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Full Length Research Paper

Angiotensin I-converting enzyme inhibitor derived from cottonseed protein hydrolysate

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Six proteolytic enzymes, including alcalase, flavourzyme, trypsin, neutrase, papain and pepsin, were employed to hydrolyze cottonseed protein to produce the hydrolysates of Angiotensin I-converting enzyme (ACE) inhibitory activity. The result indicated that the cottonseed protein hydrolysate (CPH) produced by papain had the highest ACE inhibitory activity. Therefore, papain was selected for enzymatic production of ACE inhibitor from cottonseed protein isolates (CPI). CPI was hydrolyzed with papain for 1 - 8 h, and the 6 h hydrolysate had the strongest ACE inhibitory ability. The product was separated into four ranges of molecular weight (UF-I, > 30 kDa; UF-II, 30 – 10 kDa; UF - III, 10 - 5 kDa; UF - IV, < 5 kDa) by using an ultrafiltration (UF) membrane bioreactor system. Among them, UF-IV showed the highest ACE inhibitory activity (IC_{50} = 0.792 mg/ml). UF-IV was further fractionated with Sephadex G-25 gel filtration chromatography into four fractions (Fra I, Fra II, Fra III and Fra IV) that were composed of peptides of >2.43 kDa, 2.43 - 0.82 kDa, 0.82 - 0.35 kDa and <0.35 kDa, respectively. Fra II exhibited the strongest ACE inhibitory ability (IC_{50} = 0.159 mg/ml) with the yield of 41.63%. It was suggested that Fra II with good ACE inhibitory activity can be a potential source of natural ACE inhibitor.

Key words: Cottonseed protein hydrolysate, peptide fractions, angiotensin I-converting enzyme inhibitory ability, ultrafiltration.

INTRODUCTION

Hypentension is now a major problem threatening people health in the world. It is a risk factor for developing cardiovascular diseases (arteriosclerosis, stroke and myocardial infraction) and end-stage renal disease, and is often called a “silent killer” because persons with hypertension are often asymptomatic for years. It was estimated that there is about 15-20% of adults suffering from hypertension (Je et al., 2005). Since the past two decades, the renin-angiotensin system (RAS) has been found to be a coordinated peptidic hormonal cascade for the control of cardiovascular, renal and adrenal functions governing fluid and electrolyte balance and arterial blood pressure (Carey and Siragy, 2003). Briefly, prorenin is converted to active renin by a trypsin-like enzyme. Renin cleaves angio- tensinogen to form angiotensin I. Angiotensin I-converting enzyme (ACE) hydrolyses both the inactive angiotensin I into vasoconstrictor angiotensin II and the vasodilator bradikinin into an inactive metabolite. Most well-known effects of angiotensin II, including vasoconstriction, are mediated by angiotensin type 1 receptors (AT1). Angio- tensin II also binds to angiotensin type 2 receptors (AT2) which are highly expressed in foetal mesenchymal tissues but poorly expressed in the adult. So angiotensin I-converting enzyme (ACE, kinase II, EC 3.4.15.1) is potentially of great importance for controlling blood pressure by virtue of the rennin-angiotensin system. Although synthetic ACE inhibitors, including captopril, enalapril and lisinopril, are remarkably effective as antihypertensive drugs, they cause adverse side effects such as dry cough, angio-oedema and many other

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Abbreviations: ACE, Angiotensin I-converting enzyme; **CPH,** cottonseed protein hydrolysate; **CPI,** cottonseed protein isolates; **UF,** ultrafiltration; **RAS,** renin–angiotensin system; **AT1,** angiotensin type 1 receptors; **AT1,** angiotensin type 2 receptors; **HHL,** hippuryl-L-histidyl-L-leucine; **DH,** degree of hydrolysis; **MWCO,** molecular weight cut-offs.

disfunctions of human organs (Fitzgerald and Meisel, 2000).

