Production and characterization of chicken blood hydrolysate with antihypertensive properties

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ABSTRACT Chicken blood has limited utilization despite its high protein content. Production of a blood hydrolysate exhibiting angiotensin I-converting enzyme (ACE)–inhibitory activity would be means of valorizing chicken blood. The optimized conditions used to produce chicken blood corpuscle hydrolysate (BCH) by Alcalase were 51.1°C, 4% enzyme, and pH 9.6 for 6 h, resulting in a 35.8% degree of hydrolysis and 37.7% ACE inhibition at a peptide concentration of 0.2 mg/mL. The permeate of a 1-kDa membrane, BCH-III, showed a 2.5-fold increase in ACE inhibition compared with that of BCH. BCH-III was resistant to in vitro gastrointestinal digestion, whereas the BCH digesta exhibited an increased ACE-inhibitory activity after digestion. Both BCH and BCH-III were rich in hydrophobic amino acids. A single administration of BCH and BCH-III to spontaneously hypertensive rats at concentrations of 600 and 100 mg/kg, respectively, lowered the systolic blood pressure by −57.7 and −70.9 mmHg, respectively, 6 h after oral administration compared with the control group. The blood pressure–lowering effect of the 600 mg/kg BCH dose was comparable with that of the 100 mg/kg BCH-III dose after 4 wk of oral administration. Both BCH and BCH-III could be developed for use as nutraceutical products with antihypertensive effects.

Key words: angiotensin I-converting enzyme (ACE), chicken blood, hypertension, protein hydrolysate, spontaneously hypertensive rat (SHR)

INTRODUCTION

Chicken meat is one of the most important sources of food protein, and approximately 92.5 million MT of chicken meat were produced in 2018 (USDA, 2018). Blood is a byproduct produced by slaughterhouse and constitutes approximately 3–5% of BW. Whole blood (WB) is composed of blood cells and plasma, which account for 40–45 and 60% of total weight, respectively (Parés et al., 2011). Most chicken blood is converted to low-value animal feed or blood cubes used as inexpensive sources of protein and iron in Asian diets. Means of increasing the value of chicken blood for human consumption should be sought.

It has been shown that protein hydrolysates possess various bioactive properties, including antihypertensive, antioxidant, immunomodulatory, antimicrobial, prebiotic, mineral binding, antithrombotic, and hypocholesterolemic effects (Arihara, 2006). Angiotensin I-converting enzyme (dipeptidyl carboxypeptidase, EC 3.4.15.1) (ACE) catalyzes the formation of the potent vasoconstrictive octapeptide (angiotensin II) from decapeptide (angiotensin I) and inactivates the vasodilative peptide, bradykinin (Cushman et al., 1977). Thus, ACE plays a key role in controlling blood pressure. The inhibition of ACE is considered to be a useful therapeutic approach for the treatment of hypertension. Peptides from various food sources, including canola, flaxseed, rice, milk, chicken skin, cod, eggs and oysters, have been reported to exhibit an ACE-inhibitory activity (Li et al., 2007; Wang et al., 2008; Udenigwe et al., 2009; Yamaguchi et al., 2009; Alashi et al., 2014; Yu et al., 2014; Girigih et al., 2015; Onuh et al., 2015). Despite the good in vitro inhibition activities of some hydrolysates/peptides, they failed to show their efficacy in vivo (Cushman and Cheung, 1971; Marques et al., 2012). Therefore, an animal study would be necessary to confirm the antihypertensive properties of protein hydrolysate.

The types of proteases and substrates are critical factors governing types of peptides and their ACE-inhibition capacity. Blood corpuscles (BC) are composed...
of various proteins, red blood cells (or erythrocytes), white corpuscles (leukocytes), and platelets and contain approximately 35% proteins, and hemoglobin is the predominant protein in red corpuscles; meanwhile, blood plasma (BP) contains more than 8% proteins, with albumin and globulins being the predominant ones (Ockerman and Hansen, 2000). The optimal hydrolysis condition is varied with blood proteins and proteases applied. Sampedro and Montoya (2014) reported that Alcalase-hydrolyzed bovine plasma showed the highest ACE-inhibitory activity, with a degree of hydrolysis (DH) of 6.7%, whereas the hydrolysate of red blood cells obtained from a mixture of trypsin, chymotrypsin, and thermolysin showed the highest ACE-inhibitory activity, with the half maximal inhibitory concentration (IC$_{50}$) of 0.58 mg/mL (Wei and Chiang, 2009). In addition, the pepsin hydrolysate obtained by hydrolyzing porcine hemoglobin at a pH of 2.0 and a temperature of 37°C for 6 h exhibited the highest ACE-inhibitory activity, with an IC$_{50}$ of 0.02 mg/mL (Deng et al., 2014). Proteases from Virgibacillus sp. SK1-3-7 have been shown to exhibit fibrinolytic activity (Montriwong et al., 2012), but their ability to produce ACE-inhibitor peptides from blood proteins has not been proven. The selection of a proper blood substrate and enzyme is a critical step for the production of hydrolysates with ACE-inhibitory activities, which have not been well established thus far. In addition, the factors affecting the hydrolysis process, including the substrate: enzyme ratio, hydrolysis time, temperature, and pH, also affect the yield and bioactivity of hydrolysates. Response surface methodology (RSM) has been proposed as an effective tool for optimizing the production of bioactive hydrolysates (Vermeirssen et al., 2002).

