Effect of legume varieties and fermentation time of tempe using *usar* inoculum on the inhibitory activity of angiotensin I-converting enzyme

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Abstract. Indrati R, Handayani MT, Rahayu NA, Pebrianti SA. 2021. Effect of legume varieties and fermentation time of tempe using *usar* inoculum on the inhibitory activity of angiotensin I-converting enzyme. *Biodiversitas* 22: 5262-5267. The effect of three varieties of legumes, namely, velvet bean (*Mucuna pruriens*), lima bean (*Phaseolus lunatus* L.), and pigeon pea (*Cajanus cajan*), on the activity of angiotensin I-converting enzyme (ACE) inhibitors was studied during tempe fermentation. Fermentation is one way to produce bioactive peptides. Furthermore, the effect of inoculum *usar* on tempe fermentation was studied. Hydrophobic amino acids are essential in binding firmly to the active site of ACE, and the research showed that pigeon pea had higher levels of hydrophobic amino acids compared with soybeans. The legumes affected the protease activity, peptide content, degree of hydrolysis, and production of ACE inhibitory (ACEI) activity during tempe fermentation. High protein levels and peptide content did not guarantee a high ACE inhibitor level. The mixed culture of *usar* caused no effect on the production of ACE inhibitors compared with a single culture of *Raprima*. Legume beans that reached optimal fermentation fast produced high levels of ACE inhibitors. Of the three types of legumes tested in the study, lima beans had the highest ACEI activity during fermentation.

Keywords: ACE inhibitor, legumes, tempe, *usar*

INTRODUCTION

Legumes are foods rich in protein and nutrients, such as antioxidant compounds, resistant starch, dietary fiber, and others (Zhang et al. 2015; Kan et al. 2017). Therefore, legumes are a healthy and inexpensive source of nutrition. Given their high protein content, legumes have been widely studied, especially regarding their health benefits. In addition, legumes have various varieties with varying levels of protein and amino acid composition. These factors will determine their usefulness as important bioactive compounds, especially bioactive peptides, for body health. Peptides with low molecular weight are often referred to as bioactive peptides because of their excellent physiological functions: (i) prevention of heart disease, cancer, osteoporosis, and menopause symptom of chronic diseases; (ii) lowering of cholesterol levels to protect against heart disease; (iii) lowering of lipid levels in the blood (lowering low-density lipoprotein levels and increasing high-density lipoprotein levels); (iv) inhibition of the angiotensin I-converting enzyme (ACE) activity to lower blood pressure (Soumya et al. 2021).

The treatment of diabetes, hypertension, and chronic diseases widely uses bioactive peptides through healthy functional foods (Xu et al. 2019). Examples include the bioactive peptides in fermented milk (Beltrán-Barrientos et al. 2016), cereal-based fermented foods from Turkey (Kabak and Dobson 2011), the fermented soy food *douche* (Zhang et al. 2006), and others. In addition, hypertension is a significant risk factor for coronary heart disease, kidney disease, and stroke (Hajar 2017). Therefore, the potential of an ingredient containing antihypertensive functional

compounds must be measured, and one method to achieve such goal is to determine their inhibitory activity on ACE.

The process for producing bioactive peptides can occur through (i) enzymatic protein hydrolysis and (ii) proteolytic degradation by microorganisms (Toldrá et al. 2018). The fermentation of protein-rich food ingredients is one way to derive products with higher nutrition, improved sensory properties, and healthy bioactive peptides due to the protein degradation by microbes involved in fermentation (Udenigwe and Aluko 2012; Martinez-Villaluenga et al. 2017).

Tempe is a traditional Indonesian food made by fermenting beans (jack bean, velvet bean, and others). Soybean tempe is often found in traditional/modern markets. In rural areas, tempe productions still use the traditional tempe inoculum (called *usar*), which contains a mixed culture of fungi in *Hibiscus* leaves. However, a commercial type of inoculum (known as *Raprima*) is used in urban areas and contains a potential fungal strain, *Rhizopus oligosporus*. Along with the increasing public awareness about body health, the consumption of tempe also increases. The increase in vegetarian/vegan communities has also led to the increased consumption of plant foods, one of which is tempe which serves as a protein source. From Indonesian Central Statistics Agency data (BPS 2020), soybean imports have increased throughout 2009-2019. From the side of tempe craftsmen, soybean raw materials, which are still dependent on imports, have revived the traditional tempe-making business, such as jack bean tempe and others that once existed in the community (Erina 2021). Several tempes made from legumes, which contain bioactive peptide ACE inhibitors, have been investigated using the commercial

inoculum *Raprima*. However, the research on ACE inhibitory (ACEI) activity in tempe from legumes inoculated with *usar* has not been reported.

