



Research Paper

Angiotensin I converting enzyme inhibitory activity of bee pupae protein hydrolysates

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ABSTRACT

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The protein isolates from bee pupae were hydrolyzed by Alcalase and Flavourzyme, and the angiotensin I converting enzyme (ACE) inhibitory activities of the hydrolysates were evaluated. The results indicated that hydrolysis could increase the ACE inhibitory activity; the maximum value of the inhibition was 66.71% (IC₅₀ = 22.70 mg/ml). The hydrolysates with high ACE inhibitory activity were also fractionated by different molecular weight cut-off (MWCO) membrane ultra filtration. The ACE inhibitory activities of the filtrates of MWCO 5~3 kDa, 3~1 kDa and < 1 kDa were found to be 69.77, 61.53 and 96.98%, respectively, and the IC₅₀ were 20.04, 31.78 and 0.66 mg/ml, respectively. The results suggest that the bee pupae hydrolysates can be used as an ingredient for nutraceuticals against hypertension and its related diseases.

Key words: Bee pupae, enzymatic hydrolysis, ultrafiltration, molecular weight cut-off (MWCO), ACE inhibitory activity.

INTRODUCTION

Angiotensin I converting enzyme (ACE, EC 3.4.15.1), which is present in many tissues and biological fluids of mammals, is a key enzyme in upregulation of blood pressure by degrading endogenous peptides such as angiotensin I (Hong et al, 2014). ACE inhibitory peptides play an important role in the inhibition effect of ACE in the rennin-angiotensin system and kallikren-kinin system (Hong et al, 2014; Pan et al, 2012).

There is a vast amount of literature investigating the plant and animal proteins that are rich in bioactive peptides, such as anticancer, anti-inflammatory, antioxidant and ACE inhibitory peptides (Chen et al, 2011; Chen et al, 2013, Vaštag et al, 2011). Bioactive peptides were released from parent proteins during digestion, food processing and enzymatic hydrolysis (Vaštag et al, 2011). A broad range of animal and plant protein hydrolysates have been used in the cosmetics and healthcare fields.

Protein hydrolysate is a mixture of proteoses, peptones,

peptides and free amino acids. It is possible, depending on enzyme specificity and degree of hydrolysis (DH) achieved, to generate hydrolysate products that exhibit enhanced or reduced functionality. There is a complex scientific process in the hydrolytic release of bioactive peptides encrypted within the primary structure of food proteins (FitzGerald and O'cuinn, 2006). Several studies have confirmed that protein hydrolysates should be rich in low-molecular-weight peptides, such as di- or tripeptides, and for high nutritional and therapeutic value, they should contain as few free amino acids as possible (Radha et al, 2008). It has been found that some protein hydrolysates are functional. Such functions include: acting as an antioxidant (Vaštag et al, 2011; Chang et al, 2003); inhibiting human leukemic U937 cell growth (Chen et al, 2011; Chen et al, 2013); impacting bacterial physiological activities (Swiatecka et al, 2010); exhibiting ACE inhibitory activity (Vaštag et al, 2011; Raghavan and Kristinsson, 2009). Protein hydrolysates with ACE

inhibitory activity have shown great promise in the development of novel therapeutics and functional food for preventing hypertension.

A large number of ACE-inhibitory protein hydrolysates have been produced from various animal and plant proteins, such as casein (Hong et al., 2014; Pan et al., 2012; Miguel et al., 2007), chickpea (Pedroche et al., 2002), and sunflower (Megias et al., 2009). To the best of our knowledge, bee pupae protein hydrolysates have not been the subject of study for bioactivity. The aim of this study was to evaluate the bioactive properties of bee pupae protein hydrolysates produced by Alcalase and Flavourzyme. The hydrolysis process and the *in vitro* ACE inhibitory activities of the hydrolysates obtained were investigated. Furthermore, the possible relationship between ACE inhibitory activities and the hydrolysate fractions obtained by ultrafiltration with the various molecular weight cut-off membranes was also evaluated.

