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Edamame protein hydrolysis using *Lactococcus lactis*, *Lactobacillus bulgaricus* and *Lactobacillus paracasei* produce short peptides with higher antioxidant potential

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Abstract. *Anggraeni SL, Jayus J, Ratnadewi AAI, Nurhayati N. 2022. Edamame protein hydrolysis using* Lactococcus lactis, Lactobacillus bulgaricus *and* Lactobacillus paracasei *produce short peptides with higher antioxidant potential. Biodiversitas 23: 3603-3612.* The high demand for protein sources for functional vegetable foods, especially proteins that have ACE (Angiotensin-Converting Enzyme) inhibitory activity has encouraged efforts to meet this need through exploration of potential protein sources and the development of food processing technology to modify the protein into derivative compounds in the form of short peptides to promote higher bioactivity than the native protein. This research focused on the hydrolysis of edamame proteins into shorter peptides through a fermentation process using a group of Lactic Acid Bacteria (LABs), that is *Lactococcus lactis*, *Lactobacillus bulgaricus* and *L. paracasei*, to gain a higher functional activity of its protein hydrolysate. Edamame milk was fermented with that LABs as starter culture until 24 hours at 37ºC. The activity of the protease released by the LABs was confirmed using the agar diffusion method based on the emergence of a clear zone surrounding the culture, while the antioxidant activity was confirmed with the ABTS and hydroxyl radical scavenging methods. The results exhibited that the population density of starter culture in edamame milk reached 10^8 CFU/mL, and the pH decreased from 6.75 to 4.15. All LABs used in fermentation were able to hydrolyze protein as indicated by the increasing degree of hydrolysis and changes in protein profile of SDS-PAGE analysis. In addition, when compared to the unfermented edamame milk, the protein hydrolysate from LABs fermentation showed an increase of ABTS and hydroxyl radical inhibitory activity, in which the highest IC_{50} values of 8.96 and 13.33 g/mL of each were found in edamame milk fermented by *Lc. lactis* InaCC B187. An increase in ACE inhibitory activity was also observed in edamame milk fermented using this microbe (9.80 g/µL). These findings indicate that *Lc. lactis* InaCC B187, *L. delbrueckii* subspecies *bulgaricus* FNCC41, and *L. paracasei* InaCC B145 have the potency as an effective starter culture to increase bioactive peptides in edamame soybeans potential for functional food sources.

Keywords: ACE inhibitory, bioactive peptide, edamame, lactic acid bacteria, protein

INTRODUCTION

The need for functional foods to improve the quality of human health is increasing, especially from vegetable-based protein. The trend of vegetable protein consumption is increasing because animal protein has a higher potential risk to cause of cardiovascular disease (Richter et al. 2017). As a source of functional vegetable protein, edamame (*Glycine max* (L) Merrill) contains more protein (41.3%) higher than other soybean varieties (35.3%) (Djanta 2020). Edamame also contains isoflavone which act as an antioxidant, and their antioxidant properties can be increased by fermentation process using LABs to produce an aglycones form of isoflavone (Lovabyta et al. 2019). Besides isoflavone modification, increasing the functional activity of edamame can also be carried out through protein hydrolysis using proteolytic microbes to hydrolyze protein into shorter peptides, as has been done by Undhad Trupti et al. (2021), where there was an increase in ACE inhibitory activity of AAU NRC-37-soybean milk after fermented by LABs. Another study stated that fermented soybean products had higher antioxidant activity than unfermented soybeans (Gomes et al. 2021). Moreover, seven times increase in antioxidant activity of soy protein isolate were observed after enzymic hydrolysis by protease from *Aspergillus oryzae* (Castro and Sato 2014). Since the bioactive peptides derived from these natural resources have been reported to display relatively less side effects in humans than synthetic food additives or pharmaceutical agents (Cabanos et al. 2021), consumers prefer to incorporate these products into their diet is growing.

Even though soy protein hydrolysis using protease enzymes is commonly used, but it is considered to be a very expensive process. A better strategy for producing peptides in food systems is fermentation by food-grade microorganisms, such as LABs fermentation (Singh et al.

2014). During the fermentation, proteins will be degraded into simpler forms such as oligopeptides, dipeptides, and tripeptides and serve as a good source of bioactive peptides (Cabanos et al. 2021). The use of proteolytic microbes to produce proteases is the cheapest compared to other methods (Cheng et al. 2020). The bioactive peptides (Val-Pro-Pro and Ile-Pro-Pro) obtained from LABs fermentation of soymilk exhibit antihypertensive activities has been reported by Miyazaki et al. (2017), while other soybean peptide (Pro-Gly-Thr-Ala-Val-Phe-Lys) has the antimicrobial activities (Dhayakaran et al. 2016). Peptides from soy glycinin, including Leu-Pro-Tyr-Pro, revealed a hypocholesterolemia activity (Lammi et al. 2015). A heptapeptide derived from the enzymatic digestion of soy proteins, soymorphin (Tyr-Pro-Phe-Val-Val-Asn-Ala), shows an antioxidant activity through ABTS scavenging ability test (Stefanucci et al. 2020).

