Running title: defensin with angiotensin converting enzyme inhibitory activity

Sweet potato storage root defensin and their tryptic hydrolysates exhibited angiotensin converting enzyme inhibitory activity *in vitro*

Guan-Jhong Huang[1,*,#], Te-Ling Lu[2,#], Chuan-Sung Chiu[1,3], Hsien-Jung Chen[4], Chieh-Hsi Wu[2], Ying-Chin Lin[5], Wen-Tsong Hsieh[6], Jung-Chun Liao[2], Ming-Jyh Sheu[2], and Yaw-Huei Lin[7*]

[1]School of Chinese Pharmaceutical Sciences and Chinese medicine resources, China Medical University, Taichung 404, Taiwan;

[2] School of Pharmacy, China Medical University, Taichung 404, Taiwan;

[3] Nursing Department, Hsin Sheng College of Medical Care and Management, Taoyuan 325, Taiwan;

[4]Department of Biological Sciences, National Sun Yat-Sen University, Kaohsiung 804, Taiwan;

[5] Nursing and management, Jen-Teh Junior college of Medicine, Taiwan;

[6] Department of Pharmacology, China Medical University, Taichung 404, Taiwan;

[7] Institute of Plant and Microbial Biology, Academia Sinica, Nankang, Taipei 115, Taiwan;

***Corresponding author:**

Dr. Yaw-Huei Lin

Institute of Plant and Microbial Biology, Academia Sinica, Nankang, Taipei 11529, Taiwan, ROC;

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

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

Dr. Guan-Jhong Huang

School of Chinese Pharmaceutical Sciences and Chinese medicine resources, China Medical University, Taichung 404, Taiwan;

Tel.: +886 4 2205 3366 ext 5508; Fax: +886 4 2208 3362.

E-mail address: gjhuang@mail.cmu.edu.tw

[#] These two authors contributed equally to this work.

MS NO. 3192

Abstract:

Sweet potato defensin (SPD1) overproduced in *E. coli* (M15) was purified by Ni^{2+}-chelate affinity chromatography. The molecular mass of SPD1 is about 8,600 Da determined by SDS (sodium dodecyl sulfate)-PAGE (polyacrylamide gel electrophoresis). Our previously paper showed that SPD1 had antimicrobial, dehydroascorbate reductase and monodehydroascorbate reductase activities. The activity of SPD1 to inhibit angiotensin converting enzyme (ACE) was shown by using N-[3-(2-furyl) acryloyl]-Phe-Gly-Gly (FAPGG) as substrate in a dose-dependent manner (27.56 ~ 52.58 % inhibition). The 50% inhibition (IC_{50}) of ACE activity required 190.47 μg/mL SPD1 while that of Captopril was 10 nM (868 ng/mL). The use of thin layer chromatography (TLC) also showed SPD1 as an ACE inhibitor. SPD1 acted as a mixed type inhibitor against ACE using FAPGG as a substrate. When 200 μg/mL SPD1 (10 μg) were added, Vmax and Km were, respectively, 0.01 △A/min and 0.69 mM; while without SPD1 they were 0.03 △A/min and 0.42 mM. Trypsin was used for SPD1 hydrolysis for different times. It was found that the ACE inhibitory activity increased from 52.47 % to about 74.38 % after 24 h hydrolysis. The results suggested that when small peptides increased by trypsin hydrolysis of the SPD1 ACE inhibitory capacity also increased up to 24 h, and then decreased which may be due to disappearance of some active ingredients. Six peptides, namely GFR, FK, IMVAEAR, GPCSR, CFCTKPC and MCESASSK, were synthesized based on the simulated trypsin digest of SPD1, then tested for ACE inhibitory activity. IC_{50} values of individual peptides were 94.25 ± 0.32, 265.43 ± 1.24, 84.12 ± 0.53, 61.67 ± 0.36, 1.31 ± 0.07 and 75.93 ± 0.64 μM, respectively, suggesting that CFCTKPC might represent the main domain for the ACE inhibition. SPD1 and its hydrolysates might be good for hypertension and other disease control when people consume sweet potato tuberous storage roots.

Keywords: Sweet Potato; Defensin; Angiotensin converting enzyme (ACE); Inhibition.

INTRODUCTION

Many bioactive peptides have common structural properties that include a relatively short peptide residue length (e.g. 2 - 9 amino acids), possessing hydrophobic amino acid residues in addition to proline, lysine or arginine groups (Lin et al., 2006). Bioactive peptides are among the many functional components identified in foods. These are small protein fragments that have biological effects once they are released during gastrointestinal digestion in the organism or by previous *in vitro* protein hydrolysis. Bioactive peptides with immunostimulating (Huang et al., 2010), antioxidant or angiotensin-converting enzyme (ACE) inhibitor (Liu et al., 2007), antithrombotic (Scarborough, 1991), bactericidal (Bellamy et al., 1993) functions were the research focus in recent years.

ACE (peptidyldipeptide hydrolase EC 3.4.15.1) is a glycoprotein and a dipeptide-liberating exopeptidase classically associated with the renin-angiotensin system regulating peripheral blood pressure (Mullally et al., 1996). ACE removes a dipeptide from the C-terminus of angiotensin I to form angiotensin II, a very hypertensive compound (Lin et al., 2008). Several endogenous peptides, such as enkephalins, β-endorphin, and substance P, were reported to be competitive substrates and inhibitors of ACE. Several food-derived peptides from α-lactoalbumin, β-lactoglobulin (Pihlanto-Leppälä et al., 1998), mucilage (Huang et al., 2006) and trypsin inhibitor (TI) (Huang et al., 2007) also inhibited ACE. Several antioxidant peptides (reduced glutathione and carnosine-related peptides) (Hou et al., 2003) and synthetic peptides also exhibited ACE inhibitor activities (Huang et al., 2006).