MATERIALS AND METHODS

Materials and chemicals

Bee pupae were obtained from Da-Lin, Chia-Yi, Taiwan. The bee pupae were freeze-dried and stored in a desiccator at -20°C until use. The enzymes used for protein hydrolysis were Alcalase and Flavourzyme (from Sigma Chemicals Co., St. Louis, MO, USA) with declared activities of 2.4 Anson units (AU)/g and 0.5 unit/g, respectively. ACE was purchased from Sigma Chemicals Co. (St. Louis, MO, USA) and its activity was determined using Hippuryl-His-Lue (Sigma Chemicals Co., St. Louis, MO, USA) as a substrate. All other chemicals and solvents are of analytical grade.

Determination of the proximate compositions of bee pupae

Moisture, ash and crude fat contents were assayed by the methods of the Association of the Official Analytical Chemists (AOAC, 2006); the methods used were 934.01, 942.05 and 920.39, respectively. Crude protein content ($N \times 6.25$) was determined by the AOAC Kjeldahl method 984.13.

Preparation of the bee pupae protein sample

According to the methods provided by Lee et al. (1999), bee pupae were dissolved in a 0.1 M phosphate buffer (pH 7.0) and centrifuged at 15,000 $\times g$ for 30 min at 4°C. The solution was precipitated with various saturations of ammonium sulfate and then centrifuged at 15,000 $\times g$ for 30 min at 4°C. The precipitates obtained were dissolved in distilled water and dialyzed for 24 h at 4°C. They were freeze-dried and stored in a desiccator at -20°C until use.

Enzymatic hydrolysis

One gram of protein sample was dissolved in 100 ml of distilled water and stirred with a stirrer for 10 min at room temperature. The reaction pH levels were adjusted to pH 8.5 for Alcalase hydrolysis (E/S = 0.5, 1.0, 1.5, and 2.0%) and pH 7.5 for Flavourzyme hydrolysis (E/S = 0.5, 1.0, 1.5, and 2.0%) with 1 N NaOH or HCl. The enzymes were added to the mixture to react for 1, 2, 4, 6, 8 and 10 h at 50°C for Alcalase and 40°C for Flavourzyme. The reactions were terminated by immersing the mixture into a water bath of 90°C for 15 min. In the two-stage hydrolysis process, Alcalase (E/S = 1.5%) was used in the first stage and the hydrolysis time was 4 h. Flavourzyme (E/S, 0.5, 1.0, 1.5, and 2.0%) was used in the second stage and the hydrolysis time was 6 h. Two-stage hydrolysis was stopped by heat treatment at 90°C for 15 min. DH of the bee pupae protein hydrolysate was calculated as (amino nitrogen/ total nitrogen) $\times 100\%$, where the total nitrogen and amino nitrogen contents were determined by the semi-micro-Kjeldahl method and the formol titration method AOAC (2006), respectively.

In vitro assay of ACE inhibition

The ACE inhibitory activity was measured using the spectrophotometric method and modification by Muguruma et al. (2009). The reaction mixture contained 5 mM Hip-His-Leu as a substrate, 300 mM NaCl and 8 mU enzyme in 100 mM sodium borate buffer (pH 8.3). A sample (150 μL , 150 mg/mL) was added to the reaction mixture referred to previously (150 μL) and mixed with 150 μL of 15 mM Hip-His-Leu containing 1 M NaCl. After incubation at 37°C for 60 min, the reaction was stopped by adding 0.5 mL of 1 N HCl. The resultant hippuric acid was extracted by the addition of 1.5 mL ethyl acetate and centrifuged (800 $\times g$, 15 min). One milliliters (1 ml) of the upper layer was transferred into a glass tube and evaporated in a vacuum (Firstek DB-101, Firstek Co., Ltd, China) at room temperature for 2 h. The hippuric acid was redissolved in 1.0 mL of distilled water and absorbance at 228 nm was measured using a spectrophotometer (Unikon 930, Kontron Instruments, Italy). The inhibition was calculated as:

$$\text{Inhibition (\%)} = (\text{Control-Sample}) / \text{Control} \times 100$$

The IC_{50} value was defined as the concentration of hydrolysate (mg/mL) required to reduce the hippuric acid peak by 50% (indicating 50% inhibition of ACE).

