Production and characterization of chicken blood hydrolysate with antihypertensive properties

W. Wongngam,[*] T. Mitani,[†] S. Katayama,[†] S. Nakamura,[†] and J. Yongsawatdigul[*,1]

[*]*School of Food Technology, Institute of Agricultural Technology, Suranaree University of Technology, Nakhon Ratchasima 30000, Thailand; and* [†]*Department of Bioscience and Biotechnology, Shinshu University, Nagano 399-4598, Japan*

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)

2020 Poultry Science 99:5163–5174
https://doi.org/10.1016/j.psj.2020.07.006

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

Received November 7, 2019.
Accepted July 18, 2020.

[1]Corresponding author: jirawat@sut.ac.th

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-inhibiting 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 ($\mathbf{IC_{50}}$) 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 thermoproteolyticus 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 (Bellefonte, 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

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:

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

where Y was the predicted response, β_0 was a constant, β_i was the linear coefficient, β_{ii} was the quadratic coefficient, β_{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:

$$\text{DH } (\%) = [(h_s - h_o) / h_{tot}] \times 100$$

where h_s was the α-amino content of the samples, h_o was the α-amino content of the sample at time 0, and h_{tot} was the total α-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 mU/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.

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

Independent variable							Response (Y_1)		Response (Y_2)	
	Coded			Actual			DH (%)[1]		ACE inhibition (%)[1]	
Run Order	X_1 (°C)	X_2 (%)	X_3 (h)	Temperature (°C)	Enzyme (%E)	Time (h)	Actual	Predicted	Actual	Predicted
1	−1	−1	−1	50	2	4	25.59	26.42	26.33	26.32
2	−1	−1	1	50	2	6	29.66	30.26	31.40	31.48
3	−1	1	−1	50	4	4	29.28	29.87	29.00	29.77
4	−1	1	1	50	4	6	33.80	34.32	36.27	36.14
5	1	−1	−1	60	2	4	21.80	22.16	22.66	23.32
6	1	−1	1	60	2	6	22.07	22.36	23.71	23.48
7	1	1	−1	60	4	4	25.12	25.40	25.59	26.04
8	1	1	1	60	4	6	26.17	26.21	26.86	27.41
9	0	0	0	55	3	5	31.18	31.42	31.95	32.20
10	0	0	0	55	3	5	30.98	31.42	33.62	32.20
11	0	0	0	55	3	5	31.95	31.42	34.51	32.20
12	0	0	0	55	3	5	32.48	31.42	31.44	32.20
13	0	0	0	55	3	5	32.00	31.42	30.32	32.20
14	0	0	0	55	3	5	29.74	31.42	31.20	32.20
15	0	−1.68	0	55	1.32	5	26.98	26.17	26.90	26.87
16	0	0	−1.68	55	3	3.32	28.71	27.90	28.45	27.60
17	0	0	1.68	55	3	6.68	32.24	31.81	32.99	33.09
18	0	1.68	0	55	4.68	5	32.74	32.31	33.79	33.08
19	−1.68	0	0	46.59	3	5	29.29	28.21	29.48	29.32
20	1.68	0	0	63.41	3	5	17.96	17.81	20.04	19.45

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).
[1]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:

$$ACE\ inhibition\ (\%) = \frac{(C_O - C_B) - (I_O - I_B)}{(C_O - C_B)} \times 100$$

where C_o 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 and then with a membrane with a molecular weight cutoff of 1 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 α-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 × *g* for 10 min. The α-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 × *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 × *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 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

