1 **Running title:** Defensin with Antioxidant Activities *in vitro* and *ex vivo*

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3 # Defensin Protein from sweet potato (*Ipomoea batatas* [L.] Lam. 'Tainong 4 57') storage roots exhibits Antioxidant Activities *in vitro* and *ex vivo*

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6 Guan-Jhong Huang[a, 1], Jeng-Shyan Deng[b, 1], Hsien-Jung Chen[c], Shyh-Shyun Huang[d],

7 Jung-Chun Liao[d], Wen-Chi Hou[e], and Yaw-Huei Lin[f, *]

8

9 [a]School of Chinese Pharmaceutical Sciences and Chinese Medicine Resources,
10 College of Pharmacy, China Medical University, Taichung 404, Taiwan
11 [b] Department of Health and Nutrition Biotechnology, Asia University, Taichung 413,
12 Taiwan
13 [c] Department of Biological Sciences, National Sun Yat-sen University, Kaohsiung 804,
14 Taiwan
15 [d] School of Pharmacy, China Medical University, Taichung 404, Taiwan;
16 [e] Graduate Institute of Pharmacognosy, Taipei Medical University, Taipei 110, Taiwan
17 [f] Institute of Plant and Microbial Biology, Academia Sinica, Nankang, Taipei 115,
18 Taiwan

19

20 ***Corresponding author:**

21 Dr. Yaw-Huei Lin

22 Institute of Plant and Microbial Biology, Academia Sinica, Nankang, Taipei, Taiwan

23 115, Republic of China

24 FAX: 886 (2) 2782-7954; TEL: 886 (2) 2787-1172

25 E-mail: boyhlin@gate.sinica.edu.tw

26

27 [1] Equal contribution to this paper.

28 ## ABSTRACT

29 This study was designed to investigate the antioxidant activities of sweet potato
30 defensin (SPD1) *in vitro* and *ex vivo*. Antioxidant status [2,
31 2'-azinobis[3-ethylbenzthiazoline-6-sulfonic acid (ABTS) assay], scavenging activity
32 against DPPH (1,1-dipheny-2-picrylhydrazyl) radical method, reducing power
33 method, Fe^{2+}-chelating ability, FTC (ferric thiocyanate) method, and protection of
34 calf thymus DNA against hydroxyl radical-induced damage were studied *in vitro*. The
35 *ex vivo* experiments revealed that SPD1 could decrease the production of intracellular
36 peroxide in HepG2 cells. Four peptides, namely GFR, GPCSR, CFCTKPC, and
37 MCESASSK for testing antioxidative activity were synthesized according to tryptic
38 hydrolysis simulation. In TEAC assay CFCTKPC performed the best (13.5 ± 0.3
39 μmol TE/g dw), even better than reduced glutathione (7.3 ± 0.2 μmol TE/g dw). In
40 scavenging activity of DPPH radical assay (%), [IC_{50} (μM) (the concentration
41 required for scavenging 50% activity)] CFCTKPC again has the highest antioxidant
42 activity (IC_{50} is 11.3 ± 3.2 μM) even better than reduced glutathione (IC_{50} is 74.3 ± 2.4
43 μM). In lipid peroxidation assay, once again CFCTKPC performed the best with an
44 IC_{50} value of 0.5 ± 0.0 μM better than reduced glutathione (1.2 ± 0.1 μM). These
45 findings mean that cysteine residue is most important in antioxidant activities. It was
46 suggested that SPD1 might contribute its antioxidant activities against hydroxyl and

47 peroxyl radicals.

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49 **Keywords:** Sweet potato; Defensin; Antioxidant;

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1. Introduction

It is commonly accepted that in a situation of oxidative stress, reactive oxygen species such as superoxide ($O_2^{\cdot -}$), hydroxyl ($OH^{\cdot}$) and peroxyl ($^{\cdot}OOH$, $ROO^{\cdot}$) radicals are generated. The reactive oxygen species (ROS) play an important role in the degenerative or pathological processes of various serious diseases, such as aging, Alzheimer's disease, atherosclerosis, cancer, cataracts, coronary heart disease, inflammation, and neurodegenerative disorders (Wettasinghe & Shahidi, 2000). The use of traditional medicine is widespread and plants still present a large source of natural antioxidants that might serve as leads for the development of novel drugs. Several antiinflammatory, antinecrotic, digestive, hepatoprotective, and neuroprotective drugs have recently been shown to have an antioxidant and/or antiradical scavenging mechanism as part of their activity (Prakash, Singh, & Upadhyay, 2007). In the search for sources of natural antioxidants and radical scavenging compounds in sweet potato during the last few years, some have been found, such as trypsin inhibitor proteins (Huang, Sheu, Chen, Chang, & Lin, 2007), phenolic compounds (Huang et al., 2011), and mucilage (Huang, Chen, Hou, & Lin, 2006).

Plant defensins have been shown to be major constituents of the immune systems of plants. This class of cysteine rich polypeptides is usually basic and contains 45-54

85 amino acids with four conserved disulfide bridges (Huang et al., 2008). The diverse

86 biological functions of plant defensins include antibacterial activities, inhibition of

87 insect or bovine trypsin and gut α-amylases (Olli & Kirti, 2006), antifungal activities,

88 inhibition of protein synthesis, blockage of sweet taste, and sodium channel

89 (Salampessy, Phillips, Seneweera, & Kailasapathy, 2010). The objectives of this work

90 were to investigate the antioxidant properties of defensin from sweet potato (SPD1) in

91 comparison with chemical compounds such as butylated hydroxytoluene (BHT) or

92 reduced glutathione in a series of *in vitro* and *ex vivo* tests.