To illustrate the antihypertensive effect, spontaneously hypertensive rats (SHR) are typically used as the animal model. A single-dose administration has been widely conducted to demonstrate the antihypertensive potential of various protein hydrolysates, including those derived from cod, peas, and flaxseed (Nwachukwu et al., 2014; Girgih et al., 2015, 2016). However, this test does not reflect the use of nutraceutical products, which consumers are expected to consume for a certain period of time or even on a regular basis. A long-term feeding trial would provide insights regarding the peptide effectiveness, which is more pertinent than single-dose tests for assessing the antihypertensive potential of protein hydrolysates for their applications in nutraceutical products. Thus far, the in vivo antihypertensive effect of chicken blood hydrolysate both in the short and long term has not been established.

The objectives of this study were to determine a proper blood substrate (WB, BP, and BC) and protease (Alcalase 2.4 L, papain, thermolysin, porcine pepsin, and Virgibacillus sp. SK1-3-7) for the production of chicken blood hydrolysate with an ACE-inhibitory activity. In addition, the optimized hydrolytic condition was determined using RSM. The antihypertensive effect experienced after a short (12 h) and long (4 wk) period of oral administration was evaluated and compared based on the SHR model.

### MATERIALS AND METHODS

#### Samples and Chemicals

Chicken blood samples were obtained from a commercial chicken slaughterhouse in Nakhon Ratchasima, Thailand. Birds were exsanguinated by stunning with electrocution before a conventional neck cut as per Genesis GAP Chicken Production standards. Blood was collected approximately 100 mL from each bird using sterile containers that contained sodium citrate solution at a final concentration of 1% (w/v), which was used as an anticoagulant. The collected blood from 50 birds was pooled and transported to the Suranaree University of Technology within 1 h upon collection. The sample was maintained at 5°C during collection and transportation. On arrival, the BP and BC were immediately separated by centrifuging the WB at 2,530 × g and 4°C for 15 min (Hitachi CR22GIII, Hitachi Koki Co. Ltd., Tokyo, Japan). All the blood fractions were lyophilized. The proximate composition including CP by Kjeldahl method, crude fat by the Soxhlet extraction, moisture, and ash content of lyophilized samples was determined as per the AOAC (2010).

Alcalase 2.4 L (EC 3.4.21.62) from Bacillus licheniformis (Novozymes Bagsvaerd, Denmark), papain (EC 3.4.22.2, 10 units/mg) from papaya latex, thermolysin (EC3.4.24.27, 40 units/mg) from Bacillus thunroproteolyticus rokko, and porcine pepsin (EC3.4.23.1, 596 units/mg) were purchased from Sigma-Aldrich (St. Louis, MO). The proteases from Virgibacillus sp. SK1-3-7 were prepared as described by Montriwong et al. (2012). Trifluoroacetic acid was purchased from Supelco (Belleville, PA). Angiotensin I-converting enzyme, hippuryl histidyl leucine, and 2,4,6-trinitrobenzenesulfonic acid (TNBS) were purchased from Sigma Chemical Co. (St. Louis, MO). Other chemicals and reagents used were of analytical grade.

#### Enzymatic Hydrolysis

**Selection of the Enzyme and Chicken Blood Fraction** Each lyophilized powder of the WB, BP, and BC was dissolved in deionized water to obtain a final concentration of 10 g/L. The pH of the suspension was adjusted to the optimum value of each enzyme using either a 1 M NaOH or 1 M HCl solution: a pH of 8 for Alcalase, Vigibacillus sp. SK1-3-7, and thermolysin; a pH of 7 for papain; and a pH of 2 for pepsin. The enzymes were added to achieve an enzyme to substrate ratio of 1:100. The mixtures were incubated in a shaking water bath at the optimum temperature of each protease: 60°C for Alcalase, Vigibacillus sp. SK1-3-7, and papain; 70°C for thermolysin; and 37°C for pepsin. The enzymatic reaction was allowed to proceed for 12 h. The pH of each reaction mixture was maintained at the optimal pH of the enzyme every 15 min during hydrolysis using 1 M HCl for pepsin and 1 M NaOH for the others. To terminate the reaction, the mixtures were heated in a water bath at 95°C for 10 min and cooled.

**Selection of the Blood Fraction** The objectives were to determine a proper blood fraction include WB, BP, and BC by using a method of a single-dose administration. The effectiveness of each blood fraction was evaluated based on the SHR model.
immediately. After hydrolysis, the pH of the solution was adjusted to 7 using either 1 M NaOH or 1 M HCl. The hydrolysates were centrifuged at 10,000 × g for 15 min. The resultant supernatants were stored at −20°C until further use.

