ABSTRACT

Kidney bean (Phaseolus vulgaris L. Chitra) and lima bean (Phaseolus lunatus L. Sweet) hydrolysates were obtained by alcalase and flavourzyme hydrolysis of the bean seed protein. Peptide in the bean hydrolysates, with hydrophobic amino acids had been studied for their inhibitory ACE-1 activity preventing transformation into ACE-2 that prevention hypertension. This study aimed to measure inhibitory ACE-1 activity of protein hydrolyzates from the bean Phaseolus genus spp. grown in Jember, and its solubility. The bean protein (19.8-20.2%) was extracted using isoelectric precipitation at pH 4-4.6. The extract were hydrolyzed at pH 8-9 for alcalase and pH 7 for flavourzyme, followed with inactivation at 80-85 °C. ACE-1 inhibitory activity was measured based on the amount of hippuric acid (HA) formed by the hydrolysis of Hippuryl-His-Leu (HHL) in spectrophotometry detection method (228 nm). The ultra chromatography evaluation showed that the protein hydrolysates of kidney bean contained higher hydrophobic amino acids (455.5 mg/g protein) compare to those of lima bean (350 mg/g protein). Protein hydrolysates of both beans from alcalase hydrolysis have higher ACE-1 inhibitory rather than those from flavourzyme. Protein hydrolysate from Phaseolus spp bean protein hydrolysis by alcalase, contain small molecular weight peptides (3.9-22.6 kDa) high ACE-1 inhibition ability (83-88%), and therefore suggested as antihypertensive nutraceuticals. Highest solubility of protein hydrolysate resulted from alcalase hydrolysis of both beans were observed at pH 8-9, while those resulted from flavourzyme hydrolysis were at pH 7.

Keywords: ACE-I inhibitory ability, alcalase, flavourzyme, anti-hypertensive, Phaseolus spp.

ABSTRAK

Tejasari, Faiqotul Aulia, Nurdiana Agustina. 2021. Aktivitas Penghambatan Angiotensin Converting Enzyme-1 oleh Hidrolisat Protein Kacang Genus Phaseolus.

Hidrolisat protein kacang merah (Phaseolus vulgaris L. Chitra) dan koro (Phaseolus lunatus L. Sweet) yang diperoleh melalui hidrolisis enzimatis – alkalse dan flavourzyme dari protein biji kacang tersebut. Peptida dalam hidrolisat kacang tersebut yang mengandung asam amino hidrofobik telah dipelajari tentang kemampuan hambat aktivitas ACE-1 yang mencegah menjadi ACE-2 sehingga menghambat hipertensi. Studi ini bertujuan untuk mempengukur kemampuan hambat ACE-1 oleh hidrolisat protein kedua kacang genus Phaseolus spp yang tumbuh di Jember dan daya larutannya. Protein kacang tersebut (19.8-22.2%) dikstrak dengan metode presipitasi isoelektik pada pH 4-4,6)Ekstrak protein tersebut dihidrolisis pada pH 8-9 oleh alkalse dan pH 7 oleh flavourzyme, selama 20-240 menit , suhu 50°C, dan diakhiri pada suhu 80-85 °C. Aktivitas hambat ACE-1 ditentukan berdasarkan jumlah asam hipurat yang terbentuk dari hidrolisis Hippuryl-His-Leu (HHL), pada metode spektrofotometri dideteksi pada panjang gelombang 228 nm. Evaluasi ultrakromatografi menunjukkan bahwa hidrolisat protein kacang merah berisi asam amino hidrofobik lebih tinggi (455 mg/g protein) dibanding hidrolisat kacang koro (350 mg/g protein).Hidrolisat dua jenis kacang yang berasal dari hidrolisis alkalse berkemampuan hambat ACE-1 yang lebih tinggi daripada hidrolisat flavourzyme. Hidrolisat kacang genus Phaseolus spp dari hidrolisis alkalse menghasilkan peptide berberat molekul rendah (3,9-22,6 kDa), memiliki kemampuan hambat ACE-1 tinggi (83-88%), dan direkomendasikan sebagai antihipertensi nutrasetikal. Kelarutan tinggi dari hidrolisat protein kedua jenis kacang terhidrolis alkalse dicapai pada kondisi basa (pH 8-9), sementara hidrolisis flavourzyme pada pH 7.