93

94 **2. Materials and Methods**

95 *2.1. Expression of defensin in E. coli.*

96 Sweet potato defensin (SPD1, Gene Bank accession number: AY552546) was

97 expressed in *E. coli*. The coding sequence was amplified from SPD1 cDNA using an

98 oligonucleotide (5´- AGGAT CCATG GCTTC ATCTC TTCGT TC-3´), with a *Bam*

99 HI site (underlined) at the putative initial Met residue, and an oligonucleotide (5´

100 -GCCTT GCTAG TTCAG TCGAC CGCTGT -3´), with a *Sal* I site at the 3´ end. The

101 PCR fragment was subcloned into pGEM T-easy vector. The plasmid was then

102 digested with *Bam* HI and *Sal* I and subcloned into pQE30 expression vector

103 (QIAexpress expression system, Qiagen). The resulting plasmid, termed pQE-SPD1,

104 was introduced into *E. coli* (M15). Cultures of the transformed *E. coli* (M15)

105 overexpressed a protein of the expected molecular mass, which was purified by

106 affinity chromatography in Ni-NTA columns (Qiagen), according to the manufacturer´

107 s instructions.

108

109 *2.2. Protein staining on 15% SDS-PAGE gels*

110 SPD1 was detected on 15% SDS-PAGE gels. Samples treated with sample buffer

111 and β-mercaptoethanol (2-ME) with a final concentration of 14.4 mM were heated at

112 100^0C for 5 min before 15% SDS-PAGE.

113

114 *2.3. Synthesis of peptides contained in SPD1 sequence*

115 Four peptides for testing antioxidative activity were synthesized according to

116 tryptic hydrolysis simulation of SPD1. The obtained peptides: GFR, GPCSR,

117 CFCTKPC, and MCESASSK were analyzed by TEAC assay (μmol TE/g dw),

118 Scavenging activity of DPPH radical (%) assay [IC_{50} (μM)], and scavenging activity

119 of lipid peroxidation assay [IC_{50} (μM)] using reduced form of glutathione (GSH) as a

120 positive control.

121

122 *2.4.1. Total antioxidant status*

123 Total antioxidant status of SPD1 was measured using 2,

124 2'-azinobis[3-ethylbenzthiazoline-6-sulfonic acid (ABTS) assay (Re, Pellegrini,

125 Proteggente, Pannala, Yang, & Rice-Evans, 1999). ABTS (7 mM) was reacted with

126 aqueous potassium persulfate (2.45 mM). The reaction mixture was left to stand at

127 room temperature overnight (12 to 16 h) in the dark before use. The resultant

128 intensely-colored ABTS$^{\bullet +}$ radical cation was diluted with 0.01 M PBS (phosphate

129 buffered saline), pH 7.4, to give an absorbance value of ~0.70 at 734 nm. Test

130 compound was treated with the ABTS solution in a total volume of 200 μL.

131 Absorbance was measured spectrophotometrically at time intervals of 1, 2, 5, 10, and

132 20 min after addition of each compound in a range of four to eight concentrations.

133 The assay was repeated at least three times. Controls containing 180 μL of PBS to

134 replace ABTS were used to measure the absorbance of the test compounds themselves.

135 The assay relies on the antioxidant ability of the samples to inhibit the oxidation of

136 ABTS to ABTS$^{\bullet +}$ radical cation. In addition, Trolox (TE) was used as a reference

137 standard, and results were expressed as TEAC values (μmol TE/g dw). These values

138 were obtained from at least three different concentrations of each extract tested in the

139 assay giving a linear response between 20 and 80% of the blank absorbance.

140

141 *2.4.2. Scavenging activity against DPPH radical*

142 The effect of SPD1 and four synthetic peptides on the DPPH radical was

143 estimated according to the method of Huang et al (Huang, Chen, Chang, Sheu, & Lin,

144 2007). An aliquot of SPD1 (10 μL) was mixed with 100 mM Tris-HCl buffer (120 μL,

145 pH 7.4) and then 100 μL of the DPPH in ethanol with a final concentration of 50 μM

146 was added. The mixture was shaken vigorously and left to stand at room temperature

147 for 20 min in the dark. The absorbance at 517 nm of the reaction solution was

148 measured spectrophotometrically. The percentage of DPPH decolourization of the

149 sample was calculated according to the equation: % decolourization= $[1- Abs_{sample}$

150 $/Abs_{control}] \times 100$.

151

152 *2.4.3. Determination of antioxidant activity by reducing power measurement*

153 The reducing power of SPD1 and glutathione was determined according to the

154 method of Yen and Chen (1995). SPD1 (0, 6.25, 12.5, 25, 50, and 100 μg/mL) and

155 glutathione were mixed with an equal volume of 0.2 M phosphate buffer, pH 6.6, and

156 1% potassium ferricyanide. The mixture was incubated at 50°C for 20 min, during

157 which time ferricyanide was reduced to ferrocyanide. Then an equal volume of 1%

158 trichloroacetic acid was added to the mixture, which was then centrifuged at 3,500 *g*

159 for 10 min. The upper layer of the solution was mixed with distilled water and 0.1%

160 $FeCl_3$ at a radio of 1 : 1 : 2, and the absorbance at 700 nm was measured to determine

161 the amount of ferric ferrocyanide (Prussian Blue) formed. Increased absorbance of the

162 reaction mixture indicated increased reducing power of the sample.