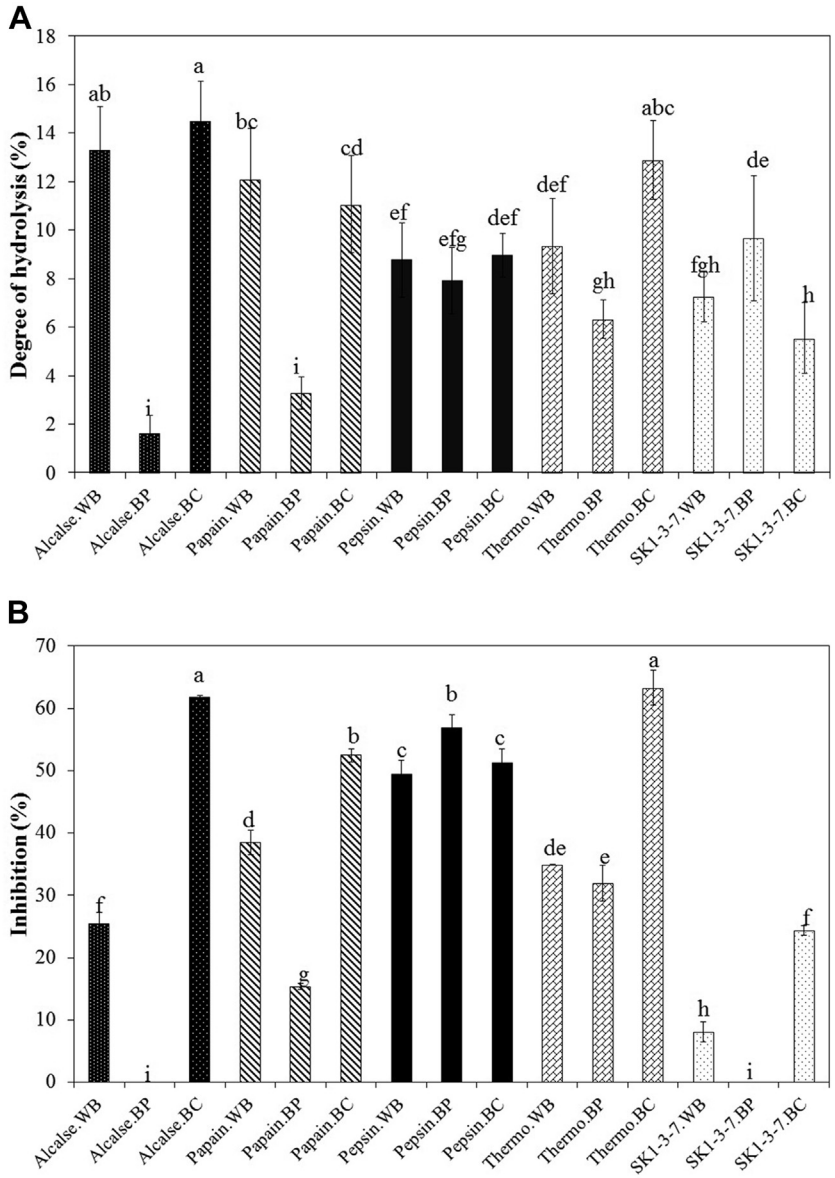

Figure 1. (A) Degree of hydrolysis and (B) ACE inhibition achieved with a 0.5 mg/mL peptide concentration of hydrolysates derived from various blood compositions and proteases and hydrolyzed for 12 h. Different letters indicate the significant differences among the treatments ($P < 0.05$). Data were expressed as the mean values (n = 3). Abbreviations: ACE, angiotensin I-converting enzyme; BC, blood corpuscles; BP, blood plasma; thermo, thermolysin; SK1-3-7, *Virgibacillus* sp. SK1-3-7; WB, whole blood.

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 ± 0.3,3 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 α_2-macroglobulin, serpins, and α_1-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 36.27% (at 0.2 mg/mL peptide). ANOVA showed an insignificant lack of fit ($P > 0.05$), suggesting that the model well described the variation of the response. The models of the DH (Y_1) and ACE inhibition (Y_2) were significant with coefficient of determination (R^2) values of 0.9719 and 0.9534, respectively, and can be described as follows:

$$Y_1 = -375.934 + 13.41011X_1 + 6.238374X_2 + 16.23549X_3 - 0.01021X_1X_2 - 0.18177X_1X_3 + 0.154559X_2X_3 - 0.11899X_1^2 - 0.11899X_2^2 - 0.77076X_3^2 \quad (1)$$

$$Y_2 = -377.091 + 12.92279X_1 + 7.047647X_2 + 21.04792X_3 - 0.03644X_1X_2 - 0.25047X_1X_3 + 0.302871X_2X_3 - 0.11043X_1^2 - 0.78526X_2^2 - 0.6548X_3^2 \quad (2)$$

where Y_1 and Y_2 are the predicted responses of the DH and ACE inhibition, respectively; X_1 is the temperature (°C); X_2 is the enzyme (%); and X_3 is time (h).

