P2	1358.6300	1358.7153	SVEGGQSVGLLGRT	2.70×10 ⁷	33.83
Cytochrome	P3	2164.0630	2164.1423	WNLGSLGLCLVAQLMTGLF	6.20×10 ⁷	79.18
	P4	1972.9020	1972.9530	EEAGAGTGWTVYPPLAGNLA	1.01×10 ⁸	66.25
	P5	2027.9430	2028.0316	GVEAGVGTGWTVPPLAGNLA	9.60×10 ⁷	21.05
	P6	869.4921	869.47930	LTIKAMGH	1.50×10 ⁸	54.94
Doublesex- and mab-3-related transcription factor 5	P7	1141.5580	1141.5590	SRGLAFMTPY	3.30×10 ⁷	60.13
Early growth response 2B	P8	2475.2050	2475.2144	GPGGGGGGSEGGPPRLPSAYSPQNLPL	1.30×10 ⁷	46.86
Estrogen receptor β	P9	1585.7190	1585.8173	SAQSRTGGSKPKTGPA	2.70×10 ⁷	29.11
Forkhead box P2	P10	1590.7860	1590.8076	PGMLGGSPGLLGGSPPT	6.00×10 ⁷	74.55
	P11	1054.6080	1054.5481	MPQVPSVLGGA	8.50×10 ⁷	44.60
	P12	1277.5860	1277.6438	PGMLGGSPGLLGGS	1.36×10 ⁸	15.43
	P13	1505.7010	1505.7548	PGMLGGSPGLLGGSP	1.56×10⁸	99.00
	P14	1708.8100	1708.8818	MPQVPSVLGGANVPSIGA	8.00×10 ⁷	51.03
	P15	926.5137	926.4896	MPQVPSVLG	4.00×10 ⁷	21.20
	P16	1523.7160	1523.6675	TGPMGGSCHGLLGGDPS	2.50×10 ⁷	33.93
Galectin	P17	1086.4800	1086.4730	SDGSNIHFNP	1.56×10⁸	89.60
Muellerian-inhibiting factor	P18	1136.5230	1136.5714	SPASSQTLFL	1.20×10 ⁸	41.71
Myocyte enhancer factor 2D	P19	1379.7060	1379.7521	GLPQRPASAGALLGG	9.50×10 ⁷	25.71

	P20	1450.7490	1450.7528	NTVSPGLPQRPASAG	8.10×10^7	33.95
NADH-ubiquinone oxidoreductase	P21	883.5083	883.5167	PFGLLQPI	8.50×10^7	38.01
	P22	939.5437	939.5753	GLVAGGILIQ	8.00×10^7	33.97
	P23	1155.6550	1155.6288	IQTPWGLTGAL	8.50×10^7	45.64
	P24	1222.6260	1222.6809	GSIISSGLLITSY	4.00×10^7	40.02
	P25	1651.9040	1651.8756	MVAIGLNQPQLAFLH	8.20×10^7	53.25
	P26	1170.6170	1170.6860	TAIGLLASLELA	1.50×10^7	81.02
	P27	1620.7920	1620.8148	GLAAVASNPSPYAALG	2.80×10^7	31.63
Prolactin receptor	P28	1646.7970	1646.7020	DKSGAPKEEQDNGSGE	8.20×10^7	28.20
Proliferating cell nuclear antigen	P29	883.5083	883.4651	VEQLGIPE	8.00×10^7	37.26
Recombinase	P30	853.5096	853.5273	ALTAVLGPI	1.16×10^8	37.96
Recombination activating protein	P31	1722.8330	1722.8424	SVSIRADGGEGETVFT	6.50×10^7	95.35
	P32	1208.6110	1208.6877	AVLGPIVAERNA	8.50×10^7	51.87
	P33	2005.9590	2005.9312	AEPQPNSSELSCKPLCLMF	1.00×10^8	70.93
Si:dkey-174m14.3	P34	1272.6150	1272.5444	WVGASPSPECSPG	2.50×10^7	35.16
Sorting nexin 33	P35	1182.5710	1182.5305	LGPQWNENPQ	6.00×10^7	69.08
Transferrin receptor 1	P36	1149.5320	1149.5455	WGPGFAASTVGT	9.10×10^7	44.07

577 ^aThe protein sources of the identified peptides are from the UniProt protein database.