163

164 *2.4.4. Determination of antioxidant activity by Fe^{2+}-chelating ability*

165 The Fe^{2+}-chelating ability was determined according to the method of Decker and

166 Welch (1990). The Fe^{2+} was monitored by measuring the formation of ferrous

167 iron-ferrozine complex at 562 nm. SPD1 (0, 6.25, 12.5, 25, 50, and 100 μg/mL) was

168 mixed with 2 mM $FeCl_2$ and 5 mM ferrozine at a ratio of 10 : 1 : 2. The mixture was

169 shaken and left to stand at room temperature for 10 min. The lower the absorbance of

170 the reaction mixture the higher the Fe^{2+}-chelating ability. The capability of the sample

171 to chelate the ferrous iron was calculated using the following equation:

172 $\text{Scavenging effect (\%)} = [1 - ABS_{sample} / ABS_{control}] \times 100$

173

174 *2.4.5. Determination of antioxidant activity by the ferric thiocyanate (FTC) method*

175 The FTC method was adopted from the method of Osawa and Namiki (1981).

176 Twenty mg/mL of samples dissolved in 4 mL of absolute ethanol (*w/v*) were mixed

177 with linoleic acid (2.51%, *v/v*) in absolute ethanol (*w/v*) (4.1 mL), 0.05 M phosphate

178 buffer pH 7.0 (8 mL) and distilled water (3.9 mL) and kept in a screw-cap container at

179 40°C in the dark. Then, to 0.1 mL of this solution was added 9.7 mL of 75% (*v/v*)

180 ethanol and 0.1 mL of 30% (*w/v*) ammonium thiocyanate. Precisely 3 min after the

181 addition of 0.1 mL of 20 mM ferrous chloride in 3.5% (*v/v*) hydrochloric acid to the

182 reaction mixture, the absorbance at 500 nm of the resulting red color {[Fe $(SCN)^{2+}$],

183 was measured every 24 h until the day when the absorbance of the control reached the

184 maximum value (Fe^{3+} was formed after lipid peroxide was produced and Fenton

185 reaction occurred). The inhibition of linoleic acid peroxidation was calculated as (%)

186 inhibition = 100 - [(absorbance increase of the sample/absorbance increase of the

187 control) x 100]. All tests were run in duplicate and analyses of all samples were run in

188 triplicate and averaged.

189

190 *2.4.6. Protection against hydroxyl radical-induced calf thymus DNA damage by SPD1*

191 *and synthetic peptide CFCTKPC*

192 The hydroxyl radical was generated by Fenton reaction according to the method of

193 Kohno et al. (Kohno, Yamada, Mitsuta, Mizuta, & Yoshikawa, 1991). The 15 μL

194 reaction mixture containing SPD1 (2.5, 5, or 10 mg/mL) or synthetic peptide

195 CFCTKPC (2.5, 5, or 10 μM), 5 μL of calf thymus DNA (1 mg/mL), 18 mM $FeSO_4$,

196 and 60 mM hydrogen peroxide were incubated at room temperature for 15 min. Then

197 2 μL of 1 mM EDTA was added to stop the reaction. Blank test contained only calf

198 thymus DNA and the control test contained all reaction components except SPD1.

199 The treated DNA solutions were subjected to agarose electrophoresis and then stained

200 with ethidium bromide and examined under UV light.

201

202 *2.5. Culture and Harvest of Human Hepatoma Cell Line*

203 HepG2 cells were cultured with Dulbecco's modified Eagle's medium (DMEM)

204 with 10% foetal bovine serum (FBS) in a T75 flask at 37°C, 5% CO_2, and 90%

205 relative humidity. To harvest cells, HepG2 cells were washed with PBS buffer and

206 treated with 2 mL of trypsin-EDTA for 3 min. The reaction was stopped by adding 8

207 mL of DMEM with 10% FBS. The mixture was then transferred into a tube and

208 centrifuged at 200 *g* at room temperature for 5 min. After removing the supernatant,

209 cell pellet was resuspended in 5 mL of DMEM with 10% FBS.

210

211 *2.5.1. Cell viability Assay*

212 **C**ell survival and proliferation were determined by a colorimetric 3-(4,

213 5-Dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT) assay

214 (Chemicon) and sulforhodamine B assay. HepG2 cells (with an initial concentration

215 of 2×10^4 cells/ well) were cultured without or with SPD1 (at various concentrations

216 in 10 μL of suspension) in a 96-well microplate (90 μL suspension/well). After 24 h,

217 10 μL of MTT solution was added to each well, and the cells were incubated at 37°C

218 for 4 h. Then, 100 μL of lysis buffer were added to each well, and the cells were again

219 incubated at 37°C for 1 h to dissolve the dark blue crystals. Each well was completely

220 pipetted, and then the absorption at 570 nm of formazan solution was measured using

221 a microplate reader. At least three repeats for each sample were used to determine the

222 cell proliferation.

223

224 *2.5.2.* Sulforhodamine B assay

225 Cell density was measured by sulforhodamine B (SRB) assay. After 24 h of

226 incubation, HepG2 cells (with an initial concentration of 2 × 10^4 cells/ well) were

227 treated without or with SPD1 (at various concentrations in 10 μL of suspension) in a

228 96-well microplate and incubated for an additional 24 h. There were three replications

229 for each treatment concentration, and the control for the experiment was cells grown

230 in media. Cells were then fixed for 1 h with 50% trichloroacetic acid and exposed to a

231 0.4% SRB solution for 30 min. The incorporated dye was solubilized in Tris-HCl

232 buffer solution (pH 7.4). The absorbance was measured by a microplate reader

233 (Molecular Devices, Orleans Drive, Sunnyvale, CA) at 570 nm. Data are reported as

234 % of control.

235

236 *2.5.3. Determination of Intracellular Peroxide in HepG2 Cells*

237 2',7'-Dichlorofluorescin diacetate (DCFH-DA), a relative specific probe for the

238 presence of hydrogen peroxide, was used as a probe for intracellular ROS formation.

