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THE ANTI HYPERTENSIVE NUTRACEUTICALS OF *VIGNA* SP BEAN PROTEIN HYDROLYZED BY ALCALASE AND FLAVOURZYME

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ABSTRACT

Peptide with hydrophobic amino acids had been studied for their inhibitory activity against angiotensin-I converting enzyme (ACE-1) transformation into ACE-2 and prevention of hypertension. The active peptides may come from alcalase and flavourzyme hydrolysis of bean protein. This study aimed to measure ACE-1 inhibitory of protein hydrolysates from *Vigna* sp. bean (mung bean and cowpea) that grew in Indonesia, and its solubility. The bean protein (22.9 - 23.6 %) was extracted using isoelectric precipitation at pH 4-4.6. The extracts were hydrolyzed at pH 8 for alcalase and pH 7 for flavourzyme, followed with inactivation at 80-85 ^{o}C. ACE-1 inhibitory activity was calculated based on the amount of hippuric acid (HA) formed by the hydrolysis of *Hippuryl-His-Leu* (HHL), in spectrophotometry detection method (228 nm). Ultra-chromatography evaluation showed that the protein hydrolysates of mungbean contained higher hydrophobic amino acids (382 mg/g protein) compared to those of cowpea (329 mg/g protein). Protein hydrolysates of both beans from alcalase hydrolysis have higher ACE-1 inhibitory activity rather than those from flavourzyme. Protein hydrolysate from *Vigna* spp bean protein hydrolysis by alcalase, contained small molecular weight peptides (3.9-4.63 kDa) and high ACE-1 inhibition ability (80-93 %), and therefore suggested as antihypertensive nutraceuticals. Highest solubility of protein hydrolysates resulted from alcalase hydrolysis of both beans were observed at pH 8, while those resulted from flavorzyme hydrolysis were at pH 7, respectively.

Keywords: *ACE-1 inhibitory ability, alcalase, anti-hypertensive, flavourzyme, vigna sp*

ABSTRAK

Peptida dengan asam amino hidrofobik telah diidentifikasi berkemampuan menghambat aktivitas angiotensin-I converting enzyme (ACE-I) menjadi ACE-2 dan mencegah hipertensi. Senyawa peptide tersebut dapat berasal dari hidrolisis protein kacang-kacangan oleh alkalase dan flavorzim. Studi ini bertujuan menilai aktivitas penghambatan ACE-1 oleh hidrolisat protein dari kacang-kacangan *Vigna* sp (kacang hijau dan kacang tunggak) yang tumbuh di Indonesia, dan juga kelarutannya. Protein kacang (22,9 -23,6%) diekstrak menggunakan metoda presipitasi sesuai titik isoelektrik pada pH 4-4,6. Ekstrak dihasilkan dari hidrolisis alkalase pada pH 8 dan flavourzyme pada pH 7, diikuti inaktivasi pada suhu 80-85 ^{o}C. Aktivitas penghambatan ACE-1 diukur berdasarkan jumlah asam hipurat (HA) yang terbentuk dari hidrolisis Hippuryl-His-Leu (HHL), dan pembacaan spektroskopi (228 nm). Evaluasi ultra kromatografi menunjukkan bahwa asam amino hidrofobik dalam protein hidrolisat dari kacang hijau (382 mg/g protein) lebih tinggi dibandingkan yang berasal dari kacang tunggak (329 mg/g protein). Aktivitas penghambatan ACE-1 lebih tinggi pada protein hidrolisat kacang *Vigna* sp yang dihidrolisis oleh alkalase daripada flavorzim. Protein hidrolisat kacang genus *Vigna* spp yang dihidrolisis oleh alkalase menghasilkan peptide molekul kecil (3,9-4,63 kDa) dan aktivitas penghambatan ACE-1 tinggi (80-93%), dan dengan demikian direkomendasikan sebagai nutrasetikal untuk antihipertensif. Kelaruratan terbaik hidrolisat protein kedua kacang dari hidrolisis alkalase adalah pada pH 8 sedangkan dari hidrolisis flavorzim pada pH 7.