**Experimental Design of Optimization** The Alcalase-hydrolyzed BC (BCH) exhibiting the highest ACE-inhibition activities were selected for the optimization using RSM. All the experiments were performed in accordance with a rotatable central composite design. Three controllable variables, including the temperature ($X_1$: 50°C–60°C), enzyme concentration ($X_2$: 2–4%), and time ($X_3$: 4–6 h), were selected for optimization. The reaction was performed at pH 9.6 ± 0.2, which was found to be optimal for BC hydrolysis. These variables were coded to 5 levels as −1.68, −1, 0, +1, and +1.68. The degree of hydrolysis (DH: $Y_1$) and ACE inhibition ($Y_2$) were used as a response of the independent variables. Twenty experimental runs with 3 replicates, resulting in the total of 60 runs, were performed. The behavior of the system can be described with a quadratic model as per the following equation:

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_i^2 X_i^2 + \sum_{i=1}^{3} \sum_{j=i+1}^{3} \beta_{ij} X_i X_j$$

where $Y$ was the predicted response, $\beta_0$ was a constant, $\beta_i$ was the linear coefficient, $\beta_i^2$ was the quadratic coefficient, $\beta_{ij}$ was the interaction coefficient, and $X_i$ and $X_j$ were independent variables as shown in Table 1. The model was applied to characterize the effect of each independent variable to the response.

### Analysis of Protein Hydrolysates

#### Degree of Hydrolysis Determination

The DH was determined by the TNBS method (Adler-Nissen, 1979). The samples (0.5 mL) were added to 0.5 mL of 0.2125 M sodium phosphate buffer (pH 8.2) and 0.5 mL of a 0.05% (w/v) TNBS reagent. The reaction mixtures were incubated at 50°C for 60 min, and the reaction was subsequently stopped by adding 1.0 mL of 0.1 M HCl. The samples were allowed to cool at room temperature for 30 min, and the absorbance was measured at 420 nm. l-Leucine (0–2.0 mM) was used as a standard. The DH (%) was calculated using the following equation:

$$DH (\%) = \frac{[h_a - h_o]}{h_{tot}} \times 100$$

where $h_a$ was the $\alpha$-amino content of the samples, $h_o$ was the $\alpha$-amino content of the sample at time 0, and $h_{tot}$ was the total $\alpha$-amino content obtained after hydrolyzing the samples with 6 M HCl at 121°C for 24 h.

### Angiotensin I-Converting Enzyme Inhibition

The in vitro ACE inhibition was assayed using hippuryl histidyl leucine as a substrate as per the method of Cushman and Cheung (1971) with slight modifications. The reaction mixture contained 50 μL of hydrolysates and 150 μL of the ACE substrate (8.3 mM hippuryl histidyl leucine in 50 mM sodium borate buffer containing 0.5 mM NaCl, pH 8.3) and was preincubated at 37°C for 10 min. Subsequently, 50 μL of 25 μM/mL ACE was added, and the mixture was further incubated at 37°C for 60 min in a shaking water bath. The reaction was terminated by adding 250 μL of 1 mM HCl. Hippuric acid (HA) was extracted with 1.5 mL of ethyl acetate.

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**Table 1.** Central composite design with experimental and predicted values of degree of hydrolysis (DH) and angiotensin I-converting enzyme (ACE) inhibition.

| Run Order | $X_1$ (°C) | $X_2$ (%) | $X_3$ (h) | Temperature (°C) | Enzyme (%E) | Time (h) | DH (%) | ACE inhibition (%) |
|-----------|-----------|-----------|----------|-----------------|-------------|----------|--------|-------------------|
| 1         | −1        | −1        | −1       | 50              | 2           | 4        | 25.59  | 26.32             |
| 2         | −1        | −1        | −1       | 50              | 2           | 6        | 29.66  | 30.26             |
| 3         | 1         | 1         | 1        | 50              | 4           | 4        | 29.28  | 29.87             |
| 4         | 1         | 1         | 1        | 50              | 4           | 6        | 33.80  | 34.32             |
| 5         | 1         | −1        | −1       | 60              | 2           | 4        | 21.80  | 22.16             |
| 6         | 1         | −1        | −1       | 60              | 2           | 6        | 22.07  | 22.36             |
| 7         | 1         | 1         | 1        | 60              | 4           | 4        | 25.12  | 25.40             |
| 8         | 1         | 1         | 1        | 60              | 4           | 6        | 26.17  | 26.21             |
| 9         | 0         | 0         | 0        | 55              | 3           | 5        | 31.18  | 31.42             |
| 10        | 0         | 0         | 0        | 55              | 3           | 5        | 30.98  | 31.42             |
| 11        | 0         | 0         | 0        | 55              | 3           | 5        | 31.95  | 31.42             |
| 12        | 0         | 0         | 0        | 55              | 3           | 5        | 32.48  | 31.42             |
| 13        | 0         | 0         | 0        | 55              | 3           | 5        | 32.00  | 31.42             |
| 14        | 0         | 0         | 0        | 55              | 3           | 5        | 29.74  | 31.42             |
| 15        | 0         | −1.68     | 0        | 55              | 1.32        | 5        | 26.98  | 26.17             |
| 16        | 0         | −1.68     | 0        | 55              | 3           | 3.32     | 28.71  | 27.90             |
| 17        | 0         | 1.68      | 0        | 55              | 3           | 6.68     | 32.24  | 31.81             |
| 18        | 0         | 1.68      | 0        | 55              | 4.68        | 5        | 32.74  | 32.31             |
| 19        | −1.68     | 0         | 0        | 46.59           | 3           | 5        | 29.29  | 28.21             |
| 20        | 1.68      | 0         | 0        | 63.41           | 3           | 5        | 17.96  | 17.81             |

Actual values are expressed as mean (n = 3). Experimental conditions according to a rotatable central composite design (RCCD).