239 HepG2 cells (2 x 10^5 cells/ well) were first cultured in 96-well tissue culture plate in

240 100 μL complete DMEM for 24 h and then treated with different concentrations of

241 SPD1 to determine its capacity to inhibit intracellular peroxide formation. The

242 reaction was initiated by the addition of 2 μM DCFH-DA (final concentration) and

243 incubated at 37°C for up to 3, 6, 9, and 12 h. The fluorescence intensity was measured

244 using a micro-plate reading fluorometer (VersaMAx, Molecular Devices) with the

245 excitation wavelength at 485 nm and the emission wavelength at 535 nm.

246

247 *2.6. Statistical Analysis*

248 Means of triplicates were calculated. Student's *t* test was used for comparison

249 between two treatments. All data (expressed as percent of control value) were means

250 ± SD. A difference was considered to be statistically significant when $p < 0.05$.

251

252 **3. Results and Discussion**

253 *3.1. Purification of expressed SPD1*

254 SDS-PAGE analysis of crude extracts from transformed *E. coli* (M15) showed a

255 high level of a polypeptide with the expected molecular mass (ca. 9,000 Da). This

256 polypeptide was found as a soluble protein in the supernatant (Fig. 1, lane 2), and was
257 absent in protein extracts obtained from *E. coli* transformed with pQE-30 vector (Fig.
258 1, lane 1). The expressed protein was purified from crude extracts by Ni^{2+}-chelate
259 affinity chromatography, which yielded highly purified His-tagged SPD1 (Fig. 1, lane
260 3).
261
262 *3.2. 2, 2'-azinobis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) assay*
263 ABTS assay is often used in evaluating total antioxidant power of single
264 compounds and complex mixtures of various plants. This method is quick and simple
265 to perform, and the results are reproducible and linearly related to the molar
266 concentration of the antioxidants (Benzie, Wai, & Strain, 1999). In Fig. 2A SPD1
267 protein exhibited a dose-dependent total antioxidant activity within the applied
268 concentrations (0, 6.25, 12.5, 25, 50, and 100 μg/mL). At the concentration of 100
269 μg/mL, SPD1 displayed the highest total antioxidant capacity.
270
271 *3.3. Scavenging Activity against 1, 1-Diphenyl-2-Picrylhydrazyl (DPPH) Radical*
272 DPPH radical is scavenged by antioxidants through the donation of a hydrogen
273 forming the reduced DPPH-H. The color changed from purple to yellow after
274 reduction, which could be quantified by its decrease of absorbance at wavelength 517

275 nm (Sharma & Bhat, 2009). Fig. 2B shows the dose-response curve for the
276 radical-scavenging activity of the different concentrations of SPD1 and glutathione
277 using the DPPH coloring method. SPD1 had the highest radical-scavenging activity
278 (27.2 ± 3.1%) at the concentration of 100 μg/mL. Free cysteine residues in whey
279 proteins (Tong, Sasaki, McClements, & Decker, 2000) were reported to have
280 antioxidant activities. These findings suggest that cysteine residues in sweet potato
281 SPD1 may also participated in the antiradical activities.
282

283 *3.4. Measurement of Reducing Power*

284 We investigated the Fe^{3+}-Fe^{2+} transformation in the presence of SPD1 to measure
285 its reducing capacity. The reducing capacity of a compound may serve as a significant
286 indicator of its potential antioxidant activity (Meir, Kanner, Akiri, & Hadas, 1995).
287 The antioxidant activities of putative antioxidants have been attributed to various
288 mechanisms. Among them are prevention of chain initiation, binding of transition
289 metal ion catalysts, decomposition of peroxides, prevention of continued hydrogen
290 abstraction, and radical scavenging (Diplock, 1997). The reducing power of SPD1 is
291 shown in Fig. 2C with reduced glutathione serving as a positive control. The reducing
292 power of SPD1 exhibited a dose-dependence ($P<0.05$) within the applied
293 concentrations (0, 6.25, 12.5, 25, 50, and 100 μg/mL).

294

295 *3.5. Measure of Fe^{2+}-chelating ability*

296 Iron and other transition metals promote oxidation by acting as catalysts of free
297 radical reactions. These redox-active transition metals transfer single electrons during
298 changes in oxidation states. Chelating compounds may also sterically hinder
299 formation of the metal hydroperoxide complex (Blokhina et al 2003). The metal
300 chelating capacity of SPD1 and standard antioxidants was determined by assessing
301 their ability to compete with ferrozine for the ferrous ions. The Fe^{2+}-chelating ability
302 of the SPD1 is shown in Fig. 2D. EDTA was used as a positive control. The Fe^{2+}
303 -chelating ability of SPD1 was lower than that of EDTA and this difference was
304 statistically significant ($P<0.05$). The doses of 6.25, 12.5, 25, 50, and 100 µg/mL of
305 SPD1 exhibited 1.25, 4.97, 9.07, 12.72, and 15.67% iron binding capacity,
306 respectively. On the other hand, the doses of 6.25, 12.5, 25, 50, and 100 µg/mL of
307 EDTA have shown 4.60, 8.24, 12.13, 22.68, and 27.03% chelating activity of iron,
308 respectively. Values obtained from Fig. 2D demonstrate that the action of SPD1, as a
309 peroxidation protector, may be related to its iron-binding capacity.

310

311 *3.6. Ferric thiocyanate (FTC) method*

312 The ferric thiocyanate method measured the amount of peroxide produced during

313 the initial stages of oxidation which was the primary product of oxidation. So far

314 low-density lipoprotein (LDL) peroxidation has been reported to contribute to

315 atherosclerosis development (Koba, Matsuoka, Osada, & Huang, 2007). Therefore,

316 delay or prevention of LDL peroxidation is an important function of antioxidants. Fig.

