

Chemical Characteristics and Activity of ACE Inhibitors on Fractionation of Tempeh *Koro kratok* (*Phaseolus lunatus*) Peptides

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ABSTRACT: Tempeh is a fermented food that is good for health and has high nutritional value. *Koro kratok* tempeh is one of tempeh which is made from non-soybean legumes. The fermentation process will convert macromolecular compounds to micromolecules thereby increasing bioavailability and providing functional properties. This study aimed to find out the chemical properties of *koro kratok* tempeh and the effect of peptide molecular weight of *koro kratok* tempeh on ACE inhibition activity. The results show that *koro kratok* seeds contained 20.66% protein which total hydrophobic amino acid was 3.32% (w/w protein). This hydrophobic amino acid was higher than that soybean, indicated that *koro kratok* (*Phaseolus lunatus*) has a potential producing ACE peptide inhibitors. The *koro kratok* seeds had ACE inhibitory activity 19.72%. This activity increased to 84.97% when the seeds were fermented for 48h to become tempeh. Peptide fractionation showed that the smaller the molecular weight of the peptide, the higher the ACE inhibitory activity.

Keywords: Tempeh, *koro kratok*, ACE-inhibitor, peptide

INTRODUCTION

Tempeh is fermented foods that popular to many people in the Java region, because of its unique taste and its nutrients. The fermentation process during tempeh production will increase bioavailability. Tempeh fermentation involves microbes, such as *Rhizopus oligosporus*, and others.

Rhizopus oligosporus is a type of mold that is commonly found in tempeh fermentation. Besides *Rhizopus oligosporus*, *Rhizopus oryzae* also found during tempeh fermentation (Nout and Kiers, 2005). *Rhizopus oligosporus* excretes protease with a higher proteolytic activity than other molds (Karmini *et al.*, 1996). Besides that, *Rhizopus oligosporus* also excretes lipase and amylase. These enzymes play a role in cutting long-chain carbohydrates, fat, and protein into shorter chains, so that the digestibility increases.

Tempeh that is sold in the market is made from soybeans. Other materials that commonly used are *koro-koroan*, such as *koro kratok* (*Phaseolus lunatus*). The *koro kratok* is a type of koro bean that originates from Central America and South America. *Koro kratok* is also found in Indonesia, especially East Java and Madura. The productivity of *koro kratok* in Indonesia reaches 2-8 tons of fresh seeds/ha depending on the type of cultivar and cultivation conditions. In the tropics, *koro kratok* has productivity of 200-600 kg/ha (in intercropping), or 1-1.5 tons/ha (single cropping). In cultivated plants, productivity reaches 2-4 tons/ha (Baudoin, 2006).

Koro kratok is known to have a high protein content, which is around 17 to 29% (Budi *et al.*, 2003). Magana *et al.*, 2015 stated that the amino acids contained in the *koro kratok* (*P. lunatus*) hydrolyzate were Asx, Glx, Ser, His, Gly, Thr, Arg, Ala, Pro, Tyr, Val, Met, Cys, Ile, Leu, Phe, Lys, Trp. Tejasari (2009) states that the essential amino

acids found in the *koro kratok* consist of phenylalanine, tyrosine, threonine, tryptophan, valine, isoleucine, leucine and lysine. Other studies on *koro kratok* concluded that *koro kratok* hydrolyzate has the potential to produce ACE inhibitor bioactive peptides. So the purpose of this study was to find out the chemical characteristics of *koro kratok* tempeh and the effect of peptide molecular weight on ACE inhibitory activity.

MATERIALS AND METHODS

Material

The research raw materials used were *koro kratok* (*Phaseolus lunatus* L.) originating from Madura, Raprima brand tempeh inoculum. Chemicals: NaCl, CaCO₃, NaOH, HCl, K₂SO₄, Na₂S₂O₃, Na₂CO₃, CuSO₄·5H₂O, TCA, distilled water, colic acid, sodium taurocholate, sodium deoxycholate, ethyl acetate, NaCl, AgNO₃ and NH₄OH, were analytical grade.