Kata kunci: *Aktivitas penghambatan ACE-I, alkalase, antihipertensif, flavorzim, vigna sp*

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INTRODUCTION

Mung beans (*Vigna radiata* (L) R. Wilzeck) and cowpea (*V. unguiculata* (L) Walph) are protein rich vegetables with more than 20% protein content. The hydrolyzed protein of the bean grown in Afrika (Adeyemi., et al, 2012), including Nigeria (Aremu et al., 2017), and also those in China (Li et al., 2005) and Mexico (Seguro-Campos et al., 2011) were found to have inhibitory activity against angiotensin-1 converting enzyme (ACE-1) that play roles in hypertension prevention. The bean protein hydrolysate was able to inhibit the ACE-I action in becoming ACE-2, that stimulated aldosterone secretion and consequently, increased blood pressure. Such beans were also grown in Indonesia, and though not yet studied before, protein hydrolysates coming from Indonesian beans may also be a potential source of natural functional ingredients or nutraceuticals with ACE-1 inhibitor or antihypertensive activities.

The angiotensin-1 converting enzyme (ACE-1) inhibitory ability arises from the action of protein hydrolysates that containing a mixture of short-chain peptides (2-15 amino acid residues) with hydrophobic amino acids at their end sites, such as Arg-Lys, Val-Ala-Pro, Phe-Val-Ala-Pro, and Try-Phe-Trp-Leu. Previous studies shown that the peptide composition in bean protein hydrolysates also consist of hydrophobic amino acids at the end side of its chain, that are leusine (Leu), phenylalanine (Phe), valine (Val) and isoleucine (Ileu). Previous studies have shown that the alcalase and flavourzyme,exogenous proteases, hydrolyzed the bean protein yielding the small peptides fragment with low molecular weight with high ACE-I inhibitory ability (Forghani et al., 2012; Hernandez-Ledesma dan Hsieh, 2013; Iwaniak et al., 2014). Various studies have shown that bean protein hydrolysates antihypertensive activity were correlated to their performance in inhibiting ACE-1 activity (Arihara et al., 2000; Daskaya-Dikem et al., 2017; Nakamura et al., 1995; Torruco-Uco et al., 2009).

Cowpea contains high protein (23-32%) (Diouf, 2011) that consist hydrophobic amino acids, namely Ala, Gly, Leu, Val, Ileu, Phe, Pro, Cys, and Met; making this bean potential yielding ACE-1 inhibitor peptides (Segura-Campos et al., 2013). Mungbean contain high protein (23.7%) (Brishti et al., 2017) and high amount of hydrophobic amino acids (Li et al, 2005) as well. Objectives of the study was to evaluate the ACE-1 inhibitory ability of alcalase and flavourzyme hydrolyzed protein from two kinds of V*igna* genus bean that are mung bean (*V. radiata* (L) R. Wilzeck) and cowpea (*V. unguiculata* (L) Walph; and therefore their potencies as antihypertensive nutraceuticals.

MATERIALS AND METHOD

Materials and instruments

The main ingredients used in the study were mung beans (*Vigna radiata* (L) R. Wilzeck) and cowpea (*V. unguiculata* (L) Walph) obtained from Tanjung Market, Jember, East Java. The enzymes used were alcalase 2.4L (2.4 U/g) (Sigma P 4860); flavourzyme 500L (500 U/g) (P6110 Sigma), ACE-I (angiotensin-I converting enzyme) from rabbit lungs (2.0 units/mg protein) (A6778 Sigma). The chemicals used were HHL (Hippuryl-L-Histidyl-L-Leucine) (H1635 Sigma), n-hexane (Merck), NaOH (Merck); 1 M HCl (Mediss); K_2SO_4; $CuSO_4$ H_2SO_4; MM-MB (MBC) Mediss); boric acid; trichloroacetic acid (TCA) (Merck); bovine serum albumin (BSA) (Sigma); Lowry A (Folin-Ciocalteau (Merck) (phosphotungstat-phosphomolybdat acid solution) with aquades 1: 1); Lowry B (Na_2CO_3 2% in 1N NaOH; 1% $CuSO_4.5H_2O$; sodium potassium tartaric (2%); sodium borate buffer pH 8.3; buffer solution pH 7 (Merck), and aquades.