317 2E shows the time-course curve for the antioxidative activity of the SPD1, BHT and

318 H_2O by the FTC method. The BHT was used as a positive control, and H_2O as a

319 negative control. The results indicate that SPD1 has antioxidative activity. SPD1 may

320 act as a significant LDL peroxidation inhibitor ($P<0.05$).

321

322 *3.7. Protecting Calf Thymus DNA against Hydroxyl Radical-Induced Damage by*

323 *SPD1*

324 Free radicals could damage macromolecules in cells, such as DNA, protein, and

325 lipids in membranes (Wu, Zhang, Kong, & Hua, 2009). The oxidative damage of

326 DNA is one of the most important mechanisms in the initiation of cancer. The damage

327 is usually caused by hydroxyl radicals. The activity of these radicals can be reduced

328 by natural antioxidants found in plants including herbs. The Fenton reaction involves

329 the reaction between hydrogen peroxide and Fe^{2+} to form hydroxyl radicals. Hydroxyl

330 radical scavenging was measured indirectly by the inhibition of the

331 Fenton reaction via reducing Fe^{3+} to Fe^{2+}. Fig. 3A shows that SPD1 protected against

332 hydroxyl radical-induced calf thymus DNA damages. The blank contained calf
333 thymus DNA only and the control contained all components except SPD1. Compared
334 to the blank and control, it was found that SPD1 added above 5 mg/mL (with a final
335 absolute amount of 75 μg) could protect against hydroxyl radical induced calf thymus
336 DNA damages during 15-min reactions. In addition, it was found that synthetic
337 peptide (CFCTKPC) added above 5 μM could protect against hydroxyl radical
338 induced calf thymus DNA damages during 15-min reactions (Fig. 3B).
339

340 *3.8. Effects of SPD1 on Cell Survival (MTT Assay) and Cell density (SRB assay).*

341 The cell survival test of HepG2 cells treated with different concentrations (0,
342 6.25, 12.5, 25, 50, and 100 μg/mL) of SPD1 for 24 h were examined, and results are
343 shown in Fig. 4A. The relative MTT activity of cells treated with 12.5, 25, 50, and
344 100 μg/mL of SPD1 were 98.41, 96.16, 90.86 and 89.95%, respectively. Cells
345 cultured with SPD1 did not change cell viability at the concentrations (0, 6.25, 12.5,
346 25, 50, and 100 μg/mL). The SPD1 concentration-dependent cytotoxic effect was also
347 confirmed by SRB assay carried out in cells treated with SPD1 under the same
348 experimental conditions (Fig. 4B). Compared to the MTT assay, the potency by which
349 SPD1 induced the cytotoxic effect was similar. In this study, the cell survival rate was
350 greater than 90% if the HepG2 cells were treated with SPD1 in the range of 0-100

351 μg/mL. Therefore, 0-100 μg/mL of SPD1 was used to study its effects on antioxidant
352 system in HepG2 cells for the following experiments.
353
354 *3.9. Effects of SPD1 on Intracellular Peroxide in HepG2 Cells*
355 Production of intracellular peroxide in HepG2 cells treated with 0, 12.5, 25, 50, and
356 100 μg/mL of SPD1 for 3 - 12 h is shown in Fig. 4B. The production of intracellular
357 peroxide in HepG2 cells after treatment with the SPD1 for 3 h was significantly
358 inhibited (P<0.05). The doses of 12.5, 25, 50, and 100 μg/mL of SPD1 inhibited 5.69,
359 11.39, 16.84 and 25.08% intracellular peroxide production, respectively. As the
360 treatment increased to 6 h, the production of intracellular peroxide in HepG2 cells
361 reached minimum value of concentration for SPD1 treatments. However, the
362 inhibitory effects of SPD1 on production of intracellular peroxide became less
363 effective after 9 h. It appeared that SPD1 could protect HepG2 cells from
364 antioxidative attack. When SPD1 was incubated with HepG2 cell at 9 h, SPD1
365 became partly denaturation or cleavage by protease. Osseni et al. also used HepG2
366 cells to study the antioxidant effects of melatonin, and they found that low doses of
367 melatonin increased glutathione concentration. They suggested that melatonin acts as
368 an antioxidant as well as a modulator of cellular signaling processes to exert its
369 oxidative-stress-protection effect (Osseni, Rat, Bogdan, Warnet, & Touitou, 2000). In

370 this study, we found that SPD1 might effectively reduce the cellular oxidative stress

371 in human hepatoma HepG2 cells.

372

373 *3.10. Determination of the Antioxidative Activities of four synthetic peptides*

374 We used four synthetic peptides to measure antioxidative activities (section *2.3.*).

375 Synthetic peptides were designed by simulating trypsin cutting sites of SPD1 gene

376 products from sweet potato (http://au.expasy.org/tools/peptidecutter/). Gly-Phe-Arg

377 (GFR), Gly-Pro-Cys-Ser-Arg (GPCSR), Cys-Phe-Cys-Thr-Lys-Pro-Cys (CFCTKPC),

378 and Met-Cys-Glu-Ser-Ala-Ser-Ser-Lys (MCESASSK) (Table I) were obtained

379 according to simulation. Our results showed that in TEAC assay CFCTKPC

380 performed the best (13.5 ± 0.3 μmol TE/g dw), even better than reduced glutathione

381 (7.3 ± 0.2 μmol TE/g dw). In scavenging activity of DPPH radical assay (%), (IC_{50}

382 (μM) (the concentration required for scavenging 50% activity) of GPCSR, CFCTKPC,

383 and MCESASSK peptides were 337.4 ± 10.1, 11.3 ± 3.2 and 136.2 ± 5.1 μM,

384 respectively. CFCTKPC again has the highest antioxidant activity (IC_{50} is 11.3 ± 3.2

385 μM) even better than reduced glutathione (IC_{50} is 74.3 ± 2.4 μM). In lipid

386 peroxidation assay, once again CFCTKPC performed the best with an IC_{50} value of

387 0.5 ± 0.0 μM better than reduced glutathione (1.2 ± 0.1 μM). These results

388 demonstrated that some simulated synthetic peptides from tryptic defensin

389 hydrolysates exhibited antioxidative activities.