Sample preparation

Koro kratok tempeh was prepared by boiling the beans for 30 min, then soaked for 12 h. After that, the epidermis skins were removed and the beans were soaked again for 24 h (water replacement every 12 h). Finally, these beans were boiled for 15 min before inoculated with raprima inoculum. Fermentation was done for 48 h of incubation at room temperature.

Peptide Extraction

Peptide extraction was carried out by the method of Mechin *et al.* (2007) and LuoAs *et al.* (2016) with a ratio between 1 g powdered tempeh mixed with 30 ml aquabides. This mixture was extracted using blender for 3 min at 8000 rpm, then incubated on waterbath at 30°C, 60 rpm for 60 min and the supernatant was collected after centrifugation at 20,000xg for 15 min.

Proximate Analysis

The protein, fat, water, and ash content were measured using the methods of AOAC (2005), whereas carbohydrate content was calculated by difference.

Analysis of Amino Acid Composition

Amino acid compositions of the *koro kratok* protein were analyzed using LC-MS/MS. The sample was hydrolyzed using 6N HCl then heated using autoclave 110°C for 12 hours. The samples were neutralized using 6N NaOH and filtered using a 0.22 µm siring filter and diluted with aquabides with a ratio of 1:50 (v/v). Two µl of the sample was injected into the LS-MS/MS (The Water Xevo TQD, Waters, USA). The solvents used are: solvent A: 0.1% pentadecafluorooctanoic acid:water/CH₃CN with 0.1% formic acid = 99.5%:0.5% and solvent B composed of 0.1% pentadecafluorooctanoic acid:water/CH₃CN with 0.1% formic acid = 10%:90%. The sample was eluted at a temperature of 50°C for 1.5 min in solvent A and 0.5 min in solvent B with a flow rate of 0.6 ml/minute.

ACE Inhibitory Activity

The ACE inhibitor activity of the hydrolyzate was measured by a UV spectrophotometer based on the rate of formation of hippuric acid from hippuryl-L-histidyl-L-leucine (HHL) (Puspitojati, 2019; Chusman and Cheung, 1971 with a modification). 50 µl of the sample solution and 50 µl of ACE solution (25mU/ml) were pre-incubated at 37°C for 10 min, then the mixture was incubated with 50 µl substrate (Hip-His-Leu 8 mM in 50 mM HEPES buffer containing 300 mM NaCl at pH 8.3) for 30 min at the same temperature. The reaction was ended with the addition of 1 M HCl (200 µl). The solution was extracted by the addition of 1.5 ml of ethyl acetate and the mixture was stirred for 2 min and then centrifuged (4000 x g) for 15 min. One ml of the supernatant was transferred to another glass tube and evaporated in boiling water for 30 min. The resulting dry sample was then dissolved in 3 ml of distilled water and absorbance was measured at λ=228 nm using a UV-VIS spectrophotometer.

ACE I activity is calculated as the percentage inhibition of ACE activity using the formula:

$$\% \text{ inhibition} = ((A-B)) / ((A-C)) \times 100\%$$

Where :

A = absorbance of the ACE enzyme and substrate
 B = sample absorbance + ACE + substrate enzyme
 C = absorbance of substrate + sample

IC50 Value Determination

The IC50 value was determined based on the relationship of linear curves between ACE inhibitory activity values (%) and the concentration of protein hydrolyzates from the sample.

Peptide Fractionation

Peptide fractionation by dialysis technique referred to Pohl (1990). The sample of 10 ml was inserted into the membrane bag (Molecular Weight Cut Off 1kDa, 3.5 kDa, and 14 kDa). Then the bag was soaked in aquabides at a

ratio between the peptides and aquabides 1:9. Dialysis was carried out for an overnight in the cold room. The resulting peptide fraction was then lyophilized.