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, hereas 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). When 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 twofold 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

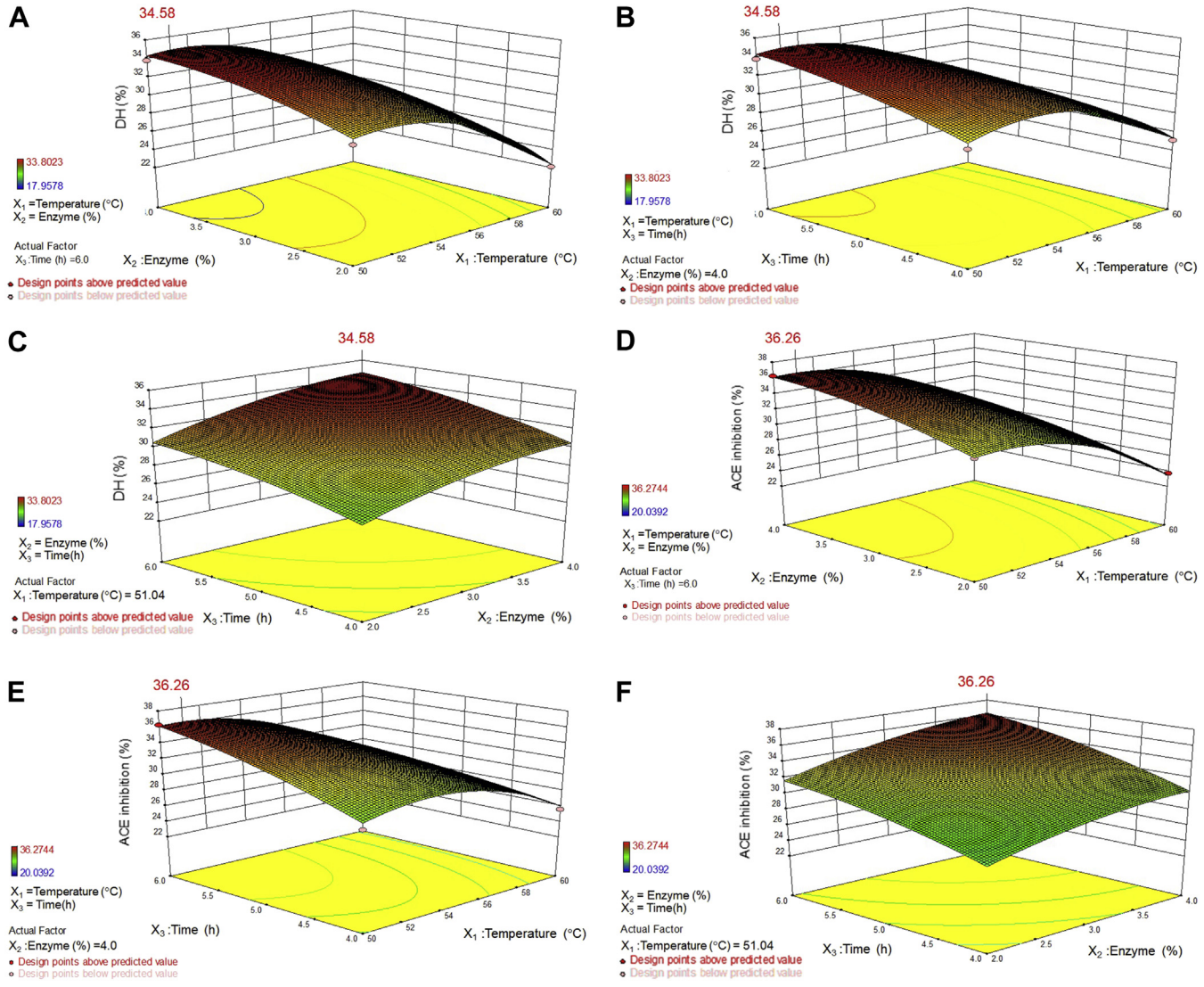

Figure 2. Response surface plots for the degree of hydrolysis as a function of (A) temperature and enzyme content; (B) temperature and time; and (C) enzyme content and time. Plots of ACE inhibition as a function of (D) temperature and enzyme content; (E) temperature and time; and (F) enzyme content and time. Abbreviations: ACE, angiotensin I-converting enzyme; DH, degree of hydrolysis.

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

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

Parameters			DH (%)[1]		ACE inhibition (%)[1]	
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	3.95	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 ($P > 0.05$).

