Changes in amino acid composition during fermentation and its effects on the inhibitory activity of angiotensin-I-converting enzyme of jack bean tempe following in vitro gastrointestinal digestion

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Summary

This study investigated changes in the amino acid composition during fermentation of jack bean tempe and its effect on the inhibitory action of angiotensin-I-converting enzyme (ACE) of the peptides produced after in vitro gastrointestinal digestion. The composition of amino acids was determined in jack bean unfermented (F0) and fermented for 48 h (F48) or 72 h (F72). Fermentation for 48 h did not increase the content of hydrophobic amino acids, but prolonging it to 72 h resulted in a significant increase. In the digestion simulation experiment, the hydrolysis level in all samples increased sharply after the addition of pancreatin. Moreover, the high degree of protein hydrolysis did not necessarily result in high ACE inhibitory activity. Furthermore, all hydrolysates were dominated by peptides with a molecular weight of < 1 kDa and all peptide fractions of F72 exhibited strong inhibition of ACE activity, except for those with molecular weights > 14 kDa. It can be concluded that the increase in the content of hydrophobic amino acids in jack bean tempe fermented for 72 h improved the ACE inhibition of peptides generated after in vitro gastrointestinal digestion, with the inhibitory value being 88.2 %.

Keywords

angiotensin-I-converting enzyme; peptide; in vitro digestion; hydrolysis, jack bean; tempe

Angiotensin-I-converting enzyme (ACE) is a zinc-dependent peptidyl dipeptidase (EC.3.4.15.1), which is the primary component of the renin-angiotensin system (RAS) playing a role in blood pressure regulation [1, 2]. Studies have reported that it is possible to inhibit the activation of RAS by ACE inhibitors using blood pressure-lowering synthetic medicines such as lisinopril and captopril [3, 4]. Action of these synthetic ACE inhibitors generally results in negative effects such as cough, higher blood potassium levels, hypotension, dermatitis, headache, flavour disorders, exhaustion or embryo abnormalities. However, naturally derived ACE inhibitors are known to have very little side effects compared with synthetic ACE inhibitory drugs [2, 5]. Therefore, recent research has shifted toward biological sources of ACE inhibitory peptides such as food [3].

Various peptides isolated from vegetable proteins have been analysed as sources of ACE inhibitory agents, including pea, walnuts, soybeans, black soybean and jack bean [5–8]. The latter are an underutilized legume with high protein content [9], proline, leucine and isoleucine being the major hydrophobic amino acids (HOAAs) in jack bean [10]. Previous research demonstrated that jack bean protein solubility increased with the increase in duration of fermentation using *Rhizopus oligosporus* [11, 12]. Furthermore, a more recent study demonstrated an increase in the peptide concentration as well [8]. During tempe fermentation, *R. oligosporus* can grow well on protein-rich

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substrates, producing proteases that can hydrolyse proteins to small peptides and amino acids [13, 14]. Another previous research reported on changes in the concentrations of essential, non-essential and HOAAs during tempe fermentation of garbanzo beans, soybeans and groundnuts [15]. Changes in the relative content of HOAAs may influence the formation of bioactive peptides during fermentation [16]. However, it was reported that the fermentation of jack beans increased the ACE inhibition of the peptides released after fermentation [8].

An earlier study reported that the peptides produced are exposed to hydrolysis during a particular gastrointestinal phase [4]. The protein substrate was not completely hydrolysed during tempe fermentation, so that high molecular weight peptides were still found in the fermented product [8]. Therefore, the peptides may be degraded during gastrointestinal digestion, which possibly inhibits their potential bioactivity. Otherwise, the biological activity may be promoted when long-chain peptide precursors produce bioactive fragments by the action of gastrointestinal enzymes [17]. The ACE inhibitory peptides are susceptible to hydrolysis during digestion due to the presence of enzymes such as pepsin, trypsin, chymotrypsin and peptidases on the epithelial cell surface. These enzymes generate peptides of various sizes with the ability to change the ACE inhibitory activity [18]. Furthermore, extensive research described conditions of stability and degradation of ACE inhibitory peptides during in vitro gastrointestinal digestion of pea seeds, whey protein, peanuts and grape skin [19–22].

