International Food Research Journal 24(4): 1799-1804 (August 2017)
Journal homepage: *http://www.ifrj.upm.edu.my*

Increasing of angiotensin converting enzyme inhibitory derived from Indonesian native chicken leg protein using *Bacillus cereus* protease enzyme

[1]Yuliatmo R, [1]Fitriyanto N. A, [2]Bachruddin Z, [2]Supadmo, [3]Jamhari, and [1*]Erwanto Y.

[1]Laboratory of By-product and Environment Technology, Faculty of Animal Science, Universitas Gadjah Mada, Yogyakarta 55281, Indonesia
[2]Laboratory of Nutritional Biochemistry, Faculty of Animal Science, Universitas Gadjah Mada, Yogyakarta 55281, Indonesia
[3]Laboratory of Meat Science and Technology, Faculty of Animal Science, Universitas Gadjah Mada, Yogyakarta 55281, Indonesia

Article history

Received: 10 March 2016
Received in revised form: 1 June 2016
Accepted: 15 July 2016

Keywords

Indonesian native chicken
Angiotensin converting enzyme
B. cereus protease
Hypertension

Abstract

Angiotensin converting enzyme (ACE) converts angiotensin I to angiotensin II, thereby increasing hypertension. In order to increase ACE inhibition from bioactive peptides in food products, native chicken leg protein was hydrolyzed by proteases extracted from three strains of *B. cereus* (LS2B, TD5B, and TD5K). The specific activities of LS2B, TD5B, and TD5K proteases were 303.57, 195.96, and 111.14 U/mg, respectively. Dissolved proteins in chicken leg hydrolysates were separated by sodium dodecylsulfate-polyacrylamide gel electrophoresis and subjected to ACE inhibition assay. LS2B strain proteases produced the highest dissolved protein content. The IC50 of chicken leg protein hydrolyzed by pepsin + trypsin, LS2B strain enzymes, and LS2B enzymes + pepsin + trypsin were 2.58 ± 0.072, 1.21 ± 0.78, and 1.092 ± 0.01 mg/mL, respectively; chicken leg protein hydrolyzed by LS2B enzymes + pepsin + trypsin purified by Sep-Pak C18 Cartridge showed an IC_{50} of 0.33 ± 0.02 mg/mL. The results indicate that enzymatic hydrolysis of chicken leg proteins via bacterial enzymes can increase the ACE inhibitory activity of resultant peptides.

Introduction

Many biologically active peptides from food proteins have been investigated. Inhibitors of angiotensin converting enzyme (ACE) have attracted particular attention for their ability to prevent hypertension (Katayama *et al.*, 2003). Recently, Erwanto *et al*. (2014) produced a potent ACE inhibitor from Indonesian native chicken leg protein. Indonesian native chickens are a major germplasm resource for Indonesia. Chicken legs are a valuable by-product that is cheap and potential to develop as they contain high amounts of protein.

Chicken legs are covered by skin comprised largely of collagen. Cheng *et al.* (2008) reported that hydrolysis of collagen by digestive enzymes can produce bioactive peptides which inhibit ACE with an IC_{50} of 0.945 mg/mL. Others have found exogenous enzymes extracted from microorganisms produce peptides with greater ACE inhibitory activity (Hati *et al*. 2015). Application of *B. cereus* proteases to increase the ACE inhibitory activity of hydrolyzed chicken leg and other food protein has not yet been investigated, and little data is available on the ACE inhibitory potency of bioactive peptides derived from proteins hydrolyzed by bacterial enzymes. Previously, peptides derived from hydrolysis of collagen protein by *Aspergillus oryzae* proteases were found to have a low IC_{50} [0.260 mg/mL] towards ACE (Saiga *et al*. 2008). Jamhari *et al*. (2013) and Erwanto *et al.* (2014) showed that hydrolyzed peptides purified further with a Sep-Pak Plus C18 Cartridge had an even lower IC_{50} towards ACE, increasing their ACE inhibitory activity by about 70%.