390 Synthetic peptides (CFCTKPC) containing cysteine (C), phenylalanine (F),
391 threonine (T), lysine (K) and proline (P) showed an effective capacity for TEAC,
392 scavenging DPPH radical and lipid peroxidation assays. The presence of three
393 cysteine (C) residues, that have been reported to exert antioxidant effects, could be
394 responsible for scavenging ABTS radical (Hernandez-Ledesma, Miralles, Amigo,
395 Ramos, & Recio, 2005). Cysteine residues with free–SH in whey proteins (Tong,
396 Sasaki, McClements, & Decker, 2000) were reported to have antioxidant activities. It
397 could be due to the localization of one of C residues at both the N-terminal and
398 C-terminal positions that could make the access to radicals easier. A number of
399 studies have been devoted to assess the antioxidant potential of soy protein fractions
400 as well as to the isolation and structural characterization of the most active peptides. A
401 number of antioxidative peptides, usually composed of 3-16 amino acid residues,
402 have been isolated and identified from these hydrolyzates, and their antioxidant
403 activities investigated to gain insight into the antioxidative mechanism of peptides.
404 Several amino acid residues, such as His, Met, Tyr, Cys, and Trp, are generally
405 accepted as antioxidants in spite of their pro-oxidative effects in some cases (Marcuse,
406 1960). These antioxidant peptides derived from soybean protein have been found to
407 contain between 5 and 16 amino acids (Chen, Muramoto, & Yamauchi, 1995). Our

408 results demonstrate that SPD1 might contribute various antioxidant properties through

409 its hydrolytic peptides as shown in Table 1. Various peptides may have specific

410 antioxidant properties. For example, among four synthetic peptides CFCTKPC (with

411 three cysteine groups) performs the best in all three assays, both GPCSR and

412 MCESASSK (with one cysteine group) perform second to CFCTKPC in lipid

413 peroxidation assay.

414 Especially, regarding to target groups, CFCTKPC was more potent for

415 scavenging hydroxyl and peroxyl radicals. It is generally accepted that the chemical

416 reactivity of hydroxyl radical is the strongest among ROS. Hydroxyl radical reacts

417 easily with biomolecules, such as amino acids, proteins and DNA. Therefore, the

418 removal of hydroxyl radical is probably one of the most effective defenses of a living

419 body against various diseases (Cacciuttolo et al., 1993). Carbon-centered radicals that

420 represent R·, RO· and ROO· could be quenched by CFCTKPC. This result agreed

421 with a lipid peroxidation inhibition assay in which CFCTKPC inhibited lipid

422 peroxidation by scavenging lipid-derived radicals.

423 In conclusion, the results from our *in vitro* experiments, including ABTS assay

424 (Fig. 2A), DPPH radical assay (Fig. 2B), reducing power method (Fig. 2C), Fe^{2+}

425 -chelating ability (Fig. 2D), FTC method (Fig. 2E), hydroxyl radical-induced calf

426 thymus DNA damage (Fig. 3), and intracellular peroxide in HepG2 cells (Fig. 4B)

427 demonstrated that SPD1 in sweet potato may have significant antioxidant activities.
428 SPD1 may also contribute significantly to complexation of di- and higher valent metal
429 ions with formation of thermodynamically stable complexes in which free metal ions
430 are bound and show no further reactions. The *ex vivo* or *in vivo* antioxidant activities
431 of SPD1 should be performed in near further.

432

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438

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522 # Figure Legends

523 Figure 1. SDS-PAGE analysis of purified recombinant sweet potato defensin protein
524 (SPD1). Crude extracts from *E. coli* (M15) transformed with pQE30 (lane 1)
525 or with pQE30-SPD1 (lane 2) were analyzed by 15% (w/v) SDS/PAGE with 5
526 μg protein in each line, and then the gel was stained with Coomassie blue
527 G-250. Molecular masses of standard proteins are indicated at the left of the
528 figure. His-tagged SPD1 was purified by Ni^{2+}-chelate affinity chromatography
529 (lane 3). The experiments were done twice and a representative one is shown.
530 M: Molecular marker.

531

532 Figure 2. Antioxidant activities of recombinant defensin (SPD1) protein from sweet
533 potato. The inhibition of the formation of $ABTS^{\cdot+}$. Glutathione was used as a
534 positive control (A). DPPH radical scavenging activity. Glutathione was used
535 as a positive control (B). Reducing power. EDTA was used as a positive
536 control (C). Fe^{2+}-chelating ability. Ascorbic acid was used as a positive
537 control (D). Inhibition of linoleic peroxidation, BHT was used as a positive
538 control (E). Concentration range includes 0, 6.25, 12.5, 25, 50, and 100
539 μg/mL. Absorbance value represents average of triplicates of different
540 samples analyzed.

541

542 Figure 3. Protecting calf thymus DNA against hydroxyl radical-induced damage by

543 recombinant defensin protein (SPD1) (2.5, 5, or 10 mg/mL) (A) or synthetic

544 peptide CFCTKPC (2.5, 5, or 10 μM) (B). Calf thymus DNA only was used

545 for blank test, and the control test contained no defensin.