Kata kunci: kemampuan hambat ACE-I, alkalse, flavourzyme, Phaseolus spp, antihipertensif nutrasetikal
INTRODUCTION

A protein, in addition to being a source of energy for the body, can also display biological activities, so is believed to be able inhibiting various diseases. One of the biological activities of protein is as an antihypertensive. However, the antihypertensive biological activity of legumes cannot be obtained when consumed as whole protein. This is because in the human body there are no specific protease enzymes that can break down the protein of legumes to produce protein fragments in the form of antihypertensive peptides, so modification is needed. One such modification is by producing protein hydrolysate by protein hydrolysis, using specific protease such as alcalase and flavourzyme.

Various studies have shown that whether or not protein hydrolysates are antihypertensive can be established by testing the performance-inhibiting activity of an ACE-I enzyme (angiotensin-I converting enzyme)\(^1\,^2\,^3\,^4\,^5\). The inhibitory activity of ACE-I in protein hydrolyzate is the ability of an inhibitor to inhibit the action of ACE-I, which is an enzyme that causes an increase in blood pressure (hypertension). Protein hydrolysates are functional ACE-I inhibitors, with characteristics containing a mixture of short-chain peptides from 2-15 amino acid residues, that are hydrophobic in their end chains, such as Arg-Lys, Val-Ala-Pro, Phe-Val-Ala-Pro and Try-Phe-Trp-Leu\(^6\). Protein hydrolyzate with these characteristics can be obtained because of the suitability of the enzyme for the protein substrate during the hydrolysis process.

Previous studies have shown that the protein hydrolyzed alcalase and flavourzyme enzymes of legumes have high ACE-I inhibitory activity. High content of hydrophobic amino acids from legumes, such as leucine, phenylalanine, valine and isoleucine, are very suitable for alcalase and flavourzyme which specifically cut peptide chains composed of the hydrophobic amino acids so that they can produce protein hydrolyzate with high ACE-I inhibition\(^5\,^7\). Two examples of types of protein-rich legumes rich in hydrophobic amino acids are kidney bean (\textit{Phaseolus vulgaris} L.) and lima bean (\textit{Phaseolus lunatus} L. Sweet). Kidney bean protein hydrolyzed enzymatically by alcalase has high ACE-I inhibitory activity\(^8\) and lima bean protein hydrolyzed alcalase, as well\(^9\). However, the value of the inhibitory activity of ACE-I protein hydrolyzate derived from lima bean and kidney bean grown in Indonesia, if hydrolyzed using alcalase and flavourzyme enzymes is valuable to evaluate. This study was conducted to 1) measure the protein content of the kidney and lima bean seed; and dissolved protein in the two bean isolates; 2) determine protein content of the kidney and lima bean seed; and evaluate. This study was conducted to 1) measure the protein hydrolysates of an ACE-I enzyme (angiotensin-I converting enzyme) established by testing the performance-inhibiting activity of ACE-I enzyme (angiotensin-I converting enzyme) and Try-Phe-Trp-Leu\(^6\). Protein hydrolyzate with these characteristics can be obtained because of the suitability of the enzyme for the protein substrate during the hydrolysis process.

Previous studies have shown that the protein hydrolyzed alcalase and flavourzyme enzymes of legumes have high ACE-I inhibitory activity. High content of hydrophobic amino acids from legumes, such as leucine, phenylalanine, valine and isoleucine, are very suitable for alcalase and flavourzyme which specifically cut peptide chains composed of the hydrophobic amino acids so that they can produce protein hydrolyzate with high ACE-I inhibition\(^5\,^7\). Two examples of types of protein-rich legumes rich in hydrophobic amino acids are kidney bean (\textit{Phaseolus vulgaris} L.) and lima bean (\textit{Phaseolus lunatus} L. Sweet). Kidney bean protein hydrolyzed enzymatically by alcalase has high ACE-I inhibitory activity\(^8\) and lima bean protein hydrolyzed alcalase, as well\(^9\). However, the value of the inhibitory activity of ACE-I protein hydrolyzate derived from lima bean and kidney bean grown in Indonesia, if hydrolyzed using alcalase and flavourzyme enzymes is valuable to evaluate. This study was conducted to 1) measure the protein content of the kidney and lima bean seed; and dissolved protein in the two bean isolates; 2) determine the characterization of the two bean protein hydrolysates in relation to dissolved protein, degree of hydrolysis (DH), peptide profile, and its solubility; and 3) determine of the ACE-I inhibitory ability of alcalase and flavourzyme hydrolyzed of kidney and lima bean protein, separately.

