Preparation of antihypertensive peptides from broad bean by compound enzymatic hydrolysis

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Abstract: Antihypertensive peptides prepared from broad bean by compound enzymatic hydrolysis were studied, assessing the impact of amino acid composition on potential antihypertensive activity. Broad bean protein concentrate (BPC) was prepared using ultrasonic assisting with alkali-solution and acid-isolation extraction method. And its amino acid composition and hydrophobic value were determined. It was proved that broad bean protein was a good source for antihypertensive peptides preparation as it had high proportion of hydrophobic amino acids, aromatic amino acids and proline, which were related to antihypertensive activity. Four enzymes of Protamex, Flavourzyme, Alcalase and Neutrase were studied to hydrolyze BPC. And the condition of compound enzymes Neutrase and Protamex under pH-spontaneous was selected as the optimum hydrolysis condition. The final degree of hydrolysis (DH) was 26.53%. All sample solution was diluted to 10 mg mL^{-1} when determined the angiotensin-converting enzyme (ACE) inhibition. And the ACE-inhibition of hydrolyzate was 54.31%. After fractionation by Sephadex G-15, 3 fractions were collected. Fraction P3 showed the strongest ACE inhibitory activity, which was 65.93%. P3 was further fractionated by semi-preparative RP-HPLC and 14 fractions were collected. Fraction F9 showed the strongest ACE inhibitory activity, which was 81.17%. Amino acid sequences of fraction F9 in RP-HPLC were analyzed by using LC-MS/MS. Five potential antihypertensive peptides were identified, they are DSTLIMQLLR, SAEYVRLY, LDWYKGPT, IYAPHWN, EGSLLLPHYNSR respectively. This study obtained broad bean hydrolysates with potential ACE-inhibitory activities, which can be used to obtain new added value functional ingredients from broad bean meal.

Keywords: antihypertensive peptides, broad bean, compound enzymatic hydrolysis, RP-HPLC, Sephadex G-15

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1 Introduction

Broad bean (*Vicia Faba L.*) is widely planted in more than 40 countries in the world. It is the world's sixth largest bean crop (McCurdy and Knipfel 1990). China is the world's largest broad bean production country, which produces around 40% of the world's supply of broad beans. Broad bean is a kind of legume foods with high content of protein and starch, low content of fattiness. Since its first use encompasses starch extraction, the remaining bulk product, rich in protein and fibre, is usually applied in animal feeding and only a small portion is further processed into various types of broad bean protein products for human consumption, e.g. broad bean protein concentrate. The BPC has many good characters, such as high nutritional value, suitable amino acid constitution and good equilibrium of the essential amino acid (Tan *et al.* 2010). As a low added value co-product, it needs to be upgraded by protein extraction or hydrolysis. Hydrolysis may generate functional ingredients with high added value, since the resulting hydrolysate frequently exhibit bioactive properties such as antioxidant or antihypertensive properties (Pena-Ramos and Xiong 2002).

Hypertension is one of the most serious chronic diseases threatening human health (Sun and Zhang 2005). Present prevention and control measure of hypertension is taking chemical-based medicines, such as captopril, enalapril and lisinopril. Clinical results showed these medicines had good effect of lowering blood pressure,

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but frequently caused side effects like coughing and ngioneurotic ed (Koklu *et al.* 2005, Guazzi and Arena 2010). Human body blood pressure is mainly regulated by Renin-Angiotensin System (RAS) and Kallikrein-Kinin System (KKS). ACE is the rate-limiting enzyme for this system (Nakagomi *et al.* 2000). ACE-inhibitory peptides prepared from food resources have drawn researchers' attention for their high antihypertensive activity, non-toxic side effects and high absorption. These peptides have strong targeting function so that they have obvious hypotensive effect on hypertensive patients, and have no deteriorated effect on hypotension (Yin *et al.* 2008, Wang *et al.* 2012).

In the current study of antihypertensive peptides prepared from legume resources, the preparation and antihypertensive activity of soy peptides are most extensively investigated. Peptides prepared from peas, green beans, black beans also have been researched generally. Native antihypertensive peptides from five soybean-based infant formulas were separated by ultra-filtration. The highest ACE-inhibitory activity in fractions was observed from 3 to 5 kDa and below 3 kDa (Puchalska *et al.* 2014). Soybean peptide fractions isolated from Korean fermented soybean paste were extracted and separated, peptide His-His-Leu (HHL) proved to have antihypertensive activity in vivo (Shin *et al.* 2001). The ACE-inhibitory activity of the extracts of bean and viscous material from the fermented black soybeans varied with extraction solvents and starter organism. But it increased as the fermentation period was extended (Juan *et al.* 2010). Pea protein isolate was hydrolyzed with alcalase, separated with cationic solid-phase extraction (SPE) and RP-HPLC. Major peptides identified as IR, KF, and EF proved to have higher potency against ACE than against renin (Li and Aluko 2010). However, very few studies have investigated broad bean proteins for the presence of ACE-inhibitory peptides as well as for the isolation and structural characterization of the most active peptides.

In the preparation of biological active peptides, protein should be hydrolyzed by chemicals, enzymes or fermentation. Chemical hydrolysis includes acid hydrolysis and alkaline hydrolysis. Amino acids are easily destroyed by chemicals and the environment is also polluted by waste liquid (Fife *et al.* 2002). Enzymatic hydrolysis is most widely used at present. Proteases prepared from microbial, plant or animal have been used in preparation of antihypertensive peptides. Compared with microbial protease, plant and animal protease are less origin and expensive. So, microbial protease is widely used in actual production. In this paper, we chose four kinds of microbial proteases which had good enzymolysis activeness. And compound enzymatic hydrolysis under pH-controlled condition and pH-spontaneous condition was studied in the hydrolysis of BPC. The hydrolyzates were separated to obtain the highest ACE-inhibitory activity fraction using the chromatographic methods including gel filtration and reversed phase high-performance liquid chromatography, and the sequences of peptides were assayed by LC-MS/MS.

2 Materials and methods

2.1 Materials

The broad bean variety Chenghudabai was obtained from Sichuan Academy of Agricultural Sciences (Sichuan, China). The commercial enzymes Protamex, Flavourzyme, Alcalase, Neutrase were purchased from sigma-Aldrich (Steinheim, Germany).