Plant defensins were originally termed γ -thionins because they have a similar size (5 kDa) and the same number of disulfide bridges (four) as α- and β-thionins. γ - Thionins are structurally different from those of α- and β-thionins and instead rather similar to

insect and mammalian defensins structurally as well as functionally. Thus this class of plant peptides was named ‘plant defensins’ (Haasen and Goring; 2010). A variety of plant defensins have been isolated and characterized from many plant species including monocots and dicots (Terras et al., 1992). Plant defensin families have been known as potent growth inhibitors of a broad spectrum of fungi and bacteria, however the antimicrobial activity of the plant defensins has been quite diverse and was classified into two main groups (A and B) sharing only 25% similarity based on amino acid sequence (Harrison, 1998). Plant defensins have been detected in different organs of plants such as leaves, flowers, seeds, and tubers (Moreno et al., 1994). Furthermore, the expression of some defensin genes is developmentally regulated, whereas that of others is greatly elevated in response to biotic and abiotic external stimuli (Epple et al., 1997).

In our previous report, SPD1 exhibited antimicrobial, dehydroascorbate reductase and monodehydroascorbate reductase activities (Huang et al., 2008a). In this work we report for the first time that SPD1 exhibited dose-dependent ACE inhibitory activity when Captopril was used as a positive control. Commercial bovine serum albumin (BSA), which was frequently found in the literature as the peptide resource of ACE inhibitors, was chosen for comparison. The K_i values of SPD1 against ACE were calculated. We also used trypsin to hydrolyze SPD1 for different times, and the changes of ACE inhibitory activity were determined. IC_{50} of ACE inhibitory activities by synthetic peptides were also determined.

MATERIALS and METHODS

Materials

Tris, electrophoretic reagents, and silica gel 60 F254 were purchased from E. Merck Inc. (Darmstadt, Germany); Captopril was purchased from Calbiochem Co. (CA, USA); Seeblue prestained markers for SDS-PAGE including myosin (250 kDa), BSA (98 kDa), glutamic dehydrogenase (64 kDa), alcohol dehydrogenase (50 kDa), carbonic anhydrase (36 kDa), myoglobin (30 kDa), and lysozyme (16 kDa) were from Invitrogen (Groningen, The Netherlands); FAPGG, ACE (1 unit, rabbit lung); Coomassie brilliant blue G-250; peptide (GL Biochem, China), and other chemicals and reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Expression of Defensin in *E. coli*

Defensin with its pre-pro-sequence (SPD1) was expressed in *E. coli*. The coding sequence was amplified from cDNA SPD1 using an oligonucleotide (5´-A GGAT CCATG GCTTC ATCTC TTCGT TC -3´), with a *Bam*HI site (underlined) at the putative initial Met residue, and an oligonucleotide (5´-GCCTT GCTAA TTCAG TCGAC CGCTG T -3´), with a *Sal*I site at the 3´ end. The PCR fragment was subcloned in pGEM T-easy vector. The plasmid was then digested with *Bam*HI and *Sal*I and the excised fragments were subcloned in pQE30 expression vector (QIAexpress expression system, Qiagen). The resulting plasmid, termed pQE-SPD1, was introduced into *E. coli* (M15). Cultures of the transformed *E. coli* (M15) overexpressed a protein of the expected molecular mass, which was purified by affinity chromatography in Ni-nitrilotriacetic acid (NTA) columns (Qiagen), according to Huang et al. (2004).

Determination of ACE inhibitory activity

The ACE inhibitory activity was measured according to the method of Holmquist et al. (1979) with some modifications. Four microliters (4 microunits) of commercial ACE (1 unit, rabbit lung) was mixed with 50 μL of different amounts of SPD1 or BSA (50, 100,

and 200 μg/mL), and then 200 μL of 0.5 mM *N*-[3-(2-furyl) acryloyl]-Phe-Gly-Gly [FAPGG, dissolved in 50 mM Tris-HCl buffer (pH 7.5) containing 0.3 M NaCl] was added. The decreased absorbance at 345 nm (Δ *A* inhibitor) was recorded during 5 min at room temperature. Deionized water was used instead of sample solution for blank experiments (Δ*A* control). Captopril (molecular mass 217.3 Da) was used as a positive control for ACE inhibitor (1.25, 2.5, 5, 10, 20, 40 and 80 nM). The ACE activity was expressed as Δ*A* 345 nm, and the ACE percent inhibition was calculated as follows: [1 - (Δ*A* inhibitor / Δ*A* control)] x 100. Means of triplicates were determined. The 50% inhibition (IC_{50}) of ACE activity was defined as the concentrations of samples that inhibited 50% of ACE activity under experimental conditions.

Determination of ACE inhibitory activity by TLC

The ACE inhibitor activity of SPD1 was also determined by TLC method (Holmquist et al., 1979). Each 100 μL of SPD1 or BSA (225 μg/mL) was premixed with 15 microunits of ACE for 1 min, and then 200 μL of 0.5 mM FAPGG was added and allowed to react at room temperature for 10 min. Then 800 μL of methanol was added to stop the reaction. The blank experiment contained FAPGG only; in the control experiment, ACE reacted with FAPGG under the same conditions. Each was dried under reduced pressure and redissolved with 400 μL of methanol, and 50 μL was spotted on a silica gel 60 F254. The FAPGG and FAP (ACE hydrolyzed product) were separated by TLC in 1-butanol-acetic acid-water, 4:1:1 (V/V/V), and observed under UV light.

Determination of the kinetic properties of ACE inhibition by Defensin

The kinetic properties of ACE (4 μU) without or with SPD1 (200 μg/mL) in a total volume of 250 μL were determined using different concentrations of FAPGG as substrate (0.1 mM to 0.5 mM). The Km (without SPD1) and Km´ (with SPD1) were calculated

from Lineweaver-Burk plots, where Km´ was the Michaelis constant in the presence of 200 μg/mL SPD1.

