Formation of ACE-inhibitory peptides during fermentation of jack bean tempe inoculated by *usar* *Hibiscus tiliaceus* leaves starter

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Abstract. Jack bean is one of the potential legumes as a source of ACE-inhibitory peptides in tempe fermentation due to its high protein content and amino acids composition. A traditional inoculum for tempe fermentation is *usar* from *Hibiscus tiliaceus* leaves. The aim of this study was to investigate the optimum tempe fermentation time inoculated using *usar* on the formation of ACE-inhibitory peptides. The study applied the prolonged tempe fermentation (120 h) at room temperature (+30°C). The change of pH, soluble protein, protease activity, and ACE-inhibitory activity during tempe fermentation were determined using standard methods. There were significant differences (α=0.05) in pH, proteolytic activity, soluble protein content, and ACE-inhibitory activity during tempe fermentation. The highest protease activity was produced at 96 h of fermentation while the highest soluble protein content was produced at 120 h of fermentation with the value of 12.71 unit/g and 3.65 mg/mL, respectively. The highest ACE-inhibitory activity was reached at 72 h of fermentation (53.89 %). Conclusion: The optimum fermentation time of the formation of ACE-inhibitory peptide was at 72 h of fermentation.

Keywords: ACE inhibitor, peptide, fermentation, jack bean tempeh, *usar*

1. Introduction

ACE-inhibitory peptides are the peptides that have an ability to inhibit ACE, an enzyme that has an important role in regulating blood pressure. They were generally short chain peptides with 3-20 amino acids [1]. Protein sources, hydrolysis conditions, the degree of hydrolysis, molecular mass, amino acid composition and the position of amino acids in the peptide sequences influenced ACE-inhibitory activity of peptides and protein hydrolysates [2][3]. ACE-inhibitory peptides commonly had hydrophobic amino acids (Tyr, Phe, Trp, Ala, Ile, Val, and Met) or positively charged amino acids (Arg and Lys) or Pro at C terminal [3]–[6]. They can be produced by the fermentation process and enzymatic hydrolysis.

Studies of ACE-inhibitory peptides through fermentation have been widely reported. Mau-tofu exhibited ACE-inhibitory activity in vitro with more total hydrophobic amino acids or proline in the
peptides [7]. The ACE-inhibitory activity of fermented douche qu by Aspergillus egyptiacus was 56.8-76.3% [8]. The antihypertensive peptides were released by fermentation of pea protein after in vitro digestion [9]. The ACE-inhibitory activity in natto and soybean tempe have been investigated [10] [11] and there was a significant decrease in blood pressure in spontaneously hypertensive rats[12].

Tempe is an authentic Indonesian fermented food. Tempe is made through the fermentation process of boiled seeds using certain fungi that can bind the seeds to resemble a cake. The most important tempe fungus is Rhizopus oligosporus, but some other fungi, such as Rhizopus oryzae and Mucor spp, may also have an important role in taste, texture and nutritional value [13]. Some traditional tempe producers in Indonesia use tempe starter derived from Waru leaves (Hibiscus tiliaceus leaves). This tempe mold starter is known as usar. Nout et al investigated that Hibiscus tiliaceus leaves harvested in Indonesia contained abundantly Rhizopus oryzae, Rhizopus microsporus var. oligosporus and a mixed flora of soil fungi. The same Hibiscus leaves harvested in Africa and Europe was found only the same soil fungi but none of Rhizopus sp [14]. It has been speculated that fungi in tempe could affect its quality. The fungi in tempe fermentation produced extracellular enzymes that diffuse and degrade the solid substrate into the soluble component. One of them is protease enzyme that can breakdown seeds protein into peptides that have the possibility as ACE inhibitory peptides.

Tempe is commonly made from soybean. Jack bean is another source of tempe making. The protein content of jack bean is 32.32 %[15], it was higher than velvet bean [16], lima bean [17] and mung bean [18]. Jack bean seeds have abundant of the hydrophobic amino acid such as leucine and isoleucine[19]. These type of amino acids have an important role in the ACE-inhibitory activity of peptides[2]. The protein content and amino acids composition of jack bean seeds influenced bioactive peptides released during tempe fermentation.

Jack bean has a protein and amino acid content that meets the requirements as a source of ACE-inhibitory peptides, but the weakness of this seed is very hard texture so that the process of jack bean tempe making requires longer seed immersion time and fermentation time. The information of tempe fermentation from jack bean especially on the formation of ACE-inhibitory peptides during fermentation is still very limited. The aim of this study was to investigate the optimum tempe fermentation time inoculated with usar on the formation of ACE-inhibitory peptides.