2.2 Preparation of BPC

Broad bean was defatted after peeling and milling. BPC was prepared from defatted broad bean powder. Proteins were dissolved in water in the ratio of powder to water of 1:12 by alkalization to pH 12 with 2.5 N NaOH(Wolf 1970). After extraction under ultrasonic condition for 30 min, the extract was clarified by centrifugation at 5000 r min^{-1} for 15 min, and then, protein was precipitated by acidification to pH 4.2 with 2 N HCl and the insoluble fraction was collected by centrifugation at 5000 r min^{-1} for 20 min. The precipitate was freeze dried and stored at 4℃ for further utilization. BPC was done in duplicate for each condition.

2.3 Determination of total protein, water, ash and starch

Total protein content was determined by the Kjeldahl Method (AOAC 2016). Water content was determined by

the direct drying method. Drying temperature was 105°C, and drying time was 4 h. Ash content was determined by combustion method. Starch content was determined by enzymatic hydrolysis method.

2.4 Determination of amino acid composition and hydrophobic value in broad bean protein

Tryptophan content was determined by alkaline hydrolysis method. 1.5 mL 4 N NaOH was added to 74.05 mg of sample material. They were hydrolyzed for 20 h under the condition of 110°C with no oxygen. After cooling down, the sample was acidized to pH 4 to pH 5 by adding 1140 μL 6 N HCl and diluted to 50 mL. Sample volumes of 20 μL were injected into the Agilent 1200 HPLC system mounted with the reversed phase column Innoval C18 (250×4.6 mm, 5 μm particle size) and eluted at 25°C, with a flow rate of 1 mL/min (Farvin et al. 2010). The mobile phase was methanol-sodium acetate buffer (5:95). Detection wavelength was set at 280 nm.

The other amino acids were determined by acidic hydrolysis method. Ten mL 6 N HCl was added to 66.78 mg of sample material. They were hydrolyzed for 24 h under the condition of 110°C, with no oxygen. After cooling down, the sample was diluted to 50 mL. One mL of this solution was transferred and blown to dry with nitrogen. Five mL 0.02 N HCl was added to re-dissolve the sample. Sample solution was filtered and analyzed by the L-8900 automatic amino acid analyzer (Hitachi Co. Ltd., Japan). The hydrophobic value was calculated according to the following Equations (1)-(2).

$$\Delta Q = \frac{AA_i / M_i}{\sum AA_i / M_i} \times \Delta f_i \quad (1)$$

$$Q = \sum \Delta Q_i \quad (2)$$

where, AA_i = amino acids content/100 g protein (g); Q = hydrophobic value of protein; M_i = molecular weight of amino acid (g/mol); Δf_i = hydrophobic value of amino-acid side chains (KJ/mol); $\Sigma AA_i/M_i$ = the total number of moles of amino acids in 100 g protein (mol).

2.5 Measurement of ACE-inhibitory effect

The ACE-inhibitory effect was measured using the modified RP-HPLC method (Cushman and Cheung 1971). HHL was dissolved in 0.1 M borax-borate buffer (pH 8.3) containing 0.3 mol/L NaCl to form 5 mM solution for further utilization. ACE working solution was diluted to 0.025 U. Sample solution was diluted to 10 mg/mL with buffer solution. All sample solution was diluted to 10 mg/mL when determined the ACE-inhibition. The experiment was performed according to Table 1.

Table 1 Determination of ACE inhibition activity in vitro

Reagent, μL	A, Sample	B, Control	C, Blank
1 M HCl	0	0	100
0.025 U ACE	160	160	160
Sample	30	0	30
0.1 M borax-borate buffer	0	30	0
37°C incubated for 10 min			
5 mM HHL	50	50	50
37°C reacted for 30 min			
1 M HCl	100	100	0
Sample	0	30	0
0.1 M borax-borate buffer	30	0	30

Note: Cooled down to room temperature, 10 μL product was filtrated through a 0.45-μm membrane, hippuric acid content was determined with RP-HPLC, ACE-inhibitory effect was determined

Hippuric acid content was determined with RP-HPLC (Wang et al., 2011). Samples were eluted at 25°C with a flow rate of 0.8 mL/min. The mobile phase was acetonitrile-water (25:75) which contains 0.1% TFA. Detection wavelength was set at 228 nm. Column temperature was 25°C. The activity of each sample was tested in triplicate. The formula applied to calculate the percentage of ACE-inhibitory activity was as:

ACE-inhibitory activity (%) =100×(C−A)/(C−B)

The letter in the formula stands for the peak area value of each group.

2.6 Enzymatic hydrolysis

2.6.1 Single enzymatic hydrolysis

Single enzymatic hydrolysis was studied under pH-controlled and pH-spontaneous conditions. Substrate concentration was 5%. Enzyme concentration was 16000 U/g protein. Firstly, single enzymatic hydrolysis was studied under their optimum reaction conditions, as shown in Table 2. Concrete operations as follows: Distilled water was added to 5 g broad bean powder to form 5% sample solution. After mixing well, it was stirred constantly in 90°C water bath for 15 min and then cooled to optimum temperature of enzyme. At the same time, 80000 U enzyme solution was preheated for 10 min by water bath which was at the optimum temperature of enzyme. After both being adjusted to optimum pH,

sample solution and enzyme solution were mixed well and put in water bath constant temperature vibrator to hydrolyze. At the 0, 15, 30, 45, 60, 75, 90, 120, 150, 180, 240, 300, 360, 420, 450 min of the hydrolysis, pH of the solution and the volume amount of 1 mol/L NaOH solution added to make the solution back to the initial pH were recorded. At the end of the reaction, solution was put in boiling water bath for 10 min to inactive the enzyme, and then clarified by centrifugation at 5000 r min^{-1} for 20 min after cooling down to room temperature. The supernatant was dehydrated using freeze drying method and stored in 4°C refrigerator for further utilization.

Table 2 Optimum reaction conditions of Protamex/ Flavourzyme/Alcalase/Neutrase

Enzyme	Optimum reaction condition	
	pH	Temperature, °C
Protamex	7.0	58
Flavourzyme	6.3	55
Alcalase	8.5	55
Neutrase	7.0	50

For the hydrolysis under pH-spontaneous condition, pH of the solution was recorded at 0, 15, 30, 45, 60, 75, 90, 120, 150, 180, 240, 300, 360, 420, 450 min. NaOH was not added to make the pH back to original state until the reaction ended. The other operations were the same as the pH-controlled condition.

2.6.2 Compound enzymatic hydrolysis

Two enzymes were selected based on the results of single enzymatic hydrolysis test. Compound enzymatic hydrolysis was studied according to the following two processes (Table 3).