Determination of the ACE Inhibitory Activity by trypsin Hydrolysates of Defensin

Six mg of SPD1 were dissolved in 1 mL of 50 mM Tris-HCl buffer (pH 7.9). Then 0.1 mL of 12 mg of trypsin was added and hydrolysis was carried out at 37°C for 8, 16, 24 and 32 h. After hydrolysis the solution was heated at 100°C for 5 min to stop enzyme reaction. The trypsin was heated before SPD1 hydrolysis for the 0 h control reaction. Each of the 60 μL SPD1 hydrolysates was used for determinations of ACE inhibition by using spectrophotometry.

Chromatograms of Tryptic Hydrolysates of defensin on a Sephadex G-50 Column

The unhydrolyzed SPD1 and tryptic SPD1 hydrolysates at 24 h were separated by Sephadex G-50 chromatography (1 x 60 cm). The column was eluted with 20 mM Tris-HCl buffer (pH 7.9). The flow rate was 30 mL/h, and each fraction contained 2 mL, the absorbance of which was determined at 570 nm.

Statistical Analysis

Means of triplicates were calculated. Student's *t* test was used for comparison between two treatments. A difference was considered to be statistically significant when $p < 0.05$.

RESULTS and DISCUSSION

Expression of defensin in *E. coli*

SDS-PAGE analysis of SPD1 crude extracts from the transformed *E. coli* (M15) showed high amounts of a polypeptide with the expected molecular mass (ca. 8,600 Da). This polypeptide was found as a soluble protein in the supernatant, and was absent in protein extracts obtained from *E. coli* transformed with pQE-30 vector. The expressed protein was purified from crude extracts by Ni^{2+}-chelate affinity chromatography, which yielded highly purified His-tagged SPD1. Preparing SDS-PAGE (Huang et al., 2008a) was used as the next step for SPD1 purification.

Determination of ACE inhibitor Activity of Defensin by Spectrophotometry

The purified SPD1 was used for determinations of ACE inhibitory activity. Fig. 1 shows time course of the effect of the different amounts of SPD1 (0, 50, 100 and 200 μg/mL) on the ACE activity (ΔA 345 nm). Compared with the ACE only (control), it was found that the higher the amount of SPD1 added the lower the ΔA 345 nm found during 300 sec reaction period. Results of Fig. 1 shows that purified SPD1 could inhibit ACE activity in a dose-dependent manner.

Effects of Defensin, BSA and Captopril on ACE Activity shown by Spectrophotometry

It was interesting to know whether BSA also exhibited the ACE inhibitory activity. Fig. 2A shows the effects of SPD1 (0, 50, 100, and 200 μg/mL), BSA (0, 50, 100, and 200 μg/mL) or Captopril (Fig. 2B; 0, 1.25, 2.5, 5, 10, 20, 40 and 80 nM; corresponding to 0, 108.5, 217, 434, 868, 1,736, 3,472 and 6,844 ng/mL, respectively) on ACE activity. It was found that BSA showed less ACE inhibitory activity (less than 25 % inhibition) and

without dose-dependent inhibition patterns. However, SPD1 exhibited dose-dependent ACE inhibitory activity (50~200 μg/mL giving, respectively, 27.56 ~ 52.58 % inhibition). From calculations, the 50% inhibition (IC_{50}) of SPD1 against ACE activity was 190.47 μg/mL compared to that of 10 nM (868 ng/mL) for Captopril, which was similar to the report (7 nM) of Pihlanto-Leppälä¨ et al. (1998); while the IC_{50} of yam dioscorin was 250 μg/mL (Hsu et al., 2002). Both BSA and purified SPD1 were proteins, but only the purified SPD1 showed specific dose-dependent ACE inhibitory activity. In the literature, the protein hydrolysates were used as sources to purify peptides as ACE inhibitors (Mullally et al., 1996). From calculations, the IC_{50} of SPD1 against ACE activity was 190.47 μg/mL, which was smaller than the synthetic peptide α-lactorphin (YGLF, 322.7 μg/mL). Several identified peptide fragments exhibited much lower IC_{50} values than our purified SPD1; for example, Tyr-Pro of whey proteins, 8.1 μg/mL (Yamamoto et al., 1999) and HHL of soybean proteins, 2.2 μg/mL (Shin et al., 2001). On the opposite, several identified peptide fragments exhibited much higher IC_{50} values than our purified SPD1; for example, hydrolysates of whey proteins (α-lactalbumin and β-lactoglobulin) were effective with IC_{50} values between 345-1,733 μg/mL (Pihlanto-Leppälä et al., 2000), LAHKAL of α-lactalbumin hydrolysates, 406 μg/mL; GLDIQK of β-lactoglobulin hydrolysates, 391 μg/mL; and VAGTWY of β-lactoglobulin hydrolysates, 1,171 μg/ mL. In our previous paper (Huang et al., 2008b), the IC_{50} of ACE activity required 187.96 μg/mL TI from sweet potato, which was lower than IC_{50} value of purified SPD1.

Determinations of ACE Inhibitor Activity of Defensin by TLC

The FAPGG and FAP (product of ACE catalyzed hydrolysis reaction) were separated by TLC using water saturated 1-butanol: acetic acid: water, 4:1:1 (V/V/V) as developing solvents according to the method of Holmquist et al. (1979). Fig. 3 shows the qualitative

results of TLC chromatograms of a silica gel 60 F254 about the effects of 225 μg/mL of commercial BSA (lane 3) or SPD1 (lane 4) on 15 microunits of ACE. Compared to the control test (lane 2), it was found that SPD1 (lane 4) inhibited ACE reaction showing less amounts of FAP production observed under UV light. However, similar amounts of FAP were found between the control test (lane 2) and BSA (lane 3). These results demonstrated again that SPD1 exhibited ACE inhibitor activity.