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548 Figure 4. Effects of recombinant defensin protein (SPD1) on cell survival (MTT assay)

549 (A), cell density (SRB assay) (B), and the production of intracellular

550 peroxide (C) in HepG2 cells. An asterisk indicates that the values are

551 significantly different from untreated cells ($P < 0.05$).

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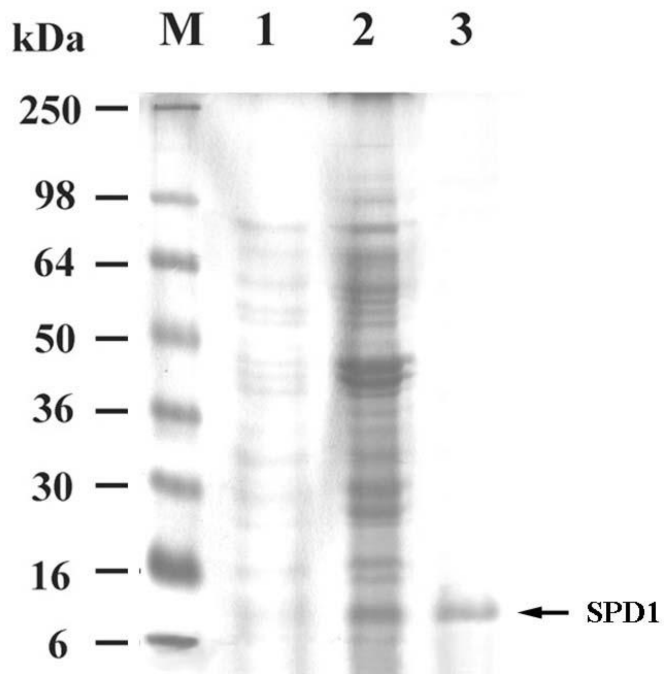
561 **Figure 1.**



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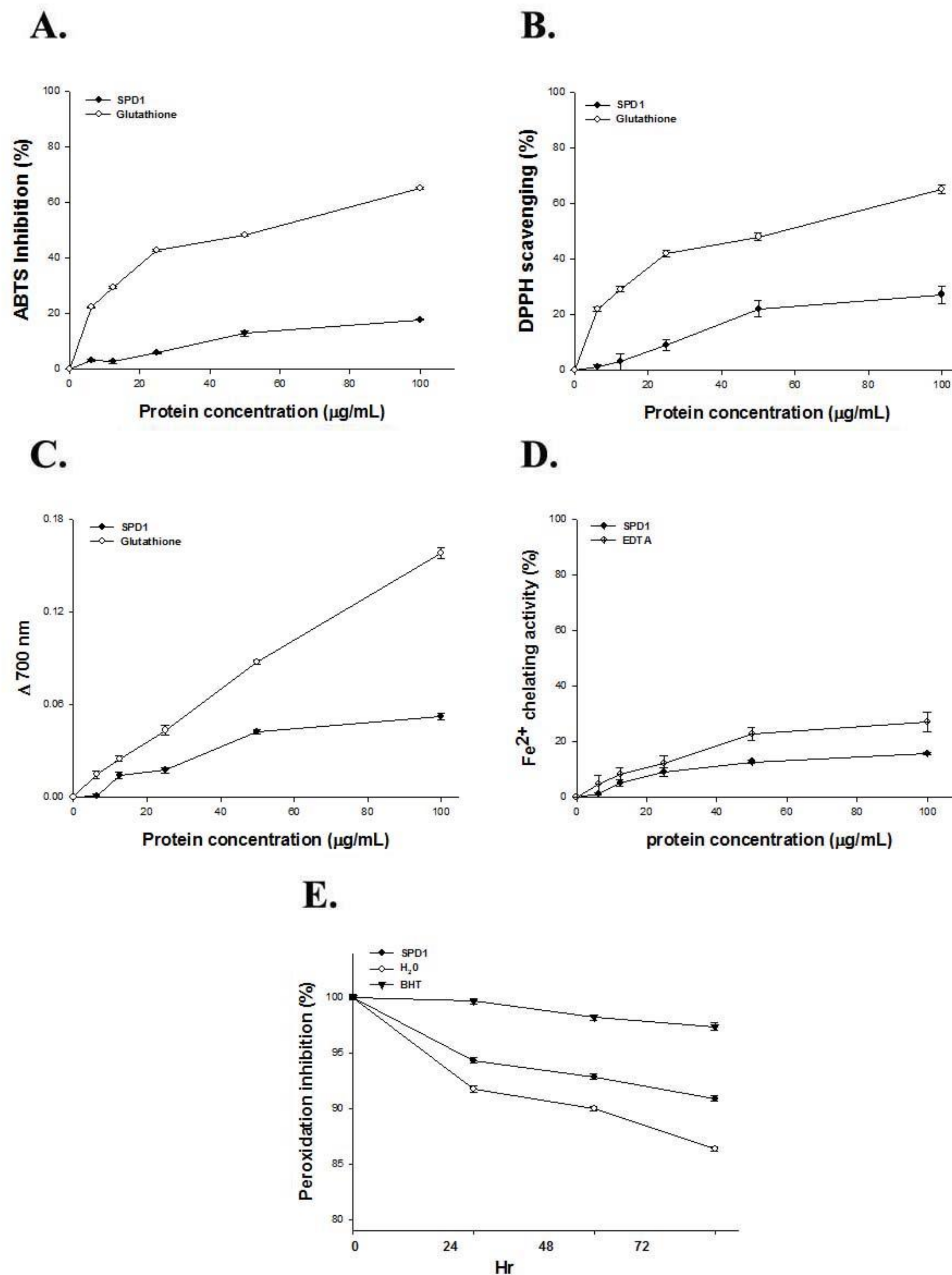
581 **Figure 2.**