Table 2 Molecular docking results of identified peptides from the simulated GI digested SHS with Keap1

Ligand	Sequence	–CIE ^a (kJ/mol)	Interactions with Keap1	Number of hydrogen bonds	Number of π bonds
TX6	(6aS,7S,10aS)-8-hydroxy-4-methoxy-2,7,10a-trimethyl-5,6,6a,7,10,10a-hexahydrobenzo[h]quinazoline-9-carbonitrile	29.5522	TYR334, GLY364, TYR525, ALA556, TYR572	1	6
P1	IVLPDEGK	72.0357	ARG380, ARG415, HIS436, ILE461, ARG483, SER508, SER555, TYR572	9	1
P13	PGMLGGSPPGLLGGSP	63.2653	GLY364, LEU365, ALA366, ARG380, ASN382, ARG415, ILE416, GLY433, ARG483, CYS434, ALA510, TYR525, LEU557, TYR572, GLY603, VAL604	17	2
P17	SDGSNIHFPN	72.8495	LEU365, ARG380, ARG415, GLY462, ARG483, ALA510, TYR525, ALA556, LEU557, TYR572, GLY603	11	4
P31	SVSIRADGGEGETVFT	—	—	—	—

579 ^a–CIE, –CDOCKER interaction energy.

580 **Figure captions**

581

582 **Fig. 1. The MW distribution (a-b) and separation (c-h) of antioxidant peptides from the**

583 **simulated GI digested SHS.** a-b, the MW distribution of peptides from simulated GI digested SHS by

584 gel filtration chromatography (a) and the standard curve of lg (MW)-elution time relationship (b). The

585 whole SHS digesta can be divided into four fractions (F-I to F-IV) by MW distribution. c-f, the DPPH

586 radical scavenging activity (c), OH radical scavenging activity (d), Fe²⁺ chelating ability (e) and

587 reducing power (f) of the simulated GI digested SHS and its ultrafiltration fractions. Different

588 lowercases above the error bar denote significant differences among antioxidant activities of the

589 simulated digested SHS sample and its different fractions ($P < 0.05$). The MWs of SHS-I, SHS-II and

590 SHS-III were > 10 kDa, 3-10 kDa and < 3 kDa, respectively. The concentration of all samples used in

591 the detection was 5 mg/mL. g-h, RP-HPLC chromatogram of peptides in SHS-III (g) and the DPPH

592 radical scavenging activities of its further separated fractions (F1 to F10) (h). Different lowercases

593 above the error bar denote significant differences among DPPH radical scavenging activities of

594 different fractions ($P < 0.05$). The concentration of all fractions used was 2 mg/mL.

595

596 **Fig. 2. The MS/MS spectra (a-d) and antioxidant activities (e-f) of the identified and synthesized**

597 **peptides.** a-d, MS/MS spectra of the five identified antioxidant peptides. a, P1, IVLPDEGK; b, P13,

598 PGMLGGSPGLLGGSP; c, P17, SDGSNIHFNP; d, P31, SVSIRADGGEGEVTVFT. The upper

599 and lower dash labeled the b ion and y ion, respectively. e-f, the DPPH radical scavenging activity (e)

600 and Fe²⁺ chelating ability (f) of synthesized antioxidant peptides. Different lowercases above the error

601 bar denote significant differences among the identified antioxidant peptides from the simulated GI

602 digested SHS ($P < 0.05$).

603

604 **Fig. 3. Molecular docking model of interactions between identified antioxidant peptides and**

605 **Keap1 (PDB ID: 2FLU).** The TX6 (Pub Chem ID: 121488089) was used as a reference. Ligand code:

606 A, TX6; B, P1, IVLPDEGK; C, P13, PGMLGGSPGLLGGSP; D, P17, SDGSNIHFNP. A1, B1, C1

607 and D1, the ligand molecule was bound to the integral Keap1. A2, B2, C2 and D2, the 2D diagram of

608 interactions between the ligand molecule and active amino acid residues of Keap1.