Determination of the Kinetic Properties of ACE Inhibition by Defensin

The Lineweaver-Burk plots of ACE (4 μU) without or with purified SPD1 (200 μg/mL) under different concentrations of FAPGG are shown in Fig. 4. The results indicated that purified SPD1 acted as a mixed type inhibitor against ACE using FAPGG as a substrate. When 200 μg/mL SPD1 (10 μg) were added, Vmax and Km were, respectively, 0.01 ΔA/min and 0.69 mM; while without SPD1 they were 0.03 ΔA/min and 0.42 mM. In conclusion, SPD1 exhibited dose-dependent ACE inhibitory activity and acted as a mixed type inhibitor with respect to the substrate (FAPGG). A similar work was reported with the calculated Km as 0.25 mM FAPGG for ACE in the presence of purified dioscorin, the calculated Km´ was 0.33 mM (Hsu et al., 2002).

The mixed-type inhibition suggests that there might be an inhibitor-binding site (or I-site) for SPD1 on the enzyme (ACE) surface in addition to the substrate-binding site (or S-site). It is noteworthy that SPD1 bind tighter to the I-site on the substrate-bound form of

ACE than that on the free form. It is also noted that the K_m values in the presence of the inhibitors are higher than that in their absence. In other words, binding of the substrate to the S-site increases the binding affinity of the inhibitor to the I-site, whereas binding of the inhibitor to the I-site decreases the binding affinity of the substrate to the S-site. The inhibitory mode of SPD1 was unique and hardly analyzed with a simple Michaelis−Menten-type interaction between the enzyme and inhibitor. The inhibitory mode of SPD1 must be examined further in the next research step kinetically and thermodynamically.

Determination of the ACE Inhibitory Activity by trypsin Hydrolysates of Defensin and their peptide Distributions. Fig. 5 shows the ACE inhibitory activity (Δ*A* 345 nm) of tryptic SPD1 hydrolysates. Fig. 5A shows the ACE inhibition (percent) of tryptic SPD1 hydrolysates collected at different trypsin hydrolysis times. From the results (Fig. 5A), it was found that the ACE inhibitory activity increased from 52.47 % (0 h) to about 74.38 % (24 h). Fig. 5B shows the chromatograms of unhydrolyzed SPD1 and tryptic SPD1 hydrolysates (24 h) on Sephadex G-50 column. It was found that smaller peptides increased with increasing trypsin hydrolytic time. The ACE inhibitor activities of tryptic SPD1 hydrolysates decreased after 24 h hydrolysis (Fig. 5A) suggesting that some active ingredients got lost after 24 h hydrolysis.

We used synthetic peptides (according to SPD1 gene sequence) to measure ACE inhibitor activity. Kohmura et al. (1989) synthesized some peptide fragments of human β-casein and found that the length of those peptides had an influence on the ACE inhibitory activity. Namely, peptides composed of 3-10 amino acids with proline on the C-terminal were necessary as ACE inhibitors. Thus the peptide Leu-Arg-Pro from food protein hydrolysates has been reported to be the most potent natural ACE inhibitor, with an IC_{50} value of 0.27 μg/mL or 1.0 μM. Byun and Kim (2002) studied the ACE inhibitory activity of a series of dipeptides, and indicated that tryptophan, tyrosine, proline, or phenylalanine at the *C*-terminal and branched-chain aliphatic amino acid at the *N*-terminal were required for a peptide to bind to ACE.

Synthetic peptides were designed by simulated trypsin cutting sites of *SPD1* gene (accession number: AY552546) products from sweet potato (http://www.expasy.org/tools/peptidecutter/). Six peptides, namely GFR, FK, IMVAEAR, GPCSR, CFCTKPC and MCESASSK, were synthesized based on the simulated trypsin digest of SPD1, then tested for ACE inhibitory activity. IC_{50} values of individual peptides were 94.25± 0.32, 265.43± 1.24, 84.12± 0.53, 61.67 ± 0.36, 1.31 ± 0.07 and 75.93 ± 0.64 μM, suggesting that CFCTKPC might represent the main domain for the ACE inhibition. These results demonstrated that simulated synthetic peptides from tryptic SPD1 hydrolysates exhibited ACE inhibitory activities. Our work suggests that (1) CFCTKPC might represent the main active site for the ACE inhibition; (2) there are marked structural similarities for peptides with antihypertensive, immunomodulatory and antioxidant activities and may be used as criteria for selecting or designing multifunctional ingredients of functional foods to control cardiovascular diseases.

In our previously paper, TI was shown to inhibit ACE activation in a dose-dependent manner. The IC_{50} of ACE activity required 187.96 μg/mL TI. And TYCQ was synthesized based on the simulated pepsin digest of TI. The IC_{50} values of

TYCQ peptide was 2.30 μM (Huang et al., 2008b). In this work the IC_{50} values of CFCTKPC peptide was 1.31 μM. This value was much better than TI and its hydrolysates. CFCTKPC peptide contains seven amino acids with three cysteine residues. So far, the structure−activity correlations among the various ACE inhibitory peptides still remain ambiguous. On the basis of some common structure patterns, it has been suggested that the most favorable amino-terminal residues are branched amino acids such as Val and Ile and the most preferred carboxyterminus residues are among Trp, Tyr, Pro, or Phe (Zhou et al., 2010).

In summary, SPD1 exhibited dose-dependent ACE inhibitory activity. SPD1 acted as a mixed type inhibitor toward ACE with IC_{50} of 1.31 ± 0.07 μM. Its peptic hydrolysates also showed ACE inhibitory activities. Some peptides derived from food proteins were demonstrated to have antihypertensive activities against spontaneously hypertensive rats (Yoshii, et al., 2001). The potential for hypertension control when people consume sweet potato deserves further investigations.

ACKNOWLEDGEMENTS

The authors want to thank the financial supports from the National Science Council (NSC 97-2313-B-039 -001 -MY3) and China Medical University (CMU) (CMU95-PH-11, CMU96-113, CMU97-232 and CMU99-S-29).

LITERATURE CITED

Bellamy, W., R.H. Wakabayashi, M. Takase, K. Kawase, S. Shimamura, and M. Tomita. 1993. Role of cell-binding in the antibacterial mechanism of Lactoferricin B. J. Appl. Bacteriol. **75:** 478-484.