The current study explored the ability of enzymes extracted from three strains of *B. cereus* to hydrolyze Indonesian native chicken leg proteins into bioactive peptides with augmented ACE inhibitory strength. The specific activity and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) profile of each *B. cereus* strain enzyme extract and hydrolyzed chicken leg proteins, as well as the ACE inhibitory activity of hydrolysates, were assessed in order to develop a source of new, highly functional food-based peptides which prevent or attenuate hypertension.

Materials and Methods

Preparation of chicken leg protein

Indonesian native chicken leg protein was prepared using a method developed by Cheng *et*

*Corresponding author.
Email: *erwantougm@gmail.com*
Tel: +62 274 513363

al. (2008) and Jamhari *et al.* (2013). Chicken legs were obtained from a restaurant in Yogyakarta city (Indonesia) then were deboned and the flesh and skin cut into small pieces. Leg pieces (50 g) were ground with 200 mL of aquadest (water) in a blender for 10 min before incubating at 70°C for 10 min in a water bath to a homogenate.

Chemical composition analysis

The moisture, fat, and protein content were analyzed using near infrared spectroscopy.

Isolation of B. cereus *enzymes*

Enzymes were extracted from three *B. cereus* strains: LS2B, TD5B, and TD5K. Strains were maintained in a glycerol stock at -20ºC. B. cereus cells (2.8 x 108) were inoculated into a medium containing 1% meat extract, 1% biological peptone, and 0.5% NaCl (pH 8.0) and then incubated for 16 h at room temperature with shaking. After, the culture filtrate was centrifuged at 13,000 x g for 10 min to remove the biomass; bacterial enzymes were located in the supernatant (Zambare *et al*., 2010).

SDS-PAGE of bacterial enzyme extracts

Bacterial enzymes were partially purified by ammonium sulfate precipitation (60% saturation) and then subjected to SDS-PAGE. SDS-PAGE was completed according to the method reported by Laemmli (1970) using a 12% resolving gel and 5% stacking gel with a constant voltage of 140 V for 3 h. Protein bands were stained with Coomassie Brilliant Blue R-250. Molecular weights of each band were calculated using the retardation factor method.

Bacterial enzyme activity assay

Bacterial enzyme specific activity was measured as described by Bergmeyer *et al.* (1983) with slight modifications. Enzyme solutions (1 mL) were incubated with 0.5% casein and 0.5 mL buffer (pH 8.0) at 37ºC for 10 min. The reaction was stopped by adding 5 mL of 5% trichloroacetic acid. The mixture was allowed to stand at room temperature for 30 min and then centrifuged at 6000 x g at 4ºC for 10 min. Supernatants (1 mL) were mixed with 2.5 mL of 0.5 M sodium carbonate and 0.5 mL of folin ciocalteu (1:2). This mixture was incubated for 10 min before measuring the absorbance at 578 nm. A standard curve was generated using 10-100 µg/mL tyrosine and used to calculate protease activity. One unit of protease activity was defined as the amount of enzyme required to liberate 1 µg of tyrosine per minute at 37ºC.

Enzymatic digestion of chicken leg homogenates

Enzymatic digestion of chicken leg homogenates was performed using a method reported by Li *et al.* (2007) with slight modifications. Chicken leg homogenates (50 mg protein) were incubated with LS2B (CL), TD5B (CTD5B), and TD5K (CTD5K) enzymes alone, in combination with 5 mL of pepsin solution (0.05 mg/mL, pH 2.0) for 3 h, or in combination with pepsin + trypsin solution. Unhydrolyzed homogenates (C) and homogenates hydrolyzed with pepsin (CP) or pepsin + trypsin (CPT) but not bacterial enzymes were used as controls. Homogenates treated with pepsin + trypsin were first incubated with pepsin solution as described above, then heated for 5 min in boiling water, and adjusted to pH 8.0 before adding 5 mL of trypsin solution (0.05 mg/mL, pH 8.0) and incubating for 4 h at 37°C. After, all hydrolysates were heated for 5 min in boiling water to terminate digestion. The extent of hydrolysis was assessed by measurement of dissolved protein concentration, SDS-PAGE, and ACE inhibition assay.