Previous studies on hydrolysis of soybean protein using LABs cultures, elucidated that the type and activities of bioactive peptide obtained depended on the variety of soybean and the LABs strain used. Soy milk fermented by *L. fermentum* showed higher ACE-inhibitory activity than that of *L. casei*, *L. helveticus*, and *Lactobacillus paracasei* (Undhad Trupti et al. 2021). Bhatnagar et al. (2018) also reported that among several bacterial strains used to hydrolyze soybean protein, only two strain *L. paracasei* CD4 and *Brevibacillus thermoruber* HM34 which were able to release peptide exhibiting ACE inhibitory activities. Different ACE inhibitory activities were observed among the fermented soymilk from different soybean varieties, as reported by Bao and Chi (2016) and Undhad Trupti et al. (2021). Even though both soymilks were fermented using the same species of *L. casei*, but its antihypertensive activities were differed because both varieties released different short peptides. Because each soybean variety can produce different ACE-inhibitory activities, it is important to study the potential of edamame soybeans to produce short peptides. This study aimed to evaluate the effect of edamame fermentation by LABs on the activity of the bioactive peptides produced.

MATERIALS AND METHODS

Materials and reagents

Edamame (green soybean vegetable) obtained from Mitra Tani 27 Co. Ltd., a producer of edamame in Jember Indonesia, MRS (de Man Rogosa Sharpe) agar and broth, Malt Extract Broth (MEB) and Agar (MEA) (Merck USA), NaCl (Merck USA), Na_2CO_3 (Merck USA), Trinitrobenzenesulfonic acid (Sigma-Aldrich, USA), Na_2SO_3 (Merck USA), Sodium dodecyl sulfate (Sigma-Aldrich, USA), 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (Merck USA), potassium persulfate (Sigma-Aldrich, USA), 2-Deoxy-D Ribose (Sigma-Aldrich, USA), EDTA (Merck USA), $FeCl_3$, H_2O_2(Merck USA), Ascorbic Acid(Merck USA), Thiobarbituric Acid (TBA)(Merck USA), Trichloro Acetic Acid (TCA)(Merck USA), Hippuryl-L-Histidyl-L-Leucine (HHL) (Bachem Inc.), L-leucine (Sigma-Aldrich, USA), acrylamide (Sigma-Aldrich, USA). N,N'-Methylenebisacrylamide (Sigma-Aldrich, USA).

Microorganisms

Lactococcus lactis InaCC B187, *L. paracasei* InaCC B145 from the Indonesia Culture Collection (InaCC) Biology Research Center LIPI, and *L. delbrueckii* subspecies *bulgaricus* FNCC41 (LB) from the Food and Nutrition Culture Collection (FNCC) the University of Gadjah Mada, Yogyakarta, Indonesia. The LABs were cultured in MRS broth media and incubated at 37ºC for 24 h.

Soymilk preparation

Edamame (500 g) was washed twice with water and soaked for eight hours. After boiling at 100ºC for 3 min, edamame was then processed using a food processor with ratio of edamame and water was 1:1 (b/v). The slurry was dried using an oven, and ground to become edamame flour. In order to remove fat, the flour (100g) was then defatted using 1L of n-hexane with continuous stirring at 4ºC for 1 hour. The solvent was then decanted to separate it from the flour followed by air-dry process to get the flour ready to be used as samples. The edamame milk was prepared by mixing 20 g of defatted edamame flour with water (1:10 w/v) and stirring it until homogenic suspension was formed. The suspension was then filtered using a cotton sheet to separate the bigger edamame's particle. The edamame milk obtained was added with 5%, (w/v) of sugar and sterilized at 121ºC for 15 min and left it for cooling to 30ºC before inoculation.

Soymilk fermentation

The prepared edamame milk was fermented with 4% starter culture aseptically and incubated at 37ºC for 24 hours. The fermentations were carried out using *Lc. lactis* (LL), *L. delbrueckii* subspecies *bulgaricus* (LB), and *L. paracasei* (LPR) separately. The fermented culture broth was centrifuged at 6000 rpm for 15 minutes to separate the cells and filtrate. The resulting filtrate was stored at -20°C.

Enumeration of viable Lactic Acid Bacteria (LAB)

The LABs were grown on MRS agar media. The microbial cells were suspended in physiological solution of 0.85% NaCl (w/v) (Xiudong et al. 2016). The enumerations of the cells were carried out at the serial dilutions of 10^{-7}, 10^{-8} and 10^{-9}. The presence of LABs was determined by the appearance of a clear zone in the medium containing CaCO3 (1% w/v). Observations were made at 0-, 12-, 24- and 36-hour incubation times. Colonies that formed the clear zone were recorded and counted in units (CFU)/mL of sample.

Determination of pH

Determination of the pH of the three samples with several types of microbial culture was measured using a pH meter and repeated three times.

Protease activity in soymilk

Determination of protease activity was carried out qualitatively and quantitatively. Qualitatively, the protease

activity was initiated by rejuvenating LABs on MRSB media for 24 hours at 37°C. The LAB cultures were inoculated on Skim Milk Agar (SMA) medium. A positive test is indicated by the presence of a clear zone around the bacterial colony. Proteolytic index can be calculated based on the following formula (Rothschild et al. 2011):

$$\text{Proteolytic index (IP)} = \frac{\text{Diameter clear zone} - \text{Diameter colony bacteria}}{\text{Diameter colony bacteria}}$$

Meanwhile, the quantitative protease activity test was carried out by mixing 5 mL of 0.65% casein solution (substrate) dissolved in 0.05 M phosphate buffer pH 7.5 with 1 mL of enzyme and incubated at 37°C for 10 min. A total of 5 mL of TCA reagent was added to stop the reaction and incubated at 37°C for 30 min. After that, the solution was filtered and 2 mL of the supernatant as the sample was added with 5 mL of Na_2CO_3 and 1 mL of Folin Ciocalteau reagent to be incubated at 37°C for 30 min. The absorbance was measured at a wavelength of 660 nm. The protease activity was calculated using a standard tyrosine curve.