Fractionation with different molecular weight cut-off (MWCO) membranes

Bee pupae protein hydrolysates, obtained by 1) 1.5% Alcalase for 10 h (2) 2.0% Flavourzyme for 10 h and (3)

1.5% Alcalase for 4 h plus 2.0% Flavourzyme for 6 h, were pumped through 5, 3 and 1 kDa MWCO membranes (IAFO®, Amicon Model 8200). The bee pupae protein hydrolysates were therefore divided into four molecular fractions: > 5 kDa, 5~3 kDa, 3~1 kDa and < 1 kDa, which were lyophilized and stored at -20°C until use.

Statistical analysis

In this study, the results from three separate experiments were given as mean $\pm$ standard deviation values. Data were subjected to one-way analysis of variance and Duncan's multiple range test. $P < 0.05$ was regarded as statistically significant in SAS (Statistic Analytic System) (2001).

RESULTS AND DISCUSSION

Proximate compositions of bee pupae

The nutrient compositions of fresh and freeze-dried bee pupae were compared, as shown in Table 1. The crude protein of fresh bee pupae was 17.16%, which is higher than the 10.3 and 16.0% reported for the edible insect *Acheta domesticus* (Pennino et al, 1991) and eri silkworm prepupae/pupae (Longvah et al, 2011), respectively. The crude protein of freeze-dried bee pupae was 67.96%, which is higher than the 54, 48.7 and 49.1-53.5% reported for eri silkworm prepupae/pupae (dry weight basis) (Longvah et al, 2011), spent silkworm pupae (Rao, 1994) and *B. Mori* L. Chrysalis toast (Pereira et al, 2003), respectively. The crude fat of fresh and freeze-dried bee pupae was 4.31 and 19.23%, respectively, which is lower than the 25% reported for larva of *Rhynchophorus phoenicis* (Ekpo and Onigbinde, 2005). The crude fat is also lower than the 8-26% reported for eri silkworm prepupae/pupae (fresh/dry weight) (Longvah et al, 2011). The high crude protein of bee pupae offers immense potential for mitigating the protein deficiency found in many people in developing countries; bee pupae represent a cheap source of good quality protein.

Yields, and ACE inhibitory activities of ammonium sulfate precipitates from bee pupae protein

Figure 1 displays the yields and ACE inhibitory activities of ammonium sulfate precipitates from bee pupae protein at various saturations. Inspection of the results presented in Figure 1 shows that ACE inhibitory activities are significantly related to the yields of ammonium sulfate precipitates from bee pupae protein at various saturations. The yields of the ammonium sulfate precipitates with various saturations of 0~10, 10~20, 20~30, 30~40, 40~50, 50~60, 60~70, 70~80, 80~90 and 90~100% were 0.66, 0.82, 2.87, 2.97, 8.41, 5.14, 5.94, 2.27, 1.68 and 2.47%, respectively; the ACE inhibitory activities were 1, 3, 12, 15,

16, 22, 21, 24, 10 and 9%, respectively. From these results it is apparent that the ammonium sulfate precipitates with saturations between 20~100% have a higher yield and ACE inhibitory activities. On the basis of these results, another results will be obtained from a further study of 20~100% of ammonium sulfate precipitates. The crude protein of freeze-dried bee pupae was 67.96% (Table 1), but the total yield of ammonium sulfate precipitates was 33.23%. Damodaran (1996) reported that plants and animals contain a certain amount of water-soluble proteins. From the above results and for the reason mentioned above, one may deduce that bee pupae protein contains much water-soluble protein, leading to limited non-polar regions of the molecular surface, which are difficult for precipitation with ammonium sulfate.