This research studied the effect of three varieties of legumes and inoculum *usar* on their ability to produce ACEI compounds. The legumes studied included velvet bean (*Mucuna pruriens*), lima bean (*Phaseolus lunatus* L.), and pigeon pea (*Cajanus cajan*). In addition, the results were compared with the tempe made from the same legumes inoculated with *Raprima* and the reported tempe made from jack bean legume (*Canavalia ensiformis*).

MATERIALS AND METHODS

Materials

Velvet beans (*Mucuna pruriens*), lima beans (*Phaseolus lunatus* L.), pigeon pea beans (*Cajanus cajan*), and *usar* were obtained from a local market in Yogyakarta, Indonesia. Hippuryl-L-histidyl-L-leucine, ACE (EC 3.4.15.1 from rabbit lung), trichloroacetic acid (TCA), and tryptone standard were from Sigma-Aldrich. Folin-Ciocalteu and *o*-phthalaldehyde (OPA) were from Merck. All other chemicals used were analytical grade.

Sample preparation and tempe fermentation

The beans were washed and soaked for 24 h. The soaked beans were boiled for 30 min followed by manual dehulling and then soaked for the second time for up to 24 h, in accordance with the method of Puspitojati et al. (2019a). Afterward, the beans were cooked for 10 min, drained, and cooled to room temperature ($\pm 29^\circ\text{C}$). Inoculation was performed using *usar* (traditional tempe inoculum, one leaf for 1 kg seed). The inoculated beans were packed in banana leaves and fermented at $\pm 29^\circ\text{C}$ for 0-120 h. The analysis was conducted every 12 h. The resulting sample at each fermentation time was lyophilized and stored for further analysis.

Protein, peptide, and protease extraction

The protein content of beans was analyzed using the Kjeldahl method (AOAC 2005). Protein, peptide, and protease extraction were carried out using the dried powder of tempe based on a modified method by Puspitojati et al. (2019b). The dried sample (1 g) was blended with 30 mL distilled water, followed by homogenization for 3 min using a food chopper, and then incubated in a water bath shaker for 60 min (60 rpm, 30°C). The supernatant containing protein, peptide, and protease was collected for further analysis after centrifugation at 20,000 g for 15 min at $\pm 29^\circ\text{C}$.

Determination of protease activity

Protease activity was measured quantitatively by the Sigma-Aldrich method with slight modifications. A total of 5 mL 0.65% casein solution was incubated for 5 min at 37°C in a water bath, added with 1 mL protease enzyme solution, and incubated at 37°C for 10 min. After incubation, 5 mL TCA reagent was added to stop the reaction, and centrifugation was performed at 6000 rpm for

10 min. Then, 2 mL supernatant was obtained and added with 5 mL sodium carbonate and 1 mL Folin. The mixture was mixed evenly, and absorbance was measured at 660 nm. Protease activity (one Unit) was expressed as the number of enzymes producing 1 μmol tyrosine per min under test conditions (Pebrianti 2019).

Determination of peptide content and degree of hydrolysis (DH)

The measurement of peptide concentration was determined by using OPA by Church et al. (1983). In addition, Lin et al.'s method (2017) with slight modifications was used to determine the DH. The sample powder was hydrolyzed with 8 M HCl (1:10 w/v) for 24 h at 110°C . Then, the sample was neutralized with 8 M NaOH, and distilled water was added to attain the total volume of 10 mL. The mixture was filtered, and the peptide concentration was analyzed using the OPA reagent. The absorbance was monitored at $\lambda=340$ nm, and tryptone was used to generate the standard curve. The calculation of DH was performed using the following formula:

$$\text{DH (\%)} = \frac{(\text{NH}_2)_{\text{tx}} - (\text{NH}_2)_{\text{t0}}}{(\text{NH}_2)_{\text{total}} - (\text{NH}_2)_{\text{t0}}}$$

Where: (NH_2) is the number of free amino groups, tx is at x min, t₀ is at 0 min of hydrolysis, and $(\text{NH}_2)_{\text{total}}$ is the total number of amino groups.