Determination of dissolved protein concentration in hydrolysates

Dissolved protein concentrations were analyzed using the Lowry method (Owusu-Apenten, 2002). Protein concentrations were obtained by comparing the absorbance of each sample at 750 nm to the absorbance of bovine serum albumin as a standard.

SDS-PAGE of hydrolysates

Hydrolysates were separated by SDS-PAGE as described above for bacterial enzyme extracts.

ACE inhibition assay

ACE inhibition assays were performed according to the method reported by Cushman and Cheung (1971) with a slight modification. Briefly, 30 µL of hydrolysates were added to 250 µL of 0.1 M borate buffer (pH 8.5) containing 7.6 mM hippuryl-L-histidyl-L-leucine (Nacalai Tesque, Inc., Kyoto, Japan) as a substrate and 0.608 M NaCl. The reaction was initiated by addition of 100 µL of 60 mU/mL rabbit lung ACE (dipeptidylcarboxypeptidase; EC 3.4.1.5.1; Sigma Chemical Co., St. Louis, MO, USA) in 0.25 M borate buffer (pH 8.5). To terminate the reaction, 250 µL of 1 M HCl was added. Ethyl acetate (1.5 mL) was added to extract the hippuric acid liberated from hippuryl-L-histidyl-L-leucine by ACE with vigorous shaking, followed by centrifugation for 10 min at 3600 x g. The ethyl acetate layer from each tube (approximately 1 mL) was collected, placed into another tube, and dried for 10 min at 100°C.

Any remaining hippuric acid was dissolved with 5 mL of 1 M NaCl before measuring the absorbance of each sample at 228 nm. ACE inhibitory activity was calculated as follows:

$$\text{Inhibition (\%)} = [(C - S)/(C - B)] \times 100$$

where S is the sample absorbance, C is the control absorbance (sample buffer only), and B is the absorbance of the blank (HCl added before ACE). Inhibitory activity was expressed as the IC50 of the sample in each assay; a decrease in IC50 indicates an increase in ACE inhibitory activity.

Purification of hydrolyzed peptides by Sep-Pak Plus C18 Cartridge

Hydrolysates were purified by Sep-Pak Plus C18 Cartridge (Waters Co., Milford, MA, USA) and eluted with 2% acetonitrile and 0.1% trifluoroacetic acid in water and then 65% acetonitrile and 0.1% trifluoroacetic acid in water. The purpose of this step was to remove nonprotein components in each hydrolysate. Filtrates were dried using a freeze dryer and then diluted with water (Erwanto et al., 2014).

Data analysis

Chemical composition, SDS-PAGE, and ACE inhibition assay were analyzed descriptively. The dissolved protein content in hydrolysates was analyzed using one-way analysis of variance. Significant differences were further analyzed by Duncan's New Multiple Range test.

Results

Chemical composition of Indonesian native chicken legs

The Indonesian native chicken legs contained an average of 64.15% moisture, 6.2% fat, and 22.91% protein.

Bacterial enzyme profiles via SDS-PAGE

SDS-PAGE of bacterial enzymes is shown in Figure 1. Enzymes extracted from LS2B, TD5B, and TD5B strains of *B. cereus* had similar bands at 35, 37, and 52 kDa. However, there were also differences in banding patterns among the three strains. In particular, only LS2B strain enzyme extracts showed a band at 100 kDa.