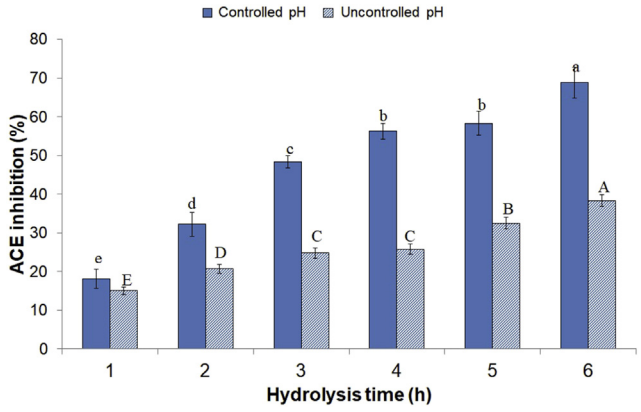

Figure 3. ACE inhibition of the hydrolysates derived from the blood corpuscles hydrolyzed by Alcalase for various durations. Different lowercase and uppercase letters indicate the significant differences among the hydrolysis times of the controlled and uncontrolled pH reactions ($P < 0.05$), respectively. Data were expressed as the mean values (n = 3). Abbreviation: ACE, angiotensin I-converting enzyme.

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 ($P < 0.05$), while that of the permeate BCH-III digesta was comparable with that of its parent hydrolysates ($P > 0.05$, Table 4). The smaller peptides generated from GI digestion appeared to possess ACE-inhibitory activities. These results suggested that the smaller peptides generated from the GI digestion of crude BCH could play a vital role in ACE inhibition. However, the ACE inhibition of the BCH-III digesta was more potent than that of the BCH digesta ($P < 0.05$). The ACE inhibition of BCH-III was not negatively affected by the GI proteases. This could be because the smaller peptides in BCH-III were resistant to GI enzymes. The ACE-inhibitory peptides of the BCH-III digesta could play an important role in the antihypertensive effect. The digesta of bovine plasma and porcine globin also showed an ACE inhibition that was compared with that of their parent hydrolysates (Yu et al., 2006; Sampedro and Montoya, 2014).

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 fro.m 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.

Table 4. ACE-inhibitory capacity of chicken blood corpuscle hydrolysates and its ultrafiltrated fraction after simulated in vitro gastrointestinal digestion.

Sample	α-amino content (mg)	IC_{50} (mg Leu eqv/mL)
BCH		
Undigested	4.31[d]	0.362[a]
Digested	6.13[b]	0.239[b]
BCH-III		
Undigested	5.16[c]	0.129[c]
Digested	7.01[a]	0.113[c]

Value are expressed as mean (n = 3). Different superscript letters in the same column indicate significant difference ($P < 0.05$).

Abbreviations: ACE, angiotensin I-converting enzyme; 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 −47.0 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 presenting in the BCH-III sample, which contained specific sequences of hydrophobic/basic

Table 5. Amino acid composition of peptides in blood corpuscle hydrolysate (BCH) and the 1-kDa permeate (BCH-III) (g/100 g powder) before and after simulated in vitro gastrointestinal digestion.

	Undigested		Digested	
Amino acid[1]	BCH	BCH-III	BCH	BCH-III
Proline (P)	8.11[x]	5.87	7.68	7.12
Glycine (G)	3.47	3.57	3.1	2.55
Alanine (A)	5.94	6.80[x]	4.19	6.47[z]
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[x]	5.78	6.02
Methionine (M)	0.72[x]	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[x]	3.50	3.37[z]	2.04
Glutamic acid (E)	6.74[x]	5.19	6.92[z]	3.68
Lysine (K)	6.10	6.67	5.02	4.02
Histidine (H)	3.64	4.35[x]	3.09	2.70
Arginine (R)	2.86	2.57	2.00	1.14
Hydrophobic (%)[2]	29.44 (42.4)	30.31 (45.8)[x]	22.38 (39.1)	23.59 (47.7)[z]
Polar (%)	12.58 (18.1)	11.61 (17.5)	9.56 (16.7)	7.62 (15.4)
Acidic (%)	10.86 (15.7)[x]	8.69 (13.1)	10.29 (18.0)[z]	5.72 (11.6)
Basic (%)	12.60 (18.2)	13.59 (20.5)[x]	10.11 (10.1)	7.86 (15.9)[z]
Estimated peptides	69.41[a]	66.25[a]	57.24[b]	49.47[c]

[x] and [z] indicate differences between BCH and BCH-III of parent hydrolysate and digesta, respectively in columns ($P < 0.05$).