Specific instruments used were a freeze dryer (CHRIST Alpha 1-2 LD plus); shaking waterbath (StuartSBS40); centrifuge (Tomy MRX-150 and Hitachi CR21GIII); pH meter (Horiba F-51); LAF (Laminar Air Flow)(Nuaire); spectrophotometer (Hitachi type U-2900 UV-Vis); kjeldahl flask (BUTCHI); destilator (BUTCHI K-355)

METHODS

Mung bean and cowpea protein extraction

The bean were cleaned from contaminants, then grinded and sieved with an 80 mesh size. The flour was then defatted according to the method of

Viernes et al. (2012), using n-hexane with a ratio of flour to solvent of 1:10 (w/v) for 1 hour in a cold room (4 ^{o}C) with constant stirring. The solvent was removed by decantation, while the remaining solvent in flour precipitate was evaporated at room temperature. The dried flour was then stored in a 4 ^{o}C storage room until being used for further processing.

Protein extraction was performed by the isoelectric precipitation method, following the method of Lie et al. (2005) for mung bean, and the methods of Salcedo-Chavez et al. (2002) for cowpea. The material in the form of flour was suspended by adding distilled water at a ratio of 1:10 (w/v) for mung bean 1:6 (w/v) for cowpea. The highest solubility pH was then adjusted to pH 9 for mung bean and pH 11 for cowpea by adding 1 dan 2 M NaOH. Constant stirring was applied during pH adjustment using a magnetic stirrer until the pH was stable, and then left for 1 -2 hour with constant stirring at room temperature. Furthermore, centrifugation process was conducted to separate the dissolved protein from the material. On the mung bean flour this was carried out for 10 minutes at 5500 rpm at 20 ^{o}C, while for the cowpea flour it was conducted for 20 minutes at 10, 000 rpm and at 4 ^{o}C. The supernatant was separated from the precipitate for subsequent precipitation by an isoelectric pH setting of 4.5 and 4.6 for the bean using 1 N HCl., respectively. Constant stirring was applied during pH adjustment using a magnetic stirrer until the pH was stable, and then left for 30 minutes to allow the protein to be completely deposited. The suspension was then centrifuged again with the same time, speed and temperature as before to separate the protein and the remaining dissolved materials. The precipitate centrifugation results were separated from the supernatant, followed by a process of washing using distilled water by means of centrifugation, as previously. The protein precipitate were separated from the supernatant and dried using a freeze dryer.

Enzymatic hydrolysis of the bean protein

The enzymatic hydrolysis process was conducted using alcalase and flavourzyme enzymes, based on the procedure followed by Li et al. (2005) for the mung bean protein material and that of (Segura-Campos et al., 2013) for cowpea protein material. The protein extract was suspended in distilled water at a ratio of 4/100 (w/v), accompanied by stirring using a stirrer. The ratio of the enzyme/substrate concentration of the mung bean protein extract was 20 µL/g protein (alcalase 0.3 AU/g; flavourzyme 50 LAP U/g). Suspension of the mung bean and cowpea protein extract was performed by adjusting to pH 8 for alcalase and pH 7 for flavourzyme, respectively, by adding 1 N NaOH with constant stirring. Furthermore, the suspension was subjected to enzymatic processes at 55 ^{o}C for 120 minutes to mung bean protein extract, and at 50 ^{o}C for 90 minutes to cowpea extract protein. The hydrolysis process was performed by a shaking water bath. The hydrolysis was terminated by enzyme inactivation using heating for 10 minutes at 85 ^{o}C, followed by a centrifugation process to separate the supernatant from the precipitate. Centrifugation was carried out at 4 ^{o}C at a speed of 10,000 rpm for 20 minutes for the mung bean and cowpea protein extract material. The resulting supernatant was a protein hydrolysate containing a mixture of peptides and amino acids. The results were then dried using a freeze dryer and stored at -20 ^{o}C until being used for further processing.

Analysis of the bean protein content (AOAC, 2001)

One gram of each mung bean and cowpea was placed into a Kjeldahl flask, followed by the addition of 7 g of K_2SO_4, 0.8 g of $CuSO_4$ and 12 ml of concentrated H_2SO_4. The mixture was then warmed in a laminar hood for ± 6 hours, followed by cooling for 10-20 minutes. After it had cooled, distilled water was added to make a total volume of 80 ml. A 50 ml 50% NaOH (w/v) was then added, which was then distillated until 150 ml of distillate was obtained. The resulting distillate was put into an Erlenmeyer flask with 30 ml of 1% H_2BO_3 (w/v) which had been dropped by 2 drops MM-MB indicator. The obtained distillate was titrated with a standard solution of 0.1 M HCl to light purple. The same treatment was performed using distilled water as a blank. The percentage of total protein content can be obtained with the following calculation: % N = ((ml sample HCl-ml HCl blank) x M HCl x

14.01) / (sample weight x 1000) x 100% % total protein =% N x conversion factor (6.25).