Bacterial enzyme specific activity

The specific activity of enzymes extracted from LS2B, TD5B, and TD5K strains of *B. cereus* were determined to be 85.00, 74.46, and 64.46 U/mL, respectively. LS2B extracts had the highest specific activity (303.57 U/mg), while that of TD5B and TD5K extracts were much lower (195.96 and 111.14 U/mg, respectively).

Table 1. Dissolved protein concentration following enzymatic hydrolysis

Protein hydrolysate	Digestion Process		
	Before digestion	Digestion by pepsin	Digestion by pepsin and trypsin
Colagen	2.69 ± 0.05^{a}	2.88 ± 0.02^{a}	3.09 ± 0.01^{a}
CLS2B	5.06 ± 0.04^{a}	4.73 ± 0.04^{ab}	4.32 ± 0.05^{b}
CTD5B	5.13 ± 0.03^{a}	4.66 ± 0.06^{b}	4.26 ± 0.04^{c}
CTD5K	4.12 ± 0.03^{a}	4.13 ± 0.02^{ab}	3.69 ± 0.04^{b}

Chicken leg homogenates digested with LS2B (CL), TD5B (CTD5B), and TD5K (CTD5K) strain *Bacillus cereus* enzyme extracts.
Different superscripts in the same column indicate significant differences (P<0.05)

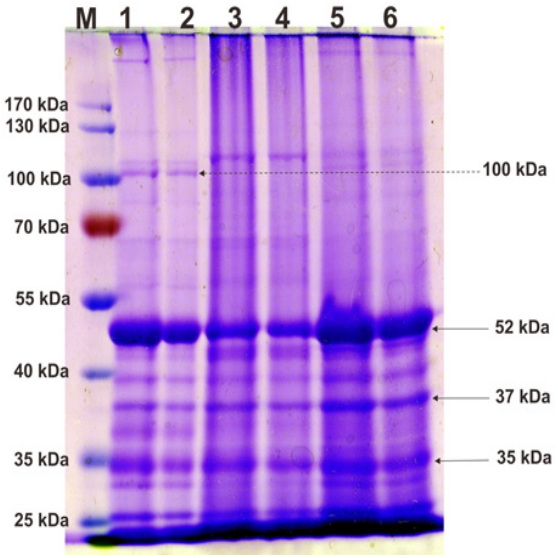

Figure 1. SDS-PAGE of enzymes extracted from B. cereus strains LS2B, TD5B, and TD5K. M: molecular weight marker; lane 1: LS2B enzymes (10 µL); lane 2: LS2B enzymes (5 µL); lane 3: TD5B enzymes (10 µL); lane 4: TD5B enzymes (5 µL); lane 5: TD5K enzymes (10 µL); lane 6: TD5K enzymes (5 µL)

Bacterial enzyme digestion

The extent of bacterial enzyme digestion was first determined by measuring the dissolved protein concentration (Table 1). There was a significant difference between C (i.e., collagen) and chicken leg protein (i.e., homogenates) hydrolyzed by LS2B, TD5B, and TD5K bacterial enzymes and/or pepsin or pepsin + trypsin. Before digestion, the concentration of collagen (2.69 mg/mL) was lower than that of other proteins in each sample (5.06, 5.13, and 4.12 mg/mL, respectively). After digestion, the collagen