Tempe is a traditional food originating from Indonesia. Currently, it is consumed worldwide because of its good taste and high nutritional value [23]. Jack bean tempe was also described as an alternative source of peptides with high ACE inhibitory capacity [8]. However, the ACE inhibition of jack beans after digestion in the digestive tract has not yet been investigated. Therefore, this study was conducted to investigate changes in the amino acid composition of jack beans during tempe fermentation and its effect on the ability of the peptides to inhibit ACE after in vitro digestion, as well as to to determine the molecular weight of the produced peptides. We hypothesized that the changes in the amino acid composition after tempe fermentation may affect the degree of protein hydrolysis by digestive enzymes, which would result in differences in the ACE inhibitory capacity of the peptides produced after digestion simulation.

MATERIAL AND METHODS

Materials

Jack bean seeds (*Canavalia ensiformis*) were obtained from farmers in Yogyakarta (Indonesia). Raprima powder tempe inoculum containing 10^6 CFU·g^{-1} of *R. oligosporus* was obtained from Aneka Fermentasi Indonesia (Bandung, Indonesia). *o*-Phthaldialdehyde (OPA) was obtained from Merck (Kenilworth, New Jersey, USA). ACE (EC.3.4.15.1) was obtained from rabbit lung, and hippuryl-L-histidyl-L-leucine, pepsin (EC.3.4.23.1 from porcine gastric mucosa (P7012)) and pancreatin (EC.232-468-9 from porcine pancreas (P7545)) were procured from Sigma-Aldrich (St. Louis, Missouri, USA). All other chemicals used were of analytical grade.

Production of jack bean tempe

Jack bean tempe was prepared according to the method described by Puspitojati et al. [8], which involved using jack bean seeds that were boiled for 30 min and inoculated with a commercial inoculum of Raprima. The inoculated seeds were wrapped in banana leaves and incubated for 0 h, 48 h or 72 h at room temperature (approximately 30 °C). The resulting tempe was termed as F0 for unfermented jack bean samples, F48 for those fermented for 48 h and F72 for those fermented for 72 h. All the samples were freeze-dried and stored at –20 °C for further analysis. All experiments were conducted in triplicate.

In vitro simulated gastrointestinal digestion

Enzymatic hydrolysis of jack bean tempe was conducted using the method described by Minekus et al. [24] and Sun et al. [25] with a slight modification. The tempe powder was mixed with distilled water in a 1:10 ratio and homogenized for 3 min. The mixture was collected and incubated for 60 min at 30 °C and centrifuged at 20 000 ×*g* for 15 min. The supernatant was collected and freeze-dried to obtain 5 mg·ml^{-1} of concentrated protein, which was quantified using the Lowry method with bovine serum albumin as a standard, and used for enzymatic hydrolysis. The protein samples were adjusted to pH 3 using 1.0 mol·l^{-1} of HCl and first hydrolysed with pepsin (EC.3.4.23.1, 2000 U·ml^{-1}) for 120 min at 37 °C and shaking at 1.33 Hz in a water bath shaker. Then, pH was adjusted to 5.3 using 0.9 mol·l^{-1} of $NaHCO_3$ and subsequently adjusted to pH 7.5 by adding 2 mol·l^{-1} of NaOH. This step was followed by the addition of pancreatin (EC.232-468-9, 100 U·ml^{-1} of trypsin) to the solution and hydrolysis for 120 min, making the total hydrolysis time to be 240 min. A sample

was taken every 30 min to monitor the progression of hydrolysis. The reaction for all the treatments was stopped by dipping the samples into boiling water for 10 min. The samples were then cooled in an ice bath, followed by centrifugation at 8000 $\times g$ and 4 °C for 15 min. The resulting supernatant was collected and then freeze-dried.