ACE inhibitory activity of bee pupae protein hydrolysates obtained by one-stage hydrolysis

Alcalase and Flavourzyme are microbial protease preparations that are widely used to improve the nutritional or functional properties of protein (Vaštag et al, 2011). Alcalase is a serine endopeptidase, while Flavourzyme is a mixture of proteases with exo- and endopeptidase activities (Vaštag et al, 2011). Figure 2 (A) displays the changes of the ACE inhibitory activities of bee pupae protein hydrolysates obtained by Alcalase ($E/S = 1\%$) of various concentrations. It was found that the ACE inhibitory activities of the hydrolysates obtained by Alcalase at 0.5, 1.0, 1.5 and 2.0% for 1 h hydrolysis were 15.04, 24.54, 29.67 and 33.58%, respectively; and the ACE inhibitory activities were 25.86, 27.85, 37.76 and 38.57%, respectively, for 8 h hydrolysis. Figure 2 (A) demonstrates that the ACE inhibitory activities increase with increasing Alcalase concentration and hydrolysis time. The results are in agreement with the findings of Raghavan and Kristinsson (2009) who reported that ACE inhibitory activities increase with increasing DH. The hydrolysate samples obtained by Flavourzyme hydrolysis ($E/S = 1\%$, enzyme concentration: 0.5, 1.0, 1.5 and 2.0%) were tested for ACE inhibitory activity (Figure 2 (B)). The results showed that ACE inhibitory activities were 12.40, 13.9, 19.81 and 21.26% with Flavourzyme hydrolysis for 1 h under 0.5, 1.0, 1.5 and 2.0% enzyme concentration, respectively. Figure 2 (B) also shows that the ACE inhibitory activities increase with increasing Flavourzyme concentration and hydrolysis time. The results are in agreement with the findings of Jang and Lee (2005) who reported that ACE inhibitory activities increase with increasing enzyme concentration.

ACE inhibitory activity of bee pupae protein hydrolysates obtained by two-stage hydrolysis

The two-stage hydrolysis of bee pupae protein by Alcalase and Flavourzyme were monitored for up to 10 h. The

Table 1. Proximate compositions of bee pupae.

Sample	Crude Protein (%)	Crude Fat (%)	Ash (%)	Moisture (%)
Raw fresh Bee pupae	17.16 ± 0.20	4.31 ± 0.11	3.23 ± 0.14	75.86 ± 0.20
Freeze-dried Bee pupae	67.96 ± 0.57	19.23 ± 0.12	5.06 ± 0.03	7.43 ± 0.03

Samples were analyzed in triplicate.