2. Materials and methods

2.1 Materials

Jack bean seeds (Canavalia ensiformis), usar, Angiotensin Converting Enzyme from rabbit lung, HHL (Hippuryl-L-Histidyl-L-Leucine) from Sigma–Aldrich Company and O- phtaldialdehyde (OPA) from Merck. All other chemicals were analytical grade.

2.2 Tempe production

Jack bean seeds were washed and soaked for 24 h, then boiled for 30 min; the ratio of water to jack bean was 4: 1. it is following by soaking for 24 h. The seeds were dehulled and cut into 4-6 parts and soaked again for 48 h. The soaking water was changed every 12 h. Sliced seeds were boiled for 30 min, then the water was discarded, the seeds were drained and cooled (+ 30°C). Usar (1 leaf for 1 kg seed) was inoculated to boiled seed. Inoculated seeds were wrapped by banana leaves and fermented at room temperatures (+30°C) for 0, 24, 48, 72, 96 and 120 h. Samples were lyophilized and kept in the freezer for further analysis.

2.3 Crude protease extraction

Protease extraction was prepared by the method of Elegado and Fujio[20],[21] with slight modifications. Jack bean tempe was homogenized in 0.05 M phosphate buffer pH 6 using a waring blender for 3 minutes. The mixture was incubated at 30°C in water bath shaker for 30 min and then centrifuged at 9391 g for 10 min at 4°C. The supernatant was collected and used as a crude enzyme.

2.4 Proteolytic activity assay
Proteolytic activity was analyzed using casein as a substrate with the method of Elegado and Fujio[21] with slight modification. Five ml of 0.65% casein solution was added and preincubated in a water bath shaker at 40°C for 5 min. One ml crude enzyme was added into the solution and incubated at 40°C for 10 min. Five ml of the TCA reagent was then added to the solution to terminate the reaction and incubated for 10 min. The mixture solution was centrifuged at 6000 g for 10 min and 2 ml of supernatant was collected. The resulting enzyme reaction was calculated using Folin’s reagent. One proteolytic unit (U) was defined as the amount of the enzyme that releases 1 μmol of tyrosine per min under assay conditions.

2.5 ACE-inhibitory activity assay
The ACE-inhibitory activity of jack bean tempe was calculated by Cushman and Cheung method with slight modifications [22]. 50 μL of peptide extract was mixed with 50 μL of substrate (Hip-His-Leu 8 mM) and incubated at 37°C for 10 min. 50 μL of ACE solution (25 mU/ml) was added and incubated at the same temperature for 30 min. 200 μL of HCl 1 M was added to terminate the reaction. The hippuric acid formed in the reaction process was extracted with 1.5 ml of ethyl acetate. The mixture was shaken vigorously and centrifuged at 4000 g for 15 min. A 1 ml amount of the clear upper layer was collected and dried out. The residue was redissolved in 3 ml of distilled water by inversion. The absorbance was determined at a wavelength of 228 nm by Dynamica Scientific Halo SB-10 Spectrophotometer UV-VIS. The ACE-inhibitory activity was calculated as follows:

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

where A was absorbance in the presence of ACE, B was absorbance in the presence of ACE and inhibitor, and C was absorbance of the reaction blank.

2.6 Total Soluble Protein
The soluble protein content was calculated by Lowry’s method [23].

2.7 pH assay
Five grams of tempe were homogenized in 50 mL of distilled water and the filtrate was filtered using filter paper. The pH was measured using a pH meter.

2.8 Statistical analysis
The data were analyzed by one-way analysis of variance (ANOVA) and mean differences between treatments were analyzed by Duncans Multiple Range Tests (DMRT) with SPSS IBM 23.

3. Results and discussion
Tempe fermentation by \textit{Rhizopus} sp. provided desirable effects on its functional properties, including the production of low molecular weight peptides which can be a good source of ACE-inhibitory peptide. Jack bean seed is one source of dietary protein that can be used as raw materials of tempe production.