Determination of molecular weight distribution

The hydrolysates were fractionated using the dialysis method, separation being conducted in stages using dialysis membranes of 1 kDa, 3.5 kDa and 14 kDa molecular weight cutoff (MWCO) for 12 h at 4 °C. The fractions obtained by this process consisted of peptides of molecular weights of < 1 kDa, 1–3.5 kDa, 3.5–14 kDa and > 14 kDa. All fractions were freeze-dried and stored at –20 °C.

Determination of ACE inhibitory activity

The ACE inhibitory activity of the peptides was measured by a slightly modified method of CUSHMAN and CHEUNG method [26] using 8 mmol·l^{-1} of Hip-His-Leu as the substrate and 25 mU·ml^{-1} of ACE solution. The ACE inhibitory activity (*IA*) was calculated using Eq. 1 and expressed in percentage as follows:

$$IA = \frac{A - B}{A - C} \times 100 \quad (1)$$

where *A* represents the absorbance in the presence of ACE without the peptide sample (inhibitor), *B* represents the absorbance in the presence of ACE and the peptide sample (inhibitor) and *C* represents the absorbance of the blank reaction [27, 28]. IC_{50} describes the peptide concentration required for 50% inhibition of ACE under the abovementioned conditions.

Determination of peptide concentration and degree of hydrolysis

The peptide concentration was determined using the OPA spectrophotometric assay [29]. Sodium tetraborate (12.5 ml of 100 mmol·l^{-1}) was mixed with 1250 μl of 20% sodium dodecylsulphate and 550 μl of OPA reagent. Distilled water was then added to make a volume of 25 ml. The OPA-based analysis was conducted by mixing 1 ml of OPA and 20 μl of the hydrolysate. The mixture was then quickly reverted and incubated in the dark for 120 s. Absorbance of the mixture was measured at 340 nm using a UV-Vis spectrophotometer (Dynamica Scientific, Livingston, United Kingdom) with tryptone as the standard [30]. The degree of hydrolysis (*DH*) was calculated as the amount of cleaved peptide bonds using Eq. 2 and expressed in percentage as follows:

$$DH = \frac{N_x - N_0}{N_t - N_0} \times 100 \quad (2)$$

where N_x represents the peptide concentration at X min, N_0 represents the peptide concentration at 0 min of hydrolysis, and N_t represents the total amount of amino groups. N_t was determined by acidic hydrolysis using 6 mol·l^{-1} of HCl for 24 h at 110 °C [31].

Determination of amino acid composition

The amino acid composition was determined by liquid chromatography tandem-mass spectrometry (LC-MS/MS) using Water Xevo TQD instrumentation (Waters, Milford, Massachusetts, USA) according to the AOAC methods with a slight modification [32]. Samples were hydrolysed using 6 mol·l^{-1} of HCl, heated in an autoclave at 110 °C for 12 h, neutralized using 6 mol·l^{-1} of NaOH and filtered using a syringe filter (pore size, 0.22 μm). The filtrate was then diluted with distilled water (1:50, v/v). The injection volume of the sample was 2 μl. Elution lasted 6 min using a gradient of mobile phase A (0.1% pentadecafluorooctanoic acid : 0.1% formic acid in water/acetonitrile at a ratio of 99.5 : 0.5) and mobile phase B (0.1% pentadecafluorooctanoic acid : 0.1% formic acid in water/acetonitrile at a ratio of 1 : 9). Elution was performed at 50 °C at a flow rate of 0.6 ml·min^{-1}, 3.5 kV capillary and a collision energy of 15 V. The positive electron ionization mode was used.

Statistical analysis

Data were statistically evaluated by one-way analysis of variance. Duncan's multiple range test was applied to determine mean differences between the samples. The statistical analysis was conducted using SPSS IBM 23 (IBM, Armonk, New York, USA) and the significant differences were declared at a 5% significance level.