MATERIALS AND METHODS

Materials

Kidney bean (\textit{Phaseolus vulgaris} L. cv Chitra) and lima bean (\textit{P. lunatus} L. Sweet) originated from Bondowoso Regency and purchased from Pasar Tanjung market Jember. After grinding each beans into 80 mesh flour using a laboratory mill, the bean flour was defatted with n-hexane for 24 h in a shaker\(^10\). Angiotensin-I converting enzyme (ACE, from rabbit lungs; 2.0 units/mg protein; A6778), hippuryl-L-histidyl-L-leucine (Hip-His-Leu; H1635), alcalase 2.4 L (activity of 2.4 U/g, P 4860), and flavourzyme 500 L (activity of 500 U/g, P6110, Sigma) Other chemicals namely n-hexane (Merck), NaOH (Merck), trichloroacetic acid (TCA) (Merck); Folin-Ciocalteau (Merck), 1 M HCl (Mediss), MM-MB (MBC) Mediss), Lowry mix, and buffer solution pH 7 (Merck) were purchased from Medialabs Chemical Co (Bogor, Indonesia).

Specific tools used were a freeze dryer (CHRIST Alpha 1-2 LD plus), shaking water bath (StuartSBS40), centrifuge (Tomy MRX-150 and Hitachi CR21GIII), pH meter (Horiba F-51); Laminar Air FlowNinaire (LAF), spectrophotometer (Hitachi type U-2900 UV-Vis), kjeldahl flask (BUTCHI); destillator (BUTCHI K-355), and a burette.

The bean flour preparation and protein extraction were done at Food and Agricultural Product Chemistry and Biochemistry Laboratory, while the protein hydrolysis and ACE-1 analysis were done at Nutraceuticals and Pharmaceutical Laboratory, Center of Advanced Science and Technology (cDAST) University of Jember. Amino acids analysis was done at PT Saraswanti Indo Genetech (The First Indonesian Molecular Biotecnology Company) at Taman Yasmin Bogor.

Methods

Protein Extraction of the Kidney (\textit{Phaseolus vulgaris} L. cv Chitra) and Lima (\textit{P. lunatus} L. Sweet) Bean\(^9\)

Protein extraction was performed by isoelectric precipitation method, The kidney bean defatted flour suspension (1:10 w/v) and lima bean defatted flour suspension (1:6 w/v) was adjusted its optimal pH (pH 9 for kidney, and pH 11 for lima bean). After pH value stable, left the each suspension for 1-2 hours with...
constant stirring at room temperature (± 20°C for kidney, ± 25°C for lima bean). Then centrifugation (at 9500 rpm, 4°C, 20 minutes for kidney, and 10,000 rpm, 4°C, 10 minutes for lima bean) were done for obtaining the supernatant. The supernatant containing soluble protein was gained thru subsequent precipitation by setting pH isoelectric at pH 4.5 for the two bean, and left for 30 minutes. The precipitate solution containing protein was again centrifuged at the same condition. The protein precipitate was washed twice with distilled water and then dried under vacuum.

**Protein Content Analysis of the Bean**

One gram of each kidney and lima bean defatted flour was placed into a Kjeldahl flask, followed by the addition of 7 g of K₂SO₄, 0.8 g of CuSO₄ and 12 ml of concentrated H₂SO₄. The solution then was warmed for ± 6 h, followed by cooling for 10-20 minutes. After cooling, distilled water was added to make a total volume of 80 ml. 50 ml 50% NaOH (w/v) was then added, and was then distilled until 150 ml of distillate was obtained. The resulting distillate was put into an Erlenmeyer flask and was distillated until 150 ml of distillate was obtained. The resulting 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 protein was calculated as : % 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). The sample and the blank was measured its protein content at three replications.