Table 3 Process of double-enzyme compound hydrolysis

Type	Compound enzymes	Temperature, °C	pH	Enzyme concentration, U/g protein	Incubation time, min
1	$enzyme_1$-$enzyme_2$	T_1-T_2	pH_1-pH_2	$enzyme_1$ & $enzyme_2$ are both 16000	0-t_1-end
2	$enzyme_2$-$enzyme_1$	T_2-T_1	pH_2-pH_1		

Notes: t_1 = incubation time of the first enzyme (Under controlled pH condition, t_1 = 210 min; Under not controlled pH condition, t_1 = 90 min).

Substrate concentration was 5%. Enzyme concentration was 16000 U/g protein. Timing started and pH was recorded after adjusting the sample solution and the first enzyme solution to the optimum condition. The volume amount of 1 mol/L NaOH solution added to make the solution back to the initial pH was also recorded. The mixture was heated by boiling water bath for 10 min to end the first period of enzyme hydrolysis. After adjusting the mixture to optimum condition for the second enzyme, same amount of second enzyme was added and timing started. NaOH solution was not added to restore the pH until the pH change was not obvious any more. The solution was put in boiling water bath for 10 min to inactive the enzyme, and then clarified by centrifugation at 5000 r/min for 20 min after cooling down to room temperature. The supernatant was freeze dried and stored in 4°C refrigerator for further utilization.

2.7 Determinations of degree of hydrolysis

The DH was determined using the modified pH-Stat method (Adlernissen, 1979). As the pH level of solution was not steady during enzymatic hydrolysis, DH was determined based on the quantity of the alkali liquor added to make the solution back to the initial pH level. The % DH was calculated according to the following Equations (4)-(6).

$$DH = \frac{B \times N_b}{\alpha \times M_p \times h_{tot}} \times 100\% \quad (4)$$

$$\alpha = \frac{10^{(\mathrm{pH-pK})}}{1+10^{(\mathrm{pH-pK})}} \quad (5)$$

$$\mathrm{pK} = 7.8 + \frac{2400(298-T)}{298T} \quad (6)$$

where, B = volume of alkali liquor, L; N_b = concentration of alkali liquor, mol/L; α = dissociation degree of α-NH_3^+; pH = pH value of reaction system; pK = mean dissociation constant of released α-NH_3^+ in reaction; M_p = total protein content in substrate, g; h_{tot} = total number of peptide bonds per protein equivalent (7.92 specific to broad bean protein). Absorbance of the eluent was monitored at 280 nm.

2.8 Fractionation by Sephadex G-15

The freeze dried hydrolyzates were dissolved in ultrapure water to a concentration of 50 mg/mL. Two mL sample solution was eluted on Sephadex G-15 at speed of 1.87 mL/min using eluent of ultrapure water. Absorbance of the eluent was monitored at 280 nm. Three peaks were detected and collected respectively.

2.9 Fractionation by semi-preparative HPLC

Fraction that had the highest ACE-inhibitory activity

was selected from fractions separated by Sephadex G-15 column. Semi-preparative HPLC was used for further isolation. Sample dissolved in ultrapure water containing 0.1% TFA to form 20 mg/mL solution and filtrated through a 0.45-μm membrane. Fractions were collected and freeze dried for ACE-inhibitory activity determination. Separation was performed on an Agilent Varian Prostar 218LC HPLC system with an Innoval C18 column (250×21.2 mm, 10 μm) at a flow rate of 10 mL/min. The compounds were separated using a linear elution gradient of (A) acetonitrile containing 0.1% TFA (v/v) and (B) ultrapure water containing 0.1% TFA (v/v) as follows: gradient of 10% to 50% A from 0 to 50 min; 10% A from 50.1 to 60 min. Detecting wavelength was at 280 nm.

2.10 Characterization and sequencing of peptides by LC–MS/MS

LC–MS/MS analysis was performed for identification of the amino acid sequences of peptides with the highest ACE-inhibitory activity. Sample was firstly separated on an ultimate 3000 HPLC system (Thermo Scientific, NY, USA) with a Waters C18 column (250×4.6 mm, 5 μm particle size) at a flow rate of 0.7 mL min^{-1}. The compounds were separated using a linear elution gradient of (A) 0.1% aqueous formic acid (v/v) and (B) acetonitrile-water (80:20) containing 0.08% formic acid (v/v) as follows: gradient of 5% to 35% B from 0 to 30 min; 95% B at 32 min; isocratic at 95% B from 32 to 37 min; 5% B at 39 min; isocratic at 5% B from 39 to 45 min. Fractions were collected and analyzed on a C18 column (120×0.15 mm, 1.9 μm particle size) with a C18 trap column(20×0.1 mm, 3 μm particle size) at a flow rate of 600 nL min^{-1}. The compounds were separated using a linear elution gradient of (A) 0.1% aqueous formic acid (v/v) and (B) acetonitrile-water (80:20) containing 0.08% formic acid (v/v) as follows: gradient of 6% to 9% B from 0 to 8 min; 14% B at 24 min; 30% B at 60 min; 40% B at 75 min; isocratic at 95% B from 78 to 85 min; 6% B at 86 min; isocratic at 6% B from 86 to 90 min. MS/MS analysis was carried out in the positive ion mode. For full MS condition, automatic gain control (AGC) target was $3E^6$, and maximum interval time (IT) was 80 ms. AGC target of dd-MS^2 was $5E^4$, and maximum IT was 45 ms. Spectra were obtained over a scan range from the mass to charge ratio (m/z) 300 to 1400. MS/MS data were acquired and analyzed by Proteome Discoverer software (Version 2.1, Thermo Scientific, NY, US).

2.11 Statistical analysis

Data were analyzed with SPSS (Version 20.0, IBM Inc., NY, US). Statistical differences between different treatments were assessed using analysis of variance, followed by Tukey's HSD post-hoc test ($p<0.05$). Data were expressed as means of at least two replicates.

3 Results and discussion

3.1 Characterization of broad bean powder and BPC

As is shown in Table 4, protein content of BPC was 2.2 times that of broad bean powder, and starch content of BPC was only 1/40 that of broad bean powder, which indicated the method of ultrasonic-assisted alkali-solution and acid-isolation could enrich protein and remove most starch in broad beans. In this way, the influence of other substances could be avoided in the further fractionation of peptides.

