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ANGIOTENSIN CONVERTING ENZYME INHIBITOR ACTIVITY OF THE SOYBEAN TEMPEH PROTEIN AS FUNCTIONAL FOOD

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ABSTRACT: Tempeh is an Indonesia's indigenous fermented food that is widely preferred by the community. Fermented food has functional food as an antihypertensive by angiotensin-converting enzyme (ACE) inhibitor. This study was aimed to measure protein activity of tempeh as ACE inhibitor. Soybean was fermented using *Rhizopus* sp. with various fermentation times of 0, 6, 12, 18, 24, 30, 36, 42 and 48 hours. Protein extraction was performed at the isoelectric point, the degree of hydrolysis was considered as trichloroacetic-dissolved nitrogen. The best fermentation time was determined by the degree of hydrolysis (DH) and protein content. Protein extract was fractionated by using an ultra membrane with 3 kDa cut of. Proximate analysis was performed by the AOAC method. The measurement of an ACE inhibitor was based on the formation of hippurate acid-H-Histidyl-L-leucine (HHL). Results of this study revealed that the lipid content of tempeh was lower than that of soybean while the water content of tempeh was higher than soybean. The best fermentation time was found at 24 hours. Protein content was 236.31 ppm and DH was 36.03%. The best fraction that was able to inhibit ACE was a fraction below 3 kDa with an inhibitory capability of 67.43%. Protein of tempeh contained proline, valine, isoleucine, histidine, teonine, tyrosine, leucine, aspartic acid, lysine, glycine, arginine, alanine, phenylalanine, glutamic acid, serine and methionine. This study concludes that tempeh was able to inhibit ACE by in vitro and is potentially continued to in vivo examination, thus tempeh can be claimed as a functional food.

Keywords: Tempeh, Indigenous fermented, Angiotension converting enzym, Isoelectric point

1. INTRODUCTION

Tempeh is a traditional Indonesian food which is made by fermentation, is widely preferred and proven to provide benefits for the health of the human body. Opportunity to develop local food such as tempeh to be recognized internationally is not impossible since Indonesia has proposed to the 34th Codex Alimentarius Commission (CAC) meeting in Geneva on July 9, 2011.

Tempeh has the potential to be developed into functional foods as antihypertensive if there is scientific data obtained through in vitro and in vivo assessment. Tempeh is made by fermentation using *Rhizopus* sp. This fungus produces several antibiotics that are active against *Bacillus* species, particularly *Bacillus subtillis* [1], has anticancer and antiangiogenesis (the formation of new blood vessels) properties which are also shown by genistein compound of isoflavone derivative extracted from tempeh [2]. Ansarullah [3] states that blood pressure of hypertension sufferers decreases after consumption of soybean drink. Tempeh is useful in inhibiting the formation of blood vessels of cancer cells [2], improving bone health [4], as an anti-bacterial agent [5**]** and active antioxidant. In addition, [6] tempeh extract is beneficial in the management and prevention of dementia and Alzheimer's disease [7]. Therefore, the functional effect of soy protein which acts as an ACE inhibitor can be enhanced by fermentation [8].

Hypertension and cardiovascular disease increase along with an unhealthy lifestyle and a low facility for prevention of hypertension [9]. Currently, there are available various synthetic drugs for the treatment of hypertension. Although those are effective, synthetic drugs have many side effects such as a chronic dry cough that causes acute respiratory distress even to death [10].

Synthetic antihypertensive drugs such as Captopril® and Lisinopril® inhibit the ACE enzyme, increase bradykinin and reduce angiotensin II, but the long-term using the medicines may cause side effects. A total of 36% of patients complained about the side effects of captopril and as many as 45% complained of amlodipine [11]. Several studies indicated that hydrolyzed soy protein could lower blood pressure in vitro basis.

The objective of this study was to determine fermentation time, the degree of hydrolysis, protein fractionation and inhibitory activity assessment of angiotensin-converting enzyme (ACE). The ACE inhibitor assessment was based on the release of hippurate acid from hippuryl-L-histidyl-L-leucine substrate [12], [13].

2. METHODOLOGY

2.1 Soybean Fermentation

The fermentation period of soybeans was varied as follows 0, 6, 12, 18, 24, 30, 36, 42 and 48 hours. A total of 1000 grams soybeans (from the traditional market) were soaked in the water with ratio of 2:1 for 18 hours, it was further removed from the skin. Cleaned soybeans were steamed using the corm for 30-40 minutes (until tender) and were then cooled 0.2 grams starter Raprima from PT Aneka Fermentasi Industri (AFI) Indonesia was inoculated by mixing the starter with steamed soybean evenly. Furthermore, it was inserted into the measuring plastic that has been given small holes. Inoculation result was incubated at room temperature until tempeh was formed.