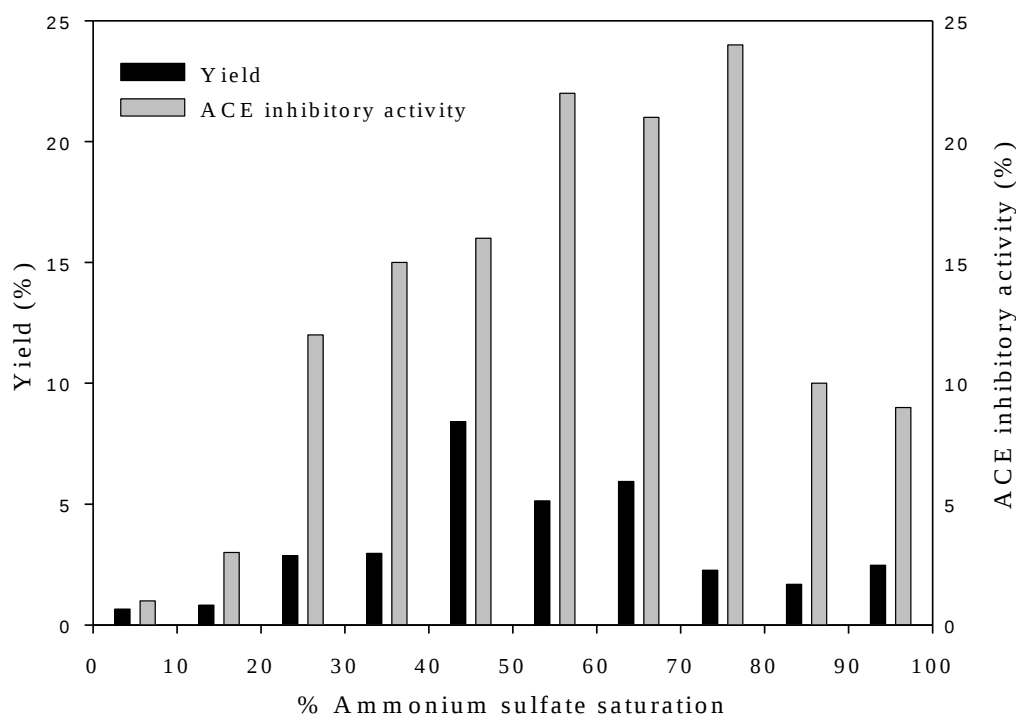


Figure 1. Yields and ACE inhibitory activities of ammonium sulfate precipitates from bee pupae protein at various saturations. ACE inhibitory activity was determined at a sample concentration of 50 mg/mL. Experiments were conducted in triplicate.

hydrolysis curves are shown in Figure 3 (A). The highest DH value was 6.86% in the first stage using Alcalase hydrolysis (hydrolysis time 4 h; E/S = 1.5%). In the second stage, Flavourzyme was added (hydrolysis time 6 h; E/S = 2.0%) and the highest DH value was 12.92%. From Figure 3 (A), it is evident that DH increases with increasing enzyme concentration. The result is in agreement with the findings of Rebeca et al. (1991), who reported that for two-stage hydrolysis of fish protein, the DH increases with increasing enzyme concentration, and that DH is positively related to hydrolysis time. Similar effects by the sequential use of Alcalase and Flavourzyme have been reported for chickpea (Clemente et al., 1999), sunflower (Megias et al., 2009), bovine colostrums (Chen et al., 2011) and pumpkin oil cake protein (Vařtag et al., 2011). From Figures 3 (A) and (B), it was found that high DH hydrolysates showed high ACE

inhibitory activity, revealing that low MW peptides exhibit higher ACE inhibitory activity than high MW peptides do. The ACE inhibitory ability of low MW peptides has also been reported for peptides derived from plant and animal protein such as pumpkin oil cake protein (Vařtag et al., 2011), tilapia protein hydrolysates (Raghavan and Kristinsson, 2009), and sunflower protein (Megias et al., 2009).

ACE inhibitory activity of fractions from bee pupae protein hydrolysates

Four fractions obtained using ultrafiltration were tested for their ACE inhibitory activity. As can be seen in Table 2, ultrafiltration significantly increased ($p < 0.05$) the ACE inhibitory activity of the fractions compared to the original