Analysis of amino acid composition

Liquid chromatography with tandem mass spectrometry (LC-MS/MS) was used to analyze the amino composition of the beans. The sample was hydrolyzed in an autoclave for 12 h at 110°C using 6 N HCl. Then, the samples were neutralized using 6 N NaOH, filtered using a 0.22 μm paper filter, and diluted with H₂O at a ratio of 1:50 (v/v). Then, 2 μL sample was injected into the LC-MS/MS equipment (ACQUITY UPLC BEH C18 Column, 130 $\text{\AA}$, 1.7 μm , 2.1 mm x 50 mm). The solvents used were as described by Chang et al. (1989) as follows: (i) A: 0.1% formic acid with 0.1% penta-decafluorooctanoic acid (PDFOA):water/CH₃CN (0.5:99.5), (ii) B: 0.1% formic acid with 0.1% PDFOA:water/CH₃CN (90:10)

The sample was eluted for 1.5 min at 50°C in solvent A and 0.5 min in solvent B at a 0.6 mL/min flow rate. The total hydrophobic amino acids for each sample were then calculated.

ACEI activity

The ACEI activity of the hydrolysate was measured by the method of Chusman and Cheung (1971). Next, 50 μL sample solution and 50 μL ACE solution (25 mU/mL) were mixed and then incubated at 37°C for 10 min. Afterward, 50 μL substrate was added (8 mM Hip-His-Leu in 50 mM HEPES buffer containing 300 mM NaCl at pH 8.3), and the incubation was continued at 37°C for 30 min. HCl (1 M, 200 μL) was added to stop the reaction. Then, the mixture was added with ethyl acetate (1.5 mL), stirred for 2 min, and centrifuged (4000 g) for 15 min. The supernatant (1 mL) was transferred to another glass tube and evaporated

in boiling water for 30 min. The resulting dry sample was dissolved in 3 mL distilled water, and absorbance was measured at $\lambda=228$ nm. The ACEI activity was calculated as the percentage inhibition of ACE activity using the formula:

$$\text{ACEI activity (\%)} = \frac{(\text{Ac} - \text{As})}{(\text{Ac} - \text{Ab})} \times 100\%$$

Where: Ac is the absorbance of the ACE + substrate (control), As is the absorbance of the substrate + ACE + sample, and Ab is the absorbance of the substrate + sample (blank). IC₅₀ is the sample concentration that can inhibit ACE activity by 50%.

Statistical analysis

Data were processed using one-way analysis of variance with a 95% significance level. A follow-up test was conducted using Duncan's multiple range test when significant differences were detected. This study was carried out with three replications, and the results were averaged.

RESULTS AND DISCUSSION

Protein and hydrophobic amino acid content of various legume seeds

Protein content will determine the number of peptides that will be produced after hydrolysis. A higher protein content is considered more accessible to use and form ACEI peptides than other sources with limited protein content (Udenigwe and Aluko 2012). Table 1 shows the protein content of the various legumes. The three legumes studied have high protein contents of about 20.06% (lima beans) to 26.75% (velvet beans). The protein content of these legumes is lower than that of jack beans and soybeans. However, these legumes are traditionally used to make tempe. To date, legume tempe can still be found, for example, velvet bean tempe, which is a typical food in the areas of Yogyakarta (Kulon Progo), Central Java (Magelang and Wonogiri), and East Java (Pacitan), which are all on the island of Java, Indonesia.

Table 1 also shows the percentage of total hydrophobic amino acids per protein weight. Hydrophobic amino acids are essential for ACE inhibition. They bind strongly to the active site, primarily hydrophobic amino acids located at the C terminal of ACEI peptides, such as Trp, Tyr, Phe, and Pro (Wu et al. 2016). Hence, the potential formation of bioactive peptides must be determined. In addition to hydrophobic amino acids, negatively charged amino acids can form bioactive peptides that inhibit ACE activity. They

can engage in an electrostatic interaction with Zn²⁺ as a coenzyme of ACE (Tuz and Campos 2017; Wu et al. 2016). The three legumes studied contain sufficiently high amounts of hydrophobic amino acids. Thus, these legumes can also produce ACE inhibitors bioactive peptides, such as those found in soybeans or jack beans. Especially, velvet bean has a high hydrophobic amino acid content similar to jack bean.