Therefore, to find safe and natural ACE inhibitors are desirable for the prevention and remedy of hypertension. Recently, peptides showing antihypertensive activity as inhibitors of ACE have been obtained from the proteolysis products of several food proteins such as casein, whey protein, gelatin, kidney bean protein, buckwheat protein, corn protein and soybean protein etc (Tauzin et al., 2002; Abubakar et al., 1998; Kim et al., 2001; Lee et al., 1999; Li et al., 2002; Suh et al., 2003). Though these peptides are less potent than synthetic ones, they do not exhibit known side effects.

Cotton is not only the most important fibre crop in the world but also the second best potential source of plant proteins after soybean (Ahmad et al., 2007). Cottonseed proteins are widely regarded as potential sources of nutrients for humans and animals, and have also been a subject for numerous investigations (Ory and Flick, 1994). In China, about 10 million tons of cottonseeds are produced annually. The presence of toxic gossypol in the cottonseed is a limiting factor for human consumption and various methods have been proposed for reduction of free gossypol content to the allowable limit of 450 ppm (FDA regulations, 1974).

Oshima et al. (1979) first reported ACE inhibitory peptides produced from food protein by digestive proteases. Afterward, many other ACE inhibitory peptides have been discovered from enzymatic hydrolysates of different food proteins. However, to our knowledge, there is no report about ACE inhibitory effect from cottonseed protein hydrolysate. In the present study, the ACE inhibitory activities of cottonseed protein hydrolysates were produced with different enzymes. The ultrafiltration membrane and gel filtration (Sephadex G-25) were used to isolate the high ACE inhibitory activity peptide fractions from enzymatic hydrolysates.

MATERIALS AND METHODS

Materials

Dephenol cottonseed meal (65% protein) was obtained from China Cotton-Unis Co. (Beijing, China). Neutrase, flavourzyme, alcalase and papain were from Novo ezymes Co. (Novo Nordisk, Bagsvaerd, Denmark). Trypsin, pepsin, ACE enzyme powder and hippuryl-L-histidyl-L-leucine (HHL) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Ultrafiltration membranes, including 5, 10 and 30 kDa, were purchased from Millipore Co. (Bedford, MA, USA). All other reagents used were of analytical grade.

Production of cottonseed protein isolates (CPI)

The CPI was prepared according to the method reported by Tunc and Duman (2007) with little modification. First, the defatted and dephenol cottonseed flour was mixed with distilled water at a ratio of 1:15 (w/v). Then the pH was adjusted to 10.5 with 0.1 M NaOH, the mixture was stirred for at least 2 h. At this point, the mixture was centrifuged at 8,000 g for 15 min. The precipitate was mixed with distilled water (1:5, w/v) and the pH was adjusted to 10.5 and then centrifuged as above. The supernatants of two centrifugations were collected and the pH was adjusted to 4.3 with 0.1 M HCl in order to precipitate cottonseed protein isolate, which was lyophilized and stored at –20°C.

Proteases hydrolysis of CPI

Alcalase, flavourzyme, trypsin, neutrase, papain and pepsin were employed in this study. 1% (w/w of CPI) protease was added to 5.0% (w/v) CPI to produce hydrolysates, and the ACE inhibitory activity of hydrolysate from different enzyme was compared. The optimum reaction conditions of temperature and pH for each enzyme were followed by the instruction of the supplier, such as 55°C and pH 9.0 for alcalase, 45°C and pH 7.0 for flavourzyme, 37°C and pH 7.0 for trypsin, 50°C and pH 7.5 for neutrase, 45°C and pH 7.0 for papain, and 37°C and pH 4.0 for pepsin, respectively. The samples were heated in a water bath at 90°C for 15 min prior to enzymatic hydrolysis and hydrolyzed with the enzyme for 6 h. Then the enzyme was inactivated by heating at 95°C for 15 min when the reaction was finished. The resulting hydrolysates were rapidly cooled to ambient temperature in the ice bath and centrifuged at 8,000 g for 15 min. The supernatants were collected to determine the ACE inhibitory activity.