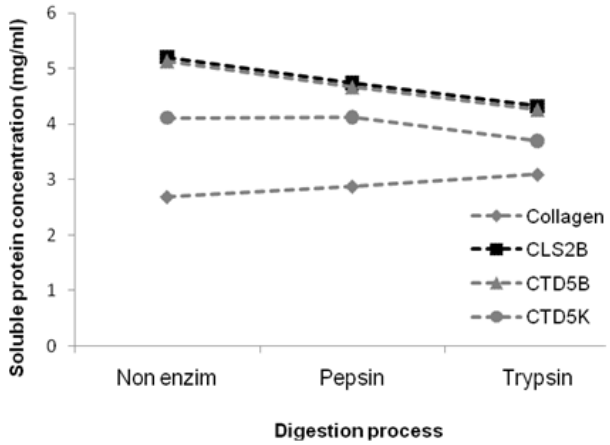

Figure 2. The protein digestion process

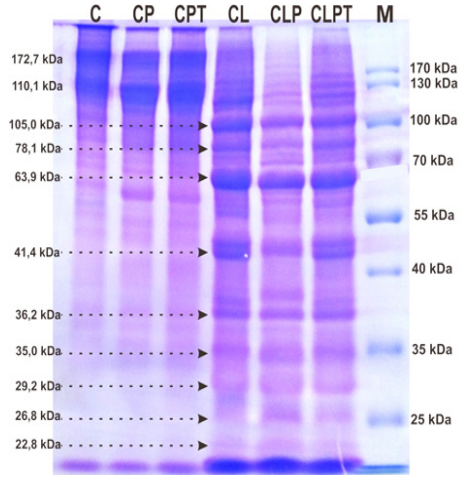

Figure 3. SDS-PAGE of hydrolyzed chicken leg protein. C: unhydrolyzed chicken leg protein; CP: pepsin-hydrolyzed chicken leg protein; CPT: chicken leg protein hydrolyzed by pepsin + trypsin; CL: chicken leg protein hydrolyzed by LS2B bacterial enzymes; CLP: chicken leg protein hydrolyzed by LS2B enzymes + pepsin; CLPT: chicken leg protein hydrolyzed by LS2B enzymes + pepsin + trypsin; M: molecular weight marker

protein concentration increased to 3.09 mg/mL, while the concentration of other proteins in each sample decreased to 4.32, 4.26, and 3.69 mg/mL, respectively. The progress of the digestion process is shown in Figure 2. Chicken leg protein hydrolized by LS2B strain enzymes had the highest dissolved protein concentration.

SDS-PAGE of hydrolysates

Figure 3 shows the shift in banding patterns before and after enzymatic digestion of chicken leg proteins. C produced bold bands at 172.7 and 110.1 kDa corresponding to collagen protein isoforms. CP and CPT produced patterns similar to that of C but with additional bands between 35.0 and 110.1 kDa. Banding patterns produced by chicken leg protein hydrolyzed by LS2B enzymes alone (CL), + pepsin (CLP), or + pepsin + trypsin (CLPT) were all significantly different from C, CP, and CPT patterns. CL, CLP, and CLPT all lacked thick bands associated with unhydrolyzed collagen proteins (172.7 and 110.1 kDa) and had many other lower molecular weight bands from 105.0 to 22.8 kDa (105.0, 78.1, 63.9, 41.4, 36.2, 35.0, 29.2, 26.8, and 22.8 kDa).

ACE inhibitory activity of peptide hydrolysates

ACE inhibition assays were carried out with CPT, CL, CLPT, and CLPT samples purified by Sep-Pak Plus C18 Cartridge (CLPT-P). IC_{50} values ± standard deviations are shown in Figure 4; a lower IC50 directly correlated with greater ACE inhibition. The results showed that the IC_{50} of CPT was highest at 2.58 ± 0.072 mg/mL, followed by that of CL (1.21 ± 0.78 mg/mL), CLPT (1.092 ± 0.01 mg/mL), and CLPT-P (0.33 ± 0.02 mg/mL).

Discussion

The chemical composition of Indonesian native chicken legs was examined by near infrared spectroscopy, which measures the intensity of infrared light absorption of a sample safely and easily. Cheng *et al.* (2008) stated the chemical composition of most material is composed of several important components, such as moisture, fat, and protein. Previously, Erwanto *et al.* (2014) measured the chemical composition of Indonesain native chickens using standard Association of Official Analytical Chemicals methods (AOAC, 2005) and reported results very similar to those obtained herein. The chemical composition of a material is usually affected by the quality of the ingredients. For example, materials other than protein can affect enzymatic hydrolysis and may also affect SDS-PAGE and ACE inhibition assay. The current study found Indonesian native chicken legs contained 22.91% protein on average. A high protein content is necessary to the survey of bioactive peptides in food products, such as those that could be used as antihypertensive agents.