Determination of the degree of hydrolysis

The degree of hydrolysis was determined according to the TNBS method (trinitro-benzene-sulfonic acid), based on the reaction between the amino group and trinitro-benzene-sulfonic acid (Noman et al. 2018). A sample of 125 µL was mixed with 2 mL of 200 mM phosphate buffer (pH 8.2) and 1 mL of 0.1% TNBS, then incubated for 30 min at 50°C. A total of 2 mL 0.1 M Na_2SO_3 was added to stop the reaction. After that, the sample was cooled at room temperature for 15 min. The absorbances of the solution were observed at 420 nm, using a standard L-leucine curve to determine the concentration of the amino acids released.

Percentage of the degree of hydrolysis (DH) was determined by the following equation (Shi et al. 2022):

$$\text{DH (\%)} = \frac{\text{AN2 - AN1}}{\text{Npb}} \text{ x } 100\%$$

Where:

DH : Degree of hydrolysis

AN1 : Amino acid group content before hydrolysis (µmol leucine/mL)

AN2 : Amino acid group content after hydrolysis (µmol leucine/mL)

Npb : Total content of peptide bonds on the substrate (µmol leucine/mL), determined after hydrolysis with 6M HCl for 24 hours at 110°C, and was then neutralized using 6M NaOH (Olivera et al. 2020).

Protein profile analysis

Protein profile analyses were undertaken by denaturing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) method (Shen et al. 2020; Laemmli 1970) using 12% resolving gel and 4% stacking gel. The 10 µg sample of fermented edamame milk to be denatured in boiling water for 3 min, was mixed with 10 µL loading buffer (95% buffer loading and 5% β-mercaptoethanol). The samples were then loaded into gel wells and electrophoresed for 2 to 4 h. To simplify marking of sample protein molecular weight, 10 µL of markers were added. After removal from the plate, the gel was immersed in a staining solution (10% Coomassie brilliant blue solution) for 3 hours. The excess color was then removed by soaking the gel in destaining solution (50 mL of distilled water, 40 mL of methanol, 10 mL of acetic acid glacial) until the gel becomes clear. The molecular weights of protein samples were then determined by interpolations of known molecular weights protein markers.

Antioxidant capacity measurement

ABTS (2,2'-azino-bis [3-ethyl benzothiazole-6-sulfonic acid]) radical scavenging ability

ABTS radical scavenging analysis was carried out based on Shalaby et al. (2013). A mix of 10 µg sample of fermented edamame milk, 950 µL ABTS (0.38 g ABTS and 0.066 g potassium persulfate dissolved in 50 mL distilled water), was added with distilled water to a volume of 1 mL. The solution was then homogenized using a vortex mixer and its absorbance was measured at 734 nm. Blanks were obtained from a mixture of 50 µL phosphate buffer saline (PBS) pH 7.4 and 950 µL ABTS solution. The measurements of absorbances were recorded for the value within the range of 0.70-0.75. Percentage of inhibition of ABTS radical scavenging ability was determined by the following equation (Jian-Wei et al. 2015).

Hydroxyl radical scavenging

The hydroxyl radical scavenging analysis was carried out based on Li et al. (2014) with modification. A mix of 50 µL 2.8 mM 2-Deoxy-D Ribose, 100 µL 1 mM EDTA, 10 µL 10 mM $FeCl_3$, 10 µL 1 mM H_2O_2, 100 µL 1 mM ascorbic acid and 20 µg sample of fermented edamame milk were added with 700 µL 20 mM phosphate buffer pH 7.4 to a volume of 1 mL and were incubated for 1 h at 37°C in a water bath. The solution was then allowed to cool to reach room temperature, prior to addition of 500 µL of TBA (1%) and 500 µL of TCA (2.8%). The solution was then incubated again for 30 min at 80°C using a dry block. The absorbances were measured using spectrophotometer at a wavelength of 532 nm. The percentage of inhibition is presented in IC_{50} using linear regression $y = ax + b$, where y is 50% inhibition and x is sample concentration (Oliveira et al. 2014).

$$\text{Inhibition (\%)} = \frac{A_{blank} - A_{sample}}{A_{blank}} \text{ x } 100\%$$

Angiotensin-I Converting Enzyme (ACE-I) inhibitor

Angiotensin-I Converting Enzyme (ACE-I) inhibitor assay was performed according to the method of Tutor et al. (2017), as the modification method of Cushman and Cheung (1971). This assay is based on liberation of hippuric acid (HA) from Hippuryl-His-Leu (HHL) catalyzed by the ACE solution from rabbit's lung. The lungs were cleaned from any remaining fatty tissues using NaCl buffer (pH 9,0) and homogenized in 3 mL 10mM of cold Tris-HCl buffer (pH 7,8) using pestle set. Homogenate was centrifuged at 12.000 rpm, 4°C for 15 min repeatedly

until no more pellets were formed. The ACE solution is stored at -80°C before being used.

