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Angiotensin I-converting Enzyme (ACE) Inhibitory Activity of ACE Inhibitory Peptides Produced during the Fermentation of Pigeon Pea (*Cajanus cajan*) Tempe

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ABSTRACT: Fermentation products are common sources of *angiotensin I-converting enzyme (*ACE) inhibitory peptides used for hypertension treatment. This research investigated the effect of fermentation time on the ACE inhibitory activity produced during the fermentation of pigeon pea tempe and aimed to determine the optimal fermentation time to obtain pigeon pea tempe with the highest ACE inhibitory activity. Seeds were inoculated with Raprima® (0.02% w/w) containing *Rhizopus oligosporus* spores and fermented for 0-96 h. Protein pattern, degree of hydrolysis (DH), soluble protein content and ACE inhibitory activity were observed during fermentation. The result from SDS-PAGE shows that protein hydrolysis occurred after 12 h fermentation, marked by the appearance and greater intensity of protein bands with low-molecular-weight (60 kDa). An increase in DH and soluble protein content were detected during the fermentation and reached a maximum of 23.99% and 3.15 mg mL^{-1} at 96 h fermentation, respectively. The ACE inhibitory activity increased with fermentation time and pigeon pea tempe fermented for 48 h (76.14%) has the highest ACE inhibitory activity with IC_{50} values of 0.65 mg mL^{-1}. It could be concluded that the optimal fermentation time to obtained pigeon pea tempe with the highest ACE inhibitory activity is for 48 h of fermentation.

Keywords: ACE inhibitory peptides, tempe, fermentation, pigeon pea

INTRODUCTION

Hypertension is one of the primary risk factors for cardiovascular diseases with 6.09 million cases and 962,400 deaths globally in 2015 (Roth et al., 2017). Control and treatment of hypertension are closely related to blood pressure regulation system in which *angiotensin I-converting enzyme* (ACE) plays an important role. An increase in blood pressure can be triggered due to the redundant production of a vasoconstrictor hormone, namely Angiotensin II derived from the hydrolysis of Angiotensin I by ACE. This enzyme also hydrolyzes and inactivates bradykinin, a vasodilator substance thus interfering with the vasodilation action to lowering blood pressure (Bernstein, et al. 2013; Su, 2014; Ames et al., 2018). Therefore, an excessive ACE activity that could account for hypertension needs to be controlled by inhibiting its catalytic activity to suppress the elevation of blood pressure.

Bioactive peptides are natural ACE inhibitors derived from food protein, mostly have low molecular weight and consist of hydrophobic as well as negatively charged amino acids. The ACE inhibitory peptides could interact directly with the structure of ACE (Wu et al., 2016, Garcia-Mora et al., 2017; Nawaz et al., 2017) or indirectly with Zn^{2+} as a cofactor of ACE (Jakubczyk et al., 2013; Aluko et al., 2015). These interactions could affect the catalytic activity and the function of ACE in regulating blood pressure. Sequentially, the disruption of ACE activity will reduce the elevation of blood pressure. Food processing such as fermentation by using microbial protease is a common way to obtain ACE inhibitory peptides. Many studies have proven the effectiveness of ACE inhibitory peptides from food-protein fermentation, especially legumes to prevent and treat hypertension (Daliri et al., 2017; Nawaz et al., 2017; Daliri et al., 2018; Puspitojati et al., 2019[a]; Pertiwi et al., 2019).

One of the well-known fermented products from legumes is tempe which is usually made from soybeans inoculated with *Rhizopus* sp. spores. Protein as a substrate of fermentation will be broken down by protease enzymes from *Rhizopus* sp. into numerous types of peptides including ACE inhibitory peptides. Okamoto et al. (1995) reported that soybean tempe contains ACE inhibitory peptides with IC_{50} up to 0.51 mg mL^{-1}. Nowadays, various high-protein legumes are being used for making tempe, like lima bean (Pertiwi et al., 2019) and jack bean which were reported to have an ACE inhibitory activity. Jack bean tempe fermented by *R. oligosporus* has an ACE inhibitory activity of 60.04% (IC_{50} 1.03 mg mL^{-1}) meanwhile jack bean tempe fermented by traditional inoculum called usar have lower ACE inhibitory activity of 53.89% (Puspitojati et al., 2019[a,b]).