Composition amino acid analysis (Genetech, 2017)

The composition of amino acids in the bean protein hydrolysate was determined using Waters Acquity UPLC H Class and H Class Bio Amino Acid Analysis System Guide year 2012. Sample was prepared using the following procedure: A 100 mg of each mung bean and cowpea protein hydrolysate was added by 5 ml HCl 6N, homogenized, then the mixture was hydrolyzed at 110ºC for 22 hours. Furthermore, the cool hydrolyzed mixture was diluted by adding aquabidest up to 50 mL, and then was filtered out with a 0.45 µm filter. A 500 µl of the filtrate was added with 40 µm AABA and 460 µl aquabidest. A 10 µl of sample solution was added 70 µl AccQ-flour Borate, and vortexed. Then, the homogenous solution was added with 20 µl reagent flour A, homogenized, hushed up for one second, and incubated for 10 second at 55 ºC. *Standard solution was prepared using the following procedure:* 40 µl amino acids mix standard was added with 40 µl AABA internal standard and 920 µl aquabidest, and homogenized subsequently. A 10 µl of the standard solution was added with 70 µl AccQ-fluor Borate and vortexed. Then, the homogenous solution was added with 20 µl reagent flour A, homogenized, hushed up for one second, and incubated for 10 second at 55 ºC. The volume of one µl of sample solution and standard solution was injected to UPLC using ACCQ-Tag Ultra C18 column at 0.7 mL per minute and 49 ºC, and detector PDA 260 nm. The amount of amino acids content can be obtained with the following calculation: Amino acid content (mg/kg) = (area standard/AABA standard x ml Final Volume x fpx x C standard) / Area sample/AABA x gr sample.

Degree of hydrolysis (Silvester et al., 2013)

A 500 µl of the bean protein was added with 500 µl of 20% TCA and then homogenized and incubated at 4 ºC for 30 minutes. The mixture was centrifuged at 6500 rpm for 20 minutes and the supernatant analyzed for dissolved protein content based on the Lowry method (Purwanto, 2014). Bovin Serum Albumin (BSA) was used as a protein standard. The percentage degree of hydrolysis was calculated using following calculation: DH (%) = (10% dissolved protein TCA (mg)) / (total protein content (mg)) x 100%

Protein solubility analysis (Muhamyankaka et al., 2013)

The bean protein hydrolysate was dissolved in distilled water (1% w/v) and the pH was adjusted (to 3, 5, 7, 8, 9, 10 and 11) by adding 1N HCl and 1N NaOH. Constant stirring was then carried out using a magnetic stirrer for 30 minutes at room temperature (25 ºC). Subsequently, the suspension was centrifuged for 15 minutes at 5000 rpm. The total protein in the hydrolyzate was analyzed by dissolving the hydrolyzate in 0.2 M NaOH (1% w/v). The content of the dissolved protein in the supernatant and suspension as a whole was measured using the Lowry method (Purwanto, 2014). The percentage of protein hydrolyzate solubility was calculated using the following formula: % Protein solubility = (Soluble protein sample) / (Total soluble protein) x 100%

Analysis of angiotensin-i converting enzyme (ACE-1) inhibitory ability (Li et al., 2005)

Evaluation of ACE-I inhibitory ability was conducted using the principle of the formation of Hippuric Acid (HA) from the reaction of Hippuryl-His-Leu (HHL) with water (H_2O). A sample of bean protein hydrolysate of 40 µl (10 mg/ml) was added with 100 µl of 5mM HHL in 0.1 M borate buffer pH 8.3 containing 300 mM NaCl, and the mixture incubated at 37 ºC for 5 minutes. A 10 µl ACE-I was then added at a concentration of 100 mU/ml and incubated for 60 minutes at 37 ºC. The reaction was stopped by adding 150 µl of HCl 1N and vortexed until homogeneous. Furthermore, 1.5 ml of ethyl acetate was added to dissolve the HA released from the HHL by ACE. The solution was then centrifuged for 10 minutes at 4000 rpm, and 1 ml of supernatant containing HA taken to evaporate the ethyl acetate. The HAobtained was further diluted by adding 3 ml of distilled water and then vortexed. The solution formed was evaluated in the spectrophotometer for its absorbance at a wavelength of 228 nm. ACE

inhibitory activity (%) was calculated by the following formula:

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

Where: A = absorbance value with the addition of ACE and sample, B = value of control absorbance (buffer replacing sample), C = blank absorbance value (HCl added before ACE)

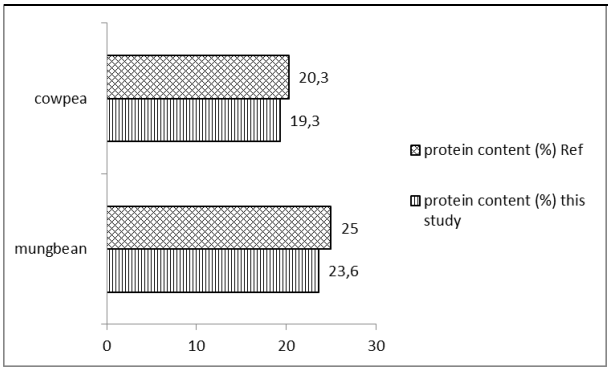

Figure 1. Comparison of protein content in mung bean and cowpea in this study and various references (Butt and Batool, 2010; Mune et al, 2013)

The levels of mung bean protein in this study were almost the same as those of Butt and Batool (2010) (23.9 percent protein content), but lower than those shown by Mubarak (2005) (25.1 percent protein content), respectively. The similarity of mung bean protein level between this study and that of Butt and Batool (2010), might be correlated to the fact that the mung bean used in the two studies were grown in Indonesia. On the other hand, Mubarak (2005) obtained higher protein content values using mung beans grown in Saudi Arabia. Carerra et al. (2011) statement, in agreement to the fact, stated that different types of climate and soil where a bean grows and develops lead to nutritional variation, including the protein content.

Total protein content of cowpea evaluated in this study (19.3 %, Figure 1) had different values compared to 20.3% protein content observed in Mune et al. (2013) and Khalid and Elharradallou (2013). The cowpea used in this research was grown in Indonesia while the other studies mentioned before used beans grown in Sudan, and Kamerun. Differences of cultivars may also play role in the nutritional variation (Habibullah et al.,2007).

Protein and hydrophobic amino acids content of mung bean and cowpea protein extract

The mung bean and cowpea protein content from the protein extract were 72.0 and 64.2 %, respectively. These results were higher (for mung bean) but lower (for cowpea) in comparison to previous studies. Comparison data of the protein content in bean protein extract and in protein hydrolysates from the results of this study and or those of others can be seen in Figure 2. Mungbean protein extract contained higher protein content value than cowpea in the results of this research. The result is in conjunction with previous studies.

The protein content of the mung bean protein extract from the results of this research were lower than previous studies (Bristhi et al.,2017; Sibt-e-Abbas et al., 2016; Butt and Batool, 2010). On the other hand, cowpea protein extract in this research consisted of protein value of (62.5 %) higher than previous studies (Mwasaru, et al.,1999; Adeyemi et al.,2012; Frota, et al., 2017). While differences in climate and soil type where the bean grown might play a role in the variation, differences in protein extraction methods might also have an influence.

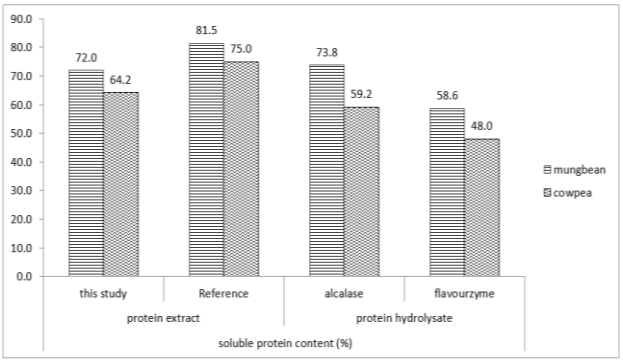

Figure 2. Comparison of protein content in extract protein and its protein hydrolysates of mung bean and cowpea in this study and other references (Bristhi et al., 2017; Khalid and Elharadallau, 2013)