The ACE-I inhibitor assay was carried out by mixinga12.5 µL of fermented edamame milk and 12.5 µL of 100 mM boric acid buffer containing 1.2 mM NaCl was hydrolyzed using 12.5 µL of the ACE solution enzyme, incubated at 37°C for 5 min, followed by the addition of 12.5 µL of 20 mM Hippuryl-His-Leu (HHL) and was incubated again at 37°C for 30 min. The reaction was terminated by the addition of 31.2 mL 1N HCl. This solution was then added with 500 µL of ethyl acetate and stirred using a vortex mixer prior to 10 min centrifugation at 800 g. The supernatant (400 µL) obtained was then evaporated at 35°C for 13 min. The concentrate was dissolved with 400µl of ddH_2O. Measurement of the absorbances was carried out using a spectrophotometer at a wavelength of 228 nm. The IC_{50} value was defined as the peptide concentration that inhibited 50% of ACE-I activity.

Amino acid composition

Amino acid composition analysis was carried out using UPLC system (Waters 2475, US) based on the method of Szkudzinska et al. (2017). A sample of 100 g was hydrolyzed with 5 mL of 6N HCl at 110˚C for 22 hours. After hydrolysis, the solution was allowed to cool before transferring to a 50 mL measuring flask using double distilled water and filtered using a 0.45µm filter. Standard used was 0.4 mL 50 mM alpha-amino butyric acid (AABA). The hydrolysate (20 µL) was injected into the UPLC using AccQ at 260 nm to identify the amino acid compositions.

RESULTS AND DISCUSSION

Growth of Lactic Acid Bacteria in edamame milk during the fermentation process

Lactococcus lactis, *Lactobacillus bulgaricus*, and *L. paracasei* in edamame milk grew rapidly within 24 hours of fermentation, as shown in Figure 1. The average cell amount in all cultures reached 10^8 CFU/mL at 37˚C. The highest cell number was observed in edamame milk fermented by *L. bulgaricus* at 24 hours, which was 7 x 10^8 CFU/mL. Even though the growth of *L. bulgaricus* was lower within the first 12 hours, but had the most biomass compared to the other 2 strains. Lovabyta et al. (2020) reported that *L. bulgaricus* reached 1.35 x 10^9 CFU/mL in edamame milk after 24 hours of fermentation. The high survival rate of LAB in soy milk is maintained by the presence of proteolytic activity which is released during its growth to break down protein into some amino acids to support its growth (Hou et al. 2015).

LABs growth decreased at 36 hours of fermentation, indicating the initial stage of its stationary phase. This observation data is in accordance with the report of Nuryana et al. (2019), that lactic acid bacteria grow fast in MRS agar for 18 to 24 hours, after 24-hour its stationary and death phases were begun. During their stationary stage, the LABs will begin to produce some secondary metabolites, such as organic acids.

Changes in the Acidity Level (pH) in edamame milk during the fermentation process

Edamame milk fermentation process using LAB produces lactic acid, acetic acid, or other organic acids as the main end-product (Ruiz et al. 2017) which causes a decrease in pH as shown in Figure 2. The pH value of fermented edamame milk decreased from 6.75 to a range of 4 after 24 hours of fermentation. Edamame milk fermented with LB had the lowest pH value, which was 4.07 with the highest number of cell growth. This observational data is similar to that of Lovabyta et al. (2020), who stated that the lower the pH value, the higher the number of cells detected. Changes in the pH value of fermented edamame milk indicated that LAB was growing well. During the fermentation process, the LABs were able to metabolize sucrose to produce energy and organic acids needed for bacterial cell growth. The organic acids produced can cause changes in medium pH (Gan et al. 2017). The number of lactic acids produced by LABs depended on the typical of organic acids production either homo- or heterofermentative. As homofermentative bacteria, *L. bulgaricus* and *Lc. lactis* produced higher amount of acid rather than *L. paracasei* which considered to be heterofermentative (Puspawati et al. 2020). Homofermentative LABs metabolized almost all substrates into lactic acid, while the heterofermentative LABs produced not only lactic acid but also ethanol, acetic acid and CO_2, so that the total acid produced by these LABs will be less than the homofermentative LABs (Papagianni et al. 2012).

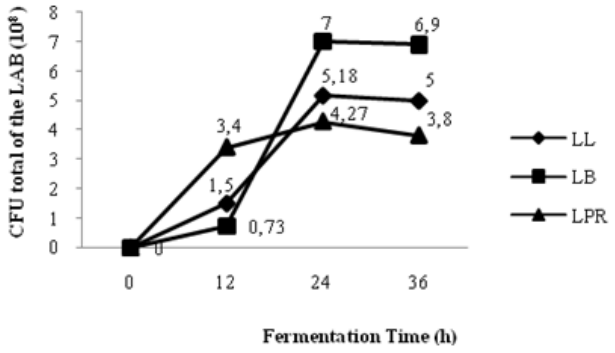

Figure 1. Growth of lactic acid bacteria in edamame milk was observed at 0, 12, 24, 36 hours of incubation time