**Enzymatic Hydrolysis of the Bean Protein**

Hydrolyzed Kidney bean protein were suspended in distilled water to obtain a 4% w/v protein solution, while stirring. The solution was then equilibrated at optimum temperature and pH for each protease (8 for alcalase and 7 for flavourzyme) with 1 N NaOH, before addition of the enzyme. Protease was then added to the solution at ratio 10g/100 mL (alcalase 0.3 U/g flavourzyme 50 U/g). Hydrolysis was performed for 2 h, at 50°C for alcalase, and 45°C for flavourzyme. The enzymatic hydrolysis was terminated by heating for 10 minutes at 85°C, followed by a centrifugation at 10.000 rpm for 20 minutes to separate the supernatant from the precipitate. The resulting supernatant were used at three replicates of ACE inhibitory activity measurement.

**Hydrolysis Degree Determination**

Degree of hydrolysis was determined by the percentage of soluble bean protein (in 10 g/mL w/v trichloro acetic acids or TCA ) in relation to the protein content of the hydrolyzed protein. The hydrolyzed bean protein aliquots of 500 μL were mixed with 500 μL of 20% w/v TCA solution to obtain soluble and insoluble fraction in 10% w/v TCA. After incubated at 4°C for 30 minutes, the mixture was centrifuged at 6500 rpm for 20 minutes and the supernatant analyzed for soluble protein content using Lowry method, and the result was expressed as mg of protein. Bovine Serum Albumin (BSA) was used as protein standard. The degree of hydrolysis was measured at three replication, and as percentage of 10% soluble protein in TCA (mg) in relation to total protein content (mg).

**Dissolved Protein Analysis**

Hydrolysed bean protein of 250 μL was reacted with a 2 ml Mix Lowry (Lowry A + Lowry B) reagent and left for 10 minutes. 250 μL ciocalteau follin was then added and left for 20 minutes. In addition, absorbance measurements were made with a spectrophotometer at 550 nm. The absorbance data were plotted on a BSA standard curve to calculate the level of dissolved protein. A standard protein solution is made by weighing 0.01 g Bovine Serum Albumin (BSA), which was then dissolved in 10 mL of distilled water so that a BSA stock solution with a concentration of 1 mg/mL was obtained. Making standar solution with concentrations of 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8 0.9 and 1 mg/mL. 250 μL BSA solution with various concentrations was taken and reacted with 2 mL Mix Lowry reagent and left for 10 minutes. 250 μL Ciocalteau follin was then added and left for 20 minutes. The absorbance was measure with a spectrophotometer at 550 nm. The dissolved protein levels were measured as percentage of the protein concentration in diluted sample (mg / mL) to the protein content (mg protein).
Protein Solubility Analysis

The bean protein hydrolyzate was dissolved in distilled water (1% w/v) and the pH was adjusted (to 3, 5, 7, 8, 9, 10 and 11) with 1N HCl and 1N NaOH, while stirring for 30 minutes at 25°C. The suspension was then centrifuged for 15 minutes at 5000 rpm. The total protein in the hydrolyzates was analyzed by dissolving it in 0.2 M NaOH (1% w/v). The content of the dissolved protein in the supernatant and suspension as a whole was measured at three replication, using the Lowry method. The soluble protein was measured as percentage of soluble protein sample in relation to soluble total protein.

Amino Acids Composition Determination

The amino acids composition of kidney and lima bean hydrolysates were determined by Ultra Performance Liquid Chromatography (UPLC) according to Waters Acquity UPLC N Class and H Class Bio Amino Acid Analysis System Guide. The hydrolyzates (0.1 g) were mixed with 5 mL HCl 6N, and were hydrolyzed for 22 minutes. The mixed was then added with 10 μl ACE-1 (100 mU/ mL) and was incubated for 60 minutes at 37°C. The reaction was stopped by adding 150 μL HCl 1N and was vortexed. An addition of 1.5 mL ethyl acetate intended to dissolve the hippuric acid released. The solution was then centrifuged for 10 minutes at 1000 x g, and then 1 mL of supernatant containing hippuric acid taken to evaporate the ethyl acetate. The hippuric acid obtained was diluted by adding 3 mL of distilled water and then vortexed, absorbance of the sample and the blank at 228 nm was measured in a spectrophotometer. For the blank experiment, sodium borate buffer was used instead of KBPH solution.