2.2 Disposal of Lipid from Tempeh Flour

The tempeh was crushed using a blender and soaked in a hexane solution at a ratio of 1: 5 (sample: hexane) for 1 hour at room temperature while stirring. The supernatant was removed and the precipitate was repeated with the same process by adding hexane solution twice with the same ratio for removing the remaining lipid. The lipid-free flour was collected and dried to liberate hexane [14] thus resulting in lipid-free tempeh flour.

2.3 Protein Extraction of Tempeh

Protein extraction was performed by precipitation method at isoelectric point [15]. Lipid-free tempeh flour was soaked in the water a ratio of 1:10 (w/v), pH 8.5 with the addition of 1N NaOH solution. The suspension was stirred using a magnetic stirrer at room temperature for 1 hour and centrifuged (6000 x g) for 20 minutes at 4°C. The supernatant was collected and adjusted to pH 4.5 with the addition of 1N HCl solution. The suspension was centrifuged at 6000×g for 20 min, a temperature of 4°C [16]. The resulting hydrolysis was lyophilized and stored in the freezer with a temperature of -20°C, thus protein hydrolysates would be obtained.

2.4 Analysis of Protein and Degree of Hydrolysis

Hydrolysate of tempeh protein was dissolved in aquadest with a ratio of 1:10 (g/v) while stirring until dissolved evenly. The mixture was centrifuged (13000 xg, 15 min, 4ºC). A total of 100 µL supernatant was piped into the test tube and added with 5 mL of Bradford solution while shaking and allowed to stand for 15 min. The absorbance was measured using a spectrophotometer at a wavelength of λ 595 nm [17].

The degree of hydrolysis was calculated using the SN-TCA method [18], a total of 2 mg of protein hydrolysate was added with 2 mL 10% (w/v) trichloroacetic acid solution. The mixture was allowed for 30 minutes to obtain the precipitate and then centrifuged (7.800 g, 4 ºC, for 15 minutes). Protein levels of supernatant and precipitate were measured by using Kjedhal method.

2.5 ACE inhibitory Examination

Enzyme activity was determined using the Hip-His-Leu (HHL) synthetic peptide as a substrate which was based on the developed method [12],[13]. A protein extract solution of 15 µl was added with 125 µl of 100 mM sodium borate buffer solution (pH 8.3) containing 7.6 mM Hip-His-Leu and 608 mM NaCl and was preincubated for 5 min at a temperature of 37 °C. The reaction begins with the addition of 50 µL ACE enzyme (rabbit lung-derived angiotensin I-converting enzyme, 500 uM) dissolved in the aquadest. The mixture was incubated for 30 minutes at temperature of 37 °C. As a blank, it was used 50 µL aquadest.

The reaction was discontinued the addition of 125 µL 1N HCl. Hippurate acid released by ACE was extracted by adding 750 µL ethyl acetate to the mixture and was immediately shaken using vortex. The mixture was centrifuged at a velocity of 13.760 x g for 10 min, a total of 500 µL top layer of the supernatant was collected and dried at 90 °C for 30 min. Furthermore, hippurate acid was dissolved in 1 mL distilled water and the absorbance was measured by spectrophotometer at 228 nm wavelength.

% Inhibitory Activity = [(C-A)/(C-B)] x100%
Description:
A= sample absorbance
B= blank absorbance
C= control absorbance (aquadest).

2.6 Amino Acid Analysis

Amino acid composition analysis determined was the result of fractionation with 3 kDa membrane. The sample was added with 5 mL 6 N HCl while stirring with a stirrer, and flowed with nitrogen and hydrolyzed at temperature of 110 °C for 22 hours. Hydrolysates obtained was then cooled at room temperature, and transferred into 5 mL measuring flask, also added with aquabidest. The solution was filtered by a filter membrane with a size of 0.45 µm. A total of 500 µl filtrate was added with 40 µL AABA (alpha aminobutyric acid) and 460 µl aquabidest. A total of 10 µl solution was added with 70 µl AccQ-fluor borate and shaken using vortex. Furthermore, a total of 20 µl fluorine A reagent was added and shaken using a vortex, and allowed to stand for 1 minute. Subsequently, the solution was