3.1 The visual of jack bean during fermentation
The visual appearance of fungi can be seen after 24 h of fermentation. Figure 1 shows that the mycelium was compactly bound at 72 h of fermentation. The growth of mycelium was slower than soybean tempe fermentation. In soybean tempe, the cottony mycelium has fully covered the bean after 24 h of fermentation. The slower growth of fungi probably because of the large size and hard texture of jack bean. Jack bean tempe has the compact structure as well as soybean tempe in longer fermentation time.
3.2 The pH during fermentation

Jack bean tempe fermentation which was inoculated with usar did not give significant pH changes until 24 h of fermentation (Figure 2). The pH significantly decreased in 48 h of fermentation ($\alpha=0.05$) and continue to increase until the end of fermentation (96 h). In general, tempe is consumed after 48-72 h of fermentation with the pH value in this research was 4.77. The low pH in the final product was influenced by the tempe starter. Usar is mixed culture starter with several fungi, including Rhizopus oligosporus, Rhizopus orizae and Mucor[24]. The total bacteria found in usar almost resemble the number of fungi that play a role in making tempe (data not shown). Widaningrum reported that there were lactic acid bacteria in the tempe fermentation process[25]. A different thing happened to jack bean tempe which was inoculated using Raprima commercial inoculum which is containing only Rhizopus oligosporus. pH increased significantly along with fungal growth to pH 7 in the final product (data not shown).

3.3 The proteolytic activity and the soluble protein during fermentation

Figure 3 shows that the soluble protein content increased significantly during the fermentation process ($\alpha=0.05$). This is indicating that there is proteolytic enzyme produced during fermentation. During the early stages of tempe fermentation, several microbial proteases were produced [26]. It was reported that both acid and neutral proteases were found during soybean fermentation [21][27]. The activity of proteolytic enzyme during fermentation was considered to be the main factor of protein
hydrolysis. It released peptides, amino acids, and decomposition products during fermentation by *Rhizopus* sp.

Figure 4 shows that the first protease activity (4.45 unit/g) was found after 24 h fermentation. Simultaneously, mycelium has grown on jack bean seeds. Proteolytic activity continued to increase up to 96 h of fermentation, it was the maximum proteolytic activity on jack bean tempe fermentation (12.71 unit/g). The increase of proteolytic activity was followed by the increase of total soluble protein.

The changes in proteolytic activity corresponded to hydrolysis of the degree of protein and contents of fungal[20]. Proteolytic activity was found dependent on the mycelial growth. There was reported in the previous study that the highest protease activity in raw soybean after 4 days fermentation produced by *Rhizopus* sp. strain F68 and F57. These strains were not able to show any noticeable fast growth testing but in the long run, they released the highest protease activity [21]. Temperature also had a significant effect on the fermentation of barley for mycelia growth. The optimum temperature of barley tempe fermentation was 30°C with a maximum proteolytic activity of 98.52 U/g [28].
Proteolytic activity influenced the breakdown of jack protein into peptides during tempe fermentation. During jack bean fermentation process, several peptides were produced. One of them has the possibility as ACE inhibitory peptides.

### 3.4 ACE inhibitory activity

Figure 5 shows the inhibitory activity of peptide during fermentation. There was a significant difference in ACE-inhibitory activity during tempe fermentation ($\alpha=0.05$). The ACE-inhibitory activity was found during the earliest stages of fermentation and continue to increase as the fermentation progressed until 72 h of fermentation, longer fermentation caused decreasing on ACE-inhibitory activity. The unfermented jack bean also had an ACE-inhibitory activity. It was maybe due to the small peptides with a molecular weight of 14.78 kD in cooked bean (data not shown). Bioactive peptides may already found in foods as natural substances or may be produced by hydrolysis[29]. The highest ACE-inhibitory activity of jack bean tempe was 53.89 % which was reached on 72 h of fermentation. At the same time, the protease enzyme was more active to hydrolyze protein of jack bean into peptides that may release biological activities such as ACE-inhibitory peptides. However, the highest protease activity was on 96 h of fermentation but there was a decline of ACE-inhibitory activity. It was may be due to the change of amino acid at C terminal of peptides.

![Figure 5. ACE inhibitory activity during tempe fermentation](image)

### 4. Conclusion

Jack bean is a legume rich in protein but still under-utilized. Tempe fermentation from jack bean used usar as a starter produced tempe structure as well as tempe in general but required a longer fermentation time. The action of proteolytic enzyme produced during fermentation hydrolyzed protein into peptides. During fermentation, ACE-inhibitory peptide was formed, with the highest inhibition was 53.89 % at 72 h of fermentation.

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