The investigation of bioactive peptides present in food products must first be hydrolyzed from their protein precursors. Previously, Saiga et al. (2008) produced bioactive peptides from food product proteins hydrolyzed by bacterial enzymes. In the present study, enzymes extracted from three B. cereus strains (LS2B, TD5B, and TD5K) were used to hydrolyze collagen proteins from chicken legs. Most enzymes have certain specificities and, therefore, produce a specific profile or "fingerprint" that can be assessed by SDS-PAGE. Bacterial enzymes were precipitated with ammonium sulfate before being

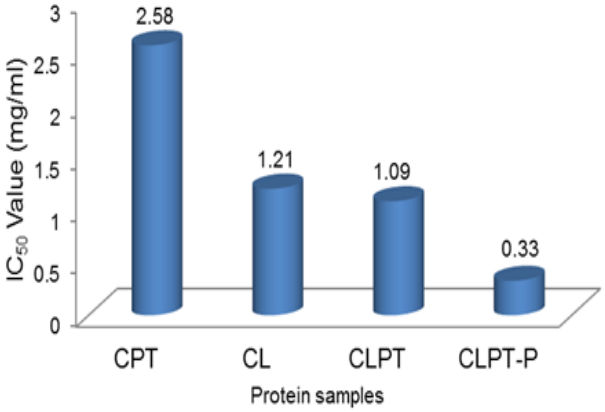

Figure 4. Determination of IC_{50} values. CPT: chicken leg protein hydrolyzed by pepsin + trypsin; CL: chicken leg protein hydrolyzed by LS2B bacterial enzymes; CLPT: chicken leg protein hydrolyzed by LS2B enzymes + pepsin + trypsin; CLPT-P: CLPT protein purified by Sep-Pak Plus C18 Cartridge

subjected to SDS-PAGE. This step is useful for purifying proteins by removing monosaccharides, oligosaccharides, nucleotides, free amino acids, and minerals.

SDS-PAGE of bacterial enzymes showed both overlap and variation in position and intensity of banding patterns for each stain of *B. cereus* (Figure 1). Moreover, several bands presented as singlets in some lanes and doublets in others, implying multiple protein isoforms. Similarities are likely due to the fact that bacterial enzymes were extracted from the same bacterial species, while variations denote strain differences. For example, LS2B and TD5K extracts both produced bands at a molecular weight >170 kDa, while extracts from TD5B did not. In addition, LS2B extracts produced a band at 100 kDa that is only faintly visible in TD5K extracts and not present at all in TD5B lanes. Hence, each enzyme from each bacterial strain has its own fingerprint. These results were similar to those reported by Berber (2004), who examined profiles of enzymes extracted from various species of *Bacillus* by SDS-PAGE.

Herein, enzymes isolated from *B. cereus* strain LS2B had the highest specific activity (303.57 U/mg). Zambare *et al*. (2010) reported the specific activity of enzymes derived from *B. cereus* strain MCM B-326 to be 0.947 U/mg, while Liu *et al.* (2010) determined the specific activity of collagenase derived from *B. cereus* strain MBL13 to be 120 U/mg. Moreover, Umayaparvathi *et al*. (2013) demonstrated the specific activity of enzymes extracted from *B. cereus* strain SU12 to be 33.23 U/mg. As with differences in SDS-PAGE banding patterns, these variations in specific activity of enzymes from the same bacterial genus are likely the result of strain differences.

