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Antioxidant Activity and Soluble Protein Content of Tempeh Gembus Hydrolysate

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ABSTRACT

Tempeh gembus is fermented soy-pulp product contains high protein and its bioactive peptide components has potential antioxidant activities. In this study bromelain enzyme was applied in tempeh gembus to break up peptides bond and released bioactive peptides and amino acids. The aim of this research was to analyse antioxidant activity and soluble protein of tempeh gembus hydrolysate. Experimental research with 4 bromelain enzymes were applied in tempeh gembus as 0 ppm, 5000 ppm, 8000 ppm, and 10000 ppm. Antioxidant activities were measured by ABTS and DPPH radicals test. Whil e soluble protein content was measured by Bradford test. In general, antioxidant activity of tempeh gembus was higher when measured by ABTS radical (63.14±1.16 – 92.85±2.28%) compared to DPPH radical (52.21±5.76 – 65.70±5.89%). Antioxidant activity of tempeh gembus hydrolysate in ABTS test differed significantly between treatment groups (p=0.001), but not on DPPH test (p=0.110). While soluble protein content of these protein hydrolysate were 0.58±0.05 – 0.78±0.11% and significantly differed between treatment groups (p=0.019). Antioxidant activity was significantly higher when measured by ABTS radical compared to DPPH radical with different protein soluble content.

Keywords: *tempeh gembus, hydrolysate, antioxidant activity, soluble protein*

Free radicals are the molecule which have one or more unpaired electrons.[1] It is known that many disease related to oxidati 27 stress is caused by excess of free radicals.[2] Diseases such as cancer, cardiovascular disease like hypertension, atherosclerosis, and neurological disease, all show strong evidence that ROS (reactive oxygen species) is involved in their pathophysiological process.[3] Human body actually has defense mechanism against the free radicals called antioxidant. Antioxidant will neutralise free radical by giving one of its free electrons, thus stop the chain reaction done by free radicals.[1]

Soybeans contain high antioxidant content, belong to *Leguminosae* family.[1] The use of soybeans as local food have been various, some of them are tempeh, oncom, tahu, and tempeh gembus. Tempeh gembus is food fermented product made from soy pulp which left over after soybean curd making process. The microorganism used to fermented this soy pulp is same with the microorganism used to fermented tempeh. In Japan, this soy pulp is known as *Okara* which is the residue of tofu or soy milk making process.[4, 5]

The nutrition components of tempeh gembus are similar with tempeh although the contents of tempeh gembus nutrition are less than tempeh. This happens because tempeh gembus is made by soy pulp from soybean curd residue making process so the nutrition contents inside have been diminished. Tempeh gembus contains nutrition contents, such as essential fatty acids, unsaturated fatty acids, protein, carbohydrate, fiber, calcium, and iron.[6, 7] The content of tempeh gembus energy is about 50% of the tempeh energy, the protein and lipid contents are less than tempeh as well, while the fiber content is on the other hand, it is three times (4.69%) richer than tempeh (1.40%). Protein content in tempeh gembus is about 3.41 gr/100 gr wet weight of tempeh gembus or 4.07 gr/100 g 15 dry weight of tempeh gembus that contains seven essential amino acids and eight non-essential amino acids.[4, 8] Another study conducted to analyse nutritional composition during tempeh gembus processing found that the total content of amino acids decreased from 34.95% in soybean to 6.7% in tempeh gembus without any changes on amino acids composition.[9] The composition of amino acids in tempeh gembus is complete enough so tempeh gembus can be a potential bioactive peptides source. Lipid content of tempeh gembus is low, it is about 0.23 gr/100 gr dry weight of tempeh gembus, but it contains essential fatty

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acids that is linoleic acid (21.51%), oleic acid (16.72%), and linolenic acid (1.82%). In addition, tempeh gembus also contains several minerals, ergosterols, and isoflavonoids.[4, 8]

Bioactive peptides are specific protein fragments that are inactive within the sequence of the parent protein and may show their physiological function when they are released from the parent protein.[10, 11] One of their physiological functions is their ability to be antioxidant. Bioactive peptides have good ability in free radicals scavenging activity because of their good ability to effectively react with the free radicals.[12] In a study, antioxidant activity showed by bioactive peptides from fermented *Okara* using *B. subtilis* B2, reaches 4.1 and 34.8 gr trolox eq/g dry *Okara* using DPPH and ABTS tests, respectively.[13]