Table 4 Main components of broad bean powder and BPC

Content	Broad bean powder	BPC
Protein,%	34.4	75.70
Water, g/100 g	10.6	7.87
Starch, g/100 g	30.2	0.76
Ash, g/100 g	6.7	24.20

BPC contained 20 kinds of essential amino acids (Table 5). 40% of these amino acids were hydrophobic amino acids (Met, Try, Phe, Val, Leu, Ile, Pro and Ala), aromatic amino acids (Tyr, Phe and Try) and Proline. It was reported that the structure-function relationship of ACE-inhibitory peptides and found ACE-inhibitory activity of peptides was often related to hydrophobicity which was associated the composition and content of hydrophobic amino acids(Gu and Wu, 2013). Hydrophobic amino acids, aromatic amino acids and Pro took a great proportion in broad bean protein. So, peptides hydrolyzed from broad bean protein could have high ACE-inhibitory activity.

ACE-inhibition of BPC was about 14.4%, which was 6.8 times that of broad bean powder (Table 6). The reason for this great difference could be that protein in broad

bean powder had complete quaternary structure. But, in the process of crude protein production, more amino acid residues and branch chains were exposed. These exposed groups could improve ACE-inhibitory activity of BPC.

Table 5 Amino acid composition and hydrophobic value in broad bean protein

Amino acid	Hydrophobic value, KJ/mol	Protein, g/100 g
Trp	12.54	0.6702
Ile	12.41	3.5560
Tyr	12.00	2.7541
Phe	11.08	3.8591
Pro	10.87	3.0680
Leu	10.12	6.3704
Val	7.06	3.8051
Lys	6.27	5.4759
Met	5.43	0.7289
Cys	4.20	0.6072
Ala	3.05	3.0437
Arg	3.05	6.2149
Glu	2.30	14.4473
Asp	2.25	8.4657
His	2.09	1.7343
Thr	1.84	2.5504
Ser	0.17	3.6178
Gly	0	2.8368

Table 6 ACE-inhibition of broad bean powder and BPC

	Broad bean powder	BPC
ACE inhibition, %	2.13±0.16	14.4±1.2

3.2 Hydrolysis

3.2.1 Changes in degree of hydrolysis of BPC under pH-controlled condition

BPC was hydrolyzed with Protamex, Flavourzyme, Alcalase and Neutrase respectively. PH condition was controlled and the DH was measured (Figure 1). At the early beginning of the reactions, DH all increased quickly. As reactions processed, changes of DH became smaller and smaller. DH of BPC treated with Protamex reached 13.19% at 15 min of reaction. It reached 18.66% at 90 min and then its changes gradually slowed down until reaction ended at 210 min with final DH of 19.91%. DH of BPC treated with Flavourzyme increased quickly and reached 12.28% at 45 min. Then, it increased slowly until ended at 14.03% at 300 min. Its DH change rate and hydrolysis efficiency were lower compared to Protamex. The DH change rate was the highest when BPC was treated with Alcalase. DH had reached 16.03% at 30 min. At 480 min, the final DH of Alcalase was 22.49%, which was higher than that of the other three enzymes. In the hydrolysis of BPC treated with Neutrase, DH increased most quickly and reached 11.00% in the first 15 min. Its final DH was 21.00% at 210 min. The final DH of Protamex, Flavourzyme, Alcalase and Neutrase were 19.91%, 14.03%, 22.49% and 21.00% respectively. And their reaction time was 210, 300, 450 and 240 min. To increase production efficiency, 210 min was chosen as the reaction time. At 210 min, DH of BPC of the four enzymes in an increasing order as: Flavourzyme, Protamex, Neutrase, Alcalase. So, Alcalase and Neutrase were selected for the compound enzymatic hydrolysis test. And 210 min was the time to add the second enzyme in hydrolysis reaction.

BPC was hydrolyzed with compound enzymes Neutrase and Alcalase added in different order under pH-controlled condition and the DH was measured (Figure 2). In the first type of compound enzymatic hydrolysis, Neutrase was added firstly. The DH reached 12% at 15 min and finally reached 22.57% as the first stage of hydrolysis ended at 210 min. At 15 min after adding Alcalase for the second stage of hydrolysis, DH reached 28.24% which was much higher than the DH of BPC treated with only Neutrase. This might be because specific recognition sites of Alcalase and Neutrase do not coincide, or there are active sites which can be reacted by the second enzyme on the peptides produced in first stage (Rui et al., 2012). The DH reached 33.48% at 450 min and finally reached 33.83% which was 1.6 times the final DH of BPC treated with only Neutrase, and 1.5 times the one treated with only Alcalase. In the second type of compound enzymatic hydrolysis, Alcalase was added firstly. DH reached 22.94% at 210 min and finally reached 28.60% at 390 min after adding Neutrase. The final DH was 1.36 times the final DH of BPC treated with only Neutrase, and 1.27 times the one treated with only Alcalase. Final DH of the first type was higher than that of the second type. It might be because Alcalase hydrolysis could inhibit Neutrase hydrolysis more than the other way around. So, the first type of compound enzymatic hydrolysis was chosen for further study.