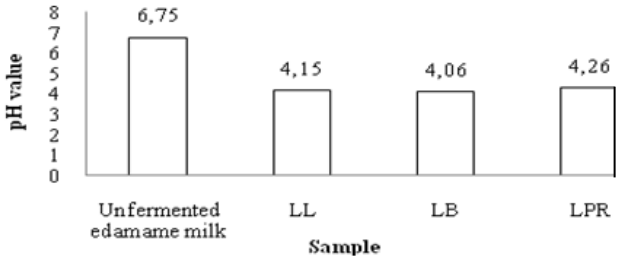

Figure 2. The pH value of protein hydrolyzate from fermented edamame milk using LL, LB and LPR for 24 hours

LABs protease activity in edamame milk

Prior to the quantitative test, a qualitative protease activity test was conducted to see the presence of proteases produced by the LABs. The results showed that *Lc. lactis*, *L. bulgaricus* (LB) and *L. paracasei* exhibit clear zones around the colonies after 24 hours of incubation time (Figure 3) indicating their ability to produce protease during their growth. At 24 hours incubation, the proteolytic index of the three LABs was higher than that of 48 hours (Table 1), this could be because secretions of the proteases ceased after 24 hours fermentation time. Other studies also showed a similar clear zone around the culture on skim milk agar media where several LABs cultures were grown, such as *Pediococcus pentosaceus* 1 W2SR04, *L. rhamnosus* R2, *L. plantarum* 1 W22408, *L. delbrueckii* BD7, *Lc. lactis* ssp *lactis* 1 BD17, *L. fermentum* R6, S206 and *L. kefiri* YK4 and JK17, which indicate the presence of protease released by those LABs (Yuliana et al. 2020). However, their proteolytic indexes were not measured.

Determination of proteolytic activities on edamame milk hydrolysate samples after being fermented with LABs for 24 and 48 hours showed that the activities were quantitatively increased compared to the unfermented milk as can be seen in Figure 4. The protease activity detected in edamame extract after being fermented using *Lc. lactis*, *L. bulgaricus*, and *L. paracasei* for 24 hours had a higher protease activity than that of 48 hours, each exhibiting 2,875; 2,809; and 2,782 Units/mL of the filtrate respectively. Based on the data obtained, it can be seen that the longer the time of cell growth, the lower the enzyme activity. This may happen because during the cell growth, the LABs can produce several proteases which can hydrolyze one to another or it may sensitive to environmental condition (Jayus et al. 2005). If the amount of substrate in the edamame milk decreases, the catalytic rate of the protease enzyme may also decrease. Based on the results of qualitative and quantitative protease activity tests, the three strains used in this study have protease activity which is similar to the observation by Quirós et al. (2005) which stated that several *Lactobacillus* strains had high protease activity, which include *L. delbrueckii* ssp. *bulgaricus*, *Lc. lactis* ssp. *diacety lactis*, *Lc. lactis* ssp. *cremoris* and *Streptococcus salivarius* ssp. *Thermophilus.*

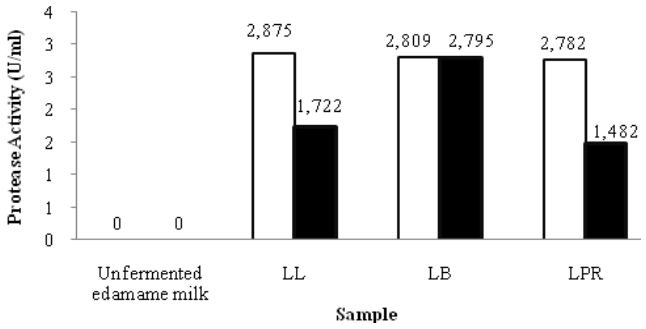

Figure 4. Protease activity of edamame milk after being fermented for 24 hours (☐) and 48 hours (■). LL: *Lactococcus lactis*, LB: *Lactobacillus bulgaricus*, and LPR: *Lactobacillus paracasei*

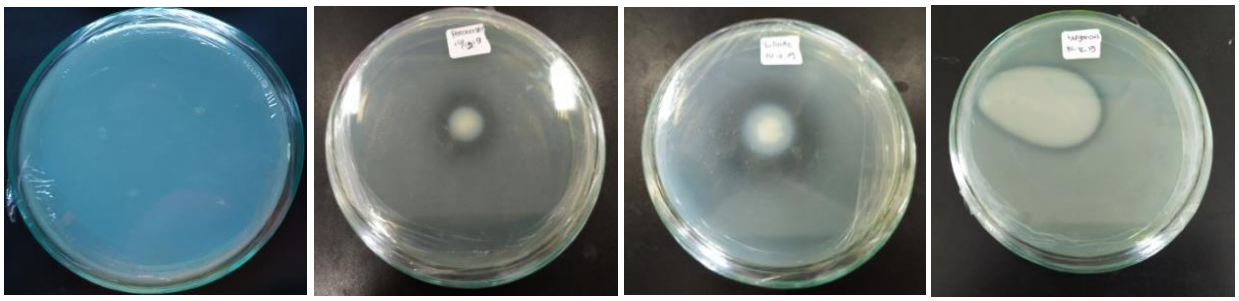

Figure 3. Protease activity of (A) Control, (B) LPR, (C) LL, and (D) LB cultures observed under agar diffusion method