[a–c]indicate differences in estimated peptides of all samples in the same row ($P < 0.05$).

Abbreviation: ND, not detected.

[1]Amino acid composition of peptides = total amino acids–free amino acids.

[2]Numbers in parentheses indicate percentage of each type of amino acids based on total amino acids in peptide.

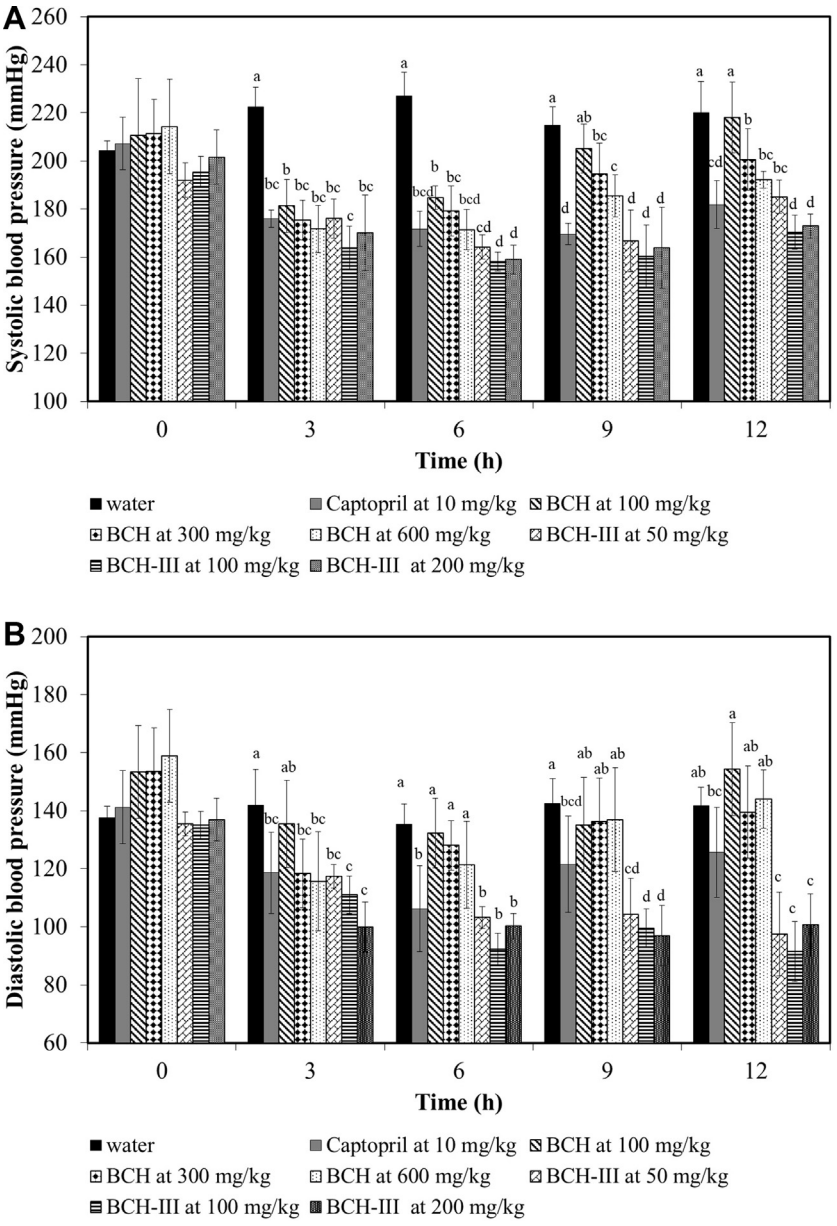

Figure 4. Changes in the (A) systolic blood pressure and (B) diastolic blood pressure after the oral administration of crude blood corpuscle hydrolysate (BCH) and its 1-kDa permeate (BCH-III) in spontaneously hypertensive rats (SHR). Different letters at the same time indicated significant differences ($P < 0.05$). Data were expressed as the mean values (n = 6).