609

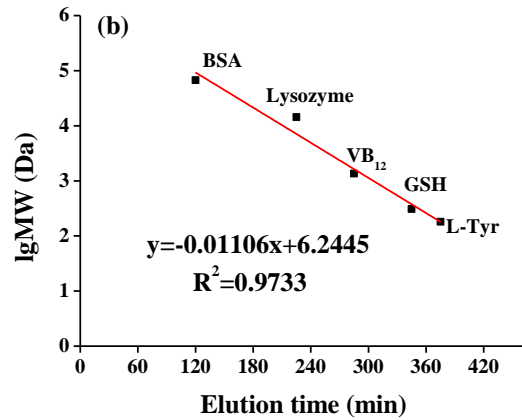
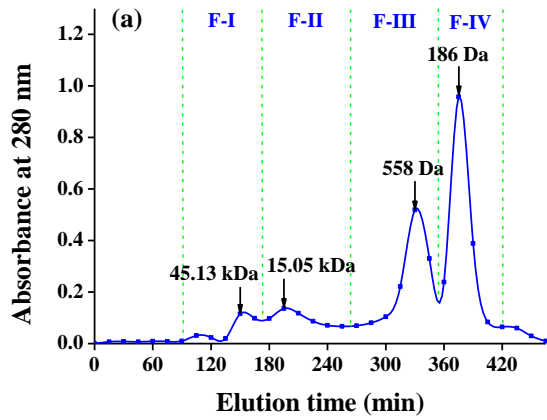
610 **Fig. 4. Protective effects of synthesized antioxidant peptides against H₂O₂-induced stress damage**

611 **in HepG2 cells.** a, the cell viability of different concentrations of synthetic peptides on HepG2 cells. b,

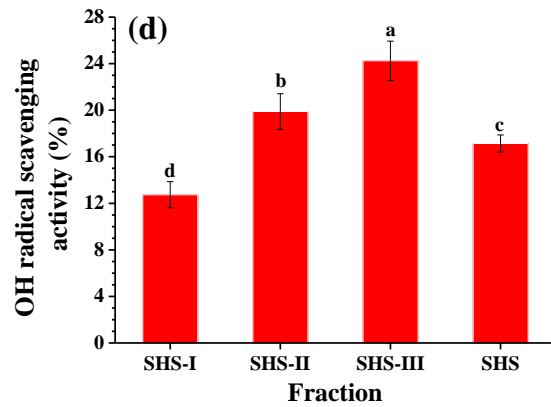
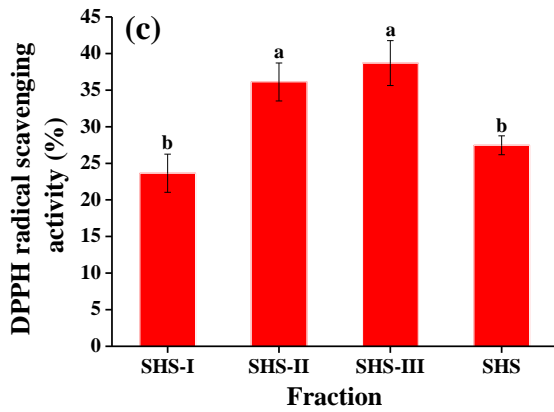
612 Protective effects of different concentrations of P1, P13 and P17 on H₂O₂-induced damage of HepG2

613 cells. Different lowercases above the error bar denote significant differences among various synthetic

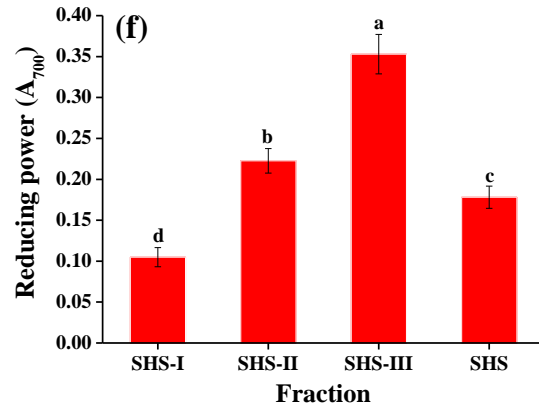
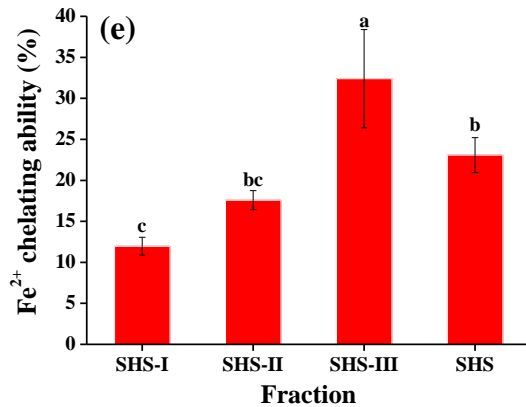
614 antioxidant peptides with different concentrations (125-1000 μ M) ($P < 0.05$).