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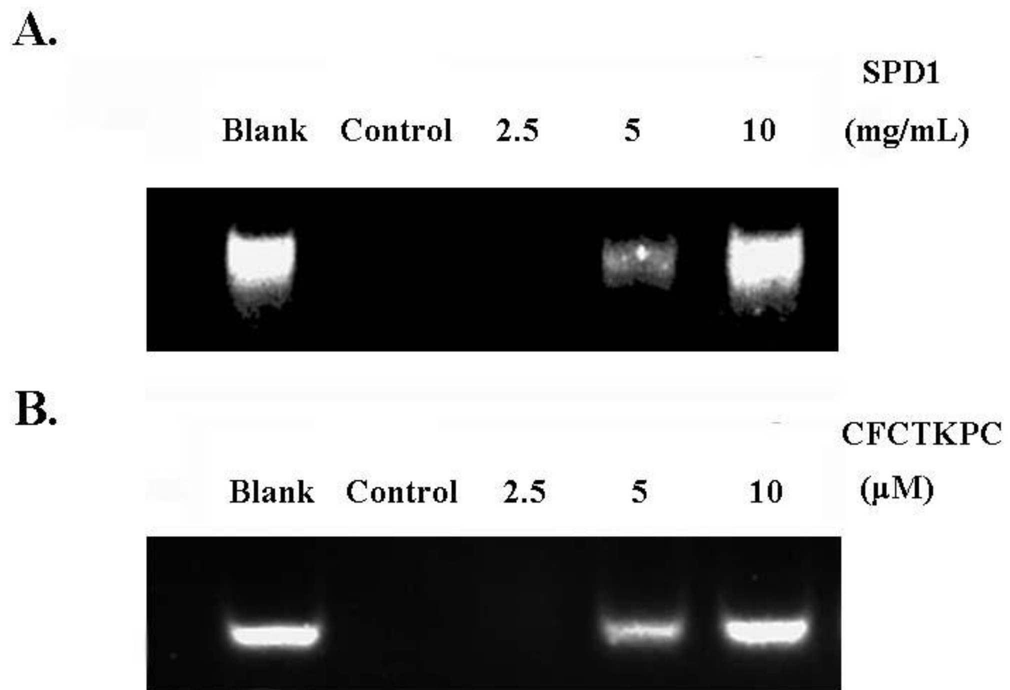
588 **Figure 3.**



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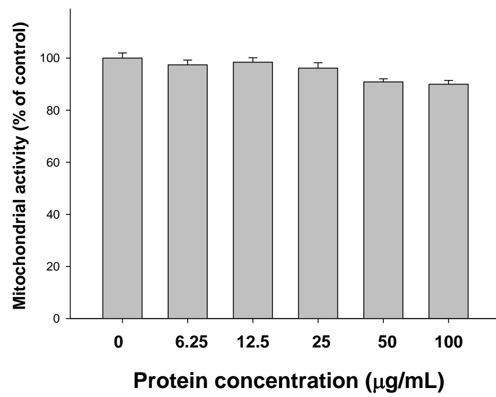
608 **Figure 4.**

609 A.



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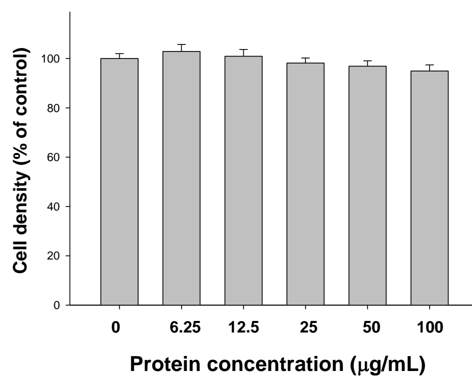

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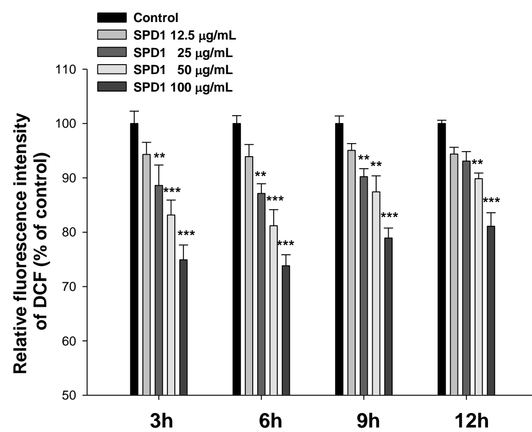

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616 Table 1: Defensin peptides with antioxidative activities[†] .

Peptide	TEAC (μmol TE/g dw)	Scavenging activity of DPPH radical (%), IC_{50} (μM)*	Scavenging activity of lipid peroxidation assay, IC_{50} (μM)*
GSH	7.3 ± 0.2	74.3 ± 2.4	1.2 ± 0.1
GFR	1.5 ± 0.1	> 1000	28.6 ± 0.5
GPCSR	4.1 ± 0.2	337.4 ± 10.1	5.6 ± 0.3
CFCTKPC	13.5 ± 0.3	11.3 ± 3.2	0.5 ± 0.0
MCESASSK	5.7 ± 0.2	136.2 ± 5.1	2.5 ± 0.2

617 Note: SPD1 has pre-pro-sequence. These sequences were retrieved from the NCBI

618 (National Center

619 for Biotechnology Information, http://www.ncbi.nlm.nih.gov) with accession

620 number AY552546.

621 [†] Each value is expressed as mean ± S.D. (n = 3).

622 *IC_{50} value was obtained by interpolation from linear regression analysis.

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