The ACE-inhibitory activity of lima bean protein hydrolysates (LBPH) was assayed as describe by Arihara et al 1, by using the same substrate as for KBPH. The inhibition percentage of ACE inhibitory activity (%) was calculated as:

\[
\% \text{ACE inhibition} = \frac{(B - A)}{(B - C)} \times 100\%
\]

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).

Statistical Analysis

All determinations experiment were performed in three replicates, each measured by triplicates analysis. The result was presented, using descriptive statistics with central tendency and dispersion measures.

RESULTS AND DISCUSSION

Protein Content of Kidney Bean (Phaseolus vulgaris cv Chitra) and Lima Bean (P. lunatus L Sweet)

Kidney (P. vulgaris cv Chitra) and lima bean (P. lunatus L Sweet) (Figure 1) are protein rich vegetables food. The analysis of the protein levels of the kidney and lima bean grown in Indonesia and measured in this study contained 19.83 ±0.18 and 20.15±0.21 percent. The content were lower compare to those in various references studies, as shown in Figure 2. The figure showed that kidney bean protein levels were slightly lower than those of lima bean in this study results, the same as in the reference. The figure shows that lima bean protein levels mentioned in the reference were higher than those of kidney bean in this study results, but they have almost the same values. The protein content difference was influenced by the differences in variety type of the bean, which results in different nutritional content 15, including protein content.
Inhibiting Activity of Angiotensin Converting Enzyme-1 by Bean Protein Hydrolysate Genus Phaseolus

Inhibiting Activity of Angiotensin Converting Enzyme-1 by Bean Protein Hydrolysate Genus Phaseolus (Tejasari, Faiqotul Aulia, Nurdiana Agustina)

**Figure 1. Kidney bean (Phaseolus vulgaris cv Chitra) and lima bean seed (P. lunatus L Sweet)**
Gambar 1. Kacang merah (Phaseolus vulgaris cv Chitra) dan kacang koro (P. lunatus L Sweet)

| Protein Content (%) | this study | Reference |
|---------------------|------------|-----------|
| Kidney              | 19.8±1.08  | Reference |
| Lima                | 21.4±2.3   | Reference |
| kidney              |            | Reference |
| Lima                |            | Reference |
| kidney***            |            | Reference |
| Lima***              |            | Reference |

**Remark/Keterangan:**
- Kidney*: Ref21
- Lima*: Ref18
- Kidney**: Ref20
- Lima**: Ref16
- Kidney***: Ref21
- Lima***: Ref14

**Figure 2. Protein content in kidney and lima bean protein in this study as compared to others findings**
Gambar 2. Kadar protein kacang merah dan kacang koro hasil studi dan referensi

The levels of kidney bean protein in this study show that their total protein content had different values to those of other studies. In this case it was 19.8±1.08 percent, while other studies showed that kidney bean had a protein content of 23±0 percent 20; 21.6±2.68 percent 21 and 15.3±0.20 percent 22. The kidney bean used in this research and in that of the reference20 is the ones that grow in Indonesia, which has a subtropical climate. As for compare, both have different protein content, even though they grow in the same soil and climatic conditions. The kidney bean in the study of the reference21 is from plants that grow in India, while those in the research of the reference23 are ones that grow in Pakistan, both two countries are subtropical. However, both have different levels of protein. The kidney bean in the reference 20 study is the ones that grow in Indonesia, as is the case with those used in this research. Although both grow in the same climate, they have different protein levels. According to the reference21, these differences can also be caused by differences in kidney bean cultivars, different cultivars result in different or varied nutritional content, including protein content.

With regard to lima bean, in this study showed that its protein content is smaller than that of in the reference 16, that obtained values of 21.4±2.3 percent. The value of these protein levels is lower than those other protein levels, as shown in the references 9,17,18, that obtained values of 23.7±0.2, 24.1±0.23 and 26±0.32 percent, respectively. The similarity of lima bean protein level results between this study and that of in the reference 16 is due to the fact that the lima bean used in the two studies grows in the tropical climate of Indonesia, so it has the same protein content. On the other hand, the other studies obtained higher protein content values because the lima bean used was one grown in Mexico, which has a subtropical climate. This is in accordance with
the reference\textsuperscript{16}, who state that different types of climate and soil where a bean grows and develops lead to the nutritional content, including the protein content, being different.