The mung bean and cowpea hydrolysate protein in this study had lower protein values than hydrolysate protein found by other researchers. A comparison of the protein content in bean protein hydrolysate can be seen in Figure 2. The data in the figure showed that alcalase hydrolyzed protein both in the mung bean and cowpea had a higher protein content than those in flavourzyme hydrolyzed protein The protein hydrolyzate content of mung bean protein and cowpea hydrolyzed alcalase were higher than those of flavourzyme because alcalase enzymes hydrolyzes peptides with broad specificity, releasing hydrophobic peptide bonds such as Phe, Tyr, Trp, Leu, Ile, Val and Met (Doucet et al., 2003), which have the potential to be ACE-I inhibitors (Li et al., 2005). In addition, the enzyme flavourzyme contains protease complex endoproteinase and exopeptidase, with greater exopeptidase activity. This enzyme is only specific in breaking the peptide bonds in the leucine amino acids located in the amine group (Fonsseca et al., 2016).

With regard to amino acid, its amount and character or composition in a peptide determine the quality and physiological role of protein. Many essential amino acids are hydrophobic, but only little of those are hydrophilic. Peptides are produced from the alcalase hydrolyzed process of extract protein that is composed of hydrophobic amino acids at the end site of its chain, which is known to have ACE-1 inhibiting ability. Mung bean extract protein hydrolyzed alcalase produced five peptides with following order Lys-Asp-Try-Arg-Leu,Phe or Val-Thr-Pro-Ala-Leu-Arg and Lys-Leu-Pro-Ala-Gly-Thr (Li et al., 2006). Data in Table 1 showed that the amount of two amino acids groups in this research were lower compared to other research findings. Furthermore, the hydrophobic amino acids content in mung bean and cowpea extract protein was lower than that of hydrophilic amino acids, similar to the reference findings. This study analysis data showed that mung bean extract protein contained higher hydrophobic amino acids than that of in cowpea (Table 1), Potentially, alcalase hydrolyzed mung bean protein produced high amount peptides composed of hydrophobic amino acids that having high ACE-1 inhibitory ability.

Table 1. Comparison of amino acids in mungbean and cowpea protein extract in this research and other studies

Amino acids group	Kind of amino acids	Content (mg/g protein)			
		[1]This Study		[2] Ref	[3] Ref
		Mungbean	Cowpea	Mungbean	Cowpea
Hydrophobic	*Isoleucine (Ile)	38.48±0.18	35.64±0.13	64.5	44.9
	*Leusine (Leu)	73.45±0.34	66.95±0.27	69.1	94.5
	*Methionine(Met)	10.87±0.03	10.47±0.04	130.0	2.2
	*Cystein (Cys)	0.88±0.01	1.10±0.01	43.0	12.4
	*Phenilalanin (Phe)	67.62±0.33	46.77±0.17	53.1	55.8
	*Trypthophan (Tryp)	5.02±0.12	6.08±0.01	2.7	2.7
	*Tyrosine (Tyr)	30.05±0.13	23.51±0.08	26.6	21.3
	*Valin (Val)	43.98±0.06	39.22±0.13	32.4	54.6
	Serin (Ser)	45.31±0.18	39.76±0.13	53.2	66.5
	Prolin (Pro)	34.28±0.07	30.77±0.01	43.0	49.1
	Alanin (Ala)	32.33±0.13	28.73±0.10	29.7	66.3
		382.24	***328.97***	***547.3***	***470.3***
Hydrophylic	Arginin (Arg)	57.19±0.22	47.24±0.14	46.0	36.5
	Aspartat (Asp)	92.67±0.29	75.07±0.29	98.0	130.3
	Glutamat (Glu)	159.20±0.85	136.38±0.46	203.0	155.6
	Glysin (Gly)	30.73±0.08	26.63±0.06	28.7	66.3
	Histidine (His)	27.02±0.21	23.47±0.07	37.5	34.5
	*Lysine (Lys)	73.14±0.33	71.33±0.25	140.2	65.0
	*Threonin (Thr)	26.52±0.13	27.25±0.05	48.8	42.5
		466.45	***407.36***	***602.2***	***530.7***

* essential amino acids [1] this study result [2] Ref: Mune et al., (2013) [3] Ref: Bristhi et al., (2017)