The potency of bacterial enzymatic hydrolysis of chicken leg protein is reflected in the concentration of dissolved proteins present following digestion (Table 1). Table 1 shows a significant difference between the concentrations of C (collagen) versus that of CL, CTD5B, and CTD5K. The concentration of collagen was nearly 50% lower than that in CL, CTD5B, and CTD5K samples. The dissolved protein concentration increased by addition of pepsin and more so by addition of trypsin, indicating increased degradation of peptide bonds. These results are in accordance with those of Kouguchi *et al.* (2010), who reported that collagen has a triple helix structure, wherein heating and hydrolysis first causes detachment of the helices to create a gelatin, followed by further structural degradation into smaller peptides with continued exposure to enzymatic digestion and/or heat. Interestingly, the concentration of dissolved proteins after hydrolysis by bacterial enzymes decreased, while that of unhydrolyzed protein increased. Nonetheless, the concentration of dissolved protein following hydrolysis by bacterial enzymes was still higher than other protein samples. Because CL samples produced the highest dissolved protein content, they were analyzed further by SDS-PAGE.

C, CP, and CPT samples showed very similar banding patterns via SDS-PAGE (Figure 3). However, some bands in lane C were thicker and slightly higher in molecular weight relative to corresponding bands in CP and CPT lanes, indicating enzymatic hydrolysis was able to produce smaller molecular weight peptides from larger ones. According to Cheng (2008), the molecular weight of collagen proteins derived from broiler chicken feet range from 97 to 200 kDa. This is in accordance with current results, wherein C, CP, and CPT lanes produced thick bands at 172.7 and 110.1 kDa. However, addition of LS2B strain enzyme extracts produced significant differences in banding patterns of CL, CLP, and CLPT compared with C, CP, and CPT. Relative to C, CP, and CPT lanes, CL, CLP, and CLPT displayed more and thinner bands spanning a greater range of molecular weights (22.8-105 kDa). The increase in bands corresponding to lower molecular weights confirms the potency of LS2B enzyme extracts.

Interestingly, some peptides resulting from enzymatic hydrolysis have ACE inhibitory activity. The IC_{50} of CL samples was lower than that of CPT proteins in accordance with a previous study by Cheng et al. (2008), who reported the IC_{50} of chicken bone proteins hydrolyzed by trypsin and pepsin to be 7.012 and 4.016 mg/mL, respectively. Saiga *et al.* (2008) reported the IC_{50} of chicken collagen hydrolyzed by Aspergillus oryzae proteases was

0.260 mg/mL. CLPT samples in the current study resulted in the lowest IC_{50} value (1.092 mg/mL), which was higher than that reported by Saiga *et al*. (2008). The higher IC_{50} values reported herein are likely due to the presence of other materials within the sample besides protein. The only sample subjected to purification was that with the lowest IC_{50} (CLPT), which generated an even lower IC_{50} value of 0.33 mg/mL (CLPT-P). The IC_{50} of CLPT-P is consistent with that reported by Jamhari *et al.* (2013) from purified protein derived from hydrolyzed Indonesian native chicken meat (0.316 mg/mL). Therefore, further purification of chicken leg protein in homogenates should be completed in future.

Conclusion

Indonesian native chicken legs have a high percentage of crude protein (22.91%). The specific activity and SDS-PAGE profile of enzymes isolated from three *B. cereus* strains were determined. Hydrolysis of chicken leg homogenates by LS2B-extracted enzymes resulted in the highest dissolved protein concentration and more low molecular weight bands via SDS-PAGE due to potent hydrolysis. CLPT peptides had the highest ACE inhibitory activity versus CL and CLP peptides. Furthermore, increased enzymatic hydrolysis, together with purification of protein, can decrease the IC_{50} of resultant hydrolyzed peptides.

References

AOAC 2005. International Official Methods of Analysis. 18th edn. Washington DC: Association of Analytical Chemists.

Berber, I. 2004. Characterization of *Bacillus* species by numerical analysis of their SDS PAGE protein profiles. Journal of Cell Molecular Biology (3): 33-37.