Abbreviations: $X_1$, Temperature (°C); $X_2$, Enzyme (%); $X_3$, Time (h).

<sup>1</sup>Actual and predict value are not different (P > 0.05).
The upper layer of ethyl acetate (1 mL) was dried at 80°C on a sand bath. The dried sample was dissolved in 1 mL of deionized water, and the absorbance was read at 228 nm in a quartz cuvette. Hippuric acid was used as an external standard. The blank was prepared by adding 0.1 mM HCl before adding ACE. The ACE inhibition (%) was calculated as follows:

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ACE\ inhibition\ (%) = \frac{(C_0 - C_B) - (I_0 - I_B)}{(C_0 - C_B)} \times 100
\]

where \(C_p\) was the HA content of the reaction of the control (without protein hydrolysates), \(C_B\) was the HA content of the control blank, \(I_0\) was the HA content of the reaction with protein hydrolysates, and \(I_B\) was the HA content of the reaction blank. Each sample was analyzed in 3 replicates.

**Ultrafiltration**

The BCH prepared under the optimized condition was fractionated by ultrafiltration first using a membrane with a molecular weight cutoff of 30 kDa (Pall, East Hills, NY). The fractions were designated as follows: BCH-I denotes the retentate of the 30 kDa membrane (>30 kDa); BCH-II denotes the retentate of the 1 kDa membrane obtained from the 30 kDa permeate (1–30 kDa); and BCH-III denotes the permeate of the 1 kDa membrane (<1 kDa). The BCH and all the ultrafiltered fractions were lyophilized and stored at −20°C until use. The \(\alpha\)-amino group content of all the fractions was determined using the TNBS method (Adler-Nissen, 1979). The ACE inhibition and molecular weight distribution of all the samples were subsequently determined.

**In Vitro Gastrointestinal Digestion**

BCH-III (permeate of 1 kDa), which was the fraction showing the highest ACE inhibition, and BCH were subjected to in vitro gastrointestinal (GI) digestion using pepsin and pancreatin as described by Cinq-Mars et al. (2008) with some modifications. The lyophilized powder (250 mg) was dissolved in deionized water (15 mL). The pH of the mixture was adjusted to 2.0 using 6 M HCl, and pepsin was added to achieve an enzyme-to-substrate ratio of 1:35 and incubated at 37°C for 1 h. Subsequently, the pH was adjusted to 5.3 with a saturated NaHCO\(_3\) solution and further to a pH of 7.5 with 10 M NaOH. The mixture was further digested by porcine pancreatin using an enzyme-to-substrate ratio of 1:25 (w/w) for 2 h at 37°C. The reaction was terminated by boiling for 10 min, which was followed by centrifugation at 10,000 \(\times\) g for 10 min. The \(\alpha\)-amino group content, ACE inhibition, and amino acid profiles of the digesta were determined.

**Amino Acid Analysis of Peptides**

The total amino acid profiles of BCH, BCH-III, and their respective digesta in the lyophilized form were determined by following AOAC 982.30 (AOAC, 2000). Total amino acid content was determined by digesting sample (25 mg) with 50 mL of 6 M HCl containing 0.1% phenol at 110°C for 24 h. The hydrolysis by performic acid and sodium metabisulfite was carried out to determine the cysteine and methionine content. The free amino acid contents of the samples were also determined by mixing the lyophilized samples with 0.1 M HCl to achieve a sample-to-acid ratio of 1:9 (w/v) and then centrifuging at 10,000 \(\times\) g for 20 min. The supernatant (1 mL) was mixed with 5% sulfosalicylic acid (1 mL) and left for 3 h before centrifuging at 10,000 \(\times\) g for 20 min. The quantitative analysis of the amino acids was performed using a Biochrom 30 Amino Acid Analyzer (Biochrom Ltd., Cambridge Science Park, England). Norleucine was used as an internal standard. The post-column derivatization of the amino acids with ninhydrin was monitored at 570 nm and at 440 nm for proline. The amino acids in net peptides were calculated as differences between total amino acids and free amino acids and expressed as g/100 g powder.