RESULTS AND DISCUSSION

Effect of fermentation on the composition of amino acids of jack bean tempe

Three samples were analysed to determine the changes in their amino acid composition during fermentation. This analysis was also meant to determine the effect of the amino acid composition of jack bean tempe on the release of the peptides with ACE inhibitory ability after gastrointestinal digestion. The results on the amino acid composition are presented in Tab. 1.

Tab. 1. Amino acid composition of jack bean tempe during fermentation.

Amino acid [mg·kg^{-1}]	Jack bean tempe			Precursor ions (*m*/*z*)	Fragment ions (*m*/*z*)
	F0	F48	F72		
Alanine	52.7 ± 4.5^{b}	51.3 ± 1.5^{b}	30.2 ± 0.7^{a}	90.00	44.16
Glycine	50.6 ± 4.3^{a}	41.6 ± 4.8^{a}	42.5 ± 1.2^{a}	76.00	30.22
Valine	34.4 ± 0.2^{a}	44.0 ± 0.8^{a}	85.4 ± 0.5^{b}	117.83	72.15
Leucine	111.7 ± 6.9^{b}	107.7 ± 0.3^{b}	80.3 ± 0.6^{a}	131.87	86.10
Isoleucine	41.2 ± 2.3^{b}	35.2 ± 0.2^{a}	86.1 ± 0.6^{c}	131.87	86.20
Proline	61.6 ± 2.2^{a}	59.6 ± 1.3^{a}	62.6 ± 0.3^{a}	115.80	70.10
Phenylalanine	64.2 ± 3.9^{a}	60.9 ± 0.8^{a}	86.8 ± 0.4^{b}	165.89	120.10
Methionine	4.5 ± 0.4^{a}	11.9 ± 0.1^{b}	21.9 ± 0.3^{c}	149.91	104.10
Cysteine	3.6 ± 1.6^{a}	2.7 ± 1.3^{a}	1.9 ± 0.2^{a}	122.00	76.04
Aspartic acid	120.5 ± 2.5^{a}	102.5 ± 0.6^{a}	84.7 ± 1.9^{a}	134.10	73.90
Glutamic acid	126.2 ± 2.6^{b}	124.6 ± 0.8^{b}	89.2 ± 1.5^{a}	148.10	84.04
Arginine	63.0 ± 1.7^{a}	65.3 ± 4.2^{a}	65.3 ± 0.4^{a}	175.00	70.00
Lycine	73.2 ± 2.9^{a}	101.4 ± 3.4^{b}	75.9 ± 4.7^{a}	146.90	84.10
Histidine	48.7 ± 5.7^{a}	51.7 ± 0.5^{a}	51.3 ± 0.9^{a}	155.86	110.10
Serine	74.1 ± 5.2^{a}	65.4 ± 0.7^{a}	84.5 ± 0.8^{b}	106.00	60.03
Threonine	53.7 ± 1.2^{c}	47.3 ± 1.1^{b}	36.3 ± 0.7^{a}	120.00	74.21
Tyrosine	16.2 ± 0.9^{a}	26.9 ± 0.5^{a}	14.9 ± 0.4^{a}	181.93	136.09
Total	1 000	1 000	999.8		
HOAA	424.4 ± 2.9^{a}	415.0 ± 1.2^{a}	497.7 ± 0.5^{b}		
HIAA	575.6 ± 5.3^{b}	585.0 ± 1.5^{b}	502.0 ± 1.4^{a}		
AAA	80.4 ± 10.9^{a}	87.8 ± 0.7^{a}	101.7 ± 0.4^{a}		
PCAA	184.9 ± 3.4^{a}	218.4 ± 2.7^{a}	192.4 ± 2.0^{a}		
NCAA	246.7 ± 12.5^{a}	227.1 ± 0.7^{a}	173.9 ± 1.7^{a}		