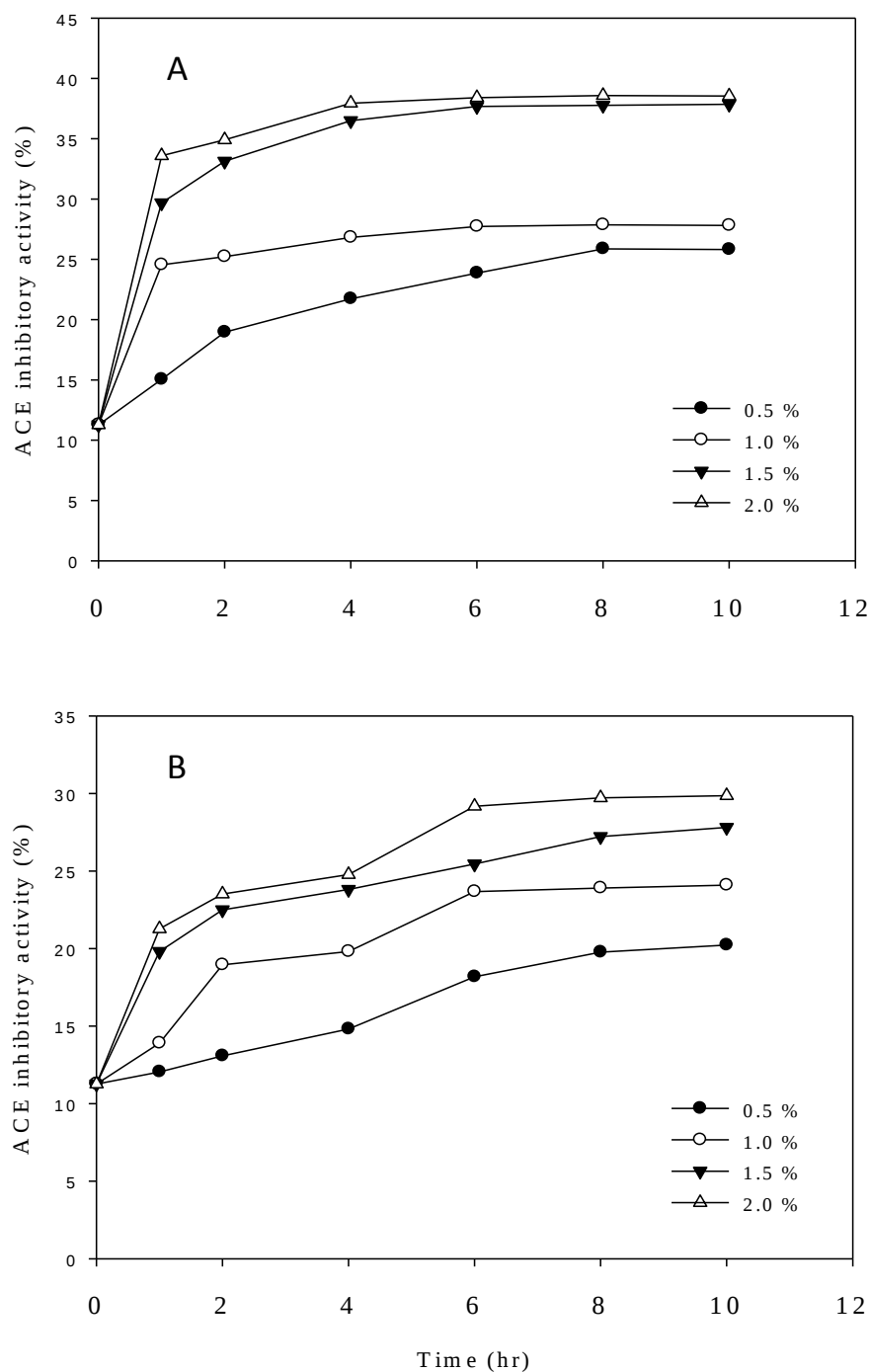


Figure 2. Changes in the ACE inhibitory activities of bee pupae protein hydrolysates during 10 h of hydrolysis by Alkalase (A) and Flavourzyme (B) of various concentrations.

hydrolysates. It is also evident from Table 2 that the 5~3 kDa fraction of Alkalase hydrolysate has the highest yield and ACE inhibitory activity (30.13 and 69.77%, respectively) and its IC_{50} value is 20.04 mg/mL. The 5~3 kDa fraction of Flavourzyme hydrolysate has the highest yield and ACE

inhibitory activity (29.32 and 61.53%, respectively) and its IC_{50} value is 31.78 mg/mL. The ACE inhibitory activity of the fractions obtained by ultrafiltration was enhanced to double that without ultrafiltration. The 3~1 kDa fraction of two-stage hydrolysate had the highest yield and ACE inhibitory