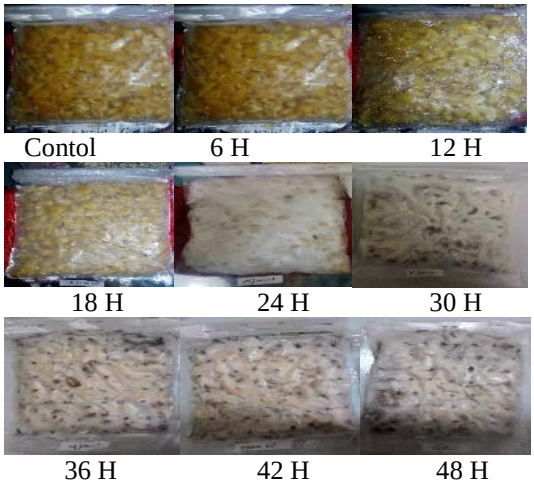
incubated for 10 minutes at a temperature of 55 ^{o}C and injected in UPLC (ultra high-performance liquid chromatography) [19].

3. RESULTS AND DISCUSSION

3.1 Tempeh

There are four steps in the making process of tempeh, namely soaking, boiling, inoculation of *Rhizopus* and incubation at room temperature [20]. The tempeh was made using a starter containing a mixture of *Rhizopus* sp. Variety of fermentation times used were 0 hours as control 6, 12, 18, 24, 30, 36, 42 and 48 hours. The best physical appearance of tempeh depends on the duration of fermentation (hour) which is based on observation of physical shape, odor and the increase of *Rhizopus mycelium* on the surface of the soybean. The physical appearance of tempeh during fermentation is shown in Fig. 1.

Fig. 1 Physical appearance of tempeh at various fermentation times (H)



The 24 hours fermentation produced tempeh with better texture and good odor in which *Rhizopus* sp. mycelium covered the surface of soybean evenly. *Rhizopus* sp. started to grow at the 18th hour and continued to next hours. The best growth of *Rhizopus* sp. was obtained at 24th-hour fermentation. *Rhizopus* mycelium of 30 hours fermentation showed a black color which indicates that tempeh began to rot. Types of *Rhizopus* which primarily found on tempeh starter are *Rhizopus oligosporus* dan *Rhizopus oryzae* [20].

Fermentation of soybean into tempeh that is widely done by Indonesian people lasts between 18 to 36 hours. After 36 hours, tempeh begins to decompose which can be seen from discoloration of tempeh such as blackening color and bad odor. Yet in this study, 30 hours fermentation had produced out of odor tempeh, thus we conclude that tempeh produced in this study is faster to decompose compared with tempeh in the other studies. Good tempeh has a fresh aroma such as the scent of mycelium and free amino acids. The quality of tempeh can be known from the growth of mycelium. If the mycelium grows evenly on the surface of tempeh, it can be declared as good tempeh. Otherwise, if the mycelium is accumulated on the certain points of soybean surface, it is declared as not good tempeh [21].

3.2 Proximate Analysis Results

Table 1 Results of proximate analysis of tempeh of 24 hours incubation

Parameter	Sample	
	Soybean	Tempeh
Water content (%)	11.81	57.42
Ash content (%)	4.95	1.64
Lipid (%)	15.28	9.86
Protein (%)	37.17	21.84
Dissolved Protein (ppm)	75.19	236.31

Based on Table 1, it is known that the lipid content of fermented soybean (tempeh) was lower than that of unfermented soybean (control) with a decrease of 64.53%. Similar with [22], lipid content of soybean was 18.38%. Meanwhile, dissolved protein content for control and tempeh was 75.19 and 236.31 ppm, respectively.

3.3 Lipid on Tempeh

Lipid disposal from tempeh in this study was aimed to obtain a higher protein extract. Wu [15] states that the samples with lipid disposal have a high protein content of 72.35% while samples without lipid disposal have a protein content of 55.88%. Results of this study indicate that fermentation can reduce lipid content in tempeh. Furthermore, the lowest lipid content obtained at 24 hours fermentation was 9.04%. We presume this lipid reduction activity is caused by lipase.

3.4 Degree of Hydrolysis and Protein Content

The degree of hydrolysis (DH) is defined as the proportion of peptide bonding broken down in protein hydrolysates. The DH value increased significantly at 18 hours fermentation and the highest value was obtained at 24 hours fermentation in which the mycelium has been seen to grow covering the surface of tempeh. The result of the calculation is presented in Table 2. The DH value increased rapidly over 18 hours fermentation since the mycelium is already grown.

A high degree of hydrolysis indicates that the protein is easily hydrolyzed (Table 2). In accordance with this study, increased DH value and protein

content are suspected as a result of protease activity produced by *Rhizopus* sp. The main function of *Rhizopus* in fermentation is the synthesis of enzymes that hydrolyze compounds in soybean [21].