Pigeon pea (*Cajanus cajan*) also could be used as raw material for tempe fermentation. In addition, protein of pigeon pea contains high amounts of hydrophobic amino acids such as phenylalanine, leucine, valine, isoleucine, proline and negatively charged amino acids (Oshodi et al, 1993; Solomon et al., 2017) which are essential for the formation of ACE inhibitory peptides (Wu et al., 2016; Daliri et al., 2017; Nawaz et al., 2017). Fermentation of pigeon pea using *Aspergillus niger* has been found to have ACE inhibitory activity with IC_{50} value of 9 µg mL-^{1} (Nawaz et al., 2017). On the other hand, the ACE inhibitory activity of pigeon pea fermented by *Bacillus subtilis* has an IC_{50} value of 1.47 mg mL-^{1} (Lee et al., 2015). These results indicate that fermentation of pigeon pea could generate ACE inhibitory peptides.

In general, protein hydrolysis increases with a longer fermentation time so it can release lower molecular weight peptides. Furthermore, the potential for the formation of ACE inhibitory peptides is also greater. However, longer fermentation will result in further hydrolysis of peptides into free amino acids which could reduce its ACE inhibitory activity (Hang and Zao, 2012; Ma et al., 2013; Puspitojati et al., 2019[a,b]). Therefore, the objective of this study was to investigate the effect of fermentation time on the ACE inhibitory activity produced during the fermentation of pigeon pea tempe and determine the optimal fermentation time to produce pigeon pea tempe with the highest ACE inhibitory activity.

MATERIALS AND METHODS

Seeds and chemicals

Pigeon pea seeds (*Cajanus cajan*) were purchased from a local farmer at Jepitu, Girisubo, Yogyakarta. Commercial tempe starter Raprima® (Bandung, Indonesia) containing *Rhizopus oligosporus* spores was purchased from a local market at Sleman, Yogyakarta. Hippuryl-L-histidyl-L-leucine (HHL), ACE (EC 3.4.15.1 from rabbit lung), sodium dodecyl sulfate (SDS), coomassie brilliant blue R-250, bovine serum albumin (BSA) standard, and tryptone standard, were purchased from Sigma-Aldrich. Folin-Ciocalteu, O-phthaldialdehyde (OPA), β-mercaptoethanol, methanol, HCl, acetic acid, and ethyl acetate were purchased from Merck. The protein marker used for SDS-PAGE was from Bio-Rad. All other chemicals used were analytical grade.

Preparation of pigeon pea tempe

Pigeon pea seeds were manually removed from any impurities and then washed, soaked for 24 h. The soaked pigeon pea seeds were boiled for 30 min followed by manual dehulling and then soaked for the second time up to 24 h. Afterward, pigeon pea seeds were boiled for 10 min, drained and cooled to room temperature. Inoculation was performed on cold pigeon pea seeds using commercial tempe starter Raprima® (0.02% w/w of cooled seeds). The inoculated pigeon pea seeds were packed in banana leaves and fermented at room temperature (±29 °C) for 0, 12, 24, 36, 48, 72 and 96 h. The resulting sample at each of fermentation time was lyophilized and stored for further analysis.

Protein extraction

Protein extraction was carried out using the dried powder of pigeon pea tempe based on a modified method by Rusdah (2016). Dried sample (1 g) was blended to 30 mL distilled water followed by homogenization for 3 min using food chopper then incubated in a water bath shaker (Sibata, WS-240, Tokyo, Japan) for 60 min (60 rpm, 30 °C). The mixture was centrifuged (Eppendorf centrifuge 5424 R, Hamburg, Germany) at 20000 g for 15 min at room temperature and the supernatant containing peptide extract was collected for further analysis.