Functional ability of protein can be modified via enzymatic hydrolysis under controlled condition. Many bioactive peptides are inactive within the parent protein and can be released during the enzymatic hydrolysis.[14] Enzymatic hydrolysis result in peptide bonds breaking so the bioactive peptides inside may release. Bromelain is one of proteases that can be used in enzymatic hydrolysis process. Bromelain is produced from pineapple fruit that spread over Indonesia. Bromelain is chosen because of its advantages that bromelain is easily obtained in Indonesia and be available from the beginning of the pineapple fruit growth till the fruit is ripe, although there is fluctuation in its proteolytic activity. Protease from other fruits such as tin and papaya are only found when the fruits are ripe. In addition, bromelain also active both in pure enzyme form and in pineapple juice form.[15] The use of bromelain for producing bioactive peptides from tempeh gembus has never been reported yet.

The intention of the present study was to determine the antioxidant activity and the soluble protein content of the tempeh gembus hydrolysate which possess anti-oxidative properties. Antioxidant activity of tempeh gembus hydrolysate in this research was measured by ABTS and DPPH tests. Both methods were chosen to be used because ABTS and DPPH are simple enough, the most used, and the most popular tests to analyse the antioxidant total capacity.[16] Besides, both ABTS and DPPH have same principle test that ABTS and DPPH undergo decolorisation to identify antioxidant activity which scavanges the ABTS and DPPH radicals.[17] Whereas, the soluble protein content was measured by Bradford test. Bradford test was chosen to confirm the process of protein hydrolysis becomes peptides or amino acids. In this method, amino acids or peptides are not able to form a complex with CBB dye so the blue color will not appear.[18]

METHOD AND MATERIALS

This was an experimental study with complete randomized experimental design using four treatments. Samples were tempeh gembus hydrolysate obtained from hydrolysis process using bromelain enzyme with concentrations were 0 ppm, 5000 ppm, 8000 ppm, and 10.000 ppm. Based on the concentration of bromelin enzyme used to hydrolyse, the tempeh gembus hydrolysate was categorised into four treatment groups. Tempeh gembus was obtained from local home industry of tempeh gembus in Semarang Indah, Central Java, using soy pulp of the soybean curd residue from Grobogan Village. The data were analysed by ANOVA.

Tools and Materials

The tools were blender, spoons, test tube, small cup, water bath incubator, stative, stirring bar, freeze dryer, kuvet, vortex, sentrifuge, magnetic stirer, pipet, pH meter, erlenmeyer, and beaker glass. The materials were tempeh gembus, aquadest, bromelain enzyme, dextrin, NaCl, NaH_2PO_4, Na_2HPO_4, ABTS, DPPH, metanol, pottasium persulfate, and Bradford reagent.

Process of making tempeh gembus hydrolysate

Tempeh gembus (300 gr) was steamed for 10 – 15 minutes, then the steamed tempeh gembus was blenderised and 450 ml aquades were added. Next, the blenderised tempeh gembus was divided into four groups, each groups was added bromelain enzyme as much as 0, 5000, 8000, and 10000 ppm, then each groups was incubated in water bath incubator for 1.5 hours at 55°C. After being incubated for 1.5 hours, each groups of blenderised tempeh gembus was added dextrin and NaCl, each of them were 1.5 gr then the groups were heated to 70°C for 10 minutes. After 10 minutes, tempeh gembus that had been thick due to the process was freeze dryed until dry.[19, 20]

Determination of antioxidant activity using ABTS test

ABTS kit solution was made by reacting 7 mM of ABTS solution with 2.45 mM of pottasium persulfate (1/1, v/v) then the mixture was dark incubated for 16 hours at room temperature. After being incubated, the mixture was diluted with metanol to obtain an absorbance of 0.700 ± 0.05 at 734 nm. Samples were tempeh gembus hydrolysate that have been dissolved before (100

µl) reacted with 900 µl of ABTS kit solution, shook for a moment, then the absorbance of samples was measured at 734 nm.[21]

Determination of antioxidant activity using DPPH test

DPPH kit solution was prepared by dissolving 7.88 mg of DPPH into 100 ml metanol to make DPPH solution with concentration was 0.2 mM. The absorbance of DPPH solution was measured at 517 nm by reacting 600 µl metanol in a kuvet then added by DPPH solution until the volume were 3 ml. Closed and shook the mixture until homogen, before measuring the absorbance. Samples were made by dissolving 2 mg of tempeh gembus hydrolysates into 4 ml of metanol, after ing dissolved, samples (600 µl) were reacted with 3 ml of DPPH solution. Closed and shook until homogen, then the absorbance of samples was measured at 517 nm.[22]