Table 2 Value of degree of hydrolysis and amount of protein on tempeh

Fermentation Time (H)	The degree of Hydrolysis (%)	Protein Level (g)
0	1.76	0.54
6	4.48	1.17
12	2.94	1.65
18	31.71	0.24
24	36.03	3.13
30	30.93	0.45

The DH value increased significantly on tempeh of 18 hours fermentation, where mycelium was seen growing on the surface of soybean (Fig.1). The bioactive peptide of the food is produced during the enzymatic hydrolysis process and fermentation or in the gastrointestinal tract. The resulting peptide has antihypertensive activity [23]. "Hrckova [24]showed that the highest degree of hydrolysis was 39.5% with lipid content had been disposed of the protease of commercial soy flour". The high dissolved protein content in this study was obtained in fermented soybean (tempeh) for 6 and 24 hours (data not shown).

3.5 ACE Inhibitor Activity

Bioactive peptide released from dietary protein is widely used for the management of hypertension based on the Angiotensin I-Converting Enzyme (ACE) inhibitory activity, which is an enzyme regulates the mechanism of regulation of Renin-Angiotensin System (RAS), which is food peptide that interacts with RAS and the vascular system and contributes to a decrease in blood pressure [25]. Peptides with sequences of Leu-Val-Tyr, Leu-Gln-Pro and Leu-Lys-Tyr of soy flour indicate Angiotensin I-Converting Enzyme (ACE) inhibitory activity [26]. Peptides with sequences of Met-Asn-Pro, As-Pro-Pro, Pro-Pro-Lys, Ile-Thr-Thr, Thr-Thr-Asn, and Thr-Asn-Pr have ACE inhibitory activity [13]. Angiotensin I-Converting Enzyme (ACE) catalyzes the conversion of angiotensin I to angiotensin II, which is a vasoconstrictor and also inactivates the antihypertensive of bradykinin as a vasodilator. Peptides derived from food proteins may have ACE inhibitory activity [27].

Some fermented foods have an ACE inhibitory activity such as brown rice [28], tofuyo extract [29], traditional Japanese food like steamed soybeans fermented by *Bacillus* (natto) [30]. By in vivo, Natto is antihypertensive [31], also pigeon pea fermented using *Bacillus subtilis* [32], a pasta made from Korean soybeans is proven to act as an antihypertensive food [33] so that the above products can be claimed as a functional food.

In order to make tempeh as a functional food, it must be obtained in vitro and in vivo data from research findings. For in vitro, it was obtained a value of 67.43%, which is the result of measurement of ACE inhibitory activity of protein extract of tempeh. This value was obtained from the fraction below 3 KDa.

3. 6 Amino Acid Composition of Protein Hydrolysates using 3 Kda Membrane

The amino acid analysis was performed to determine the type and level of amino acid found in soybean hydrolysate. Results of the amino acid analysis using UPLC are presented in Table 3. The result showed that tempeh contains both essential and non-essential amino acids. Predominant amino acids are glutamic acid, lysine, leucine, alanine, valine and phenylalanine, while predominant essential amino acids are Thr, Leu, Lys, Val, Met and Phe.

Table 3. The amino acid content of tempeh hydrolysate using 3 KDa membrane

No	Type of Amino Acid	Amino Acid Content (ppm)
1	L-Histidine	1106.79
2	L-Threonine	1442.295
3	L-Proline	1636.755
4	L-Tyrosine	2907.28
5	L-Leucine	4774.595
6	L- Aspartic acid	895.45
7	L-Lysine HCl	3688.065
8	Glycine	1516.235
9	L-Arginine	2276.61
10	L-Alanine	3257.36
11	L-Valine	2235.715
12	L-Isoleusine	2053.84
13	L-Phenylalanine	3905.97
14	L- Glutamic acid	6936.845
15	L-Serine	1005.745
16	L-Methionine	614.43
17	L- Cysteine	-
18	L-Tryptopane	-

"Kitts [34] revealed that bioactive peptides generally have low molecular weight consisting 2-9 amino acid residues and are resistant to digestive enzymes have hydrophobic amino acid residues from the type of proline, lysine or arginine". In this study, it was obtained hydrophobic amino acids such as alanine, valine, isoleucine, proline, phenylalanine, and methionine, thus authors presume that the functional properties of tempeh as ACE inhibitors are obtained from the above amino acids.

4. CONCLUSIONS

Fermentation for 24 hours produced tempeh with the best texture, low lipid and high protein content. Protein extract showed a high ACE inhibitory capacity with a total of 16 essential and non-essential amino acid compositions. So tempe has the potential to be developed as a functional food.

5. ACKNOWLEDGMENT

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