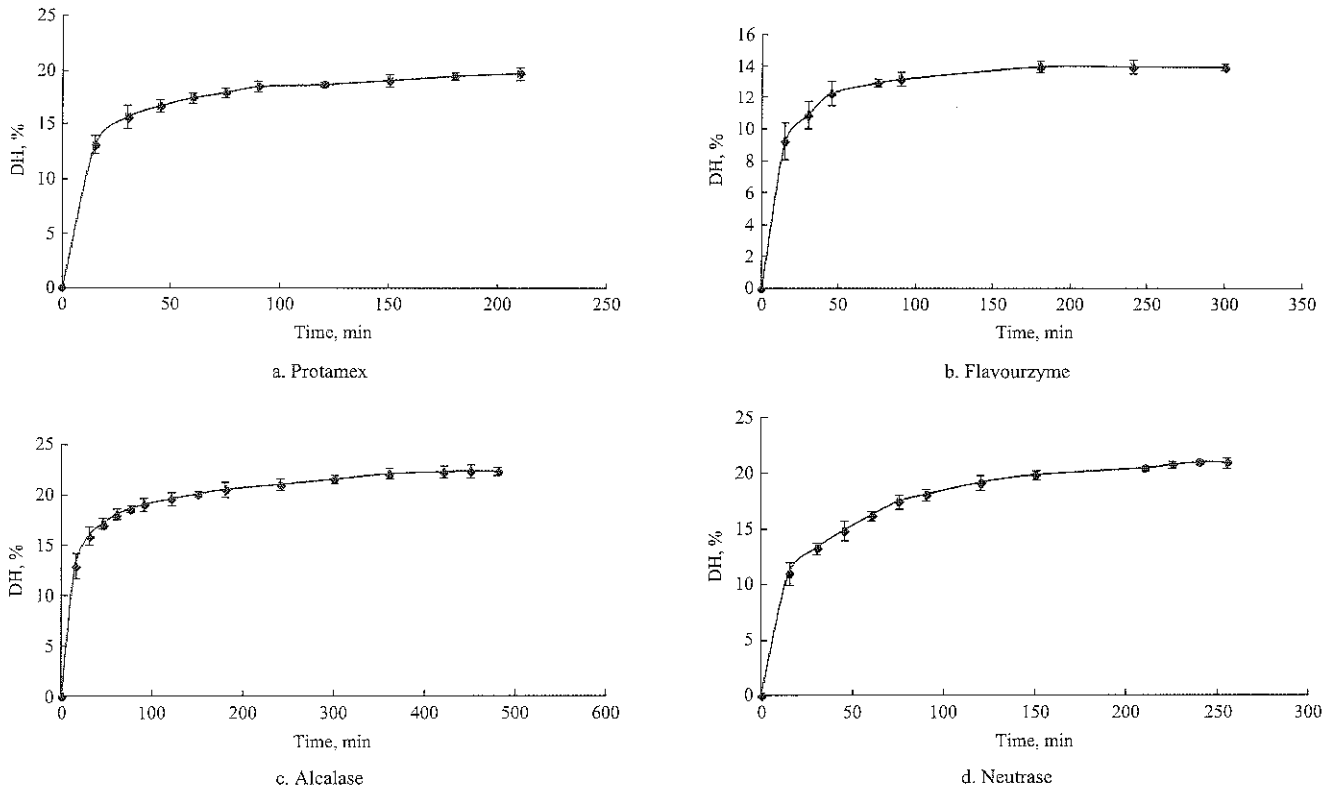


Figure 1 Changes in degree of hydrolysis of BPC treated with Protamex/Flavourzyme/Alcalase/Neutrase under pH-controlled condition

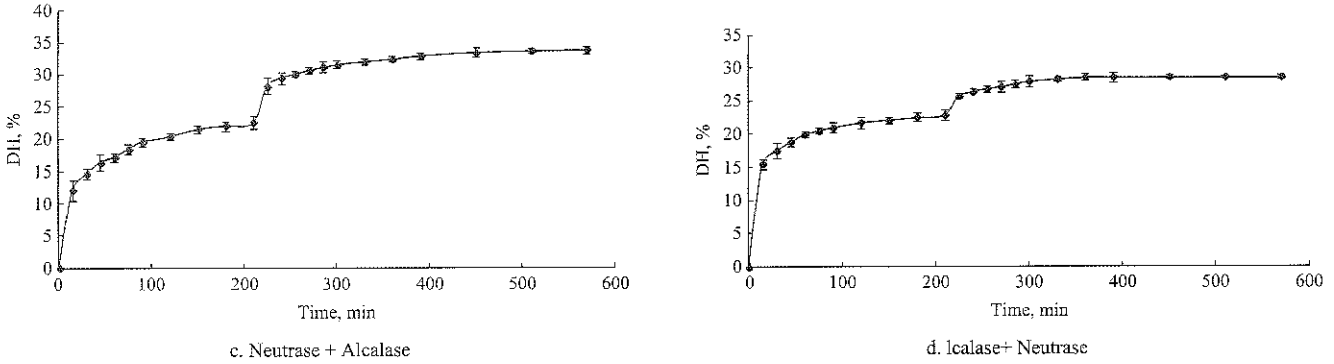


Figure 2 Changes in degree of hydrolysis of BPC treated with Neutrase + Alcalase / Alcalase+ Neutrase under pH-controlled condition

3.2.2 Changes in pH of hydrolyzates of BPC under pH-spontaneous condition

Under pH-controlled condition, enzymes could maintain highly active in solution of optimum range of pH. But, large amount of alkali salt was added to control pH. This made it harder to purify the peptides in the further study. Alkali salt was not added in the reaction under pH-spontaneous condition and DH could be lower than that of pH-controlled reaction.

BPC was hydrolyzed with Protamex, Flavourzyme, Alcalase and Neutrase respectively under pH-spontaneous condition, and the pH of hydrolysates was measured (Figure 3). At the beginning of Protamex hydrolysis, pH decreased greatly from 7 to 5.97 at 5 min. The decline rate of pH gradually decreased from 10 min to 45 min. The pH was 5.76 at 45 min and decreased to 5.74 by the end of reaction at 90 min. The final DH was 18.81% which was 94% of that of Protamex hydrolysis under pH-controlled condition (Table 7). In the hydrolysis process of Flavourzyme, pH decreased quickly from 6.3 to 5.81 at 5 min. Then, it decreased slowly to 5.65 at 30 min and almost unchanged till the end at 90 min. The final DH was 10.53% which was 75% of that of Flavourzyme hydrolysis under pH-controlled condition. This proved Flavourzyme was more relying on pH condition than Protamex. In the hydrolysis process of Alcalase, pH decreased quickly from 8.47 to 6.57 at 5 min. Then, it decreased slowly to 6.2 at 75 min and almost unchanged till the end at 180 min. The final DH was 18.53% which was 82% of that of Alcalase

hydrolysis under pH-controlled condition. Like the other three enzyme hydrolysis, in the hydrolysis process of Neutrase, pH decreased quickly from 7 to 6.23 at 5 min. Then, it decreased slowly to 5.93 at 60 min and almost unchanged till the end at 120 min. The final DH was 19.43% which was 92% of that of Neutrase hydrolysis under pH-controlled condition. PH-dependence of the four enzymes in an increasing order as: Protamex, Neutrase, Alcalase , Flavourzyme. Under pH-spontaneous condition, DH of the four enzymes in an increasing order as: Flavourzyme, Alcalase, Neutrase, Protamex. So, Neutrase and Protamex were selected for the compound enzymatic hydrolysis test.