Protein hydrolysis degree and the solubility of mung bean and cowpea protein hydrolysates

The degree of protein hydrolysis can be expressed as an indicator of the success of the hydrolysis process. The higher the percentage, the better the hydrolysis. Figure 3 shows that the values of the degree of hydrolysis in mung bean and cowpea protein hydrolyzed by alcalase were higher than with the use of flavourzyme (43>23% and 49>34%). The difference was due to the enzyme specificity of the substrate. Mung bean protein is rich in protein sources of hydrophobic amino acids such as Phe, Tyr, Trp, Leu, Ile, Val and Met. Different types of enzymes used in the hydrolysis process will produce different degrees of hydrolysis. Alcalase is an alkaline protease that is able to produce bioactive peptides with ACE-I inhibitory activity.

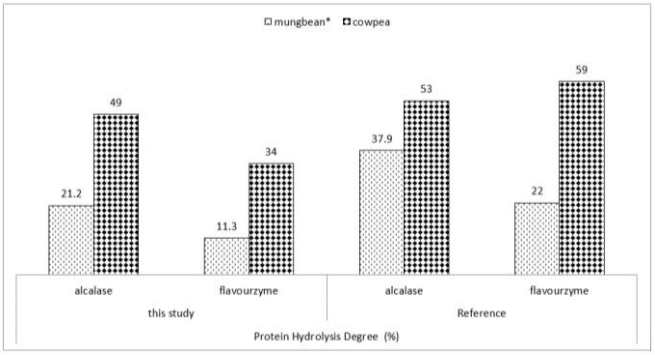

Figure 3. The degree hydrolysis values of mung bean and cowpea protein hydrolysed by alcalase and flavourzyme

Alcalase enzymes hydrolyses peptides with broad specificity, releasing bonds of hydrophobic peptides such as Phe, Tyr, Trp, Leu, Ile, Val and Met (Doucet et al., 2003), which have the potential to be ACE-inhibitors (Li et al., 2005). On the other hand, the enzyme flavourzyme contains protease complex endoproteinase and exopeptidase, with greater exopeptidase activity. This enzyme is only specific in breaking the peptide bonds in the leucine amino acids located in the amine group (Fonsseca et al., 2016). It can therefore be concluded that mung bean and cowpea were good substrates for alcalase enzymes to produce antihypertensive peptides. Solubilities of mung bean and cowpea protein hydrolyzed by alcalase were highest at pH 8. While the solubilities of those hydrolyzed by flavourzyme were highest at pH 7 (Figure 4).

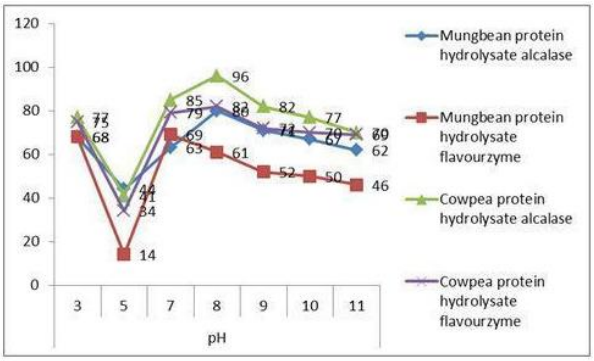

Figure 4. The solubility of mungbean and cowpea protein hydrolyzed by alcalase and flavourzyme

The SDS-PAGE electrophoresis of mungbean and cowpea protein hydrolysates showed that the fractions or peptides molecular weight ranged in between 22.6-46.3kDa. However, after alcalase hydrolyzed, the mung bean and cowpea protein hydrolysates fractions or peptides were having smaller molecular weight ranged in between 3.0-8.7 kDa (Figure 5).