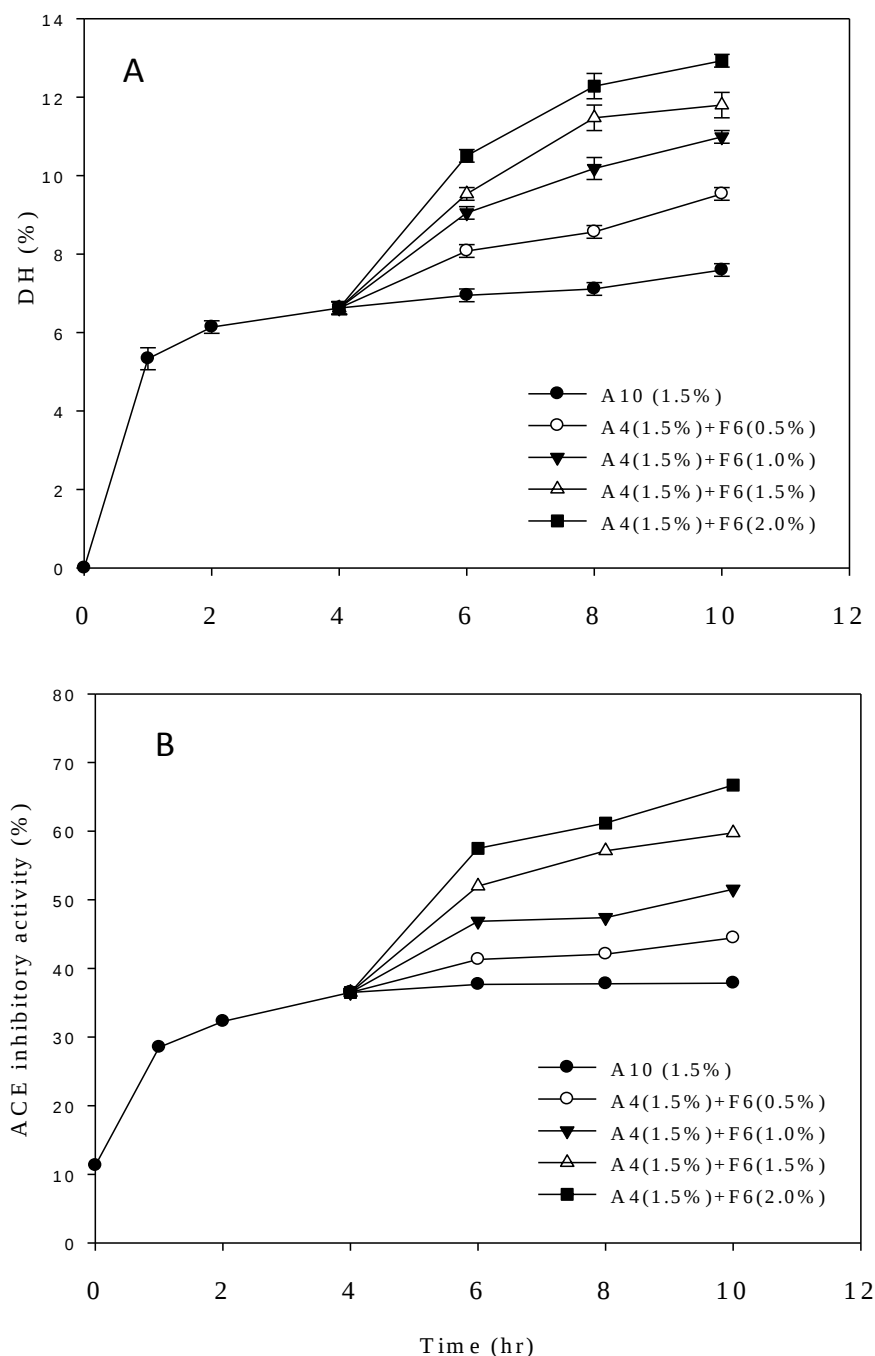


Figure 3. Changes in the DH (A) and ACE inhibitory activities (B) of bee pupae protein hydrolysates during two-stage hydrolysis using Alcalase and Flavourzyme of various concentrations.