Degree of hydrolysis (DH)

In this study, the DH of cottenseed protein hydrolyzed with papain was determined by using a pH-stat method (Alder-Nissen, 1986) at 1 h intervals based on the equation:

$$DH = (h / h_{tot}) \times 100\%$$

Where, $h = B \times N_b \times 1/a \times 1/M_P$, B = base consumption (ml), N_b = concentration of base (1 M NaOH), 1/a = calibration factors for pH-stat, M_P = mass of protein (g), and h = hydrolysis equivalents. For cottonseed protein, h_{tot} = 7.21 mmol/g.

ACE inhibitory activity assay

ACE inhibitory activity was measured by the hippuric acid method (Cushman and Cheung, 1971) with modification. The reaction mixture contained 100 µl of 5 mM HHL as a substrate, 150 µl of ACE solution (0.025 U/ml) and 50 µl of the sample solution. The reaction was carried at 37°C for 60 min, and terminated by addition of 250 µl of 1 N HCl. The hippuric acid formed was extracted with ethyl acetate (1.5 ml), and after removal of ethyl acetate by heat evaporation, hippuric acid was redissolved in 1 M NaCl (3 ml) and the absorbance was measured at 228 nm by UltroSpec 4300 pro UV/visible spectrophotometer (Pharmacia Co, USA). The inhibition activity was calculated using the following equation:

$$\text{Inhibition activity (\%)} = [(Ac - As / (Ac - Ab)] \times 100$$

Where, *Ac* is the absorbance of the buffer (control), *As* is the absorbance of the reaction mixture (sample), *Ab* is the absorbance when the stop solution was added before the reaction occurred (blank). The IC_{50} value was defined as the concentration of peptide in mg/ml required to reduce 50% of ACE activity, which was determined by regression analysis of ACE inhibition (%) versus peptide concentration.

Ultrafiltration preparation of cottonseed protein hydrolysate (CPH)

Cottonseed protein was hydrolyzed with papain adjusting substrate/enzyme ratio to 100:1 (w/w) at 45°C for 8 h (pH 7.0). The enzyme was inactivated by heating at 95°C for 15 min when the reaction was finished. The resultant CPH was fractionated into 4 parts (UF-I, UF-II, UF-III, UF-IV) by the ultrafiltration membrane bioreactor system with 30, 10 and 5 kDa of molecular weight cut-offs (MWCO). UF-I was not passed through the 30 kDa membrane. UF-II was passed through the 30 kDa membrane but not passed through the 10 kDa membrane. UF-III passed through the 10 kDa membrane but not the 5 kDa membrane. UF- IV was passed through the 5 kDa membrane. All of UF fractions were lyophilized in a freeze-drier for ACE inhibitory activity tests.

Table 1. ACE inhibitory activities of CPH fractionated by ultrafiltration.

Sample	IC_{50} (mg protein/ml)
CPH	5.26 ± 0.140
UF-I (>30 kDa)	7.83 ± 0.352
UF-II (30 – 10 kDa)	2.76 ± 0.030
UF-III (10 – 5kDa)	1.73 ± 0.036
UF- IV (<5 kDa)	0.792 ± 0.079

Values were expressed as mean ± SD.

Size exclusion chromatography

UF-IV with the highest activity among the fractions (Table 1) was separated on a Sephadex G-25 (1.5 × 90 cm) column by using AKTA purifier (Amersham pharmacia biotech Co, USA). Phosphate buffer solution (10 mM, pH 7.4) was used to equilibrate the column and to elute the proteins at a flow rate of 0.2 ml/min. A fixed amount of sample (1.0 ml) at a protein concentration of 10 mg/ml was applied to the column, and the absorbance of the effluent was measured at 215 nm. A molecular weight calibration curve was made according to the following standards: bovine serum albumin (66 kDa), lysozyme (14.4 kDa), insulin from bovine pancreas (5.7 kDa), vitamin B_{12} (1.355 kDa), oxidized glutathione (0.612 kDa) and reduced glutathione (0.307 kDa). The fractions of CPH were lyophilized and used for ACE inhibitory activity tests.