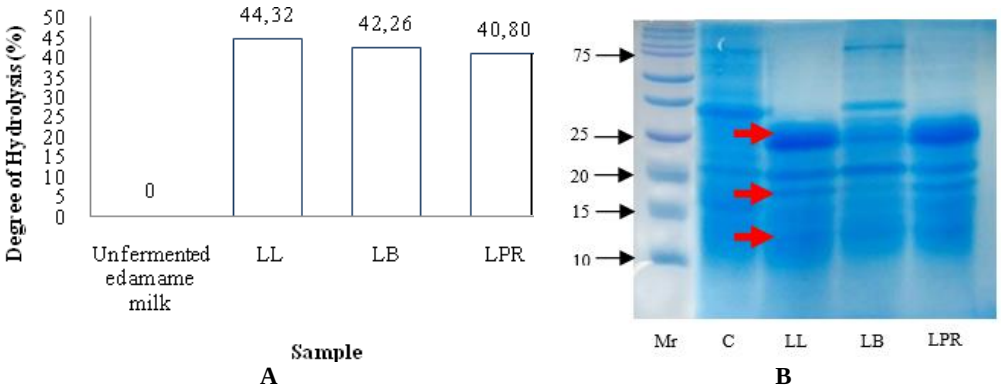

Figure 5. Degree of hydrolysis of edamame milk protein fermented LL, LB, and LPR for 24 hours. A) Degree of hydrolysis in edamame milk, B) Effect of fermentation on protein profile (SDS-PAGE) in edamame milk. Mr: Protein Marker, C: Unfermented edamame milk, LL: *Lactococcus lactis*, LB: *Lactobacillus bulgaricus*, and LPR: *Lactobacillus paracasei*

Table 1. Proteolytic indexes of *Lactococcus lactis*, *Lactobacillus bulgaricus* (LB) and *Lactobacillus paracasei* measured from agar diffusion method clear zone

LABs	Proteolytic index	
	24 h incubation	48 h incubation
Lactococcus lactis	1.98	1.77
Lactobacillus bulgaricus	1.68	1.43
Lactobacillus paracasei	1.75	1.57

Table 2. Antioxidant capacity of fermented edamame protein hydrolysate using LL: *Lactococcus lactis*, LB: *Lactobacillus bulgaricus*, and LPR: *Lactobacillus paracasei*

Sample	IC_{50} (µg/mL)	
	ABTS radical scavenging	Hydroxyl radical scavenging
Unfermented soymilk	30,86	28,23
LL	8,96	13,33
LB	10,16	14,37
LPR	10,24	15,53
GSH	0,16	8,18

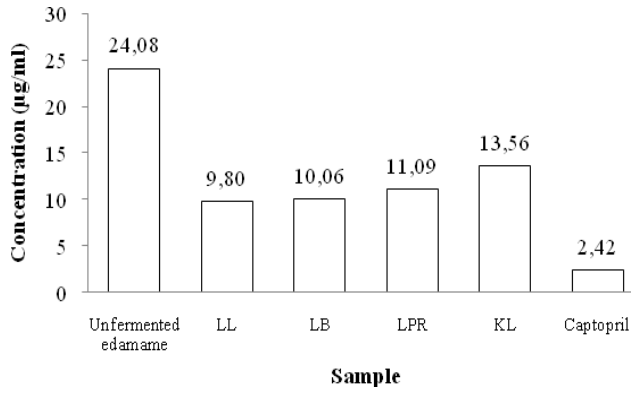

Figure 6. ACE inhibitory activity of fermented edamame milk using LL: *Lactococcus lactis*, LB: *Lactobacillus bulgaricus*, and LPR: *Lactobacillus paracasei*

Degree of hydrolysis of edamame milk protein during fermentation

Enzymatic hydrolysis of edamame milk protein fermented by *Lc. lactis* for 24 hours reached the highest DH value of 44.32%. This is in accordance with the results of protein profile analysis using SDS-PAGE, where the fermented protein bands were different from the unfermented soymilk protein bands. During fermentation, proteins were degraded into smaller molecular sizes of newform band (Figure 5.B). The amount of hydrolyzed protein as the product of degraded peptide bonds into smaller peptides and or single amino acids, were depended on the degree of protease hydrolysis which is influenced by substrate concentration, enzyme concentration, pH and temperature (Ramakrishna and Ramakrishna 2006). The protease enzymes produced by *Lc. lactis*, *L. bulgaricus*, and *L. paracasei* are endopeptidase types, which are able to hydrolyze peptide bonds randomly within the protein chains to produce oligopeptides and or smaller polypeptides (Kieliszek 2021). Endopeptidase produces short-chain hydrophobic amino acids that can increase ACE inhibitory activity have been reported by Mótyán (2013). Daliri et al. (2018) also stated the presence of new novel bioactive peptides with ACE-inhibitory activity from the fermented soybean protein isolates YX 2000 using *Pediococcus pentosaceus* SDL1409.