Dissolved Protein of Kidney and Lima Bean Protein Hydrolysates

The results of this study showed that the dissolved protein in kidney bean protein hydrolyzate (62.5±1.07\%) was lower than that of in lima bean protein hydrolyzate (68.2±0.11\%). However, in other study, the dissolved protein of the kidney bean protein isolates (77.8±0.06 \%)\textsuperscript{21} was higher compare to that of in the lima bean protein isolates (69.9±0.0 \%)\textsuperscript{17}. Comparative of the dissolved protein data in the bean protein isolates from this study and those of others can be seen in Figure 3.

Remark/Keterangan :
Ref\textsuperscript{21} for kidney bean; Ref\textsuperscript{17} for lima bean
Alc= alcalase ; Fla= flavourzyme

Figure 3. Comparison amount of the dissolved protein in kidney and lima bean protein isolates and hydrolysates from the results of this study and those of others

Gambar 3. Perbandingan jumlah protein terlarut dalam isolat dan hidrolisat protein kacang merah dan koro pada studi ini dan referensi

The dissolved protein content variations were caused by differences in the climate and soil type where the bean grows. The lima bean used in the study of \textsuperscript{17} originated from Mexico, while the kidney bean in the study of \textsuperscript{21} grew in India, with subtropical climate conditions in both countries. This is in accordance with the statement by \textsuperscript{19}, that different types of climate and soil where the bean grows and develops influence its nutritional content, including the protein content.

The dissolved protein in the kidney bean protein isolates from this study (Figure 3) was lower than those found in reference\textsuperscript{21}, valued by 62.5±1.07 percent compared to 77.8±0.06 percent. Like kidney bean protein isolates, that of lima bean in this study also showed the protein value (68.2±0.11\%) lower than the value (69.9±0.0\%) obtained by the reference\textsuperscript{17}. This difference was not only influenced by variations in climate and soil type where the bean grows, but also by differences in protein extraction methods. In the study of the reference \textsuperscript{21}, the extraction method used was a suspension filtering process using a filter with 75 µm mesh before adjusting the pH of the protein solubility, which served to separate the starch and fiber contained in the flour so that the extraction of the protein produced a higher protein content than the kidney bean protein extract in this studies that refer to the method of \textsuperscript{11} that without a screening process.

In this study, the dissolved protein in kidney and lima bean protein-alcalase hydrolysed was higher than that of in the bean protein-flavourzyme hydrolysed. The comparison of the dissolved protein content in the two bean protein hydrolysed by alcalase and flavourzyme can be seen in Figure 3. The data in the figure showed that kidney bean protein hydrolyzed by each alcalase and flavourzyme enzymes were 76.2±1.62 and 71.1±1.44 percent, respectively. Meanwhile, levels of lima bean protein hydrolyzed by alcalase and flavourzyme was at levels of 50.34±0.13 and 40.21±0.03 percent, respectively. Alcalase hydrolyzed protein both in the kidney bean and lima bean had a higher protein content than those in flavourzyme hydrolyzed protein. The dissolved protein content of kidney bean and lima bean protein hydrolyzed by alcalase are higher than those of flavourzyme because alcalase enzymes hydrolyze peptides with broad specificity, releasing hydrophobic peptide bonds such as Phe, Tyr, Trp, Leu, Ile, Val and Met\textsuperscript{24}, which have the potential to be ACE-I inhibitors\textsuperscript{3}. In addition, the flavourzyme contains protease complex endoproteinase and exopeptidase, with greater exopeptidase activity. This enzyme is only specific in breaking the peptide bonds in the amino acids located in the amine group\textsuperscript{25}.

The dissolved protein content in kidney bean hydrolyzate still has a close value to the protein in kidney bean in the reference\textsuperscript{17}. Meanwhile, the protein in lima bean protein hydrolysates have lower values than those in the research of the reference\textsuperscript{17}, both using alcalase (70.4±1.13 \%) and flavourzyme (71.1±0.35\%). Differences in the protein content in its hydrolyzate of the same type of beans and hydrolyzing enzymes can be caused by differences in the protein contained in each bean. The chemical composition of bean is influenced by differences in the type of climate and soil where they grow and develop, leading to different nutritional content, including that of protein\textsuperscript{19}. 
Degree of Hydrolysis

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 4 showed that the values of the degree of hydrolysis in kidney bean and lima bean protein hydrolyzed by alcalase were higher than with the use of flavourzyme (31.4±1.24 >21.4±0.2 % and 30.63±0.05 > 16.84 ±0.32%). The similar figure was also found by other study. Enzymatic alcalase hydrolysis produced the bean hydrolysates with DH values above 30 percent that require for practical application as antihypertensive nutraceuticals used in functional food.