Statistical analysis

Data were obtained as the mean and standard deviation (SD) and analyzed using one-way analysis of variance (ANOVA) by the statistical package for the social sciences (SPSS) version 13.0 for windows. Difference in mean values were considered significant when $P < 0.05$.

RESULTS AND DISCUSSION

Effect of different protease hydrolyses on ACE inhibitory activity

CPI was hydrolyzed by alcalase, flavourzyme, trypsin, neutrase, papain and pepsin for 6 h, respectively. Figure 1 shows the ACE inhibitory activities of the different protease hydrolysates. The hydrolysate produced by hydrolyzing CPI with papain exhibited strongest ACE inhibitory activities (85.61%), and the others in turn were pepsin (74.38%), flavourzyme (54.93%), neutrase (52.88%), trypsin (51.23%) and alcalase (35.03%). Therefore, the choice of proteases for generating ACE inhibitory activity is very important. Papain is a commercial protease for industrial use and has been employed to hydrolyze many kinds of proteins, such as whey, plasma, albumin, casein, gelatin and some plant proteins to produce bioactive peptides, so it was selected for further study on enzymatic production of ACE inhibitor from CPI.

DH and ACE inhibitory activity of papain-hydrolysate

In the protein hydrolysis, it is necessary to have a measure to determine the extent of hydrolytic degradation. The degree of DH is defined as the percentage ratio of the number of peptide bonds broken (h) to the total number of bonds per unit weight (h_{tot}). DH is generally used as a proteolysis monitoring parameter, and it is the most widely used indicator for comparison among different protein hydrolysates (Alder-Nissen, 1986). The DH of hydrolyzing CPI with papain increased almost linearly ($P < 0.05$) with increasing hydrolysis time in the first 4 h, and reached a plateau at 26.2 – 27.5% after 4 h (Figure 2). The ACE inhibitory activity increased when CPI was hydrolyzed by papain from 1 to 6 h, but decreased steadily from 6 to 8 h. Further treatment may result in the hydrolysis of the ACE inhibitory peptides into amino acids with a lost the ACE inhibitory activity. As a result, 6 h was the optimum hydrolysis time in our work, which was consistent with the result reported by Mullally et al. (1997), they also found that the ACE inhibitory activity was released during the first hydrolysis reaction time and further digestion did not result to an increased development of inhibitory activity.

ACE inhibitory activities of the hydrolysate and ultrafiltration fractions

CPH prepared from hydrolysis of CPI with papain was collected for further fractionation of active peptides by ultrafiltration. As shown in Table 1, CPH was fractionated into UF-I (>30 kDa), UF-II (30–10 kDa), UF-III (10–5 kDa) and UF-IV (below 5 kDa). Table 1 also shows that ACE inhibitory activities of UF fractions varied with the molecular mass distribution, the ACE inhibitory activity increased with decreasing cut-off MW, indicating the relatively low MW of ACE inhibitory peptides. The UF-IV with MW below 5 kDa showed the most potent ACE inhibitory activity with an IC_{50} value of 0.792 mg/ ml. The membrane module with 5 kDa of molecular cut-off was applied for production of ACE inhibitor from CPI.

Won-Kyo et al. (2006) reported that yellowfin sole (*Limanda aspera*) frame protein hydrolysate were frac-

tionated into three ranges of molecular weight (YFPH-I, 3 0-10 kDa; FPH-II, 10-5 kDa; YFPH-III, below 5 kDa) using an ultrafiltration membrane bioreactor system. It was found