Determination of pH value

The pH value during the fermentation of pigeon pea tempe was measured with a digital pH meter. The sample was crushed and mixed with distilled water at 5:45 (v/v) then the pH value was measured after homogenization for 5 min.

Protein pattern (SDS-PAGE)

Observation of protein hydrolysis during fermentation of pigeon pea tempe was carried out based on the protein pattern using SDS-PAGE following method by Laemmli (1970). The gel was made by 5% stacking gel and 13% resolving gel. Buffer sample was prepared by mixing tris HCl (0.5 M, pH 6.8), glycerol (87% w/v), SDS (10% w/v), bromophenol blue (0.5% v/w) and distilled water. A 950 µL β-mercaptoethanol was added to a 50 µL buffer sample and the peptide extract was then dissolved in the buffer sample (ratio 1:2) then heated at 100 °C for 4 min. A total of 20 µL sample and 5 µL protein markers were injected into each well of stacking gel. Electrophoresis was carried out in Bio-Rad PowerPac™ Basic Mini Electrophoresis System (California, UK) at a constant ampere, 220 V for about 1 h. Staining was done by soaking the gel for 24 h in 0.2% coomassie brilliant blue R-250 containing 50% methanol, 40% distilled water and 10% acetic acid. The gel was then soaked in a destaining solution (50% methanol, 40% distilled water and 10% acetic acid) until the gel background became clear.

The molecular weight (MW) of the protein band from SDS-PAGE was determined by following the Bio-Rad protocol (Bio-Rad Bulletin 3133). The R_f value was calculated for each of the targeted protein bands. The R_f value is explained as the migration distance of the protein through the gel divide by the migration distance of the dye front. The measurement of the migration distance was done from the top of the resolving gel up to the targeted protein band. The standard curve of R_f and log MW of the protein marker was used for the MW determination of the targeted protein bands.

Degree of hydrolysis

The degree of hydrolysis (DH) was carried out using the peptide extract based on the OPA method described by Church et al. (1985). A total of 40 mg OPA was mixed with 1 mL methanol and 100 µL β-mercaptoethanol to make an OPA solution. Afterward, 25 mL sodium tetraborate (100 mM), 2.5 mL SDS (20% w/v in distilled water) and 21,4 mL distilled water was mixed evenly with OPA solution to make a final volume of 50 mL of OPA reagent. A peptide extract of 30 µL was mixed evenly with 1 mL OPA reagent and allowed to react for 2 min. The measurement of the absorbance was done at 340 nm using Spectrophotometer UV-VIS (Dynamica Scientific Halo SB-10, Livingston, UK). DH was calculated based on the percentage of cleaved peptide bond following the equation:

$$\text{Degree of hydrolysis (\%)} = \frac{(NH_2)_{tx} - (NH_2)_{t0}}{(NH_2)_{total} - (NH_2)_{t0}}$$

Where $(NH_2)_{tx}$ is the number of free amino groups at X min, $(NH_2)_{t0}$ is the number of free amino groups at 0 min of hydrolysis and $(NH_2)_{total}$ is the total number of amino groups in pigeon pea protein determined by acidic hydrolysis using HCl (6 N) at 110 °C for 24 h (Lin et al., 2017).

Soluble protein content

Soluble protein content was analyzed based on the Lowry et al. (1951) method using Folin-Ciocalteu reagent and complex-forming reagent (CFR) consisting of Na_2CO_3, $CuSO_4.5H_2O$, and K-Na-Tartrate. CFR was made by mixing 2% Na_2CO_3 (w/v in distilled water) with 1% $CuSO_4.5H_2O$ (w/v in distilled water) and 2% K-Na-Tartrate (w/v in distilled water) at 100:1:1 v/v). A peptide extract of 0.4 mL was added to 0.4 mL NaOH (2 N) and hydrolyzed at 100 °C for 10 min then cooled to room temperature. Next, a total of 1 mL CFR was added into the sample and incubated for 10 min at room temperature. After that, 0.4 mL Folin-Ciocalteu reagent (1 N) was added, mix thoroughly and incubated in the dark for 50 min at room temperature. Measurement of absorbance was done at 750 nm using Spectrophotometer UV-VIS (Dynamica Scientific Halo SB-10, Livingston, UK).