Determination of soluble protein content using Bradford test

Bradford reagent was made by mixing 10 mg of Coomassie Briliant Blue (CBB) into 50 ml of metanol, then the mixture was dissolve into 100 ml of phosphate acid. After dissolving, aquades were added following 1:2 ratio. Stored in a dark closed bottle. Samples were prepared by reacting 20 µl of tempeh gembus hydrolysate (the tempeh gembus hydrolysate has been dissolved before) with 1 ml of Bradford reagent, then the mixture was incubated for 1 hour. The absorbance of samples was measured at 595 nm.[23]

RESULTS

Antioxidant activity

All groups of tempeh gembus hydrolysate showed radical scavenging activity against ABTS and DPPH radicals presented as antioxidant activity percentage (%) and shown on Table 1. There was significant different over the four treatment groups assessed by ABTS test (p=0.001) but not significant to DPPH test (p=0.110). Antioxidant activity of tempeh gembus hydrolysate assessed by ABTS test showed increase as the concentration of bromelain enzyme used was increased, while assessed by DPPH test, its antioxidant activity decreased with the same treatments. Tempeh gembus hydrolysate showed the highest of antioxidant activity when bromelain enzyme used reached 10000 ppm concentration (92.85 ± 2.28%) using ABTS test. Whereas using DPPH test, the highest antioxidant activity of tempeh gembus hydrolysate (65.70 ± 5.89%) achieved when no bromelain enzyme was added (0 ppm).

Soluble protein content

Table 2 shows the soluble protein content of tempeh gembus hydrolysate. It was found that the soluble protein content of tempeh gembus hydrolysate showed significant decrease as the use of bromelain enzyme was increased (p=0.019). The highest value was 0.78 ± 0.11 when the concentration of bromelain enzyme used in tempeh gembus hydrolysate was 5000 ppm.

DISCUSSIONS

Antioxidant activity of tempeh gembus hydrolysate

Antioxidant activity of tempeh gembus hydrolysate significantly increased based on ABTS test but decreased in DPPH test. Enzymatic hydrolysis process which used bromelain enzyme as hydrolisis agent may cause antioxidant activity increased through several mechanisms. It was clear that hydrolysis brokedown peptide bonds of tempeh gembus protein and may cause the bioactive peptides of tempeh gembus released. Bioactive peptides were considered to be responsible to the increase of antioxidant activity of tempeh gembus hydrolysate. Bioactive peptides which had antioxidant activity generally contained 5 – 16 amino acids residue which may had different characteristics in composition, structure, and hydrofobicity.[11] A study stated that radical scavenging activity of β – conglycinin and glycinin increased 3 – 5 times after both of them enzymatically digested[7, 24, 25]

Some studies mentioned amino acids such as tyrosine, methionine, histidine, lysine, cystein, and tryptophan have been generally accepted as antioxidant amino acids.[24] A study conducted to identified nutrition and composition of tempeh gembus stated that the highest amino acids existed in the tempeh gembus were threonine, tyrosine, and histidine (0.95%, 0.72%, and 0.60%, respectively).[8] The others stated that tyrosine, histidine, phenilalanine, lysine, and leucine amino acids showed and improved the peptide antioxidant actvity.[15, 26]

Certain amino acids have structural characteristics that may enhance the antioxidant activity in the tempeh gembus hydrolysate. During the soybean protein hydrolysate process, the structure of soybean protein underwent changes and amino acids in R group will be more actively exposed, resulted in the higher of antioxidant activity of soybean peptides produced from the hydrolysis process than the intact protein.[11, 27] Amino acids with aromatic residue such as tyrosine, typtophan, and phenilalanine amino acids were known could enhance antioxidant activity because of their capability to

easily release their proton to the radical that lacked of electron and directly played a role in radical scavenging activity. Histidine also indicated strong enough of radical scavenging activity and could act as metal – ion chelators, hydrogen donation, and oxygen active and radical hydroxyl scavenging.[24, 26, 28] It happened because histidine decomposed its imidazol ring during reacting.[28, 29] Cystein has sulfihidril (-SH) residue that allowed cystein to act as antioxidant activity as well by directly reacting with the radical.[28]