The values are expressed per kilogram of protein. Data are mean ± standard deviation (n = 2). In each row, the values with the same letter are not significantly different.
F0 – unfermented jack beans, F48 – jack beans fermented for 48 h, F72 – jack beans fermented for 72 h.
HOAA – total hydrophobic amino acids (alanine, glycine, valine, leucine, isoleucine, proline, phenylalanine, methionine, cysteine), HIAA – total hydrophilic amino acids (aspartic acid, glutamic acid, arginine, lycine, histidine, serine, threonine, tyrosine), AAA – total aromatic amino acids (phenylalanine, tyrosine), PCAA – total positively charged amino acids (arginine, histidine, lysine), NCAA – total negatively charged amino acids (glutamic acid and aspartic acid).

The duration of fermentation affected the content of certain amino acids but did not significantly change the nitrogen concentration. In general, the amino acid composition in the three samples was different in terms of glutamic acid, one of the amino acids whose levels became higher in all the samples. Consistent with the duration of fermentation, the content of glutamic acid significantly decreased in tempe fermented for 72 h (F72). However, glutamate could play a role in promoting the transfer of aminogroups in the synthesis of other amino acids through transamination [33], which is a biochemical mechanism that results in the release of large amounts of amino acids during fermentation [14]. In addition, decomposition of proteins to amino acids may decline in the stationary phase of growth [34].

There was no change in the percentage of HOAAs for up to 48 h of fermentation, but it increased significantly at 72 h. Fermentation for 72 h using the inoculum of *R. oligosporus* increased the levels of valine, isoleucine, phenylalanine and methionine in jack bean tempe, isoleucine being increased by 2-fold to 8.6% during this period.

Similar to HOAAs, there was no change in the percentage of aromatic amino acids (AAAs) during fermentation, except that the content of phenylalanine increased after 72 h of fermentation. However, previous studies reported an increase in the relative amounts of HOAAs and AAAs during the fermentation of soybeans, groundnuts and garbanzo beans using *R. oligosporus* for 24 h and 30 h of fermentation [15]. The increase in the total amount AAAs was also found in chickpea fermented for 51.3 h with *R. oligosporus* [35]. The percentage increase in the content of HOAAs in the fermented jack beans took longer than that for legumes in general. This phenomenon was probably caused by fungal growth on jack beans, which required a longer adapta-

tion time because of the presence of hard seeds in jack beans. In addition, it was reported in previous studies that the proteolytic activity in jack beans fermented by *R. oligosporus* was optimal at 96 h of fermentation, which was longer than in case of barley or grass pea fermentation [8, 36, 37]. Moreover, *Rhizopus* spp. also cause changes in the amino acid composition during fermentation and some strains with a high proteolytic activity were able to produce almost five times more amino acids than other strains. However, a higher proteolytic activity results in increased degradation of proteins into smaller peptides, thereby causing an increase in the total content of amino acids and an increase in solubility of protein [14].

Peptide concentration and degree of hydrolysis during gastrointestinal digestion

The concentrated proteins from the three samples were hydrolysed sequentially using pepsin and pancreatin, the progress of hydrolysis being monitored by evaluating *DH* through changes in peptides. The results demonstrated that *DH* and the concentration of peptides increased during hydrolysis in the simulated digestion (Fig. 1, Tab. 2).

The use of pepsin in the process of hydrolysis for 90 min resulted in similar characteristics amongst the three samples. The peptide concentration and *DH* of the three samples were also not statistically different ($p > 0.05$). The unfermented jack bean (F0) had a higher hydrolysis rate than the other samples after 120 min, which could be due to the differences in the amino acid composition amongst them. It has been reported that pepsin could specifically cleave the C-terminal phenylalanine, leucine and glutamic acid but had no effect on valine, aspartic acid and glycine in this respect, whereas other amino acid residues could be cleaved at varying rates [38]. Unfermented jack beans showed a higher percentage of leucine and glutamic acid than the fermented jack bean and it may be speculated that it could lead to increased cleavage of peptide bonds, thus contributing to the higher *DH*.