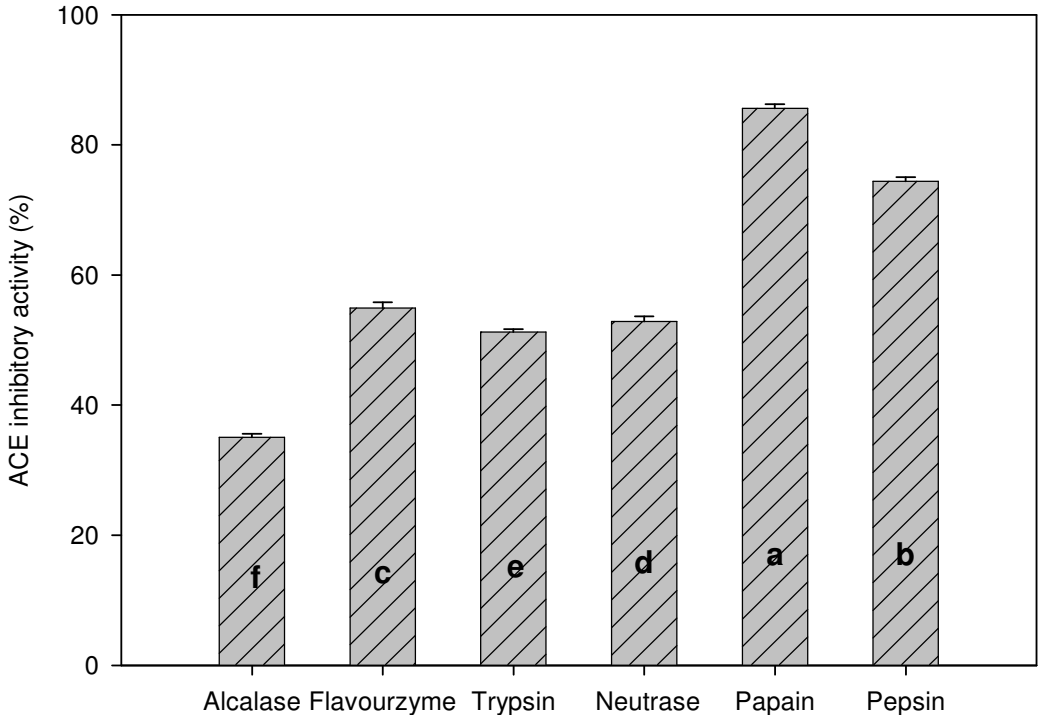

Figure 1. The ACE inhibitory activity of different protease hydrolyses. Means with different letters (a – f) were significantly different ($P < 0.05$).