**Antihypertensive Effect**

The SHR (9–12 wk of age, male, 220–250 g BW, and blood pressures more than 180 mmHg) were purchased from Charles River Japan, Inc. (Yokohama, Japan). The SHR were housed individually in steel cages with a controlled room temperature (25°C ± 1°C), humidity of 55 ± 5%, and 12L:12D cycle. The SHR were allowed to freely access food and water. The rats were acclimatized in the aforementioned conditions for approximately 2 wk before the experiment. The experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The experimental protocols were approved by the Ethics Committee on Animal Experimentation of Shinshu University. For the single-administration experiment, the SHR were randomly divided into 8 groups with 6 rats in each group. The rats were orally administered BCH and BCH-III at dosages of 100, 300, and 600 mg/kg BW/day; whereas BCH-III was administered at dosages of 50, 100, and 200 mg/kg BW/day. The negative control group was administered tap water, whereas captopril was administered at 10 mg/kg BW/day for the positive control. The rats of each treatment group were orally administered their daily dose at 8 am, and their blood pressure was measured first at 11 am and, then, every 3 h onwards for 12 h by the tail-cuff method (Cushman and Cheung, 1971). The rat was placed in a restraining device that fit the rat securely and allow for protrusion of the tail (MK2000ST; Muromachi Kikai Co., Ltd., Tokyo, Japan). A cuff was placed around the tail to measure the systolic blood pressure (SBP) and diastolic blood pressure (DBP).

In a separate experiment, the SHR were randomly divided into 4 groups with 6 rats in each group. The rats were daily administered BCH and BCH-III at dosages of 600 and 100 mg/kg BW/day, respectively, for 4 wk. These were effective concentrations determined
from a single-dose study. The negative control group was administered tap water, whereas captopril was administered at a dosage of 10 mg/kg BW/day for the positive control. Daily oral administration was carried out at 8 am, while the SBP and DBP were recorded weekly at 2 pm during the 4-wk feeding trial by a tail-cuff method.

**Statistical Analyses**

ANOVA was performed to determine the effect of the substrate, enzymes, and incubation time on the DH and ACE inhibition. Duncan’s multiple range test was used to determine the differences between the mean at $P < 0.05$. A statistical analysis was performed using the SPSS package (SPSS 17.0 for Windows, SPSS Inc., Chicago, IL). The response data for RSM were analyzed by Design Expert v. 8.0.6.1 (Stat-Ease, Inc., Minneapolis, MN).

**RESULTS AND DISCUSSION**

**Selection of the Enzyme and Chicken Blood Fractions**

The CP content of the lyophilized WB, BP, and BC was 75.55 ± 0.12, 47.55 ± 0.3, and 90.79 ± 0.45% dry basis, respectively. All the samples also contained a relatively low-fat content of 0.45–0.67% dry basis. The DH and ACE inhibition of chicken blood hydrolysate varied depending on the blood fractions and proteases applied (Figure 1). The highest DH value of 14% was found for the hydrolysates prepared from the BC hydrolyzed by either Alcalase or thermolysin ($P < 0.05$). The Alcalase-hydrolyzed WB also had a comparable high DH of 13% (Figure 1A). In general, the chicken plasma exhibited a relatively low DH,
particularly when it was hydrolyzed by Alcalase and papain. The BC consist of hemoglobin, which is the major protein, and serve as a main substrate for hydrolysis. The limited hydrolysis of blood plasma proteins could be partly attributed to endogenous protease inhibitors, such as α2-macroglobulin, serpins, and α1-antitrypsin, which are present in BP (Aubry and Bieth, 1976).

Alcalase is a known serine endopeptidase derived from *B. licheniformis* and has a broad specificity (Nagodawithana and Reed, 1993), whereas thermolysin produced from *Bacillus thermoproteolyticus* was specific toward hydrophobic amino acid residues including phenylalanine (F), isoleucine (I), leucine (L), or tyrosine (Y) (Matsubara and Feder, 1971). *Virgibacillus* sp. SK1-3-7 and pepsin were able to hydrolyze the plasma proteins to a greater extent than papain and Alcalase. Montriwong et al. (2012) reported that *Virgibacillus* sp. SK1-3-7 endopeptidases showed a rather broad specificity and a high activity toward fibrin and fibrinogen, which are important proteins in BP. These results demonstrated that the extent of hydrolysis largely depends on the nature of the substrate and the type of protease.

The highest ACE inhibition of 61.8 and 63.8% was found in the BC hydrolyzed by Alcalase and thermolysin, respectively, with concentrations of 0.5 mg/mL peptide (*P* < 0.05, Figure 1B), and these values corresponded to the highest DH. These results suggested that the smaller peptides exhibited higher ACE-inhibitory activities. Deng et al. (2014) reported that porcine hemoglobin hydrolyzed by pepsin exhibited the highest ACE-inhibitory activity at a 9.3%DH, whereas Sampedro and Montoya (2014) found that bovine plasma hydrolysates obtained from Alcalase 2.4 L showed the maximum ACE-inhibitory activity of 50.2% at a 6.7% DH. The porcine BC hydrolyzed by a mixture of thermolysin, chymotrypsin, and trypsin also had an IC_{50} value for ACE-inhibitory activity of 0.58 mg/mL (Wei and Chiang, 2009). Blood proteins, particularly BC, appeared to be good sources of substrates for ACE-inhibitor peptides. Therefore, BC and Alcalase were selected for further optimization.

### Optimization of the Hydrolysis Process

The predicted and experimentally measured responses obtained with 20 runs and 3 replicates and performed in accordance with the experimental design are shown in Table 1. The DH (*Y_1*) ranged from 17.96 to 33.80%, whereas the ACE inhibition (*Y_2*) ranged from 20.04 to 68.8%, which was a two-fold increase compared with that achieved with the same peptide concentration (Figure 3). The liberation of protons by peptide cleavage leads to a decrease in the pH of the reaction in which the pH falls below the particular condition. For this reason, the hydrolysis process was carried out under the optimized condition with the pH controlled at 9.6 ± 0.2, the ACE inhibition increased to 68.8%, which was a two-fold increase compared with that achieved with the same peptide concentration (Figure 3).