Furthermore, *DH* was increased sharply in all samples after the addition of pancreatin and the increase continued for up to 210 min. After

Tab. 2. The degree of protein hydrolysis during in vitro gastrointestinal digestion.

Hydrolysis time [min]	Degree of hydrolysis [%]		
	F0	F48	F72
30	5.9 ± 1.2^{aA}	4.1 ± 1.0^{aA}	4.6 ± 0.2^{aA}
60	5.6 ± 0.7^{aA}	5.9 ± 1.3^{aAB}	4.0 ± 0.8^{aA}
90	9.5 ± 1.6^{aB}	7.4 ± 1.2^{aB}	7.6 ± 1.3^{aA}
120	16.7 ± 0.4^{bC}	8.7 ± 0.8^{aB}	7.3 ± 0.9^{aA}
150	53.1 ± 3.5^{aD}	57.0 ± 2.9^{aC}	59.9 ± 2.5^{bB}
180	59.9 ± 0.4^{aE}	60.4 ± 0.9^{aD}	65.8 ± 3.6^{bC}
210	65.7 ± 2.2^{aF}	72.9 ± 2.7^{bE}	69.9 ± 2.6^{aD}
240	67.9 ± 1.5^{aF}	74.5 ± 2.0^{bE}	68.0 ± 1.3^{aCD}

The samples were hydrolysed using pepsin for 120 min and followed by pancreatin for 120 min.
Data are mean ± standard deviation ($n = 3$). Mean values with small alphabet superscripts within columns and those with capital alphabet superscripts within rows are significantly different ($p < 0.05$).
F0 – unfermented jack beans, F48 – jack beans fermented for 48 h, F72 – jack beans fermented for 72 h.

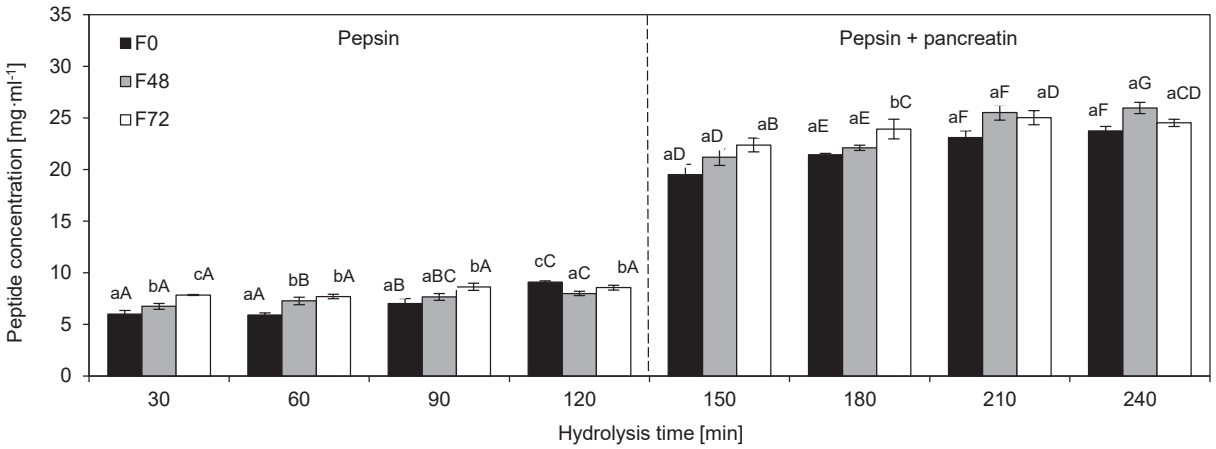

Fig. 1. Peptide concentration during hydrolysis using gastrointestinal enzymes.