RESULT AND DISCUSSION

Proximate *Koro kratok* beans

The proximate analysis of *koro kratok seeds* can be seen in Table 1. The protein content (20.66 %) was within the range protein content of *koro kratok* reported by Maesan and Somaatmadja (1993) (18-25 %). Just like protein, the fat content of *koro kratok* in this study was also within the range of fat content reported by Maesan and Somaatmadja (1993) (0.2-3 %). These data indicated that *koro kratok* was a source of protein that has the potential to become a peptide after fermentation.

Table 1. Proximate of *koro kratok seeds (Phaseolus lunatus)*

Component	%
Moisture content	12.04 ± 0.07
Ash content	3.97 ± 0.17
Lipid content	1.28 ± 0.01
Protein content	20.66 ± 0.24
Carbohydrate by difference	62.71 ± 0.50

Amino Acid Composition

Table 2 shows the amino acid composition of *koro kratok*. leucine content was the highest among the hydrophobic amino acids. This amino acid had a strong effect on ACE bonding (Daskaya-Dikmen *et al.*, 2017). As seen in Table 2, the content of hydrophilic amino acids was higher than hydrophobic ones.

Table 2. Amino acid composition of *koro kratok seeds (Phaseolus lunatus)*

Amino acids content	%(g/100 g protein)
Alanine	0.51
Glycine	0.49
Valine	0.45
Leucine	0.71
Isoleucine	0.36
Proline	0.34
Phenylalanine	0.41
Methionine	0.03
Cysteine	0.02
Hydrophobic Amino Acids	3.32
Aspartic acid	0.94
Glutamic acid	1.18
Arginine	4.40
Lycine	0.40
Histidine	0.27
Serine	0.70
Threonine	0.45
Tyrosine	0.13
Hydrophilic Amino Acids	8.47

The amino acid of arginine was dominant in the hydrophilic amino acids group. L-arginine could be converted to nitric oxide by the enzyme nitric oxide synthase so that it has the potential to be a vasodilator (Lee and Hur 2017).

ACE Inhibitory Activity

Bioactive peptides that have ACE inhibitory activity were expressed as percentage inhibition of ACE. ACE inhibition

activity in non-fermented koro seeds as control and *koro kratok* tempeh shown in Table 3.

Table 3. Inhibitory ACE activity in non-fermented and *koro kratok* tempeh

Sample	Inhibitory ACE Activity (%)	IC ₅₀ (mg/mL)
Non-Fermented	24.1 ± 8.5	1.86
Tempeh	85.0 ± 2.9	0.763

ACE inhibitory activity was detected in non-fermented *koro kratok*, which was 24.1%. This inhibitory activity may be caused by the presence of small molecular weights of peptides (14-18 kDa) which were naturally present in the seed (Chel-Guerrero *et al.*, 2007). These bioactive peptides can be found in food ingredients as natural ingredients or as hydrolyzate products (Malaguti *et al.*, 2014).

ACE inhibitory activity in *koro kratok* tempeh was 85.0%, increased compared to non-fermented seeds. This increase could be understood because, during fermentation, the protein was hydrolyzed to the small peptide by protease produced by the mold of *Rhizopus oligosporus*. These peptides may possess ACE inhibitory activity.

ACE inhibitory peptides are affected by hydrophobic amino acids. *Koro kratok* tempeh has a hydrophobic amino acid composition of 4.78 g/100 g protein with. According to Pan *et al.* (2011), the mechanism of inhibition of ACE activity was as followed: the active site of ACE was bond to amino acid residues in peptides through hydrogen bonding, hydrophobic interactions, hydrophilic interactions,

electrostatic interactions, or Zn²⁺ binding. Besides, the size of the peptide will also influence the ACE inhibitory activity. Some researchers reported that peptides having ACE inhibitory activity can be dipeptide (Pan *et al.*, 2011), tripeptides (Pan *et al.*, 2011), heptapeptides (Toopcham *et al.*, 2015) and oligopeptides (Balti *et al.*, 2015). These peptides might be formed during *koro kratok* tempeh fermentation so that resulted in a higher ACE inhibitory activity compared to that non-fermented.