Figure 4. Comparison data of the protein hydrolysis degree of kidney and lima bean protein from the results of this study and references

Gambar 4. Perbandingan derajat hidrolisis protein kacang merah dan koro terhidrolisis alkalase dan flavorzim studi ini dan referensi

The difference was due to the enzyme specificity of the substrate. Kidney 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 functions to produce bioactive peptides with ACE-I inhibitory activity. Alcalase enzymes hydrolyze peptides with broad specificity, releasing bonds of hydrophobic peptides such as Phe, Tyr, Trp, Leu, Ile, Val and Met, which have the potential to be ACE-inhibitors. 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. It can therefore be concluded that kidney bean and lima bean were good substrate for alcalase enzymes to produce antihypertensive peptides.

Solubility of the bean protein hydrolysates

The determination of protein solubility was expected to be used as a basic reference in the practical application of processed food products, which also produce biological activity in the body due to the presence of bioactive compounds in it. The solubility of kidney bean and lima bean protein hydrolyzed by alcalase was higher than that by flavourzyme. A comparison of the hydrolysates solubility of hydrolyzed protein by alcalase and flavourzyme can be seen in Figure 5. The results of the study showed that the kidney bean protein hydrolyzed by alcalase and flavourzyme have the high solubility at the range pH 3 and 7-11, at 70.46±0.54 up to 98.35±1.71 and at 59.62±0.09 up to 80.22±0.76 percent, respectively. In lima bean protein hydrolyzed using alcalase and flavourzyme have high solubility in the pH 7-11, at 82.73±0.29 up to 86.27±0.29 percent, and 48.36 ± 0.69 up to 61.72±0.19 percent, respectively. The lowest pH solubility in lima bean hydrolyzate and kidney beans was found in the pH of both hydrolysed alcalase and flavourzyme. The lowest pH values of the hydrolyzate protein of kidney bean protein hydrolyzate hydrolyzed by alcalase and flavourzyme were 34.27 ±0.07 and 26.93±0.19 percent, respectively, while lima bean hydrolyzed alcalase and flavourzyme were 54.48±0.33 and 41.54±0.63 percent, respectively, and were similarly low values as those found by other references.

Figure 5. The protein solubility of kidney bean and lima bean hydrolyzed by alcalase and flavourzyme

Gambar 5. Kelarutan protein kacang merah dan koro terhidrolisis alkalase dan flavorzim

In acidic and basic pH conditions (pH 3, 7, 8, 9, 10 and 11), the two hydrolysates sourced from these two bean show a higher solubility and produce a “V” pattern of the curve because the hydrolysis produces a new peptide that has a different electrical charge from non-hydrolyzed proteins. This was consistent with the findings by that protein hydrolyzate can dissolve at pH 3, having positive, and at pH 8-9, having negative charge. Figure 5 showed that alcalase hydrolyzed protein has a higher solubility than that hydrolyzed by flavourzyme. This was influenced by the degree of hydrolysis value. Hydrolyzate with a high hydrolysis value contains...
peptides with low molecular weight, so it is more soluble. The smaller the peptide, the greater the number of amino acids with hydrophilic groups that bind to the water molecules. The hydrolysis process can open bonds between hydrophobic groups, causing the groups to change into hydrophilic groups by producing carboxyl and amino acids that easily bind to water.

ACE-I Inhibiting Activity of Kidney and Lima bean Protein Hydrolysates

Angiotensin-I converting enzyme (ACE-I) is a peptidyl peptide hydrolase enzyme that plays a role in increasing blood pressure in the body. ACE-I is responsible for converting angiotensin I (Ang I) to the powerful vasoconstrictor angiotensin II (AngII) and inactivating vasodilator peptide bradikinin (BK) by removal of C-terminal dipeptide. In functional sense, therefore, the enzymatic action of ACE-I potentially cause increased vasoconstriction and decreased vasodilation. Inhibition of ACE-I prevents conversion of Ang I into Ang II, making it becomes one of the most effective methods for suppressing increases in blood pressure.