The DH gradually increased with increasing amounts of enzyme in the reaction and reached a maximum at 4% enzyme and after 6 h of hydrolysis, whereas increasing the temperature up to 60°C appeared to lower the DH (Figures 2A, 2B). The ACE inhibition also showed the same trend as the DH (Figures 2D–2F). High DH indicated extensive hydrolysis that yielded shorter peptides. Shorter peptides (<10 amino acid residues) have been reported to exhibit higher ACE-inhibitory activity (Ruiz-Ruiz et al., 2013). The strong correlation observed between the DH and ACE inhibition agreed with the observations made by van der Ven et al. (2002) who reported that a high DH led to a high ACE inhibition of whey protein hydrolysates. Deng et al. (2014) also found that the ACE inhibition of porcine hemoglobin hydrolyzed by Alcalase increased as the DH increased. To confirm the validity of the quadratic regression model, some conditions within the design space were evaluated to compare the responses with the predicted values (Table 2). The obtained responses were comparable with the predicted values (*P* > 0.05), suggesting that the models satisfactorily and reliably described the ACE-inhibitory activity and DH of chicken BC hydrolysate.

Based on the optimized condition in which the samples were hydrolyzed at 51.1°C for 6 h with 4% enzyme, the hydrolysis process yielded a maximum DH of 35.8% and an ACE inhibition of 37.7% with a peptide concentration of 0.2 mg/mL peptide (Table 2). The hydrolysis process was carried out under the optimized conditions with the pH controlled at 9.6 ± 0.2, the ACE inhibition increased to 68.8%, which was a two-fold increase compared with that achieved with the same peptide concentration (Figure 3). The liberation of protons by peptide cleavage leads to a decrease in the pH of the reaction in which the pH falls below the
optimal pH of Alcalase. The pH control process would help maintain the enzyme activity during the course of hydrolysis, resulting in a significant increase in the DH and ACE inhibition.

### Ultrafiltration and In Vitro GI Digestion

BCH-III, the permeate of the 1-kDa membrane, showed the highest ACE inhibition, with an IC$_{50}$ value of 0.138 mg/mL ($P < 0.05$, Table 3). The ACE-inhibition

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**Table 2.** Validation of the cubic models of DH and ACE inhibition within the design space.

| Parameters | DH (%) | ACE inhibition (%) |
|------------|--------|--------------------|
| Temperature (°C) | Enzyme (%) | Time (h) | Actual | Predict | Actual | Predict |
| 50 | 4 | 6 | 34.55 | 34.32 | 37.01 | 36.31 |
| 50.31 | 4 | 6 | 34.64 | 34.42 | 36.85 | 36.38 |
| 50 | 3.77 | 6 | 33.96 | 34.16 | 35.84 | 36.03 |
| 51.07 | 4 | 6 | 35.83 | 34.58 | 37.70 | 36.50 |
| 50 | 2.78 | 6 | 32.33 | 32.58 | 33.77 | 34.09 |
| 52.22 | 3.97 | 5.99 | 34.82 | 34.53 | 36.15 | 36.33 |
| 51.81 | 3.97 | 5.95 | 34.91 | 34.56 | 36.51 | 36.36 |

Actual values are expressed as mean (n = 3). **Bold** indicates the optimized hydrolysis condition obtained from RSM. Abbreviations: ACE, angiotensin I-converting enzyme; DH, degree of hydrolysis; RSM, response surface methodology.

$^1$Actual and predict value are not different ($P > 0.05$).
potency of BCH-III increased by 2.5 times compared with that of crude BCH. Peptides with smaller molecular weights could be responsible for inhibiting ACE. Cinq-Mars and Li-Chan (2007) also reported that the hydrolysate derived from Pacific hake (Merluccius productus) and obtained from ultrafiltration showed a higher ACE inhibition than crude hydrolysate.

The GI digestion process increased the α-amino content of the samples ($P < 0.05$, Table 4). The increase in the α-amino content of BCH-III was less than that of crude BCH. This was due to the limited proteolysis of smaller peptides in BCH-III. The ACE inhibition of the BCH digesta increased by approximately 1.5 times ($P < 0.05$), respectively. Data were expressed as the mean values ($n = 3$). Abbreviation: ACE, angiotensin I-converting enzyme.