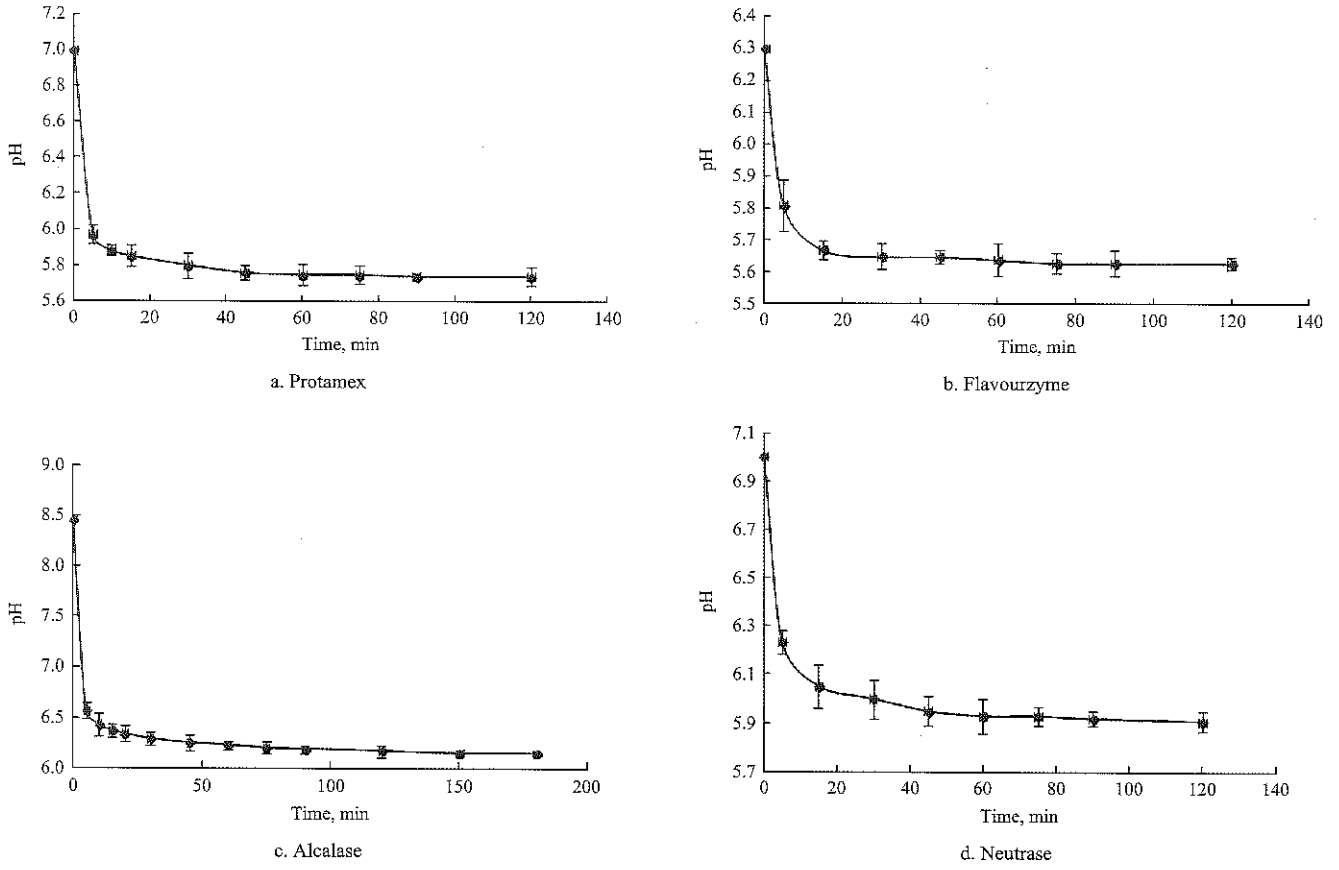


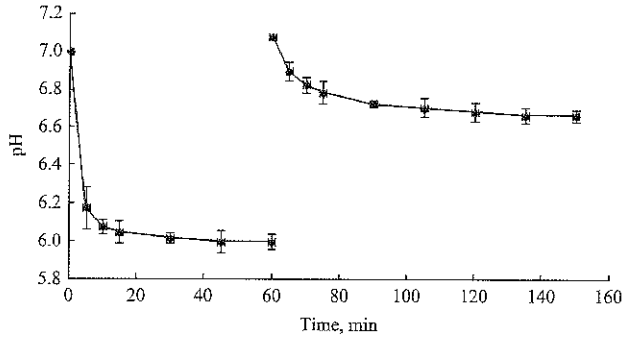
Figure 3 Changes in pH of hydrolyzates of BPC treated with Protamex/Flavourzyme/Alcalase/Neutrase under pH-spontaneous condition

Table 7 Degree of hydrolysis of BPC treated with single protease under pH-spontaneous condition

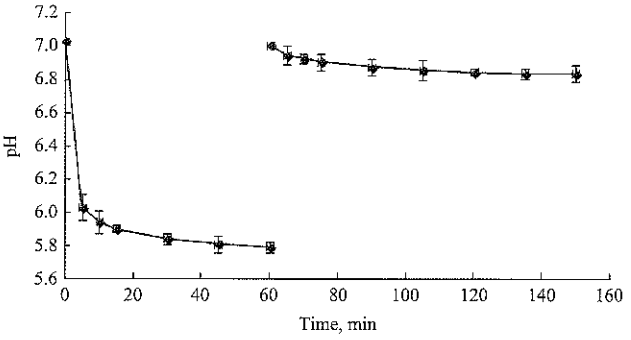
Enzyme	DH, %
Protamex	18.81
Flavourzyme	10.53
Alcalase	18.53
Neutrase	18.53

BPC was hydrolyzed with compound enzymes Neutrase and Protamex under pH-spontaneous condition and the pH of hydrolyzates was measured (Figure 4). In the first type of compound enzymatic hydrolysis, Neutrase was added firstly. The DH reached 17.10% as the first stage of hydrolysis ended at 60 min. At 5 min after adding the second enzyme Protamex, pH decreased quickly from 7.08 to 6.90. The decrease range was only 17% of the one treated with only Protamex. This might be because specific recognition sites of Protamex were destructed greatly by Neutrase hydrolysis(Rui et al., 2012). The pH decreased slowly from 65 min to 90 min. The final DH was 26.53% at 150 min (Table 8). In the second type of compound enzymatic hydrolysis, Protamex was added firstly. DH was 17.66% at 60 min by the end of first stage. In the second enzyme hydrolysis, pH decreased only 0.17 and DH only increased 1.51%, which meant that hydrolysis effect of the second enzyme was very limited. The final DH of the second type of compound enzymatic hydrolysis under pH-spontaneous condition was 19.17%, which was higher than the DH of only Protamex, but lower than the DH of only Neutrase. It was also lower than the DH of the first type of compound enzyme under pH-spontaneous condition. So,

the first type of compound enzyme was selected.



a. Neutrase +Protamex



b. Protamex + Neutrase

Figure 4 Changes in pH of hydrolyzate of BPC treated with Neutrase +Protamex / Protamex + Neutrase under pH-spontaneous condition