ACE inhibitory activity

The modified method of Chusman and Cheng (1971) was applied to determine the ACE inhibitory activity during fermentation of pigeon pea tempe by using HHL as a substrate. The ACE inhibitory activity was measured based on the formation of hippuric acid from the conversion of HHL by ACE. Peptide extract of 50 µL with a concentration of 1 mg mL^{-1} was mixed with 50 µL HHL (8 mM) previously dissolved in 50 mM Hepes buffer (pH 8.3) containing 300 mM NaCl, preincubated for 10 min at 37 °C. A total of 50 µL ACE solution (25 mU mL^{-1}) was added and allowed it to react for 30 min at 37 °C. The enzyme reaction was then terminated by adding 200 µL HCl (1 M).

The hippuric acid released from the reaction was extracted with an addition of 1.5 mL ethyl acetate followed by shaking the solution for 2 min and centrifuged for 15 min at 6526 rpm, 4 °C. A total of 1 mL of supernatant from the upper layer was taken and evaporated at 100 °C for 25 min. The residues obtained were dissolved in 3 mL distilled water then homogenized using a vortex. The absorbance was measured at 228 nm and the ACE inhibitory was calculated following the equation:

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

where A is the absorbance of ACE and HHL solution without ACE inhibitory peptides (sample), B is the absorbance of ACE and HHL solution with ACE inhibitory peptides (sample), and C is the absorbance of the blank solution. The IC_{50} value was expressed as the peptide concentration required to inhibit 50% of the ACE activity.

Statistical analysis

All data were obtained from three replications and analyzed using one-way ANOVA. Determination of the differences between treatments was analyzed using Duncans Multiple Range Test (DMRT). The confidence level of 95% was used in all analyses. The statistical analysis was completed using SPSS IBM version 22.0 software.

RESULTS AND DISCUSSION

Change of pH value during the fermentation of pigeon pea tempe

The change of pH during fermentation of pigeon pea tempe occurred as a result of protein hydrolysis by protease enzyme from *R. oligosporus*. Free amino acids and ammonia as the products of protein hydrolysis could increase the pH value during fermentation of tempe (Ruiz-Teran and Owens, 1996; Sparringa and Owens, 1999; Muzdalifah et al., 2017). Figure 1 illustrated the change of pH value during the fermentation of pigeon pea tempe. At the beginning of fermentation, the observed pH value was 5.82 and slightly decreased to 5.34 at 24 h fermentation due to the growth of mold that has not yet entered the log phase (Nurdini et al. 2015).

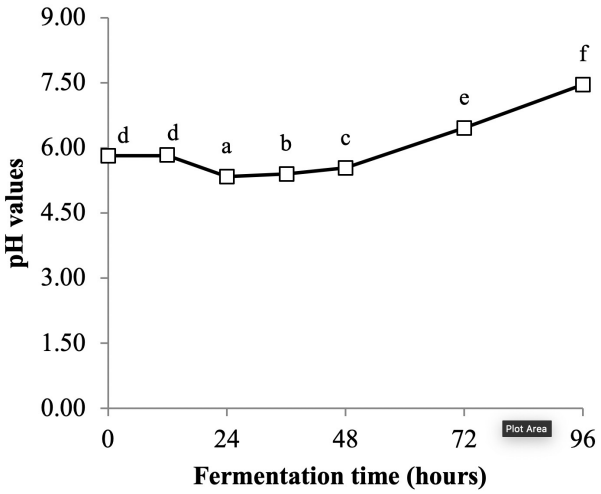

Figure 1. Change of pH values during the fermentation of pigeon pea tempe. Different superscript letters indicate statistical difference ($P < 0.05$).