Hydrophobicity was the important factor that played a role in antioxidant activity of peptides. It was a tendency of amino acids to not interact with water at physiological pH.[30] Antioxidant activity of peptides was more related to the total hydrophobic amino acids content than peptides size.[15] A study conducted about refinery and characterisation of antioxidant peptide from a soy protein hydrolysate indicated that the content of hydrophobic amino acids increased 7% to 60% while the hydrophilic amino acids extremely decreased from around 59% to 9%. Hydrophobic amino acids were known to play a role in the inhibition of peroxidation by increasing peptide solubility in fat and acting as an antioxidant which increases peptide solubility in non-polar environments so as to facilitate the interaction of amino acids (especially in hydrophobic amino residues) with radical compounds by reducing the radical activity better.[31-33]

These three things may lead to the improvement of antioxidant activity of tempeh gembus hydrolysate.

Whereas the decrease of antioxidant activity of tempeh gembus hydrolysate assessed by DPPH test in the authors' result seemed to be correlated to several literatures. DPPH radical was relatively more stable than ABTS radical so it was harder to be neutralised. Some antioxidant agent reacted more quickly when assessed used other tests and reacted slower to DPPH resulting in a slower reaction range.[15] ABTS test was known more sensitive to assess radical scavenging activity done by peptides and amino acids because it was more reactive to peptides and amino acids.[32]

A study stated that DPPH test indicated the insensitivity of DPPH may caused antioxidant activity assessed by DPPH test was smaller. Only cystein having SH-group with strong reduction capability was able to show DPPH radical scavenging activity compared to the others amino acids assessed by DPPH test.[34] When DPPH test was compared to ABTS and ORAC tests to assess antioxidant activity, tyrosine and tryptophan indicated the strongest antioxidant activity and were thought to be responsible for the antioxidant activity of the peptides. Dipeptides that contained cystein showed DPPH radical scavenging activity around 0.14 – 0.25 µmol TE/mol, while all peptides containing tyrosine or tryptophan showed very weak DPPH radical scavenging activity with TE value around 0.00 – 0.02 µmol TE/mol. Whereas when assessed by ABTS and ORAC tests, dipeptides containing cystein indicated moderate radical scavenging activity and very strong radical scavenging activity to dipeptides containing tyrosine or tryptophan. Try – Gly dipeptides showed TE value up to 5.05 µmol TE/mol using ABTS test.[34] This insensitivity may allow DPPH test showed smaller result of antioxidant activity of tempeh gembus hydrolysate. As mentioned before, amino acids found high in the soy protein hydrolysate were tyrosine, histidine, isoleucine, phenilalanine, lysine, leucin, and valine.[26, 31] Whereas amino acids found high in tempeh gembus were threonine, tyrosine, histidine, glutamic acid, and aspartic acid, while cystein were found in very low levels.[4, 8] DPPH radical were unable to react quickly towards this such of peptides and amino acids resulted in decrease of antioxidant activity.

Soluble protein content of tempeh gembus hydrolysate

The soluble protein content of the tempeh gembus hydrolysate tended to decrease as the concentration of bromelain enzyme used increased. Bromelain is an endopeptidase enzyme which has sulfihidril group (-SH) on the active site so the bromelain is an cysteine protease.[15, 35] Bromelain break the protein on the carbonyl end of lysine, alanine, tyrosine, and glycine amino acids. Bromelain produces protein hydrolysate that contains mix of bioactive and non-bioactive peptides. [15] Hydrolysis peptide bonds and polypeptides by bromelain generated peptide with small size and amino acids. Hydrolysis will reduce the molecular weight of protein and enrich the number of polar groups.[20] Actually, the hydrolysis of protein changes non soluble protein into soluble protein due to the decrease in molecular weight of the protein so the solubility increase, but further hydrolysis will expose the hydrophobic protein that used to be interior protein which makes the solubility decrease.[19, 20] This literature explained the fluctuation of results of the soluble protein content of tempeh gembus hydrolysate. Hydrolysing using 5000 ppm of bromelain enzyme showed the highest soluble protein content because protein hydrolysis process changed the non soluble protein into soluble protein, thus the solubility and its value increased, but the results of soluble protein

content using 8000 ppm and 10000 ppm of bromelain enzyme decreased. The decrease was most likely caused by the further hydrolysis that produced the hydrophobic protein which made the solubility and its values decreased as well.