The samples were hydrolysed using pepsin for 120 min and followed by pancreatin for 120 min.
Mean values with small alphabet superscripts within samples and those with capital alphabet superscripts within hydrolysis time are significantly different ($p < 0.05$).
F0 – unfermented jack beans, F48 – jack beans fermented for 48 h, F72 – jack beans fermented for 72 h.

Tab. 3. Angiotensin-I-converting enzyme inhibitory activity of jack bean tempe hydrolysates.

Hydrolysate	Inhibitory activity [%]
HF0	75.1 ± 2.9^{a}
HF48	81.8 ± 0.9^{b}
HF72	88.2 ± 1.1^{c}
Captopril (control)	93.3 ± 0.3^{d}

Data are mean ± standard deviation ($n = 3$). Mean values with different superscripts are significantly different ($p < 0.05$).
HF0 – hydrolysate of unfermented jack beans, HF48 – hydrolysate of jack bean tempe fermented for 48 h, HF72 – hydrolysate of jack bean tempe fermented for 72 h.

this time, there were no significant changes in all samples till the end of hydrolysis at 240 min. The difference in *DH* obtained with pepsin and pancreatin was influenced by the hydrolytic action of each enzyme. Pancreatin consists of several endopeptidase and exopeptidase enzymes such as trypsin, chymotrypsin, elastase and carboxypeptidase, having a broader cleavage site specificity than pepsin [39]. At the end of hydrolysis using pepsin, pancreatin probably well hydrolyzed the pepsin hydrolysates resulting in a rapid increase *DH*.

Angiotensin-I-converting enzyme inhibitory activity of hydrolysates

As shown in Tab. 3, each hydrolysate had a significantly different ACE *IA* ($p < 0.05$), and the strongest ACE inhibition was obtained with hydrolysate of jack bean tempe that was fermented for 72 h (HF72), with a value of 88.2 % (IC_{50} = 0.60 mg·ml^{-1}). The strongest ACE *IA* of the peptides found in this study did not correlate with the highest *DH*. At this point, degradation of bioactive peptides during gastrointestinal digestion may release new peptides to increase *IA* against ACE.

Previous studies reported that extending the duration of incubation for tempe fermentation affected the changes in the ACE inhibition of the peptides produced after fermentation. It was observed that unfermented jack beans showed the minimum ACE *IA* of 13.9 %, whereas jack beans fermented for 48 h and 72 h showed 46.1 % and 60.0 % of ACE *IA*, respectively [8]. *IA* was remarkably higher in the HF72 sample, followed by hydrolysate fermented for 48 h (HF48) and unfermented hydrolysate (HF0) samples. In addition, ACE inhibition increased in all samples after hydrolysis using gastrointestinal enzymes. Comparison of the fermented jack beans with the unfermented ones revealed that the fermentation process could increase ACE *IA* of the hydrolysates, and the activity in the HF72 sample was found to be higher than that in the HF48 sample before and after the digestion stage. Moreover, the amino acid composition of the initial protein source significantly influenced the release of ACE inhibitory peptides, and the level of activity was also found to be influenced by HOAAs. Jack bean tempe fermented for 72 h (F72) had a higher HOAA content than those fermented for 48 h (F48) or the unfermented ones (F0). This could be associated with the easiness with which fermented jack beans are digested to produce stronger inhibitory peptides after the digestion simulation. The same phenomenon was found in the fermentation of pea seeds using *Lactobacillus plantarum* 299v, which could increase ACE *IA* after the digestion simulation [19].

Angiotensin-I-converting enzyme inhibitory activity of hydrolysate fractions

The fractions of the three hydrolysate samples were primarily peptides with a molecular weight (*MW*) of <1 kDa, as shown in Tab. 4. The higher number of peptides with *MW* <1 kDa might be related to the high *DH* (approximately 67.9–74.5 %) in all samples.