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

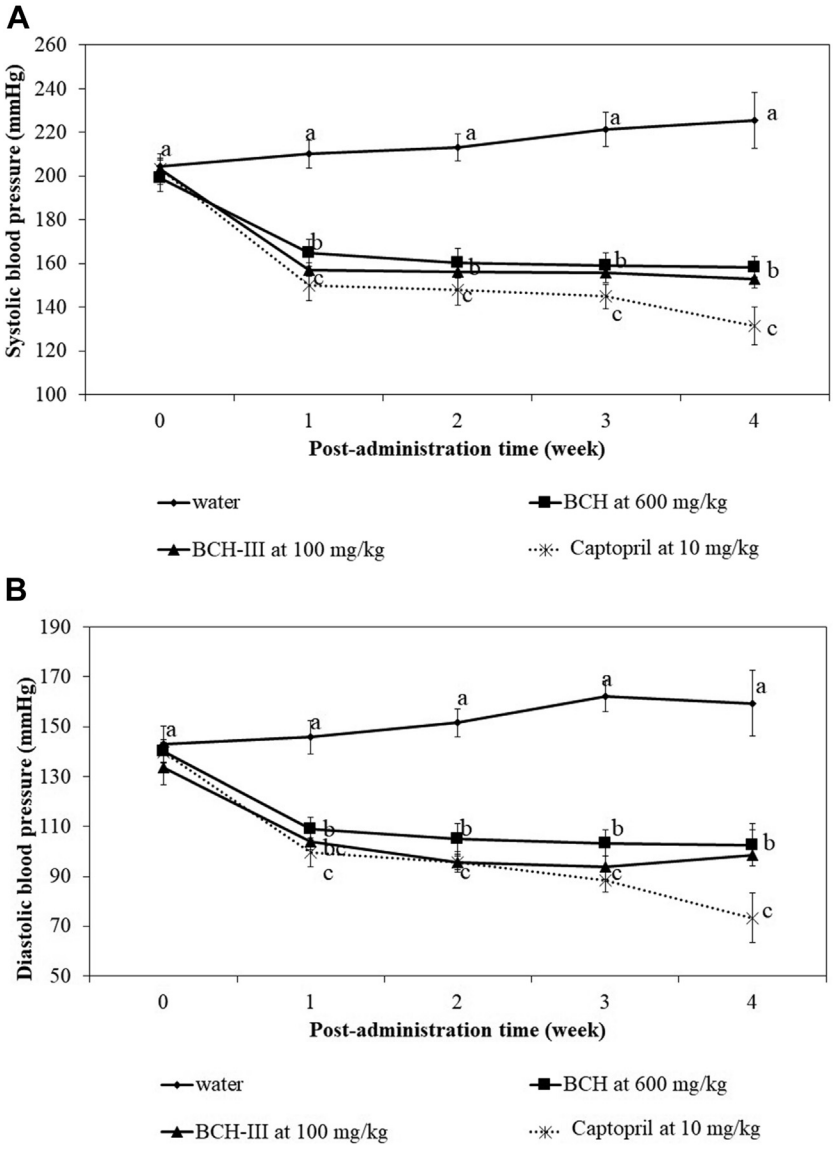

Figure 5. Changes in the (A) systolic blood pressure and (B) diastolic blood pressure of spontaneously hypertensive rats administered crude chicken blood corpuscle hydrolysate (BCH) and its 1-kDa permeate (BCH-III) for 4 wk. Different letters at the same time indicated significant differences ($P < 0.05$). 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.

ACKNOWLEDGMENTS

This research was financially supported by Thailand Research and Researcher for Industry (RRi) Scholarship (Grant no. PHD56I0024) and Agricultural Research Development Agency, Thailand (Public Organization, Grant no. CRP5805011560).

Conflict of Interest Statement: All authors have no conflict of interest to declare.

REFERENCES

Adler-Nissen, J. 1979. Determination of the degree of hydrolysis of food protein hydrolysates by trinitrobenzenesulfonic acid. J. Agric. Food Chem. 27:1256–1262.

Alashi, A. M., C. L. Blanchard, R. J. Mailer, S. O. Agboola, A. J. Mawson, R. He, S. A. Malomo, A. T. Girgih, and R. E. Alukod. 2014. Blood pressure lowering effects of Australian canola protein hydrolysates in spontaneously hypertensive rats. Food Res. Int. 55:281–287.

AOAC International. 2010. Official Methods of Analysis. 18th ed. Association of Official Chemists, Gaithersberg, MD.