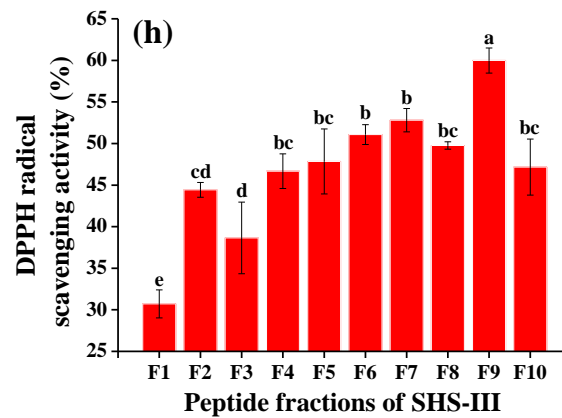
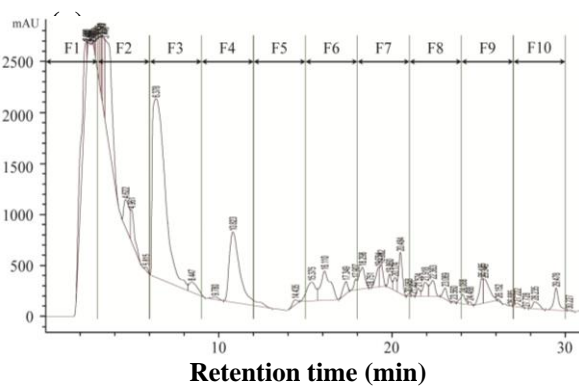
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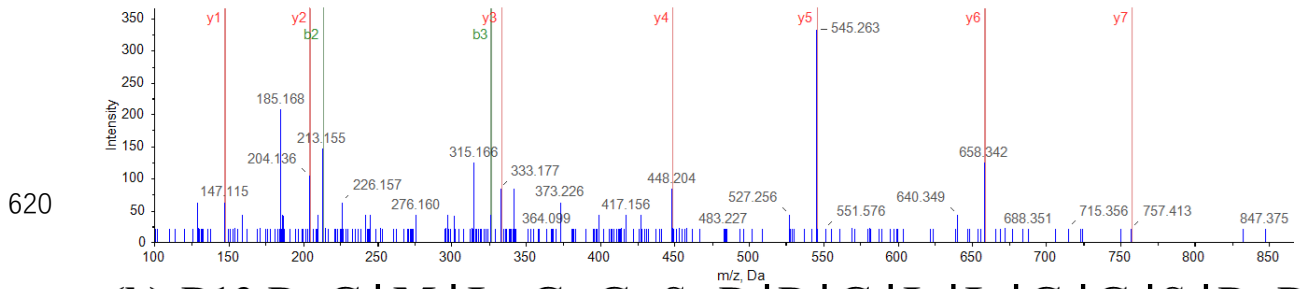
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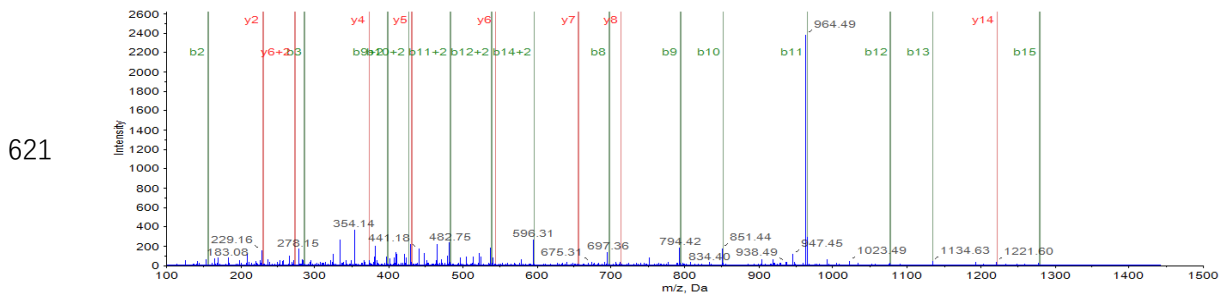
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619 **Fig. 1**