Meanwhile, lima beans have the lowest protein content and low levels of hydrophobic amino acids. The seed varieties, ecological diversity, different harvest, post-harvest handling, and storage conditions influence the differences in the quantity and quality of protein in beans in different geographic locations (Sridhar and Seena 2006; Kachare et al. 2019). Seidu et al. (2015) reported that the hydrophobic amino acid content of lima beans ranged from 40.61% to 42.14% by protein weight, with a protein content of 14.53%-15.93% dry weight. These reported data differed from those in this study.

Effect of legume varieties on tempe characteristics during fermentation

Given their high protein content, three legumes were used to prepare tempe using *usar* inoculum. Every 12 h, several parameters were observed (Figure 1). Figure 1A shows the changes in the protease activity pattern during the fermentation of three types of legumes. The protease activity, which increased during fermentation, showed the same pattern. Pigeon pea beans had the highest protease activity, whereas lima beans exhibited the lowest. In general, in regulating metabolism, the presence of substrate encourages microbes (in this case, fungi from *usar* inoculum) to produce their degrading enzymes. Given this condition, high protein levels result in high protease levels. As the mycelium of fungi appeared thick, and the texture of pigeon pea tempe became compact, the proteolytic activity also continually increased significantly ($P<0.05$) with fermentation time. The same pattern of proteolytic activity was also reported by Ruiz-Teran and Owens (1996) in soybean tempe, Starzyńska-Janiszewska et al. (2015) in grass pea tempe fermented by *Rhizopus microsporus* var. *Chinensis* and *Aspergillus oryzae*, and Puspitojati et al. (2019b) in jack bean tempe fermented by commercial starter *Raprima* containing *Rhizopus oligosporus*. Thus, this result indicates that no difference occurred in the proteolytic activity patterns in either a single culture of *Raprima* or mixed culture of *usar*. The proteolysis during tempe fermentation is essential and will significantly affect the formation and generation of ACE inhibitor peptides (Puspitojati et al. 2019b).

Table 1. Protein content and hydrophobic amino acids of several legume varieties

Substance	Legumes				
	Velvet bean	Lima bean	Pigeon pea	Jack bean ¹	Soybean
Protein (db, % w/w)	26.75 ± 1.29	20.06 ± 0.20	22.78 ± 0.51	31.33 ± 1.52	35.35-39.80 ²
Hydrophobic amino acid (db, % w/w protein)	27.44 ± 0.05	6.96 ± 0.39	24.55 ± 0.08	28.78 ± 0.07	37.70 ³

Note: db: dry basis; ¹Puspitojati et al. (2019a); ²Ciabotti et al. (2016); ³Sridhar and Seena (2006)

The protease activity data in Figure 1A are in line with those on the DH shown in Figure 1B, that is, pigeon peas had the highest DH. However, at 12 h, the value was still lower than that of velvet beans. When the pigeon peas were fermented for 120 h, the tempe texture became very soft and dark in color due to over-fermentation. Therefore, pigeon peas were fermented for 96 h only. Starzyńska-Janiszewska et al. (2015) showed that the DH increased along with fermentation time in their experiment on fermenting grass pea tempe using *Rhizopus* spp. and *Aspergillus*. This report confirms that protease activity affects the hydrolysis rate; the higher the protease activity, the more peptides are produced, resulting in a higher DH. Another factor is the variety of the legume itself. Figure 1C shows that pigeon peas had the highest peptide content and protease activity. However, the case was slightly different for lima beans. At 96-120 h of fermentation, the peptide content of lima beans was higher than that of velvet beans, although the protease activity was low. During this time, the DH increased sharply (Figure 1B).