Arihara, K. 2006. Functional properties of bioactive peptides derived from meat proteins. Pages 245–273 in Advanced Technologies for Meat Processing. L. Nollet and F. Toldrá, eds. CRC Press, Boca Raton, FL.

Aubry, M., and J. Bieth. 1976. A kinetic study of the inhibition of human and bovine trypsins and chymotrypsins by the inter-alpha-inhibitor from human plasma. Biochim. Biophys. Acta 438:221–230.

Cing-Mars, C. D., and E. C. Y. Li-Chan. 2007. Optimizing angiotensin I-converting enzyme inhibitory activity of pacific hake (*Merluccius productus*) fillet hydrolysate sing response surface methodology and ultrafiltration. J. Agric. Food Chem. 55:9380–9388.

Cinq-Mars, C. D., C. Hu, D. D. Kitts, and E. C. Y. Li-Chan. 2008. Investigations into inhibitor type and mode, simulated gastrointestinal digestion, and cell transport of the angiotensin I-converting enzyme-inhibitory peptides in Pacific hake (*Merluccius productus*) fillet hydrolysate. J. Agric. Food Chem. 56:410–419.

Cushman, D. W., H. S. Cheung, E. F. Sabo, and M. A. Ondetti. 1977. Design of potent competitive inhibitors of angiotensin I-converting enzyme: carboxyalkanoyl and mercaptoalkanoyl amino-acids. Biochemistry 16:5484–5491.

Cushman, D. W., and H. S. Cheung. 1971. Spectrophotometric assay and properties of the angiotensin-converting enzyme of rabbit lung. Biochem. Pharmacol. 20:1637–1648.

Daliri, E. B. M., F. K. Ofosu, R. Chelliah, M. H. Park, J. H. Kim, and D. H. Oh. 2019. Development of a soy protein hydrolysate with and antihypertensive effect. Int. J. Mol. Sci. 20:1496.

Deng, H., J. Zheng, H. Zhang, Y. Wang, and J. Kan. 2014. Isolation of angiotensin I-converting enzyme inhibitor from pepsin hydrolysate of porcine hemoglobin. Eur. Food Res. Technol. 239:933–940.

Girgih, A. T., A. Alashi, R. He, S. Malomo, and R. E. Aluko. 2014. Preventive and treatment effects of a hemp seed (*Cannabis sativa L.*) meal protein hydrolysate against high blood pressure in spontaneously hypertensive rats. Eur. J. Nutr. 53:1237–1246.

Girgih, A. T., I. D. Nwachukwu, F. Hasan, T. N. Fagbemi, T. Gill, and R. E. Aluko. 2015. Kinetics of the inhibition of renin and angiotensin I-converting enzyme by cod (*Gadus morhua*) protein hydrolysates and their antihypertensive effects in spontaneously hypertensive rats. Food Nutr. Res. 59:29788.

Girigh, A. T., I. D. Nwachukwa, J. O. Onuh, S. A. Malomo, and R. E. Aluko. 2016. Antihypertensive properties of a pea protein hydrolysate during short- and long-term oral administration to spontaneously hypertensive rats. J. Food Sci. 81:H1281–H1287.

Li, G. H., G. W. Le, Y. H. Shi, and S. Shrestha. 2004. Angiotensin I-converting enzyme inhibitory peptides derived from food proteins and their physiological and pharmacological effects. Nutr. Res. 24:469–486.

Li, G. H., M. R. Qu, J. Z. Wan, and J. M. You. 2007. Antihypertensive effect of rice protein hydrolysate with in vitro angiotensin I-converting enzyme inhibitory activity in spontaneously hypertensive rats. Asia Pac. J. Clin. Nutr. 16:275–280.

Marques, C., M. M. Amorim, J. O. Pereira, M. E. Pintado, D. Moura, C. Calhau, and H. Pinheiro. 2012. Bioactive peptides—are there more antihypertensive mechanisms beyond ace inhibition? Curr. Pharm. Des. 18:4706–4713.

Matsubara, H., and J. Feder. 1971. The Enzymes. Academic Press, New York.

Montriwong, A., S. Kaewphuak, S. Rodtong, S. Roytrakul, and J. Y. Yongsawatdigul. 2012. Novel fibrinolytic enzymes from Virgibacillus halodenitrificans SK1-3-7 isolated from fish sauce fermentation. Process. Biochem. 47:2379–2387.