After 24 h fermentation, pH value generally increased and reached a maximum of 7.46 at the end of fermentation (96 h). The increase of pH value was accompanied by a stronger ammonia odor particularly at 72 and 96 h fermentation. It indicates that the protein or peptides of pigeon pea as a substrate were hydrolyzed into lower MW peptides and free amino acids during fermentation (Ruiz-Teran and Owens, 1996; Sparringa and Owens, 1999; Muzdalifah et al., 2017). With the generation of low MW peptides, the formation of ACE inhibitory peptides could be expected. However, extended fermentation time of tempe could lead to further hydrolysis of peptides into free amino acids (Muzdalifah et al., 2017) which would affect the ACE inhibitory activity of the peptides (Puspitojati et al., 2019[b]).

Protein pattern during the fermentation of pigeon pea tempe

As shown in Figure 2, the change in protein pattern was first detected after 12 h fermentation. This result illustrated that the hydrolysis of pigeon pea protein started in the earlier stage of fermentation. Protein hydrolysis was characterized by the presence of new protein bands of 60 kDa (12 h fermentation). The greater intensity of the protein band under 34 and 17 kDa was also detected. These indicated that the concentration of protein with lower MW

has increased due to protein hydrolysis during the initial stage of pigeon pea tempe fermentation.

The protein bands with MW of 94 kDa, 78 kDa, and 60 kDa were disappeared after 48 h fermentation (Fig. 2). New protein bands with MW of 73 kDa, 40 kDa, and 28 kDa were appeared and last until the end of fermentation except for protein band with MW of 73 kDa. This result shows that large MW of protein was degraded by protease enzyme during the fermentation of pigeon pea tempe. The greater intensity of the protein bands especially at 96 h fermentation illustrated that the protein hydrolysis continues until the end of fermentation to produce lower molecular weight peptides. It indicated the potential for the presence of ACE inhibitory peptides during the fermentation of pigeon pea tempe.

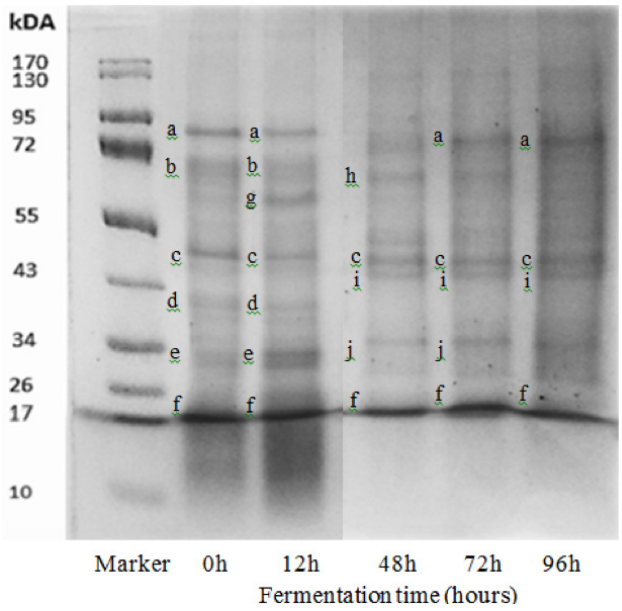

Figure 2. Protein pattern during the fermentation of pigeon pea tempe. Letters indicate MW a. 94; kDa; b. 78; c. 45 kDa; d. 34 kDa, e. 25 kDa; f. 18 kDa; g. 60 kDa, h. 73 kDa; i. 40 kDa; j. 28kDa.

Protein hydrolysis during the fermentation of pigeon pea tempe

Endopeptidase-class protease enzyme was reported to be produced by *R. oligosporus* during fermentation of tempe (Baumann and Bisping 1995; Heskamp and Barz 1998). Endopeptidase acts to break the peptide bonds from the inside or within the polypeptide chain and produce lower molecular peptides (Souza et al., 2015). Protein hydrolysis and peptides production during fermentation could be characterized by observing the degree of hydrolysis (DH). Typically, high DH will produce more peptides, including those that are likely to be ACE inhibitory peptides (Puspitojati et al., 2019[a]).