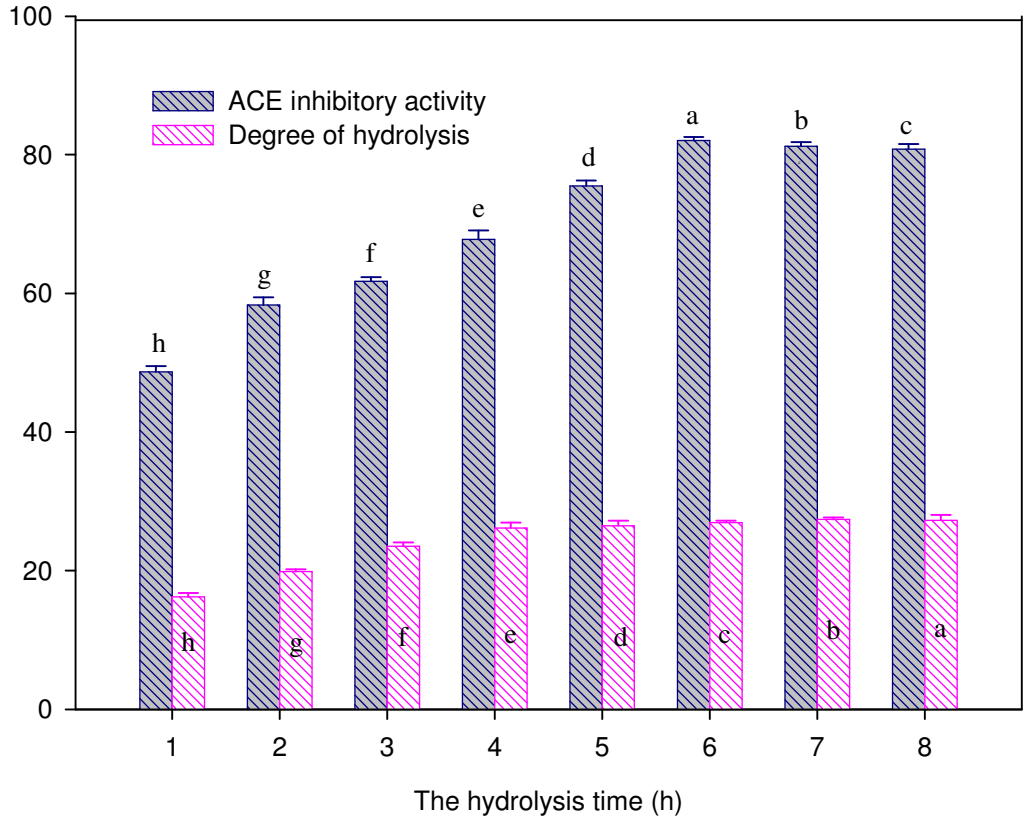

Figure 2. DH of CPI treated with papain and ACE inhibitory activity of the hydrolysates. Means with different letters (a – h) are significantly different (P < 0.05).

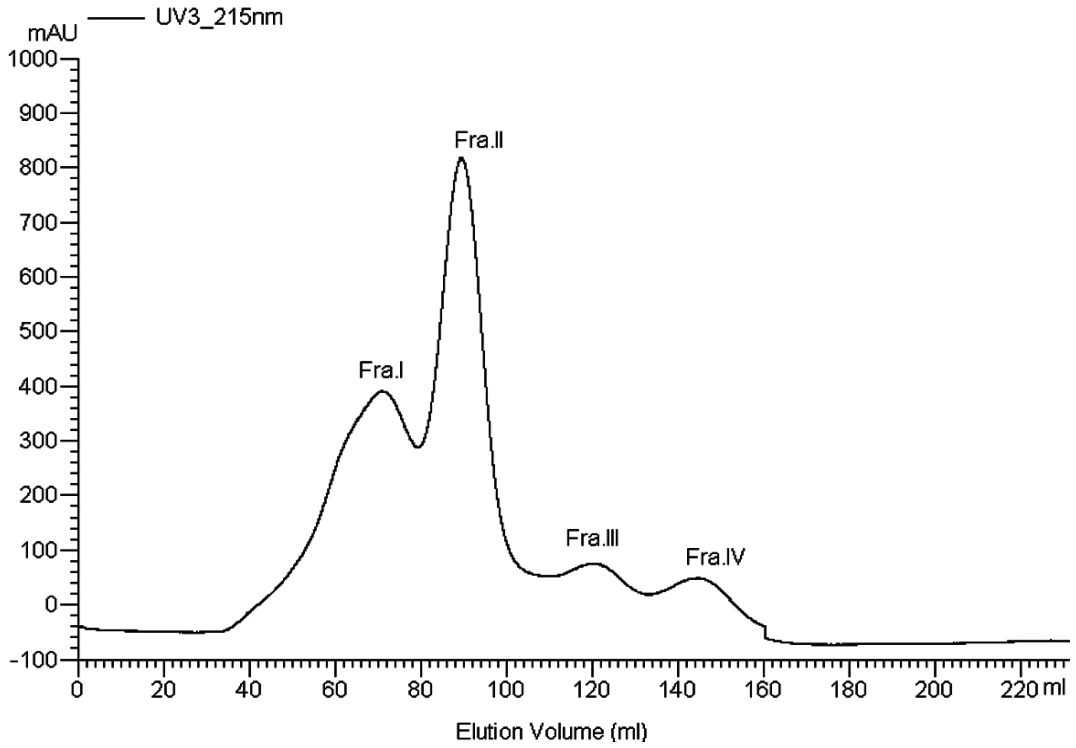

Figure 3. The pattern of UF-IV fraction separated by gel filtration on Sephadex G-25.

Table 2. The ACE inhibitory activity and yield of the fractions obtained on a Sephadex G-25 column.