ACE-I inhibitory activity analysis is used to determine the potential of an ACE-I inhibitor for use as an antihypertensive agent. The ACE-I enzyme that 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 is performed by reacting the protein hydrolyzate with Hippuryl-His-Leu (HHL) and involves 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 agent.

Comparison of the ACE-I inhibitory activity of kidney bean and lima bean protein in this study and other’s research findings were presented in Table 1.

Table 1. Inhibitory ACE-I Activity of kidney and lima bean protein hydrolysates

| Hydrolysates   | ACE-I inhibitory activity (%) | Sources |
|----------------|-------------------------------|---------|
|                | Acalase                       | Flavoryzme |
| Kidney bean    | 88.28±0.81 81.28±0.43         | This study |
| Lima bean      | 82.76±1.19 70.87±1.62         | This study |

Remark/Keterangan : Acalase = alcalase ; Flavoryzme = flavoryzme

From the data in Table 1, the inhibition values of ACE-I lima bean protein hydrolyzed by alcalase and flavoryzme were 83 and 71 percent, respectively. While the ACE-I inhibition of kidney bean protein hydrolyzed by alcalase and flavoryzme were 88 and 81, percent respectively. These ACE-I inhibition values proved that the lima bean protein and that of kidney bean protein hydrolyzed by both enzymes were rated as its high ability, since the value are above 70-80%.

The high ACE-I inhibitory activity of lima bean and kidney bean hydrolyzate protein may be due to the presence of peptide content with short peptide chains (2-5 amino acid), with C-terminal proline or hydroxyproline residues having a stronger inhibitory effect, having been proven to bind 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.

The amino acid analysis in this study showed that the kidney bean protein hydrolysate contains hydrophobic amino acids higher (405.03 mg/g protein) than that of lima bean protein hydrolysate (308.53 mg/g protein) (Table 2).
Inhibiting Activity of Angiotensin Converting Enzyme-1 by Bean Protein Hydrolysate Genus Phaseolus
(Tejasari, Faiqotul Aulia, Nurdiana Agustina)

Figure 6. Kidney and lima bean protein hydrolysates profile from electrophoresis results
Gambar 6. Profil hidrolisat protein kacang merah dan koro hasil elektroforesis

Kidney bean and lima bean protein hydrolyzed by alcalase have a higher inhibitory activity than those hydrolyzed by flavourzyme. This was because enzymatic hydrolysis of proteins using alcalase tend to produce peptides with C-terminal amino acids in aromatic and aliphatic side chains, such as Ile, Leu, Val, Met, Phe, Try and Trp^{24}. The alcalase enzyme is a group of endopeptidase enzymes that cut peptide bonds in the middle of the chain of hydrophobic amino acids. In addition, the flavourzyme has the ability of an 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)\(^{25}\).

CONCLUSIONS

Kidney bean (Phaseolus vulgaris CV Chitra) and lima bean (P. lunatus L.Sweet) grow in Jember Indonesia contain high protein, amounting to 19.83 ±0.18 and 20.15±0.21 percent, therefore considered as rich protein vegetable. Kidney and lima bean protein were hydrolyzed by alcalase greater than by flavourzyme (76.2±1.62 versus 50.34±0.13 %; and 71.1±1.44 versus 40.21±0.03 %). The alcalase and flavourzyme hydrolyzed protein of the kidney and lima bean were able to inhibit activity of angiotensin converting enzyme (ACE) with values higher than 80 percent. The amount of hydrophobic amino acids in the kidney protein hydrolyzed by alcalase, were higher than that of in lima bean protein hydrolysate (405.03 versus 305.53 mg/g protein). This might play a role in the higher ability of ACE-1 inhibitory by kidney bean protein hydrolysate (88.28±0.81 %) than that by lima bean protein hydrolysate (82.76±1.19). The ACE-1 inhibitory activity of these two bean protein hydrolysates were considerably high, and may be potential for further exploration for the clinical study and its practical application in functional food. The solubility values of the bean protein hydrolysates is needed for the application. The solubilities of alcalase hydrolyzed kidney and lima bean protein were highest at base condition – pH 8 and pH 11, respectively, the same as those flavourzyme hydrolyzed.

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