Table 8 Degree of hydrolysis of BPC treated with compound enzymatic hydrolysis under pH-spontaneous condition

Compound enzyme	DH, %
Neutrase + Protamex	26.53%
Protamex + Neutrase	19.17%

3.2.3 Analysis of hydrolysates treated with double-enzyme hydrolysis under pH-controller and pH-spontaneous condition

As is shown in Table 9, the protein content of hydrolysates of compound enzyme Neutrase and Alcalase under pH-controlled condition was lower than that of hydrolysates of compound enzyme Neutrase and Protamex under pH-spontaneous condition. This might be because protein content was determined by Kjeldahl Method and it was calculated on basis of nitrogen content. During enzymatic hydrolysis, peptide bonds were cut off and combined with a water molecule, forming carboxyl group and amino group. So, when amino acid number stayed same, total quality of peptides increased for combining with water molecules. And protein content of higher hydrolytic degree sample was lower when calculating the nitrogen content of sample of the same quality. In the hydrolysis of compound enzyme Neutrase and Alcalase under pH-controlled condition, DH was higher, which meant more proteins were hydrolyzed to peptides. So, its protein content was lower.

The ash content of hydrolysates under pH-controlled condition was 1.3 times that of hydrolysates under pH-spontaneous condition and 1.4 times that of BPC. This was because much more alkali salt was added in the reaction under pH-controlled condition. And an amount of alkali salt was also added in the reaction under pH-spontaneous condition to calculate DH using pH-stat method and adjust optimum pH before adding enzymes. So, the second process in Table 9 should be selected to simplify the further fractionation process.

Table 9 Main components of hydrolysates of two hydrolysis process

Process	Protein, g/100 g	Ash, g/100 g
Neutrase + Alcalase under pH-controlled condition	49.9	34.3
Neutrase + Protamex under pH-spontaneous condition	66.7	26.0
BPC	75.7	24.2

Figure 5 shows the ACE-inhibitory activity of hydrolysates treated with double-enzyme hydrolysis under pH-controlled and pH-spontaneous condition. The ACE-inhibitory activity of hydrolysates treated with Neutrase and Protamex under pH-spontaneous condition was 54.3% which was 3.76 times that of BPC. The ACE-inhibitory activity of hydrolysates treated with Neutrase and Alcalase under pH-controlled condition was 63.20% which was 4.39 times that of BPC. This indicated that compound enzymatic hydrolysis was an effective way to produce ACE inhibition peptides.

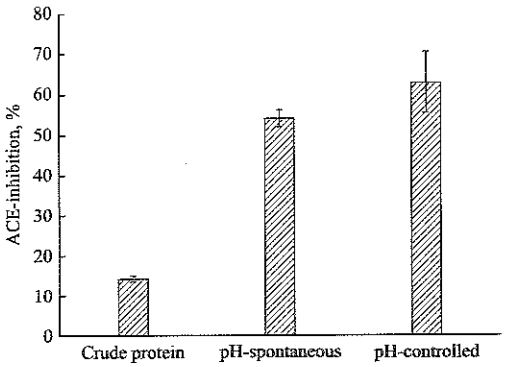


Figure 5 ACE-inhibitory activity of hydrolysates treated with double-enzyme hydrolysis under pH-controlled and pH-spontaneous condition

ACE-inhibitory activity of hydrolysates treated with compound enzymes was significantly higher than that of BPC (Table 10). The ACE-inhibitory activity of hydrolysates treated with Neutrase and Alcalase under pH-controlled condition was higher than that of hydrolysates treated with Neutrase and Protamex under pH-spontaneous condition, but the difference was not significant. It was reported that the tripeptide with C-terminal of aromatic amino acid and N-terminal of hydrophobic amino acid had higher ACE-inhibitory activity (Wu et al., 2006). So, ACE-inhibitory activity is associated with amino acid variety and branched chain type. But enzyme species and enzymatic hydrolysis processes are often different, branched chains and residues produced in hydrolysis are different correspondingly. So, although DH of hydrolysates treated with Neutrase and Alcalase under pH-controlled condition was higher than that of hydrolysates treated with Neutrase and Protamex under pH-spontaneous condition, the difference of ACE-inhibitory activity was not significant.

Table 10 Significance analysis of ACE-inhibitory activity of hydrolysates treated with double-enzyme hydrolysis under pH-controlled and pH-spontaneous condition

(I) VAR00001	(J) VAR00001				95% confidence interval	
		Mean difference (I-J)	Standard error	Significance	Lower limit	Upper limit
1	2	−39.90000*	3.68661	0.000	−48.9208	−30.8792
	3	−48.80000*	3.68661	0.000	−57.8208	−39.7792
2	1	39.90000*	3.68661	0.000	30.8792	48.9208
	3	−8.90000	3.68661	0.052	−17.9208	0.1208
3	1	48.80000*	3.68661	0.000	39.7792	57.8208
	2	8.90000	3.68661	0.052	−0.1208	17.9208

Notes: * at 0.05 significance level. 1. BPC, 2. Neutrase + Protamex hydrolysates under pH-spontaneous condition, 3. Neutrase + Alcalase hydrolysates under pH-controlled condition.

In addition, there were more differences in the operations of the three processes. First, alkali salt solution should be added continually in the pH-controlled process, which increased the difficulty of the experimental operations. Second, the alkali salt added in the hydrolysis process should be removed in the further fractionation stage, which would increase time and economic cost. Third, pH-controlled process required more time than pH-spontaneous process under the condition of same substrate amount and enzyme concentration.

So, the process of compound enzymes Neutrase and Protamex under pH-spontaneous condition was selected to hydrolyze BPC. The first stage of hydrolysis ended at 60 min and the second stage ended at 150 min with the DH of 26.53%. The hydrolysates were freeze dried for further fractionation.

3.3 Fractionation by Sephadex G-15 and semi-preparative HPLC

After fractionation by Sephadex G-15, three fractions were collected. ACE-inhibitory activity increased as molecular weight of fractions decreased. Fraction P3 which had the highest ACE-inhibitory activity was selected for further study (Figure 6).