The degree of hydrolysis measurement of edamame protein fermented by LABs was carried out to ensure that the fermentation process gave a high proteolytic activity compared to the unfermented milk. The more proteolytic activities occurred, the more amino acids will be released, especially amino acids that act as antioxidants such as arginine, lysine, histidine, and tyrosine. Based on the results of protein profile analysis, it can be seen that there were new bands of molecular weight between 10-15 kDa and 15-20 kDa appear in the three LABs cultured edamame milk. From edamame milk cultured by *Lc. lactis* and *L. paracasei* were observed even thicker band, which appeared in between 20-25 kDa of marker protein, indicating more massive protein hydrolysis occurred within these two microbial cultured edamame milks. The lower molecular weight band of peptide below 15 kDa was also observed from the soymilk cultured by the three LABs. This hydrolysate may also contribute to the antioxidative activity increments of the fermented soymilk. Wattanasiritham et al. (2016) reported a similar finding of low molecular weight peptides, which was even lower than 10 kDa size. This low molecular size peptide had been found to be more effective as an antioxidant as well as acting as antihypertensive peptide compared to its native higher molecular weight peptides.

Table 3. Amino acid composition of edamame milk protein before and after fermentation using *Lactococcus Lactis*

Parameter	Mean (mg/100g)	
	unfermented	LL
L- Serine	42,67	92,90
L-Glutamic acid	185,94	370,05
L-Phenylalanine	26,10	57,48
L-Isoleusine	24,75	61,07
L-Valine	23,51	67,09
L-Alanine	33,80	85,64
L-Arginine*	47,16	98,06
Glycine	30,44	63,77
L-Lysine*	63,09	154,93
L-Aspartic acid	94,57	229,94
L-Leucine	46,04	120,93
L-Tyrosine*	16,19	35,05
L-Proline	40,36	86,10
L-Threonine	30,09	70,89
L-Histidine*	18,88	31,81
L-Tryptophane	7,55	14,67
TAA (mg/100 g)	731,14	1640,39
TAntAA (mg/100 g)	145,32	319,84
TAntAA/TAA (%)	19,88	19,50

Mean: Average (mg/100g), SD: Standard Deviation, CV: coefficient of variance, *antioxidant amino acids, TAA: Total Amino Acids, TAntAA: Total Antioxidant Amino Acids

Antioxidant activity of post-fermented edamame Milk

Observations of the antioxidant activity of 24-hour fermented soybeans milk, analyzed using the both ABTS and HRS method showed that the IC_{50} value of edamame milk fermented by *Lc. lactis*, *L. bulgaricus*, and *L. paracasei* were lower when compared with that of unfermented milk (30.86 µg/mL). This indicates that those three fermented milks showed a higher potency as antioxidant agent, even though their IC_{50} is still higher than the glutathione (GSH) as a positive control (0.16 µg/mL) (Table 2).

Long et al. (2021) also observed the presence of peptide exhibiting the ABTS free radical scavenging ability, obtained from *L. bulgaricus* fermented soybean milk (40.88%), which was also stronger than that of unfermented soybean milk (26.59%). Meanwhile, hydroxyl radical scavenging ability of fermented soybean milk by *L. bulgaricus* (35,82%) was stronger than unfermented soybean milk too (15.54%). An increase in antioxidant activity of 52,27% was also shown in the fermented goat milk by *L. fermentum* M4 in the study by Panchal et al. (2020). Another study also observed that fermented soy milk by *L. paracasei* KUMBB005 had an IC_{50} value of 27 mg/mL higher than the standard (Rani and Pradeep 2015). The antioxidant activity of fermented edamame milk increased due to the presence of bioactive components such as bioactive peptides, isoflavones and other bioactive components produced during LABs fermentation. In this study, the free amino acid content in fermented edamame milk is able to react with free radicals and be converted into a more stable product and stop the free radical chain reaction which is characterized by a lower IC_{50} value than the control (unfermented edamame).

Angiotensin-I Converting Enzyme (ACE) inhibitor

Based on the result of ACE inhibitory test, all fermented edamame milk by *Lc. lactis*, *L. bulgaricus*, and *L. paracasei* showed a higher ability to inhibit ACE activity compared to the unfermented milk. *Lactococcus lactis* fermented protein had the highest ACE inhibitory activity, which was 9.80 µg/mL, while the IC_{50} value of unfermented edamame milk was 24.08 µg/mL (Figure 6). Even though the fermented soymilk exhibited a higher ACE inhibitory activity, their activities were still lower compared to captopril as a positive control as can be seen in Figure 6. Previous studies also explained that soy milk fermented by specific strains of LABs can be a good source of ACE inhibitory peptides (Singh and Vij 2017; Tsai and Chen 2006). Wang et al. (2014) stated that IC_{50} value of ACE inhibitory peptides obtained from commercial defatted soybean meal fermented by *L. plantarum* Lp6 for 24 hours was 1 mg/mL. Additionally, Mishra et al. (2019) reported that approximately 80% increase in ACE inhibition derived from lactobacillus strain fermented soy milk after 5 days of cold storage. Bhatnagar et al. (2018) also found an increase in ACE inhibitory activity up to 41.66% in the hydrolysates of fermented soy milk *L. paracasei* CD4, while Yuliana et al. (2020) showed that 50 µl of fermented soy milk *L. delbrueckii* BD7 and *Lc. Lactis* ssp *lactis* 1 BD17 had inhibitory activity of 4.99 and 9.06%, respectively. Another study also found that ACE inhibitory activity was also found in milk fermented by *L. delbrueckii* subsp. *bulgaricus* SS1 and those fermented by *Lc. lactis* subsp. *cremoris* FT4 for 96 hours (Gobbetti et al. 2000). Based on these finding, it is apparent that The ACE inhibitory activities of peptides from soybean depend on the variety of soybean and the microbial strain used.