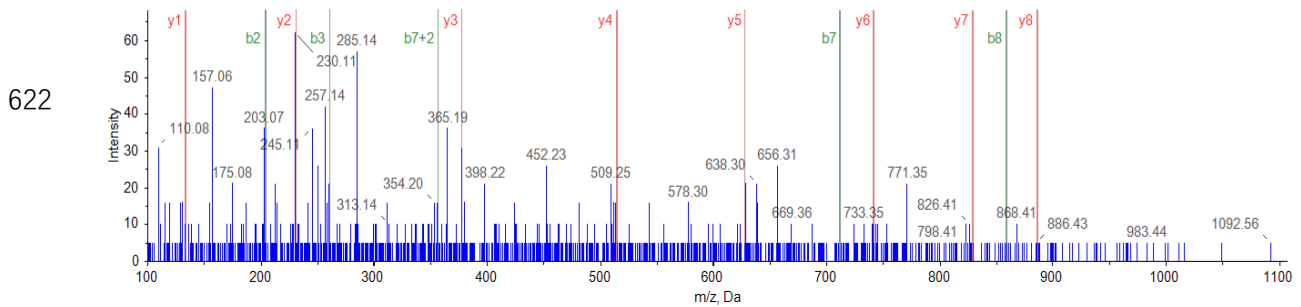
(a) P1 I-V-L-P-D-E-G-K



(b) P13 P-G-M-L-G-G-S-P-P-G-L-L-G-G-S-P-P



(c) P17 S-D-G-S-N-I-H-F-P-N



(d) P31 S-V-S-I-R-A-D-G-G-E-G-E-V-T-V-F-T

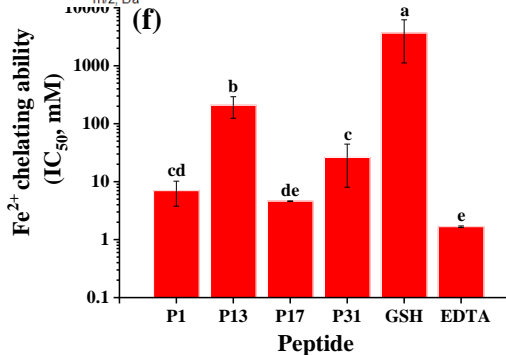
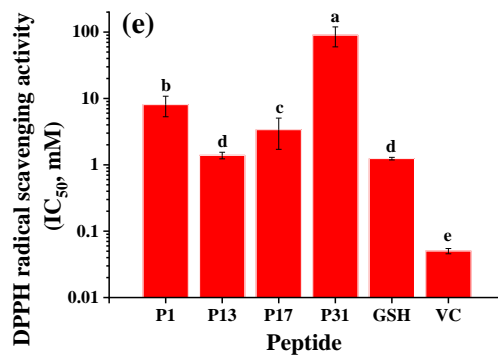
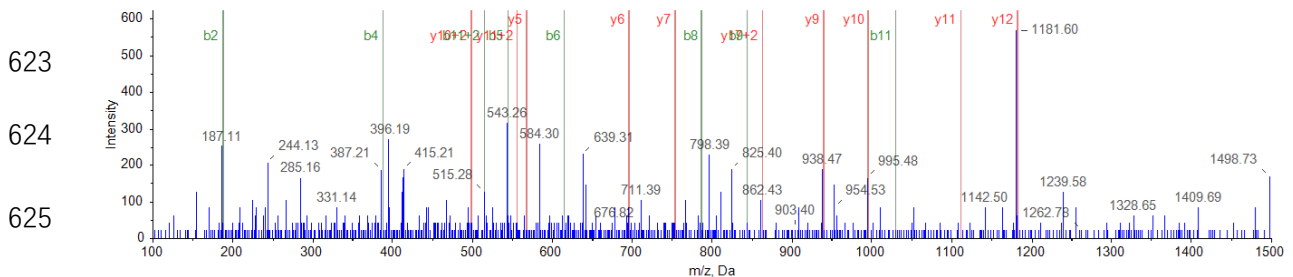