Nagodawithana, T., and G. Reed. 1993. Enzymes in Food Processing. 3rd ed. Academic Press, San Diego, CA.

Nwachukwa, I. D., A. T. Girgih, S. A. Malono, J. O. Onuh, and R. E. Aluko. 2014. Thermoase-derived flaxseed protein hydrolysates and membrane ultrafiltration peptide fractions have systolic blood pressure-lowering effects in spontaneously hypertensive rats. Int. J. Mol. Sci. 15:18131–18147.

Ockerman, H. W., and C. L. Hansen. 2000. Animal By-Product Processing and Utilization. CRC Press, Boca Raton, Fla.

Ondetti, M. A., and D. W. Cushman. 1982. Enzymes of the renin-angiotensin system and their inhibitors. Annu. Rev. Biochem. 51:283–308.

Onuh, J. O., A. T. Girgih, S. A. Malomo, R. E. Aluko, and M. Aliani. 2015. Kinetics of in vitro renin and angiotensin converting enzyme inhibition by chicken skin protein hydrolysates and their blood pressure lowering effects in spontaneously hypertensive rats. J. Funct. Foods 14:133–143.

Ruiz-Ruiz, J., G. Davila-Ortiz, L. Chel-Guerrero, and D. Betancur-Ancona. 2013. Angiotensin I-converting enzyme inhibitory and antioxidant peptide fractions from hard-to-cook bean enzymatic hydrolysates. J. Food Biochem. 37:26–35.

Parés, D., E. Saguer, and C. Carretero. 2011. Blood by-products as ingredients in processed meat. Pages 218–242 in Processed Meats: Improving Safety, Nutrition and Quality. J. P. Kerry and J. F. Kerry eds. Woodhead Publishing Ltd., Cambridge, UK.

Sampedro, L. J. G., and J. E. Z. Montoya. 2014. Effects of hydrolysis and digestion in vitro on the activity of bovine plasma hydrolysates as inhibitors of the angiotensin I-converting enzyme. Braz. Arc. Biol.Technol. 57:386–393.

Udenigwe, C. C., Y. S. Lin, W. C. Hou, and R. E. Aluko. 2009. Kinetics of the inhibition of renin and angiotensin I-converting enzyme by flaxseed protein hydrolysate fractions. J. Funct. Foods 1:199–207.

USDA. 2018. Livestock and Poultry: Poultry & Grain Market News. Accessed May 2018. https://www.ams.usda.gov/market-news/livestock-poultry-grain/.

van der Ven, C., H. Gruppen, D. B. A. De Bont, and A. G. J. Voragen. 2002. Optimisation of the angiotensin I-converting enzyme inhibition by whey protein hydrolysates using response surface methodology. Int. Dairy J. 12:813–820.

Vermeirssen, V., J. V. Camp, and W. Verstraete. 2002. Optimisation and validation of an angiotensin I-converting enzyme inhibition assay for the screening of bioactive peptides. J. Biochem. Biophys. Methods 51:75–87.

Wang, J., J. Hu, J. Cui, X. Bai, Y. Du, Y. Miyaguchi, and B. Lin. 2008. Purification and identification of a ACE inhibitory peptide from oyster proteins hydrolysate and the antihypertensive effect of hydrolysate in spontaneously hypertensive rats. Food Chem. 111:302–308.

Wei, J. T., and B. H. Chiang. 2009. Bioactive peptide production by hydrolysis of porcine blood proteins in a continuous enzymatic membrane reactor. J. Sci. Food Agric. 89:372–378.

Yamaguchi, N., K. Kawaguchi, and N. Yamamoto. 2009. Study of the mechanism of antihypertensive peptides VPP and IPP in spontaneously hypertensive rats by DNA microarray analysis. Eur. J. Pharmacol. 620:71–77.

Yu, Y., J. Hu, Y. Miyaguchi, X. Bai, Y. Du, and B. Lin. 2006. Isolation and characterization of angiotensin I-converting enzyme inhibitory peptides derived from porcine hemoglobin. Peptides 27:2950–2956.

Yu, Z., Y. Yin, W. Zhao, F. Chen, and J. Liu. 2014. Antihypertensive effect of angiotensin I-converting enzyme inhibitory peptide RVPSL on spontaneously hypertensive rats by regulating gene expression of the renin-angiotensin system. J. Agric. Food Chem. 62:912–917.