Test sample	Sample molecular weight	ACE IC_{50} (mg/ml)	Recovery yield (%)
Fraction I	>2430 Da	1.197 ± 0.008	36.43 ± 0.121
Fraction II	816 - 2430 Da	0.159 ± 0.007	41.63 ± 0.192
Fraction III	345 - 816 Da	0.815 ± 0.020	10.88 ± 0.078
Fraction IV	<345 Da	1.086 ± 0.014	9.36 ± 0.069

Values were expressed as mean ± SD.

that the 5 kDa permeate fraction had strongest ACE inhibitory activity with an IC_{50} value of 0.883 mg/ml. Moreover, Wang et al. (2007) reported that wheat gluten hydrolysate prepared by papain was se- parated through an ultrafiltration membrane with a molecular weight cut-off of 5 kDa and found that the 5 kDa permeate fraction had the strongest antioxidant activity. The results mentioned above indicated that the 5 kDa ultrafiltration membrane was often used to produce bioactive peptides.

ACE inhibitory activities of the fractions fractionated with gel filtration

The UF-IV fraction (below 5 kDa) with highest ACE inhibitory activity was fractionated by gel filtration column chromatography on Sephadex G-25 at 215 nm (Figure 3). The UF-IV was successfully separated into four fractions (Fra I, Fra II, Fra III and Fra IV), corresponding to molecular weights of >2.42 kDa, 2.4 – 0.82 kDa, 0.82 – 0.35 kDa, and <0.35 kDa (Table 2). The fractions asso- ciated with the peaks were collected, concentrated and evaluated for ACE inhibitory activity. Table 2 shows the peptide yield as well as the ACE inhibitory activity for the various fractions. When the ACE inhibitory activity was tested in all fractions, fraction II (2.4 – 0.82 KDa) exhibited the highest activity with an IC_{50} value of 0.159 mg/ml and its yield was 41.63%. Matsui et al. (2002) reported that single oral administration of royal jelly protein hydroly- sates with IC_{50} value of 0.099 mg protein/ml in 10-week spontaneously hypertensive rats resulted in a significant reduction of systolic blood pressure. Wen-Dee et al. (2006) found that soy protein hydrolysate prepared by alcalase could reduce its IC_{50} value from 66.4 to 1.79 mg

protein/ml in 1 h of hydrolysis. Mullally et al. (1996) concluded that the tryptic casein of the IC_{50} values ranging

from 0.130 to 0.201 mg/ml had potential application as nutraceuticals in the prevention of hyper- tension. Wu and Ding (2002) characterized the soy protein derived ACE inhibitor peptides and suggested that the most economic way to market the peptides was as contained in the crude protein hydrolysate for consum- ption on a long-term basis for desired therapeutic effects. In our work, fraction II derived from cottonseed protein with strong ACE inhibitory activity (IC_{50} = 0.159 mg/ml) and higher yield (41.63%) could mediate an antihy- pertensive effect and have a potential use in function food.

Conclusion

ACE inhibitors have been developed to prevent angiotensin II production in cardiovascular diseases and utilized in clinical applications since the discovery of ACE inhibitor in snake venom (Ferreira et al., 1970). In this study, the peptides were produced by hydrolysis of cottonseed protein with different protease, including alcalase, flavourzyme, trypsin, neutrase, papain and pepsin. The most potent ACE inhibitory activity hydrolysate was obtained by papain hydrolysis of CPH. Further, the papain-hydroylsate was separated with ultrafiltration into 4 parts (UF-I, UF-II, UF-III, UF-IV); the ACE inhibitory activity (IC_{50}) of the hydrolysate decreased from 5.26 to 0.792 mg protein/ml. The UF-IV was successfully fractionated into four fractions (Fra I, Fra II, Fra III and Fra IV) by gel filtration column chromatography on Sephadex G-25. The results revealed that fraction II has the highest ACE inhibitory activity (IC_{50} = 0.159 ml/mg) and higher yield (41.63%), and might be useful for health food and medicine.

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