### Amino Acid Composition of Peptides

Crude BCH and BCH-III contained approximately 66–70% peptides, and the major amino acids of the peptides in both the samples were hydrophobic amino acids including F, glycine, alanine (A), valine (V), I, and L (Table 5). In general, the amino acid profile of BCH-III peptides was comparable with that of crude BCH, although that contents of A, L, and histidine in BCH-III were higher than those in crude BCH. Common residues for ACE inhibition are F, glycine, A, V, I, and L (Ondetti and Cushman, 1982; Li et al., 2004). After the in vitro GI digestion process was simulated, the peptides still remaining in both the digesta accounted for 49–57% of the total weight (Table 5). Hydrophobic amino acids were the most abundant amino acids in the peptides of the digesta, and polar amino acid residues were the second most abundant. The BCH-III digesta contained more hydrophobic peptides (47.7%) than the BCH digesta (39.1%). Phenylalanine, Y, proline, A, V, and L have been reported to be the most favorable amino acids for the antepenultimate position (S1) for ACE inhibition (Ondetti and Cushman, 1982). This might partly explain the higher ACE-inhibition potency of the BCH-III digesta (Table 4).

### Antihypertensive Effect

The BCH and BCH-III at all the studied concentrations decreased the SBP from 3 to 6 h after administration ($P < 0.05$, Figure 4A). The potency of BCH was comparable with that of BCH-III within 6 h of administration ($P > 0.05$). The maximum reduction in the SBP was achieved with a dose of 600 mg/kg BCH and 100–200 mg/kg BCH-III, which resulted in an SBP that was comparable with that achieved in the group administered with 10 mg/kg captopril ($P > 0.05$) within 6 h of oral administration. However, the hypertensive effect of BCH was reduced after 6 h, leading to an increase in the blood pressure after 6 h. In contrast, administering BCH-III maintained the SBP at the lowest values for the 12 h after the oral administration of a 100–200 mg/kg dose. The group administered with captopril also showed an SBP reduction for up to 12 h. The antihypertensive effect appeared to correspond to the

### Table 3. IC$_{50}$ value of ACE inhibition and peptide yield derived from chicken blood corpuscle hydrolysate obtained from sequential ultrafiltration.

| Sample         | α-amino content (mg) | IC$_{50}$ (mg Leu eqv/mL) | Purification fold | Yield (%) |
|----------------|----------------------|----------------------------|-------------------|-----------|
| BCH            | 317.5                | 0.341$^a$                  | 1                 | 100.0     |
| BCH-I (>30 kDa)| 46.1                 | 0.323$^a$                  | 1.06              | 17.5      |
| BCH-II (1–30 kDa) | 299.3               | 0.259$^b$                  | 1.32              | 94.6      |
| BCH-III (<1 kDa) | 150.5               | 0.138$^c$                  | 2.47              | 47.4      |

Value are expressed as mean ($n = 3$).
Different superscript letters at the column indicate significant difference ($P < 0.05$).
Abbreviations: ACE, angiotensin I-converting enzyme; BCH, blood corpuscle hydrolysates; IC$_{50}$, the half maximal inhibitory concentration.
ACE-inhibition effect achieved from the in vitro assay (Table 4). These results suggested that the smaller peptides in BCH-III were likely to be effective ACE inhibitors in vivo.

The reduction of the DBP in all the treatment groups was greater than that observed in the negative group 3 h after administration except for the 100 mg/kg BCH-treated group (P < 0.05, Figure 4B). Six h after administration, the greatest reduction of the DBP of 247.0 mmHg was found in the SHR administered with 100 mg/kg BCH-III, and this DBP reduction was comparable with that observed in the captopril-treated group (P < 0.05). The DBP values of the BCH-III-treated groups remained stable up to 12 h after administration. Therefore, the oral administration of BCH-III at a dose as low as 100 mg/kg effectively reduced the blood pressure and achieved longer lasting effects than that of BCH (P < 0.05).

This study demonstrated that a single dose of BCH-III produced antihypertensive effects up to 12 h after administration, and the dose of 100 mg/kg BCH-III was as effective as captopril. The crude BCH was only effective at a dose that was 6 times higher than that of BCH-III and only within 6 h of oral administration. The chicken blood protein hydrolysate evaluated in this study was comparable or more effective than protein hydrolysates obtained from other sources, as previously reported. Administering a BCH dose of 600 mg/kg reduced the SBP (−57.7 mmHg 6 h post-administration) more than rice protein hydrolysate administered with the same dose (−25.0 mmHg) (Li et al., 2007). In addition, the SBP reduction achieved by administering 100 mg/kg BCH-III was comparable with that achieved by the 3-kDa permeate of the pea protein hydrolysate administered at the same dose (Girigh et al., 2016). A similar SBP reduction was also observed for the flaxseed protein hydrolysate administered with a 200 mg/kg dose (Nwachukwu et al., 2014). These results suggested that the smaller peptides in BCH-III were likely to be more effective ACE inhibitors in vivo. The digesta of BCH-III contained a higher content of A than BCH (P < 0.05, Table 5). In addition, the total hydrophobic and basic amino acid content of the digesta of BCH-III was slightly higher than that of BCH (P < 0.05, Table 5). The short peptides generated from GI digestion and/or those originally present in the BCH-III sample, which contained specific sequences of hydrophobic/basic