Byun, H.G., and S.K. Kim. 2002. Structure and activity of angiotensin I converting enzyme inhibitory peptides derived from Alaskan pollack skin. J. Biochem. Mol. Biol. **35:** 239-243.

Casaretto, H.Z., and P. Jolles. 1984. Immunostimulating hexapeptide from human case in amino acid sequence, synthesis and biological properties. Eur. J. Biochem. **145:** 677-682.

Haasen, K.E., and D.R. Goring. 2010. The recognition and rejection of self-incompatible pollen in the *Brassicaceae*. Botanical Studies. **51:** 1-6.

Epple, P., K. Apel, and H. Bohlmann. 1997. ESTs reveal a multigene family for plant defensins in Arabidopsis thaliana. FEBS Lett. **400:** 168-172.

Harrison, P.F. and J. Lederberg. 1998. Antimicrobial Resistance. National Academies Press.

Holmquist, B., P. Bunning, and J.F. Riordan. 1979. A continuous spectrophotometric assay for angiotensin converting enzyme. Anal. Biochem. **95:** 540-548.

Hou, W.C., H.J. Chen, and Y.H. Lin. 2003. Antioxidant peptides with angiotensin converting enzyme inhibitory activities and applications for angiotensin converting enzyme purification. J Agric Food Chem. **51:** 1706-1709.

341 Hsu, F.L., Y.H. Lin, M.H. Lee, C.L. Lin, and W.C. Hou. 2002. Both dioscorin, the tuber
342 storage protein of yam (*Dioscorea alata* cv. Tainong No. 1), and its peptic
343 hydrolysates exhibited angiotensin converting enzyme inhibitory activities. J Agric
344 Food Chem. **50:** 6109-6013.

345 Huang, D.J., C.D. Lin, H.J. Chen, and Y.H. Lin. 2004. Antioxidant and antiproliferative
346 activities of sweet potato (*Ipomoea batatas* [L.] Lam. 'Tainong 57') constituents.
347 Bot. Bull. Acad. Sin. **45:** 179-186.

348 Huang, D.J., W.C. Hou, H.J. Chen, and Y.H. Lin. 2006. Sweet potato (*Ipomoea batatas*
349 [L.] Lam 'Tainong 57') storage roots mucilage exhibited angiotensin converting
350 enzyme inhibitory activities *in vitro*. Botanical Studies. **45:** 397-402.

351 Huang, G.J., H.J. Chen, Y.S. Chang, M.J. Sheu, and Y.H. Lin. 2007. Recombinant
352 Sporamin and its synthesized peptides with Antioxidant Activities *in vitro*. Botanical
353 Studies. **48:** 133-140.

354 Huang, G.J., H.C. Lai, Y.S. Chang, M.J. Sheu, T.L. Lu, S.S. Huang, and Y.H. Lin. 2008a.
355 Antimicrobial, dehydroascorbate reductase and monodehydroascorbate reductase
356 activities of defensin from sweet potato (*Ipomoea batatas* [L.] Lam. 'Tainong 57')
357 storage roots. Journal of Agricultural and Food Chemistry **56:** 2989-2995**.**

358 Huang, G.J., Y.L. Ho, H.J. Chen, Y.S. Chang, S.S. Huang, H.J. Hung, and Y.H. Lin.
359 2008b. Sweet potato storage root trypsin inhibitor and their peptichydrolysates
360 exhibited angiotensin converting enzyme inhibitory activity *in vitro*. Botanical
361 Studies. **49:** 101-108.

362 Huang, C.Y., C.L. Wen, Y.L. Lu, Y.S. Lin, L.G. Chen, and W.C., Hou. 2010.
363 Antihypertensive activities of extracts from tissue cultures of *Vitis thunbergii* var.
364 *Taiwaniana*. Botanical Studies. **51:** 317-325.

365 Kohmura, M., N. Nio, K. Kubo, Y. Minoshima, E. Munekata, and Y. Ariyoshi. 1989.
366 Inhibition of angiotensin-converting enzyme by synthetic peptide fragments of
367 human β-casein. Agric. Biol. Chem. **53:** 2107-2114.

368 Lin, C.L., S.Y. Lin, Y.H. Lin, and W.C. Hou. 2006. Effects of tuber storage protein of yam
369 (*Dioscorea alata* cv. Tainong No. 1) and its peptic hydrolyzates on spontaneously
370 hypertensive rats. J. Sci. Food Agric. **86:** 1489-1494.

371 Lin, S.Y., C.C. Wang, Y.L. Lu, W.C. Wu, and W.C. Hou. 2008. Antioxidant,
372 anti-semicarbazide-sensitive amine oxidase, and antihypertensive activities of
373 geraniin isolated from *Phyllanthus urinaria*. Food Chem. Toxicol. **46:** 2485-2492.

374 Liu, Y.H., M.T. Chuang, and W.C. Hou. 2007. Methanol-soluble,β-elimination products
375 from preparations of alginic acid hydroxamate exhibited DPPH scavenging and
376 angiotensin converting enzyme inhibitory activities. Botanical Studies. **48:** 141-146.

377 Moreno, M., A. Segura, and F. Garcia-Olmedo. 1994. Pseudothionin-St1, a potato peptide
378 active against potato pathogens. Eur. J. Biochem. **223:** 135-139.

379 Mullally, M.M., H. Meisel, and R.J. Fitzgerald. 1996. Synthetic peptides corresponding to
380 α-lactalbumin and β-lactoglobulin sequences with angiotensin-I-converting enzyme
381 inhibitory activity. J. Biol. Chem. **377:** 259-260.

Pihlanto-Leppälä, A., T. Rokka, and H. Korhonen. 1998. Angiotensin I converting enzyme inhibitory peptides derived from bovine milk proteins. Int. Dairy J. **8:** 325-331.

Pihlanto-Leppälä, A., P. Koskinen, K. Piilola, T. Tupasela, and H. Korhonen. 2000. Angiotensin I-converting enzyme inhibitory properties of whey protein digest: concentration and characterization of active peptides. J. Dairy Sci. **67:** 53-64.