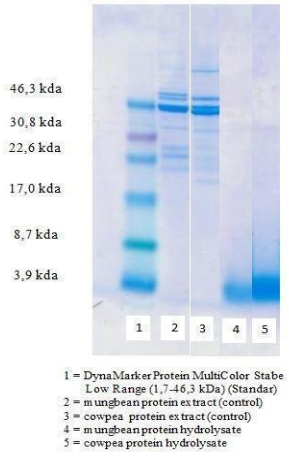

Figure 5. Fractination of mung bean and cowpea protein hydrolysed by alcalase on the molecular weight basis

ACE-I inhibiting ability

Angiotensin-I converting enzyme (ACE-I), a peptidyl peptide hydrolase enzyme plays a role in increasing blood pressure in the body. ACE-I inhibitory activity determines the potential of an ingredient utilization as an antihypertensive agent. The ACE-I enzyme used in this study was isolated from rabbit lungs and commercialized for research purposes may be used in testing the antihypertensive potential of an inhibitor in vitro. ACE-I inhibitory activity analysis was performed by reacting the protein hydrolyzate with Hippuryl-His-Leu (HHL) and involved an inhibitor; in this case the inhibitor used was in the form of protein hydrolyzate in order to obtain a percentage value of the ACE-I inhibition of an inhibitor. Comparison of the ACE-I inhibitory activity of mung bean protein and cowpea protein in this research and other studies can be seen in Table 2.

Table 2. Comparison of the ACE-I inhibitory activity of mung beanand cowpea protein in this research and other studies

Bean hydrolysates	ACE-1 inhibitory (%)		Source
	Alcalase	Flavorzyme	
Mungbean (*Vigna radiata* L. R. Wilczek) (VIMA-1)	91	72	this study
Mungbean *(Vigna radiata)*	79	-	Li et al. (2005)
Cowpea (*Vigna unguiculata* L. Walph)	80	77	this study

From the data in Table 2, the inhibition values of ACE-I mung bean protein hydrolyzed by alcalase and flavourzyme were 91 and 72 percent, respectively. While, the ACE-I inhibition value of cowpea protein hydrolyzed by alcalase and flavourzyme were 80 and 77 percent, respectively. The ACE-I inhibition of mung bean and that of cowpea protein hydrolyzed by alcalase and flavourzyme in this study were higher than previous studies. The evaluation in this study confirmed that the protein of mung bean and cowpea grown in Indonesia, and hydrolyzed by both enzymes had considerably high ACE-I inhibitory ability, since the values were above 70 percent.

The high ACE-I inhibitory activity of mung bean and cowpea hydrolyzate protein may be due to the presence of peptide content with short peptide chains (2-5 amino acid) and C-terminal proline or hydroxyproline residues, peptide chain that have stronger inhibitory effect, since theybind to ACE-I more strongly. Proline, lysine and arginine are the preferred C-terminal substrates for ACE-I, contributing greatly to the inhibition of ACE-I (Erdmann et al., 2008). These enzymatic hydrolyzed peptides have a strong affinity with the active side of the ACE-I enzyme and can interfere with its catalytic activity in hydrolyzing the hippuril-histidyl-leucine (HHL) substrate in in vitro tests, giving high percentage inhibition (Ryan et al., 2011).

Mung bean and cowpea protein hydrolyzed by alcalase expressed higher inhibitory values than those hydrolyzed by flavourzyme. This might due to the fact that enzymatic hydrolysis of proteins using alcalases tends to produce peptides with C-terminal amino acids in aromatic and aliphatic side chains, such as Ile, Leu, Val, Met, Phe, Try and Trp (Doucet et al., 2003). The alcalase enzyme is a group of endopeptidase enzymes that can cut peptide bonds in the middle of the chain of hydrophobic amino acids. In addition, the enzyme flavourzyme also has the ability of both enzyme endopeptidase and exopeptidase, which can cut peptide bonds in the middle or at the end of the chain of combined amino acids, especially in leucine amino acids (Leu) (Fonsseca et al., 2016).

CONCLUSION

The alcalase and flavourzyme hydrolysed protein of mungbean and cowpea grown in Indonesia, were

able to inhibit the activity of angiotensin-I converting enzyme (ACE-I) with inhibitory values greater than 70 percent. The amount of hydrophobic amino acids in mungbean protein extract that hydrolysed by alcalase, was higher than that of in cowpea protein extract. This might play a role in the higher ability of ACE-1 inhibitory by mung bean protein hydrolysate (91%) than that of cowpea protein extract (80%). The solubilities of alcalase hydrolyzed mung bean and cowpea protein were highest at pH 8. While the solubilities of those hydrolyzed by flavourzyme were highest at pH 7. The ACE-I inhibitory activity of these two bean protein hydrolysates were considerably high, and may be potential for further exploration as antihypertensive nutraceuticals.

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