The result of DH is shown in Figure 3. The DH was significantly increased ($p<0.05$) with fermentation time and reached a maximum after 96 h fermentation. At the beginning of fermentation (12 h), DH was 0.048% and increased significantly to 3.165% (24 h), 18.889% (48 h) and reached a maximum of 23.99% (96 h). The increase in DH was previously reported by Weng and Chen (2011) during the two-step fermentation of soybean tempe using *R. oligosporus* and *Bacillus subtilis*. A similar result was also reported by Starzynska-Janiszewska et al. (2015) during the fermentation of grass pea tempe using *Rhizopus* spp. and *Aspergillus*. Both results revealed that the DH value increased along with fermentation time.

The result of soluble protein content also shows the same pattern as the result of DH (Figure 3). During fermentation, the protein will be hydrolized and broken down into peptides or free amino acids that are more soluble than protein in the complex form. Therefore, longer fermentation time will result in higher soluble protein content (Puspitojati et al., 2019[b]). As observed, the DH increased sharply during 12-48 h fermentation and began to slow down as it heads towards the end of fermentation. The soluble protein content increased from 0.385 mg mL^{-1} (12 h) to 2.708 mg mL^{-1} (48 h). Afterward, the soluble protein content increased from 2.708 mg mL^{-1} (48 h) to 3.041 mg mL^{-1} (72 h) and reached

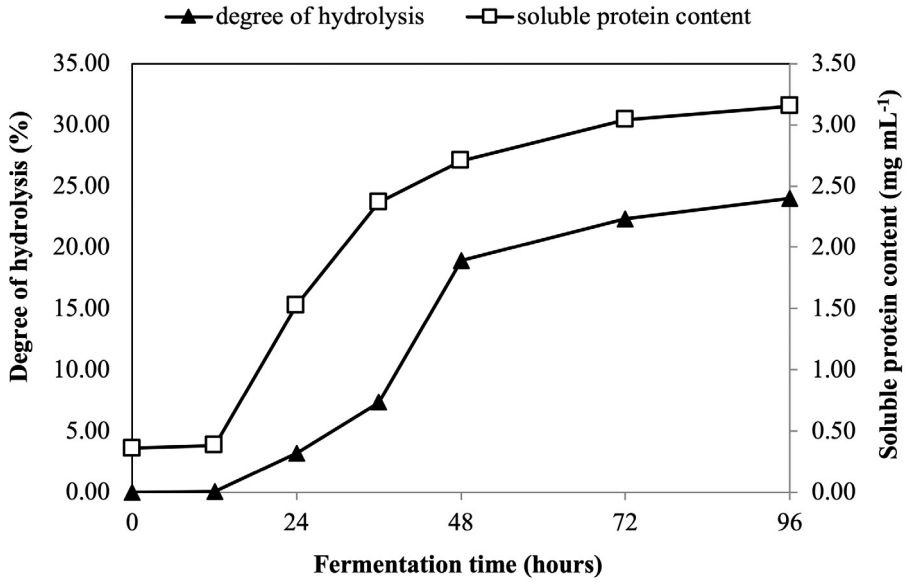

Figure 3. Protein hydrolysis during the fermentation of pigeon pea tempe.

a maximum of 3.152 mg mL^{-1} (96 h). These results of the increase in soluble protein content were lower than the results obtained at 12-48 h fermentation.

Based on the results, it could be concluded that a high DH value will result in the increase of hydrolysis products including low MW of protein indicated by an increase in soluble protein content. As seen in Figure 2, pigeon pea fermented for 96 h has the greatest intensity of protein bands. These results were positively correlated with the results of the increase in DH and soluble protein content along with fermentation time. Furthermore, an increase in the soluble protein content could enhance the potential for producing more peptides including ACE inhibitory peptides.

The ACE inhibitory activity

The ACE inhibitory activity present even in unfermented pigeon pea at 0 h fermentation, which was 37.52% (Figure 4). It was due to the soaking and boiling process in the preparation of tempe which could cause protein hydrolysis (Nogata et al., 2009; Nout and Kiers, 2005). The result from SDS-PAGE also shows the presence of low molecular peptides below 18 kDa at 0 h fermentation (Figure 2) which was likely to have ACE inhibitory activity.