Bergmeyer, H.U., Grassl, M. and Walter, H.E. 1983. Biochemical reagents for general use. In Bergemeyer, H.U. (Eds). Methods of enzymatic analysis, p. 126-328. Weinheim: Verlag Chemie.

Cheng, F. Y., Liu, Y. T., Wan, T. C., Lin, L. C., and Sakata, R. 2008. The development of Angiotensin I-Converting Enzyme Inhibitor derived from chicken bone protein. Journal of Animal Science (79): 122–128.

Cheung, H. S., Wang, F. L., Ondetti, M. A., Sabo, E. F. and Chusman, D. W. 1980. Binding of peptide substrater and inhibitors of Angiotensin Converting Enzymes. Journal of Biological Chemistry (225): 401-408.

Cushman, D. W. and Cheung, H. S. 1971. Spectrophotometric assay and properties of the Angiotensin-Converting Enzyme of rabbit lung. Journal of Biochemical Pharmacology (20): 1637–1648.

Erwanto, Y., Ismanto, A., Jamhari, Prasetyo, A., Yuliatmo R. and Wijayaningsih, R.A.. 2014. The Potency of bioactive peptide of native chicken leg as an anti-hipertency agent. Proceedings of the 16th AAAP Animal Science Congress. (2): 2632-2635. Yogyakarta : Gadjah Mada University.

Fricke, B., Drößler, K., Willhardt, I., Schierhorn, A., Menge, S. and Rücknagel, P. 2001. The cell envelope-bound metalloprotease (camelysin) from *Bacillus cereus* is a possible pathogenic factor. Biochimica et Biophysica Acta (1537): 132–146.

Jamhari, Yusiati, L.M., Suryanto, E., Cahyanto, M.N., Erwanto, Y. and Murugama, M. 2013. Purification of Angiotensin Converting Enzyme inhibitory peptide derived from Kacang Goat meat protein hydrolysate. Journal of Indonesian Tropical Animal Agriculture 38(4): 293-246.

Hati, S. , Sreeja V, Solanki, J. and Prajapati, J. B. 2015. Significance of proteolytic microorganisms on ACE-inhibitory activity and release of bioactive peptides during fermentation of milk. Indian Journal Dairy Science 68(6) : 584-591.

Laemmli, V.K. 1970. Cleavage of Structural protein during the assembly of the head of bacteriophage t4. Nature (227): 680-685.

Li, G. H., Qu, M. R., Wan, J. Z. and You, J. M. 2007. Antihypertensive effect of rice protein hydrolysate with *in vitro* Angiotensin I-Converting Enzyme inhibitory activity in spontaneously hypertensive rats. Asia Pacific Journal of Clinical Nutrition (16): 275-280.

Liu, L., Ma, M., Cai, Z., Yang, X. and Wang W. 2010. Purification and properties of a collagenolytic protease produced by *Bacillus cereus* MBL13 strain. Journal of Food Technology and Biotechnology (48): 151-160.

Owusu-Appenton, R.K. 2002. Food Protein Analysis Quantitative Effects on Processing. New York: CRC Press

Saiga, A., Iwai, K., Hayakawa, T., Takahata, Y., Kitamura, S., Nishimura, T. and Morimatsu F. 2008. Angiotensin I-Converting Enzyme-Inhibitory peptides obtained from chicken collagen hydrolysate. Journal of Agriculture Food Chemistry (56): 9586–9591.

Umayaparvathi, S., Meenakshi, S., Arumugam, M. and Balasubramanian, T. 2013. Purification and characterization of protease from *Bacillus cereus* SU12 isolated from oyster Saccoctrea cucullata. African Journal of Biotechnology 12(40): 5897-5908.

Zambare, V., Nilegaonkar, S. and Kanekar, P. 2010. Application of protease from *Bacillus cereus* MCM B-326 as a bating agent in leather processing. IIOAB Journal 1 (4): 18-21.