Figure 1D shows that the patterns of ACEI activity in the three legumes were similar and followed the growth pattern of fungal mycelia (data not shown). The ACEI activity was optimum at 48-72 h of fermentation. The growth pattern of this fungus was similar to that of other

fermented tempe made from soybeans (optimal at 32 h; Nout and Kiers 2005) or koro beans (optimal at 72 h; Puspitojati et al. 2019a). Thus, the pattern of ACEI activity did not follow that of the peptide levels, which increased with fermentation time (Figure 1C). ACE inhibitors are bioactive peptides with specific protein fragments and low molecular weight (2-20 amino acids). The constituent amino acids are hydrophobic (Himaya et al. 2012). The highest inhibitory activity was observed in the lima bean tempe fermented for 48 h (83.9%). These data indicate that a high peptide content does not always guarantee the most increased ACEI activity, as suggested by Puspitojati et al. (2019b). Likewise, high protein and hydrophobic amino acid content do not necessarily result in high ACEI activity, such as that in velvet pea (Table 1 and Figure 1D). Table 1 shows that lima beans had the lowest protein and hydrophobic amino acids among the three legumes. This result may be related to peptides with negatively charged amino acids (Asp and Glu) found in lima bean ($4.48\% \pm 0.51\%$ dry protein). Several researchers (Udenigwe and Mohan 2014; Daskaya-Dikmen et al. 2017; Lin et al. 2017) reported that negatively charged amino acids can increase the ACEI activity due to the binding of Zn atoms by these negatively charged amino acids. Zn atoms are essential for ACE activity.

Figure 1. Effect of legume varieties on tempe characteristics during fermentation using *usar* as an inoculum. A. Changes in protease activity, B. Changes in a DH, C. Changes in peptide content, D. Changes in ACEI activity

Table 2. Optimum fermentation time, production of ACEI and IC₅₀ of tempe made from several legume varieties inoculated with traditional inoculum (*usar*) compared with the reported commercial inoculum

Legumes	Inoculum					
	<i>Usar</i> ¹			<i>Raprima</i> ²		
	ACEI (%)	Time (h) ³	IC ₅₀ (mg/mL)	ACEI (%)	Time (h) ³	IC ₅₀ (mg/mL)
Velvet Bean	55.6 ± 6.7	72	1.07	59.2 ± 1.9 ⁴	72	0.86
Lima Bean	83.9 ± 3.6	48	0.77	85.0 ⁵	48	0.76
Pigeon Pea	72.3 ± 0.8	48	0.74	76.1 ⁶	48	0.65
Jack Bean ^{7,8}	53.9	72	NA	60.04	72	1.03

Note: NA: data not available. ¹*usar*, traditional inoculum (this study); ²*Raprima*, commercial inoculum; ³time (h), optimum fermentation time (h); ⁴Rahayu 2019 (for *Raprima* data); ⁵Handayani 2019 (for *Raprima* data); ⁶Pebrianti et al. 2019 (for *Raprima* data); ⁷Puspitojati et al. 2019a (for *usar* data); ⁸Puspitojati et al. 2019b (for *Raprima* data)

Effect of type of inoculum on ACEI activity and IC₅₀

Table 2 shows that the difference in inoculum did not affect the production of ACEI activity. The more decisive factor was the variety of legumes used. Velvet bean tempe had the lowest ACE inhibitor, resulting in the highest IC₅₀. Its protein and hydrophobic amino acid content were the highest among the three legumes studied. This ACEI activity of velvet beans is similar to that reported for jack bean tempe (Puspitojati 2019a; 2019b). The optimal fermentation time is affected not by the type of inoculum but by the variety of legumes, especially the hardness of beans. Velvet bean tempe required a longer fermentation time (72 h) to reach its optimum value than the other two legumes. The results are similar to the fermentation time of jack bean tempe (Puspitojati 2019a; 2019b). Compared with soybean tempe, which was optimal at 32 h incubation (Nout and Kiers 2005), the tempe of the studied legumes needed a longer fermentation period. The seeds were more difficult to degrade and more prominent in size than soybeans. Table 2 shows that when the beans reached optimum fermentation fast, high levels of ACE inhibitor production and low IC₅₀ were observed (lima beans and pigeon pea).

In conclusion, this study concluded that the types of legumes affect the protease activity, peptide content, DH, and production of ACEI activity during the tempe fermentation process. Mixed cultures (*usar*) showed no effect on the production of ACE inhibitors in the same legume variety compared with a single culture. The fast optimum bean fermentation resulted in a high ACE inhibitor production. High protein levels and peptide content do not guarantee a high level of ACE inhibitor. Of the three types of legumes tested in the study, lima beans produced the highest ACEI activity during fermentation.

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