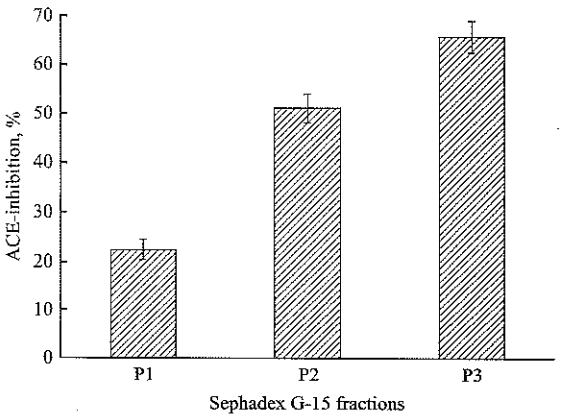
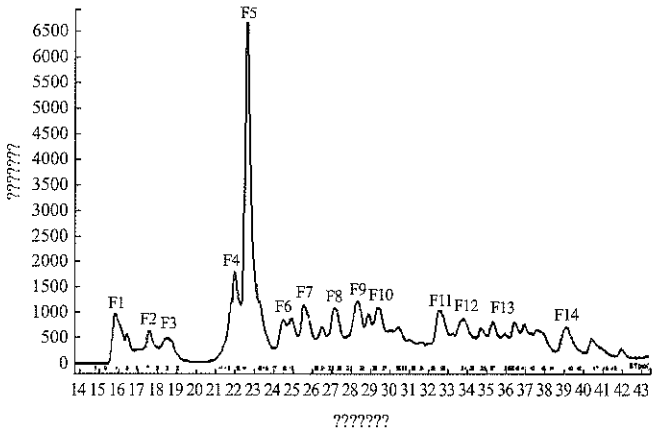


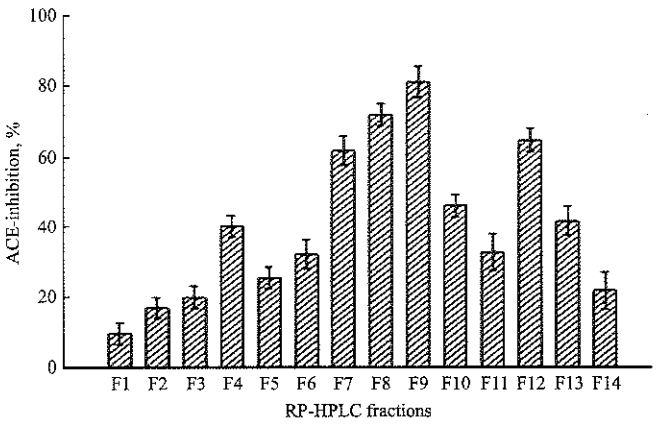
Figure 6 ACE inhibition of Sephadex G-15 fractions

Fraction P3 that had the highest ACE-inhibitory activity was selected from fractions separated by Sephadex G-15 column. Then it was separated by semi-preparative HPLC (Figure 7). Fourteen main components were collected. Hydrophobicity of components increased over elution time. And fraction F9 had the highest ACE-inhibitory activity of 81.17%. F9 was collected by repeating injection and freeze dried for

determination of amino acid sequences. ACE-inhibitory activity of peptides was often related to hydrophobicity, composition and content of hydrophobic amino acids(Farvin et al., 2016). But as shown in Figure 10, ACE-inhibitory activity did not increase over elution time, which indicated that hydrophobicity of amino acids did not affect ACE-inhibitory activity. So, amino acid sequences of peptides should be determined to analyze the structure-function relationship of ACE-inhibitory peptides.



a. Chromatography of fraction P3 separated by semi-preparation RP-HPLC



b. ACE-inhibitory activity of each fraction in chromatography of RP-HPLC

Figure 7 Fractionation by semi-preparative HPLC and ACE-inhibitory activity assay

3.4 Characterization and sequencing of peptides by LC–MS/MS

Fraction F9 which had the highest ACE-inhibition was analyzed by LC-MS/MS. Amino acid sequences were deduced by analyzing ESI-MS chromatograms of the major peptides (Table 11). By comparing the data base of Proteome Discoverer, the amino acid sequences of five peptides were selected, namely, DSTLIMQLLR, SAEYVRLY, LDWYKGPT, IYAPHWN, EGSLLLPHYNSR. These showed a marked hydrophobic character being rich in amino acids as leucine (L), proline (P) and isoleucine (I). It was reported that ACE showed preference for competitive inhibitors that contain a hydrophobic amino acid at the third position from the C-terminal (Fang et al., 2008, Lau et al., 2013). This is in accordance with the amino acid sequence of peptides identified. It was established that a tyrosine residue (Y) at the amino terminus was a common motif of the peptides with the most potent vasodilator activity when assayed in mesenteric arteries of rats (Garcia-Redondo et al., 2010).

Table 11 Predominant peptide sequences identified by LC-MS/MS

Mass obs. $[M+H]^+$	Retention time, min	MS(2) fragments	Putative sequence
1189.66	67.00	600.38, 773.47, 355.07, 286.10 *a.m.o*	DSTLIMQLLR
1000.51	39.21	131.08, 159.08, 713.39 *a.m.o*	SAEYVRLY
979.49	42.00	159.09, 207.11, 302.11, 402.23 *a.m.o*	LDWYKGPT
900.43	36.37	136.07, 249.16, 456.20 *a.m.o*	IYAPHWN
1385.71	36.58	773.36, 694.30, 487.15, 263.10 *a.m.o*	EGSLLLPHYNSR

4 Conclusions

The results obtained in the present study suggest that method of ultrasonic-assisted alkali-solution and acid-isolation could enrich protein and remove most starch of broad beans. The analysis of amino acid composition and hydrophobicity indicated that BPC had high proportion of hydrophobic amino acids, aromatic amino acids and Proline, which made it good resource of antihypertensive peptides. The process of compound enzymes Neutrase and Protamex under pH-spontaneous condition was selected to hydrolyze BPC. The first stage of hydrolysis ended at 60 min and the second stage ended at 150 min with the DH of 26.53%. The ACE-inhibition of hydrolyzates was 54.31%. Three fractions were collected by fractionation on Sephadex G-15 using eluent of ultrapure water at speed of 1.87 mL min^{-1} and fraction P3 proved to have the highest ACE-inhibition, which was 65.93%. P3 was further fractionated by semi-preparative RP-HPLC and 14 fractions were collected. Fraction F9 proved to have the highest ACE-inhibition, which was 81.17%. Amino acid sequences of fraction F9 in RP-HPLC were analyzed by LC-MS/MS. The result showed F9 mainly consisted of five peptides, namely,

DSTLIMQLLR, SAEYVRLY, LDWYKGPT, IYAPHWN, EGSLLLPHYNSR. These results suggested that broad bean peptide hydrolysates had potential antihypertensive activity and could be developed into new functional ingredients with this enzymatic food grade preparation. However, further studies need to be performed in future using other models to reinforce and confirm the results obtained.

Acknowledgments

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