Scarborough, R.H., J.W. Rose, M.H. Hsu, D.R. Phillips, V.A. Fried, A.M. Campbell, L. Manniaai, and I.F. Charo. 1991. Barbourin A GpIIb-IIIa specific integrin antagonist from the venom of sistrurus *M. barbouri*. J. Biol. Chem. **266:** 9359- 9360.

Shin, Z.I., R. Yu, S.A. Park, D.K. Chung, C.W. Ahn, H.S. Nam, K.S. Kim, and H.J. Lee. 2001. His-His-Leu, an angiotensin I converting enzyme inhibitory peptide derived from Korean soybean paste, exerts antihypertensive activity *in vivo.* J. Agric. Food Chem. **49:** 3004-3009.

Terras, F.R., I.J. Goderis, F. Van Leuven, J. Vanderleyden, B.P. Cammue, and W.F. Broekaert. 1992. *In Vitro* Antifungal Activity of a Radish (*Raphanus sativus* L.) Seed Protein Homologous to Nonspecific Lipid Transfer Proteins. Plant Physiol. **100:** 1055-1058.

Yamamoto, N., M. Maeno, and T. Takano. 1999. Purification and characterization of an antihypertensive peptide from a yogurt-like product fermented by *Lactobacillus helveticus* CPN4. J. Dairy Sci. **82:** 1388-1393.

Yoshii, H., N. Tachi, R. Ohba, O. Sakamura, H. Takeyama, and T. Itani, 2001. Antihypertensive effect of ACE inhibitory oligopeptides from chicken egg yolks.

Comp. Biochem. Physiol. C Pharmacol. Toxicol. Endocrinol. **128:** 27-33.

Zhou, F., Z. Xue, and J. Wang, 2010. Antihypertensive effects of silk fibroin hydrolysate by alcalase and purification of an ACE inhibitory dipeptide. J Agric Food Chem. **58:** 6735-6740.

Figure Legends

Figure 1. Inhibitory activity of different amounts of defensin (SPD1) (0, 50, 100 and 200 μg/mL) from sweet potato storage root on the ACE activity (Δ*A* 345 nm).

Figure 2. The effects of SPD1, albumin and Captopril on ACE activity determined by spectrophotometry. SPD1 (0, 50, 100 and 200 μg/mL) or bovine serum albumin (0, 50, 100 and 200 μg/mL) was used. The inhibition of ACE (%) was calculated according to the equation [1-(ΔA inhibitor ÷ ΔA control)] × 100 %.

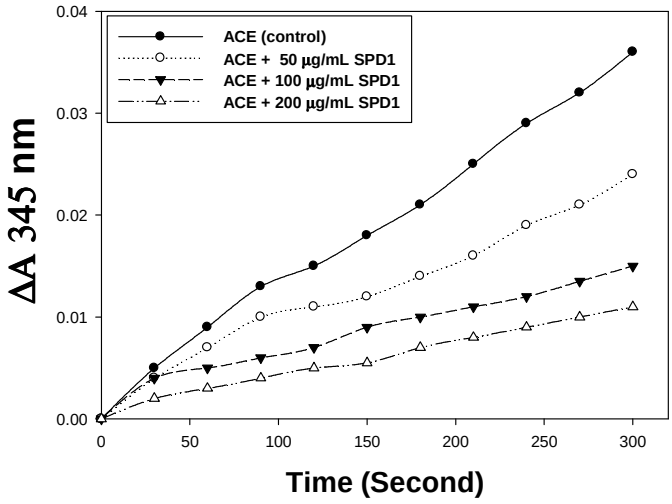
Figure 3. The TLC chromatograms of a silica gel 60 F254 showing the effects of defensin from sweet potato storage root or bovine serum albumin on ACE activity. Lane 1, blank test (FAPGG only); lane 2, control test (ACE reacted with FAPGG to produce FAP); lane 3, 225 μg/mL bovine serum albumin added; lane 4, 225 μg/mL SPD1 added. Each solution was dried under reduced pressure and redissolved with 400 μL methanol. Each 50 μL was spotted on a silica gel 60 F254. The FAPGG and FAP were separated by water saturated 1-butanol : acetic acid : water, 4:1:1 (V/V/V). Arrows indicated the positions of both FAP and FAPGG.

Figure 4. The Lineweaver-Burk plots of ACE (4 μU) without or with SPD1 (200 μg/mL) from sweet potato storage root using different concentrations of FAPGG (0.1 to 0.5 mM).

Figure 5. ACE inhibitor activity of tryptic hydrolysates of sweet potato SPD1. The plot shows the ACE inhibition (%) of tryptic SPD1 hydrolysates obtained at different trypsin hydrolysis time (A). The proteins and the inhibition of ACE (%) were shown (B). The inhibition of ACE (%) was calculated according to the equation [1-(ΔA inhibitor ÷ ΔA control)] × 100 %.