The fractions with *MW* <1 kDa had the strongest *IA* in the range of 84.8–89.4 % amongst all the hydrolysate samples, whereas in contrast, those with >14 kDa had low inhibitory activities (Fig. 2). It was previously reported that the peptide size and the amino acid composition as well as their sequence contributed to ACE *IA*. Bioactive peptides with smaller sizes may have a greater chance during intestinal absorption to exert their bioactivity in the target organ [16].

In this study, no significant difference was recorded in ACE *IA* between fractions with *MW* of 1 kDa, 1–3.5 kDa and 3.5 kDa in the hydrolysate tempe fermented for 72 h (HF72). However,

Tab. 4. Molecular weight distribution of peptides produced by in vitro gastrointestinal digestion.

Molecular weight [kDa]	Distribution [%]		
	HF0	HF48	HF72
<1	64.4 ± 1.4^{aD}	80.4 ± 2.5^{bD}	79.3 ± 4.3^{bC}
1–3.5	6.4 ± 0.3^{aC}	11.9 ± 1.5^{bC}	11.9 ± 2.0^{bB}
3.5–14	27.9 ± 1.2^{cB}	5.8 ± 0.8^{aB}	8.2 ± 0.5^{bB}
>14	1.2 ± 0.1^{aA}	1.8 ± 1.8^{bA}	1.0 ± 0.1^{aA}

Data are mean ± standard deviation ($n = 3$). Mean values with small alphabet superscripts within columns and those with capital alphabet superscripts within rows are significantly different ($p < 0.05$).
HF0 – hydrolysate of unfermented jack beans, HF48 – hydrolysate of jack bean tempe fermented for 48 h, HF72 – hydrolysate of jack bean tempe fermented for 72 h.

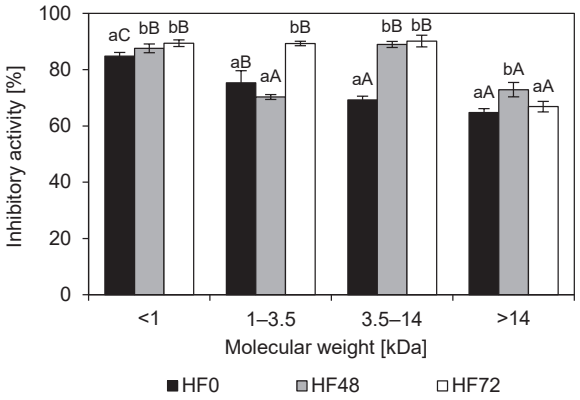

Fig. 2. Angiotensin-I-converting enzyme inhibitory activity of hydrolysates based on the peptide size.

Mean values with small alphabet superscripts within molecular weights and those with capital alphabet superscripts within samples are significantly different ($p < 0.05$).
HF0 – hydrolysate of unfermented jack beans, HF48 – hydrolysate of jack bean tempe fermented for 48 h, HF72 – hydrolysate of jack bean tempe fermented for 72 h.

the strongest inhibition of ACE in the HF48 sample was achieved by the fraction with *MW* of 3.5–14 kDa, with the fraction with the *MW* of 1–3.5 kDa having a much lower *IA*. This demonstrated that peptides of a smaller size did not always exhibit stronger ACE *IA*. Therefore, amino acid sequence may play a more substantial role in the bioactivity of peptides, compared with the peptide size.

CONCLUSIONS

This study showed that jack bean tempe fermented for 72 h released a relatively high amounts of HOAAs. This demonstrated the capacity to increase the formation of ACE inhibitory peptides after the in vitro digestion simulation. The sample also generated large amounts of low *MW* peptides with strong ACE inhibitory activities in the peptide fractions of <1 kDa, 1–3.5 kDa and 3.5–14 kDa. The duration of fermentation was found to be an important factor in the formation of ACE inhibitory peptides after digestion. Further research is needed to demonstrate the correlation between ACE *IA* of jack bean tempe and the ability to reduce blood pressure in experimental animals in vivo.

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