Even though the amino acid sequence of peptides obtained from edamame has not yet identified, many of the active soybean peptides were reported to be preceded by amino acid of Leucine. As has been reported by Hanafi et al. (2018), peptides that have ACE-inhibitory activity from enzymatic hydrolysis of Malaysian green soybean were identified to be Arg-Gly-Gln-Val-Ser and Phe-Ile-Thr-Ala-Phe-Arg. Other finding by Gu et al. (2013) also shown similar pattern of amino acid sequences, identified Leu-Leu-Phe, Leu-His-Phe, Leu-Glu-Phe and Leu-Ser-Trp.

Higher antihypertensive peptides were mostly detected in fermented processed soy foods such as soy sauce (Nakahara et al. 2010), natto (Shimakage et al. 2012) and tempeh (Tamam et al. 2019) and other fermented products (Shimakage et al. 2012). In soy sauce, peptide identified as Ala-Trp, Gly-Trp, Ala-Tyr, Ser-Tyr, Gly-Tyr, Ala-Phe, Val-Pro, Ala-Ile, Val-Gly were found to be antihypertensive peptides. In natto Leu-Tyr-Tyr and Leu-Phe-Tyr (Shimakage et al. 2012). Surprisingly, tempeh as Indonesia's traditional food also contains antihypertensive peptides, identified as Ala-Val and Pro-Leu (Tamam et al. 2019). Therefore, it is importance to note that in order to increase the bioactivities which include antihypertensive activities of soybean derived food, soybean need to be fermented before consumed as part of our diet.

Even though the protein hydrolysate obtained from the edamame in this study has not yet been identified, its ACE inhibitory activity may confirm its bioactivity which will contribute to the potency of this hydrolysate as the source of functional and nutraceutical food. Pharmacokinetic studies of some protein hydrolysate derived from soybean fermentation were reported to exhibit opioid activity which could produce several benefits against some health problems, such as obesity, cardiovascular diseases, type II-diabetes and immune disorders such as soymorphins (Stefanucci et al. 2018; Stefanucci et al. 2020).

Amino acid composition of edamame milk fermented by *Lactococcus lactis*

The observations of dissolved amino acid content in fermented edamame milk showed that their amino acid contents increased compared to that of unfermented milk. In accordance with the value of the degree of hydrolysis, the highest degree of hydrolysis edamame milk fermented by *Lc. lactis*, its amino acids compositions were recorded to be higher in their content as can be observed in Table 3. The increment of all individual amino acids content in *Lc. lactis* fermented edamame milk indicating that during fermentation, *Lc. lactis* was able to release proteases and peptidases, where these such enzymes have been reported were able to break down proteins into tripeptides and dipeptides which serve as good sources of bioactive peptides (El-Ghaish et al. 2011). The bioactive peptides obtained can have several activities such as antimicrobial,

antioxidative, antihypertensive, and immunomodulatory (Agyei 2015). Several single amino acids are also reported to be the constituents of bioactive peptides revealing antioxidant activity (Ranamukhaarachchi et al. 2013). The presence of single active amino acid in fermented edamame milk may also formed from the metabolite produced by the LABs used during fermentation, beside from the accumulation of the proteolytic hydrolysis product. As can be seen in Table 3, glutamic acid (Glu) and aspartic acid (Asp) were observed higher in fermented edamame milk which possibly *Lc. lactis* has the ability to release those amino acids as the extracellular metabolite. *Lactococcus lactis* strain HY708 have been reported to produces significant amounts of arginine, isoleucine, phenylalanine, serine and glutamic acid (Lee et al. 2021).

The inhibitory properties of ACE are related to peptides consisting of hydrophobic amino acids (Daskaya-Dikmen et al. 2017), such as proline, glycine, phenylalanine and leucine. Other studies have also revealed that the antihypertensive effect of bioactive peptides is due to the presence of the amino acids valine, histidine, proline, tryptophan or methionine in the peptide sequence (Mujtaba et al. 2021). Proline existed in the bioactive peptide can effectively interact with amino acid residues at the ACE active site. This interaction causes Zn (II) distortion and inactivates ACE (Wu et al. 2015). Based on Table 3, it can be seen that the fermented edamame milk contains the amino acid proline higher than that of the unfermented, which may give arise the possibility of the proline containing bioactive peptide to form hydrogen bonds with Zn in ACE and inhibits its activity. Beside proline, the *Lc. lactis* fermentation on edamame milk also released higher content of valine, histidine, and tryptophan just about double of its content in the unfermented prepared edamame milk. Since the LABs strain are considered as GRAS (generally recognized as safe) microorganism, the application of such organisms in edamame derived food or beverages will provide a safe and economical bioactive peptide as the alternative solution for hypertensive patients.

In conclusion, *Lc. lactis* InaCC B187, *L. bulgaricus*FNCC41, *and L. paracasei* InaCC B145 were able to hydrolyze edamame protein into some shorter peptides. The shorter peptides from the three LABs strains had higher antioxidant activity than that of their native protein from edamame. The shorter peptides were also revealed the ability to inhibit ACE-converting enzyme, which indicates the potential of the short peptide as an antihypertensive compound. This finding will trigger the effort to explore further the bioactive peptide from different varieties of soybean and also the fermentation method used, especially the microbial strain selection in order to obtain a more bioactive peptide derived from soybean.

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