| Amino acid      | Undigested | Digested | Undigested | Digested |
|-----------------|------------|----------|------------|----------|
| Proline (P)     | 8.11       | 5.87     | 7.68       | 7.12     |
| Glycine (G)     | 3.47       | 3.57     | 3.1        | 2.55     |
| Alanine (A)     | 5.94       | 6.80     | 4.19       | 6.47     |
| Valine (V)      | 5.80       | 5.28     | 4.23       | 4.10     |
| Isoleucine (I)  | 2.77       | 2.27     | 2.30       | 2.01     |
| Leucine (L)     | 7.28       | 8.57     | 5.78       | 6.02     |
| Methionine (M)  | 0.72       | 0.62     | 0.54       | ND       |
| Threonine (T)   | 2.94       | 3.30     | 2.60       | 2.42     |
| Serine (S)      | 2.51       | 1.87     | 1.75       | 1.56     |
| Tyrosine (Y)    | 2.23       | 2.00     | 1.89       | 1.2      |
| Phenylalanine (F)| 4.18      | 3.82     | 2.78       | 2.44     |
| Aspartic acid (D)| 4.12      | 3.50     | 3.37       | 2.04     |
| Glutamic acid (E)| 6.74      | 5.19     | 6.92       | 3.68     |
| Lysine (K)      | 6.10       | 6.67     | 5.02       | 4.02     |
| Histidine (H)   | 3.64       | 4.35     | 3.09       | 2.70     |
| Arginine (R)    | 2.86       | 2.57     | 2.00       | 1.14     |
| Hydrophobic (%)  | 29.44 (42.4) | 30.31 (45.8) | 22.38 (39.1) | 23.59 (47.7) |
| Polar (%)       | 12.58 (18.1) | 11.61 (17.5) | 9.56 (16.7) | 7.62 (15.4) |
| Acidic (%)      | 10.86 (15.7) | 8.69 (13.1) | 10.29 (18.0) | 5.72 (11.6) |
| Basic (%)       | 12.60 (18.2) | 13.59 (20.5) | 10.11 (10.1) | 7.86 (15.9) |
| Estimated peptides| 69.41     | 66.25    | 57.24      | 49.47    |

* and † indicate differences between BCH and BCH-III of parent hydrolysate and digesta, respectively in columns (P < 0.05).
* † indicate differences in estimated peptides of all samples in the same row (P < 0.05).
Abbreviation: ND, not detected.
1Amino acid composition of peptides = total amino acids–free amino acids.
2Numbers in parentheses indicate percentage of each type of amino acids based on total amino acids in peptide.
amino acids, could contribute to the hypertensive effects observed in the SHR. The identification of the antihypertensive peptide derived from BCH-III would shed light on the relationship between the structure of peptide(s) and their antihypertensive properties.

For a long-term study of 4 wk, administering BCH at a 600 mg/kg dose and BCH-III at a 100 mg/kg dose produced antihypertensive effects in terms of both the SBP and DBP throughout the 4 wk ($P < 0.05$, Figures 5A–5B). A reduction in the blood pressure was observed in the first week after the daily oral administration of either BCH or BCH-III, and BCH-III was more effective than BCH ($P < 0.05$, Figure 5A) in terms of the dose (100 vs. 600 mg/kg) and the extent of the SBP reduction (−53.0 vs. −45.3 mmHg). From the second week onward, BCH and BCH-III showed a comparable efficacy in reducing the SBP ($P > 0.05$, Figure 5A). BCH-III appeared to be more effective than BCH in reducing the DBP by the third week ($P < 0.05$, Figure 5B). By the fourth week, BCH and BCH-III exhibited comparable potencies, and their efficacy was lower than that of captopril ($P < 0.05$, Figures 5A–5B). Our study reveals that BCH-III was more effective than BCH when a single dose was administered, but both showed a comparable antihypertensive effect by the fourth week of oral administration at the studied concentrations. The effect of the long-term antihypertensive effects has been reported in hemp seed protein, soybean protein hydrolysate, and a permeate fraction of pea protein (Girigh et al., 2014, 2016; Daliri

Figure 4. Changes in the (A) systolic blood pressure and (B) diastolic blood pressure after the oral administration of crude blood corpuscle hydrolysate (BCH) and its 1-kDa permeate (BCH-III) in spontaneously hypertensive rats (SHR). Different letters at the same time indicated significant differences ($P < 0.05$). Data were expressed as the mean values ($n = 6$).
et al., 2019). The GI digestion of BCH was likely to generate active peptides that could be readily absorbed, exerting a comparable effect on BCH-III during the long-term study. These results suggested that both BCH and BCH-III could be developed as nutraceutical products with blood pressure-lowering properties. The identification of the peptide(s) responsible for the antihypertensive effect of BCH/BCH-III would be needed.

**CONCLUSIONS**

The hydrolysis of BC by Alcalase yielded hydrolysates with antihypertensive properties. The optimum condition was achieved using 4% enzyme at a temperature of 51.1°C and a controlled pH of 9.6 throughout the course of 6 h. BCH-III exhibited a higher ACE inhibition than crude BCH. Both BCH and BCH-III were rich sources of F, glycine, A, V, I, and L and showed antihypertensive effects 6 h after a single oral administration. BCH-III was more effective than BCH in lowering the blood pressure when a single oral dose was administered, but the former administered at a 100 mg/kg dose and the latter administered at a 600 mg/kg dose showed comparable efficacies during a 4-wk feeding trial. Thus, both BCH and BCH-III showed potential to be developed as nutraceutical products for lowering blood pressure.

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