441

442 **Figure 1.**



443

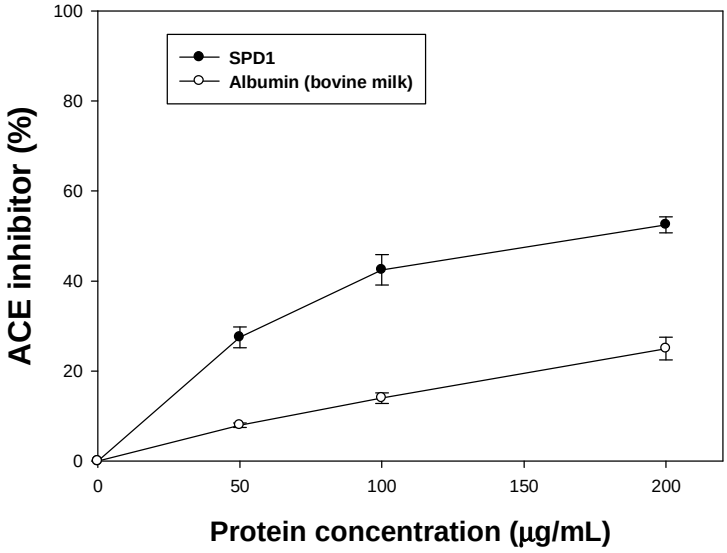
444

445

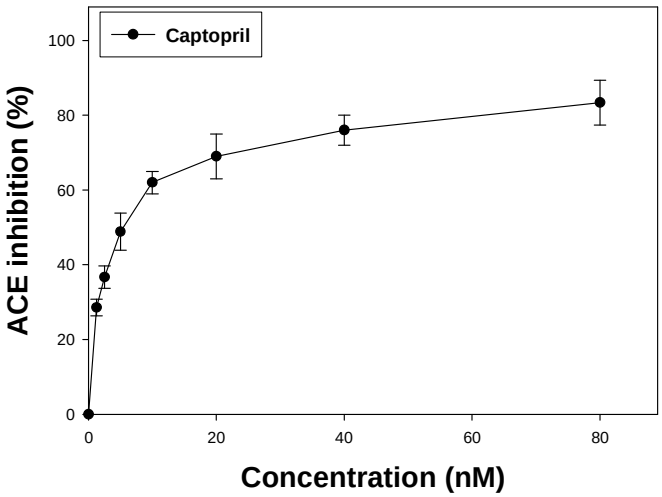
446

Figure 2.

A.

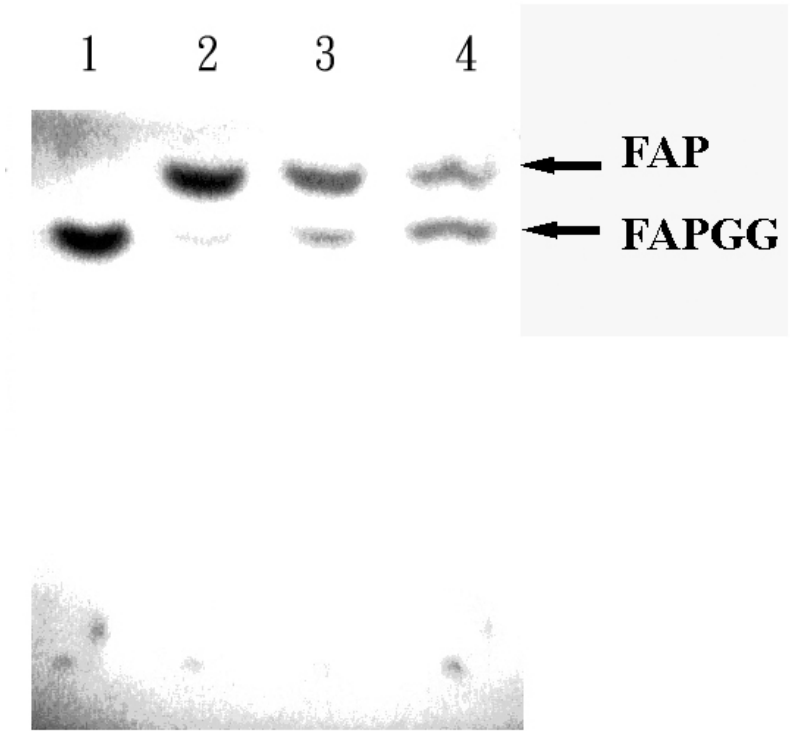

SPD1
Albumin (bovine milk)
ACE inhibitor (%)
Protein concentration (µg/mL)
0
20
40
60
80
100
0
50
100
150
200

B.


Captopril
ACE inhibition (%)
Concentration (nM)
0
20
40
60
80
100
0
20
40
60
80

455 **Figure 3.**

456



457

458

459

Figure 4.

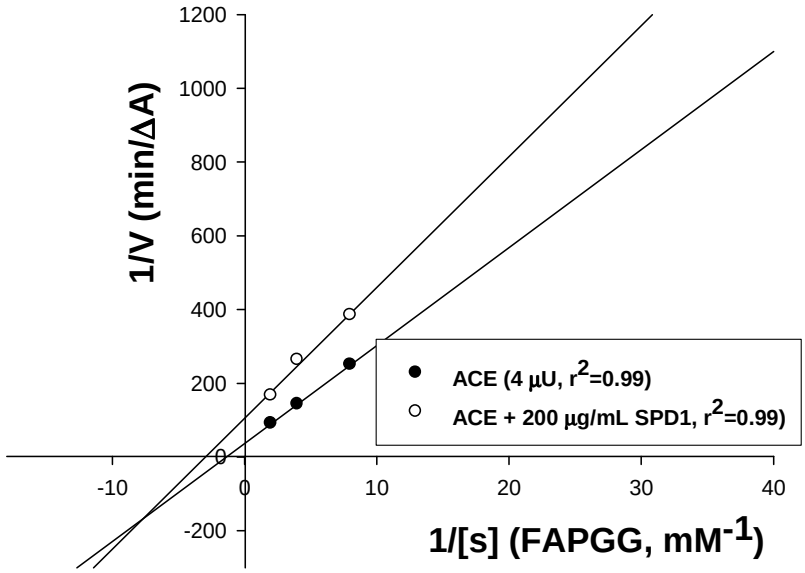
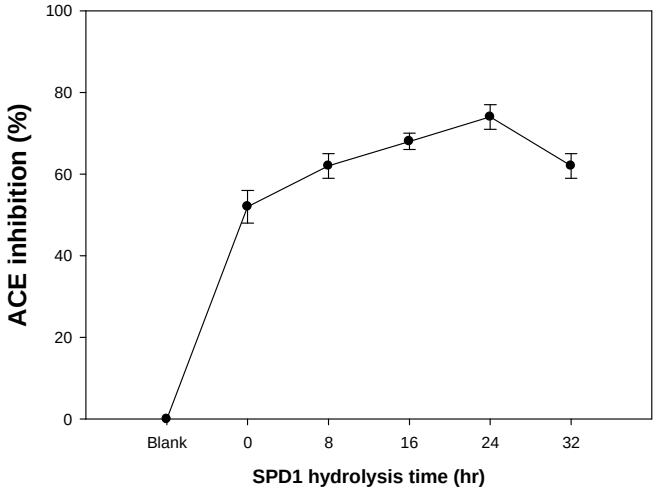

1200
1000
800
600
400
200
0
-200
-10
0
10
20
30
40
1/V (min/ΔA)
1/[s] (FAPGG, mM-1)
ACE (4 µU, r2=0.99)
ACE + 200 µg/mL SPD1, r2=0.99)

Figure 5.