High free amino acids, peptides, and proteins with low molecular weight contents of the tempeh gembus hydrolysate that less than 3000 Da were unable to form complex with CBB *(Coomassie Brilliant Blue)* dye so the blue color did not appear resulted in the soluble protein content of tempeh gembus hydrolysate read as low.[18, 36] In addition, the probability of the appearance of hydrophobic protein due to further hydrolysis also could decrease the soluble protein content of tempeh gembus hydrolysate. So, this result was correlated with the result of the antioxidant activity of tempeh gembus hydrolysate that showed as high in the result of ABTS test.

The use of protein hydrolysate has been various. Protein hydrolysate is generally used for flavor-lasing, but now its usage starts shifting becomes functional food product.[7]

A study mentioned the use of tempeh hydrolysate to develop a sport drink formula as a beverage to recover the athletes' muscle damage. The high antioxidant content in the hydrolysate was useful in the development of sport drink, to prevent free radical activity causing lysis of muscle cell membrane, which was produced during and after strength exercise.[37] This study may open the opportunity for tempeh gembus hydrolysate to be developed as a basic or fortification ingredient in sport drink to increase the peptides and amino acids antioxidant content there in.

A study conducted on rats fed an atherogenic diet successfully found that giving of tempeh gembus with additional bromelain enzyme to the rats fed an atherogenic diet had the lowest mean value of homocysteine level. This result was due to a higher antioxidant content produced from protein hydrolysis process was able to protect the endothelium from oxidative stress as the endothelium produced nitrit oxide optimally that would detox homocysteine induced by the atherogenic diet. High level of homocysteine can cause oxidative stress due to excessive ROS and is considered as factor of independent risk for the cardiovascular disease and not constituent of normal diet.[7] This study may lead to the use of tempeh gembus hydrolysate as functional food to prevent the cardiovascular diseases. In addition, antioxidant content in tempeh gembus hydrolysate also can be used to inhibit the oxidation process in high fat foods and stabilize food products which need stabilisation.[38]

Tempeh gembus also has another functional properties. Tempeh gembus known to be potential source of microbial fibrinolytic protease that could be used to produce functional food or medicine to treat cardiovascular diseases. A study stated that *B. pumilus* confirmed as microbial fibrinolytic from isolate of tempeh gembus known to be have proteolytic and fibrinolytic activities that might be able to act as antithrombotic.[39, 40]

In conclusion, there were significant different on antioxidant activity using ABTS test and soluble protein content of tempeh gembus hydrolysate among the four treatment groups (0 ppm, 5000 ppm, 8000 ppm, and 10.000 ppm). ABTS test indicated higher antioxidant activity of tempeh gembus hydrolysate than DPPH test. Antioxidant activity of tempeh gembus hydrolysate increased as the concentration of bromelain enzyme increased based on ABTS test but not on DPPH test. The soluble protein content of tempeh gembus hydrolysate tend to decrease as the concentration of bromelain enzyme increased.

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Table 1. Antioxidant activity of tempeh gembus hydrolysate

Tempeh Gembus Hydrolysate (TGH)	Mean ± SD of Antioxidant Activity	
	ABTS test (%)	DPPH test (%)
TGH 0 ppm	63.14 ± 1.16[a]	65.70 ± 5.89
TGH 5000 ppm	75.56 ± 9.43[b]	58.58 ± 7.43
TGH 8000 ppm	88.56 ± 4.13[c]	55.87 ± 9.25
TGH 10.000 ppm	92.85 ± 2.28[c,d]	52.21 ± 5.76
p value*	0.001	0.110

*Based on One Way ANOVA test.

[a, b, c, d]Different letters in the same column showed significant differences based on the LSD post hoc test ($p < 0.05$)

Table 2. Soluble protein content of tempeh gembus hydrolysate

Tempeh Gembus Hydrolysate (TGH)	Mean ± SD of Soluble Protein Content	p value*
TGH 0 ppm	0.60 ± 0.05[a]	0.019
TGH 5000 ppm	0.78 ± 0.11[b]	
TGH 8000 ppm	0.58 ± 0.10[a]	
TGH 10.000 ppm	0.58 ± 0.05[a]	

*Based on One Way ANOVA test.

[a, b, c, d]Different letters in the same column showed significant differences based on the LSD post hoc test ($p < 0.05$)

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