activity (29.15 and 96.98%, respectively), and its IC_{50} value was 0.62 mg/mL. The ACE inhibitory function and the IC_{50} value of the fractions from plant and animal protein hydrolysates through ultrafiltration, such as tilapia protein hydrolysate (Raghavan and Kristinsson, 2009), pumpkin oil

cake protein hydrolysate (Vařtag et al, 2011) and soybean protein hydrolysate (Kuba et al, 2005) have also been reported. The above results show that bee pupae protein hydrolysates could achieve higher ACE inhibitory activity through ultrafiltration. The ACE inhibitory activity of low

Table 2. Yields, IC₅₀ and ACEI activities of the filtrates of bee pupae protein hydrolysate fractionated by membranes with different molecular weight cut-offs (MWCO).

	Hydrolysates								
	Alcalase (10 h, 1.5%)			Flavourzyme (10 h, 2.0%)			Alcalase + Flavourzyme (4 h, 1.5% + 6 h, 2.0%)		
	Yield (%)	Inhibitory activity* (%)	IC ₅₀ (mg/mL)	Yield (%)	Inhibitory activity* (%)	IC ₅₀ (mg/mL)	Yield (%)	Inhibitory activity* (%)	IC ₅₀ (mg/mL)
Hydrolysate		38.71 ^{e**}	68.89		30.86 ^e	87.45		66.37 ^d	22.70
>5 kDa	23.70	54.38 ^d	43.92	22.94	46.39 ^d	55.56	25.21	74.96 ^c	11.57
5~3 kDa	30.13	69.77 ^a	20.04	29.32	61.53 ^a	31.78	19.80	96.13 ^a	0.66
3~1 kDa	28.53	58.17 ^c	37.96	23.03	55.09 ^c	42.12	29.15	96.98 ^a	0.62
<1 kDa	19.83	61.31 ^b	33.06	22.84	57.33 ^b	39.95	23.25	91.91 ^b	0.78

*ACE inhibitory activity was determined at a sample concentration of 50 mg/mL. **Values with the same letter in the same column are not significantly different at $P > 0.05$.

MW fraction (5~3 kDa, 3~1 kDa and <1 kDa) obtained through two-stage hydrolysis was more than 90% and the IC₅₀ value was less than 0.8 mg/mL. The result was in agreement with the findings of Miyoshi et al. (1991), Wang et al. (2008), and Matsui et al. (1993), who reported that ACE inhibitors are mostly for short-chain peptides of 2 ~ 14 amino acids.

Conclusions

Hydrolysates prepared from bee pupae protein exhibited significant ACE inhibitory activity. Compared to Flavourzyme, Alcalase was more effective in the hydrolysis of bee pupae protein, and the inhibitory activity increases with an increasing percentage DH and hydrolysis time. Bee pupae protein hydrolysates obtained by two-stage hydrolysis showed higher ACE inhibitory activity and a lower IC₅₀ value than the hydrolysates obtained by one-stage. A further study, fractionating bee pupae protein hydrolysates using ultrafiltration

into various molecular weight fractions did enhance the ACE inhibitory activity and reduced the IC₅₀ value of the fractionates compared to the original hydrolysates. Yamamoto et al. (2003) reported that ACE inhibitors, isolated from natural food protein did not show the same side effects when compared to the ACE inhibitors from chemical synthetic antihypertensive drugs. The present results confirm that the bee pupae protein hydrolysates and fractionates have ACE inhibitory activity; however, the active components and mechanism are still unclear. Thus, further studies will be done on purification, characterization of the individual peptides responsible and on investigating the mechanism for ACE inhibitory activity of bee pupae protein hydrolysates.

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