A.


ACE inhibition (%)
100
80
60
40
20
0
Blank
0
8
16
24
32
SPD1 hydrolysis time (hr)

B.

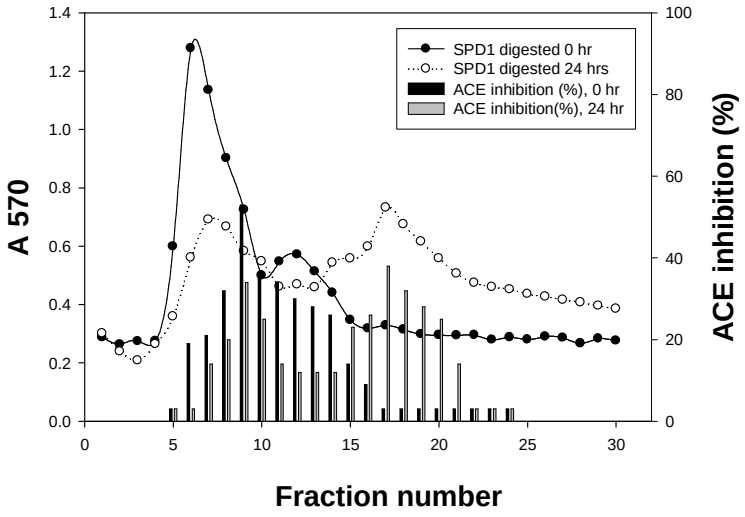

SPD1 digested 0 hr
SPD1 digested 24 hrs
ACE inhibition (%), 0 hr
ACE inhibition(%), 24 hr
A 570
ACE inhibition (%)
Fraction number

Table 1. SPD1 peptides with ACE inhibition activity.

SPD1 peptides	IC_{50} (μM)
GFR	94.25 ± 0.32
FK	265.43 ± 1.24
IMVAEAR	84.12 ± 0.53
GPCSR	61.67 ± 0.36
CFCTKPC	1.31 ± 0.07
MCESASSK	75.93 ± 0.64

Note: The sequence of SPD1 contains pre-pro-sequence. These sequences were retrieved from the NCBI (National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov) with the following accession numbers AY552546.

甘藷塊根中防禦素及其合成之胜肽具有血管收縮素轉化腖抑制活性

黃冠中[1] 陸德齡[2] 邱傳淞[1,3] 陳顯榮[4] 吳介信[2] 林穎志[5] 謝文聰[6] 廖容君[2] 許明志[2] 林耀輝[7]

[1]中國醫藥大學中國藥學暨中藥資源系

[2]中國醫藥大學藥學系

[3]新生醫護管理專科學校護理科

[4]中山大學生命科學系

[5]仁德醫護管理專科學校護理科

[6]中國醫藥大學醫學系藥理學科

[7]中央研究院植物暨微生物研究所

在大腸桿菌(M15)中大量表現甘藷塊根之重組蛋白質防禦素(SPD1)，利用鎳離子螯合之親和性管柱純化。SPD1 經 SDS-PAGE 分析其分子量約為 8,600 Da. 由以前的研究發現 SPD1 具有抗微生物活性，去氫抗壞血酸還原酶，單去氫抗壞血酸還原酶的活性。SPD1 以 *N*-[3-(2-furyl) acryloyl]-Phe-Gly-Gly (FAPGG)為受質，利用分光光度計的方法分析抑制血管收縮素轉化酶 (angiotensin converting enzyme, ACE) 的能力，其效果隨劑量增加而增加 (50 到 200 μg/mL SPD1，分別抑制 27.56～52.58% 血管收縮素轉化酶活性)。SPD1 對於血管收縮素轉化酶之 50% 抑制濃度 (IC_{50}) 為 190.47 μg/mL，對照組 Captopril 為 10 nM (868 ng/mL)。另外利用螢光 silica TLC 偵測 FAPGG 及其水解產物 FAP，結果也顯示 SPD1 對於血管收縮素轉化酶有抑制的效果。SPD1 對於血管收縮素轉化酶是屬於混合型抑制。利用胰蛋白腖以不同時間水解 SPD1 時，發現反應 24 小時時其血管收縮素轉化酶活性有抑制的效果可以從 52.47 % (0 h) 增加到 74.38 % (24 h)。由結果可知小分子的胜肽會隨著水解時間增加且血管收縮素轉化酶活性抑制也有增加，但水解時間超過 24 h 時，血管收縮素轉化腖活性抑制會降低，可能是由於一些胜肽的結構被破壞。利用電腦模擬胰蛋白酶水解 SPD1 的結果，得到六種人工合成具有抑制血管收縮素轉化酶活性胜肽: GFR, FK, IMVAEAR, GPCSR, CFCTKPC 和 MCESASSK，測定其 IC_{50} 為 94.25± 0.32, 265.43± 1.24, 84.12± 0.53, 61.67 ± 0.36, 1.31 ± 0.07 和 75.93 ± 0.64 μM。結果發現 CFCTKPC 具有很好的抑制血管收縮素轉化酶活性。當人們食用甘藷塊根時，SPD1 及其胜肽也許對於高血壓和其他疾病的控制是有益的。

關鍵詞: 甘藷; 防禦素;血管收縮素轉化酶;抑制作用。