Fig. 2

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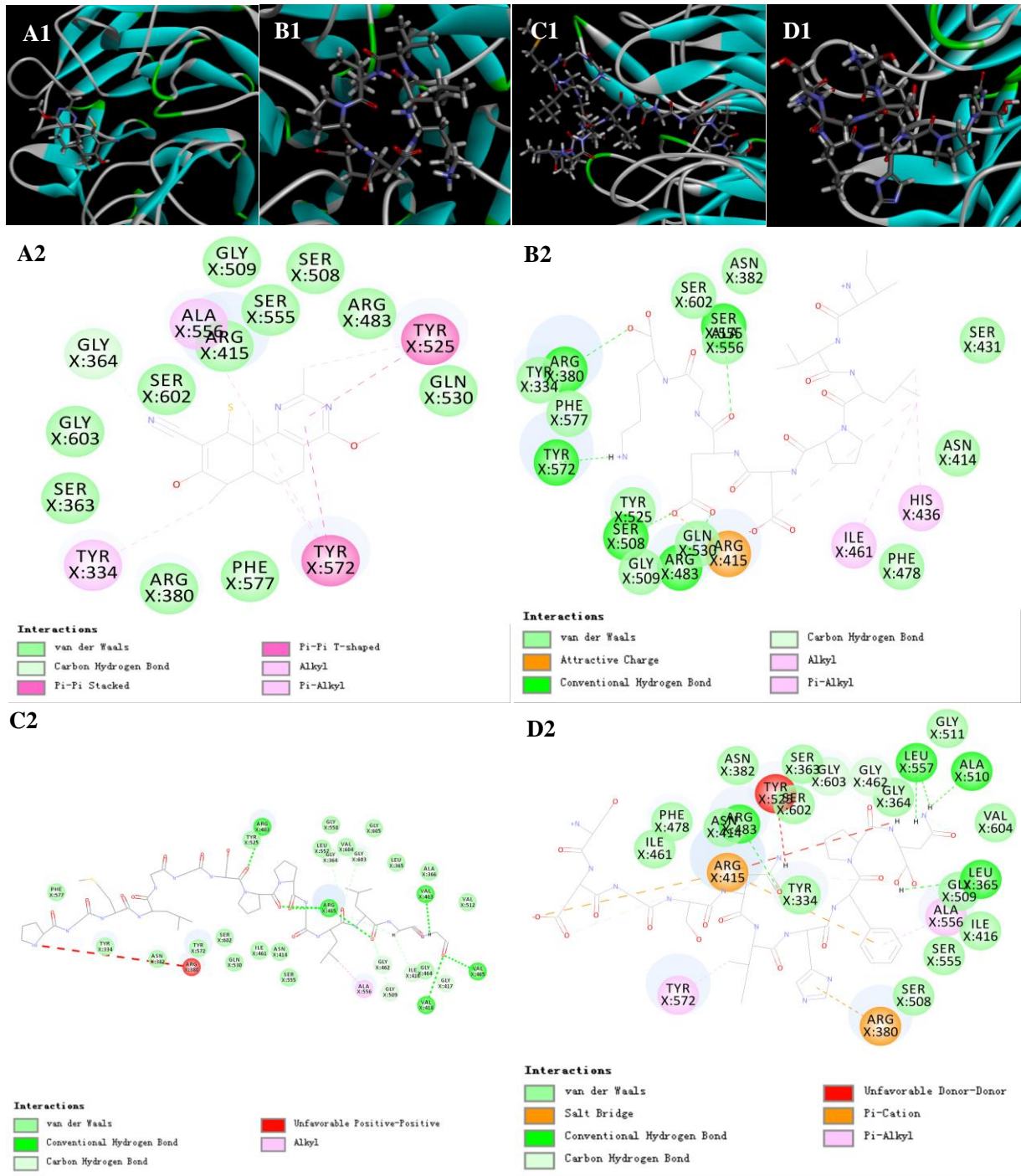