Protein hydrolysis by protease enzymes during pigeon pea tempe fermentation was more effective to produce ACE inhibitory peptides rather than the boiling or soaking process. Until the end of fermentation, the ACE inhibitory activity significantly increased ($p<0.05$) compared to the unfermented pigeon pea at 0 h. After 24 h fermentation, the ACE inhibitory activity was 43.17% and increased to 68.76% after 36 h fermentation. The ACE inhibitory activity reached a maximum of 76.14% at 48 h fermentation with an IC_{50} value of 0.65 mg/mL and then decreased significantly to 61.00% at the end of fermentation (96 h).

Despite the greater intensity of the lower molecular protein bands (Figure 2) also the increase in soluble protein content (Figure 3), the ACE inhibitory activity of pigeon pea tempe was decreased after 48 h fermentation. It is likely due to the hydrolysis of peptides into free amino acids which could change the composition and amino acid sequence of ACE inhibitory peptides produced at 48 h. These changes might result in a decrease in ACE inhibitory activity (Hang and Zao, 2012; Ma et al., 2013; Puspitojati et al., 2019[b]). This result indicated that protein hydrolysis by *R. oligosporus* used in the fermentation of pigeon pea tempe is capable to produced ACE inhibitory peptides and the optimal fermentation time to obtain pigeon pea tempe with highest ACE inhibitory activity is for 48 h fermentation.

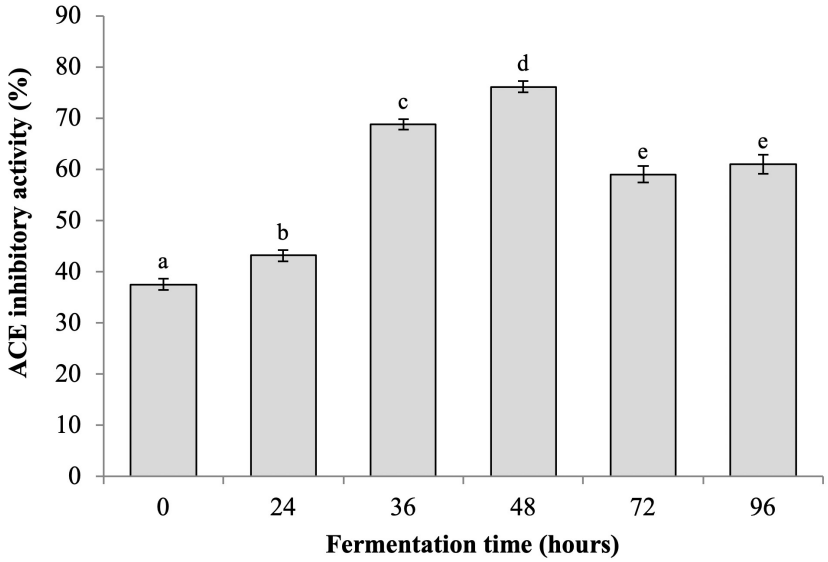

Figure 4. The ACE inhibitory activity during the fermentation of pigeon pea tempe. Different superscript letters indicate statistical difference ($P < 0.05$).

CONCLUSION

Fermentation significantly increased the ACE inhibitory activity of pigeon pea tempe compared to unfermented pigeon pea. These results suggest that fermentation of pigeon pea by using tempe starter Raprima® containing *R. oligosporus* could produce ACE inhibitory peptides. The optimal fermentation time to obtain pigeon pea tempe with the highest ACE inhibitory activity is for 48 h with 76.14% of ACE inhibitory activity and an IC_{50} value of 0.65 mg/mL. Pigeon pea tempe fermented for 48 h could be recommended for consumption as a functional food for hypertension after 48 h fermentation. However, further study is needed to evaluate the fate of ACE inhibitory peptides from pigeon pea tempe in the gastrointestinal tract.

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