Koro kratok tempeh has a smaller IC₅₀ value compared to non-Lermented *koro kratok*, which was 0.763 mg/mL (Table 3). This IC₅₀ value was still bigger compared to the *koro kratok* which was hydrolyzed for 90 min using alcalase and Flavourzy, each of 0.056 and 0.0069 mg/mL, respectively (Uco *et al.*, 2009).

ACE Inhibitory Activity on Fractionation of Peptide Tempeh Koro Kratok

The tempeh peptides were fractionated using the dialysis method. The fraction obtained was then analyzed for ACE inhibitory activity and the results were shown in Figure 1.

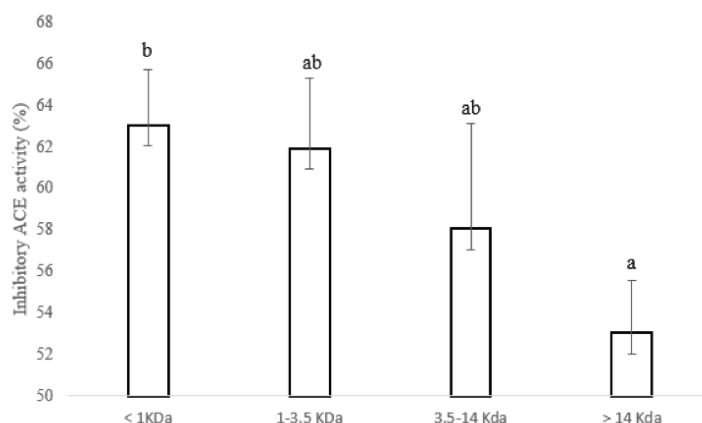


Figure 1. ACE inhibitory activity of peptide fraction from *koro kratok* tempeh

Figure 1. shows peptides having a molecular weight (MW) less than 1 kDa had ACE inhibitory activity of 63.06%, whereas fractions with MW of 1-3.5 kDa, 3.5-14 kDa, and more than 14 kDa had an inhibitory activity of 61.94%, 58.06%, and 53.06% respectively. The results of this study indicate that the smaller the MW of the peptide, the greater the ACE inhibitor activity. The results of this study were similar to that of Natesh *et al.* (2003); Toopcham *et al.* (2014); Darewicz *et al.* (2014); Power *et al.* (2014) which they stated that fractions with small MW peptides have high ACE inhibitory activity.

The results of this study indicate that the smaller the MW of the peptide the greater the inhibitory activity. Natesh *et al.* (2003) state that long-chain peptides are more difficult to bind to the active site of ACE so that reduced ACE inhibitory activity. This is in line with the statement of Tamam *et al.* (2018) that peptides that have ACE inhibitory activity have a smaller peptide chain and MW than other peptides. However, statistical calculation indicated that the fractions of MW <1 kDa not significantly differed ($p < 0.05$) from the peptide fraction of MW between 1 - 3.5 kDa and 3.5 - 14 kDa. The results of this study are supported by

the results of previous studies that the MW of peptides which have the highest ACE inhibitory activity ranges from 200 - 850 Da (Wang *et al.*, 2017; Sornwatana *et al.*, 2015; Moayedi *et al.*, 2018; Lee *et al.*, 2004; White *et al.*, 2014).

CONCLUSION

Koro kratok tempeh has the potential to be a source of bioactive peptides because of protein content and amino acid composition. The fermentation could increase the ACE inhibitory activity because fermentation hydrolyzed protein to become small peptides. Low molecular weight peptides contained in the *koro kratok* tempeh had higher ACE inhibitory activity.

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