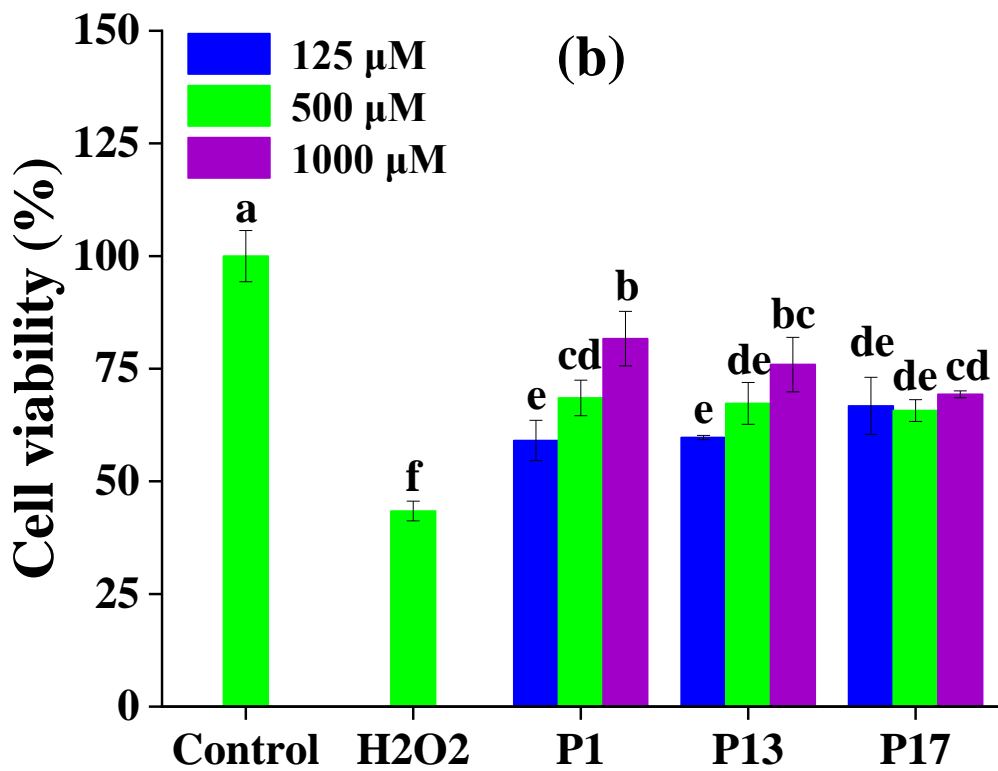
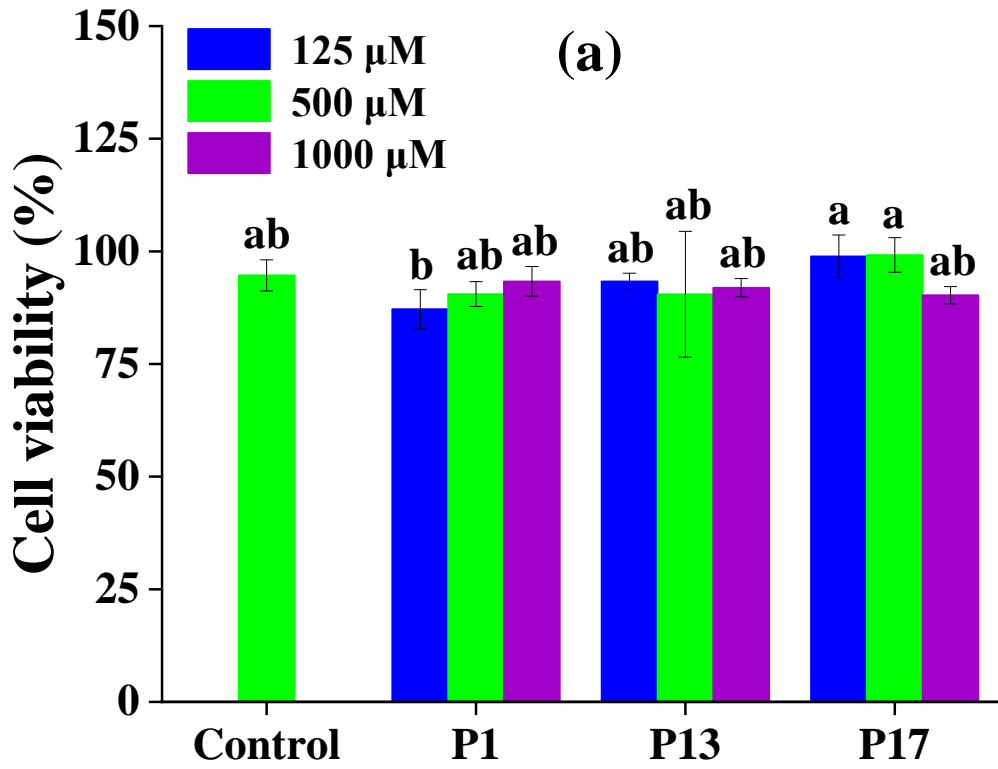


Fig. 3



649 Fig. 4