PJN
ISSN 1680-5194

PAKISTAN JOURNAL OF NUTRITION

ANSInet
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan
Mob: +92 300 3008585, Fax: +92 41 8815544
E-mail: editorpjn@gmail.com

OPEN ACCESS Pakistan Journal of Nutrition

ISSN 1680-5194
DOI: 10.3923/pjn.2020.295.302

Research Article
Proteolytic Activity of Indigenous Lactic Acid Bacteria and Angiotensin-I-Converting Enzyme (ACE) Inhibitory Activity in Fermented Soy Milk

[1,2]Yuliana Tandi Rubak, [1,3]Lilis Nuraida, [4,5]Dyah Iswantini and [1,3]Endang Prangdimurti

[1]Department of Food Science and Technology, IPB University (Bogor Agricultural University), IPB Dramaga Campus, Bogor 16680, Indonesia
[2]Departement Agrotechnology, Faculty of Agriculture, Universitas Nusa Cendana Kupang, UNDANA Lasiana Campus, Kelapa Lima, Nusa Tenggara Timur (NTT) 85228, Indonesia
[3]Southeast Asian Food and Agricultural Science and Technology (SEAFAST) Center, IPB University (Bogor Agricultural University), IPB Dramaga Campus, Bogor 16680, Indonesia
[4]Department of Chemistry, IPB University (Bogor Agricultural University),IPB Dramaga Campus, Bogor 16680, Indonesia
[5]Tropical Biopharmaca Research Center, IPB University (Bogor Agricultural University), IPB Taman Kencana Campus, Bogor 16128, Indonesia

Abstract

Background and Objective: Lactic acid bacteria with its proteolytic system hydrolyzes proteins to produce angiotensin-I-converting enzyme inhibitor during fermentation. A total of 108 indigenous lactic acid bacteria isolated from fermented food were screened based on proteolytic activity for the potential formation of angiotensin-I-converting enzyme inhibitory activity in fermented soy milk. **Materials and Methods:** Indigenous lactic acid bacteria (from tempe, kefir and breast milk) were screened based on proteolytic activity. Semi-qualitative screening of proteolytic activity of lactic acid bacteria was performed on skim milk agar. Thirty lactic acid bacteria isolates were further selected based on the formation of peptides in 11% reconstituted skim milk. A total of 10 lactic acid bacteria isolates with high proteolytic activity were selected as starter cultures for soy milk fermentation at 37°C until pH 4.6 was reached. Evaluation of fermented soy milk was performed by enumeration of lactic acid bacteria population, analyses of titratable acidity, soluble protein content, peptide content and determination of angiotensin-I-converting enzyme inhibitory activity. **Results:** Of 108 lactic acid bacteria isolates, 13.8% isolates had strong proteolytic activity and 15 isolates (13.8%) had moderate proteolytic activity based on clear zones formed surround the colony on skim milk agar after 48 h of incubation. The amount of peptide produced by isolates with strong and moderate proteolytic activity and the ability to reduce pH of soy milk varied among isolates. The pH value of 4.6 of fermented soy milk was reached after 24-48 h of incubation. Of 10 selected isolates, *Lactobacillus plantarum* 1W22408 and *Lactobacillus fermentum* R6 produced the highest angiotensin-I-converting enzyme inhibitory activity in fermented soy milk. **Conclusion:** Proteolytic activity and acidification ability of the lactic acid bacteria varied between isolates. Lactic acid bacteria isolates of *Lactobacillus plantarum* 1W22408 and *Lactobacillus fermentum* R6 were potential to be used as a starter culture to produce fermented soymilk which has angiotensin-I-converting enzyme inhibitory activity.

Key words: ACE inhibitory activity, fermented soy milk, indigenous lactic acid bacteria, peptides, proteolytic activity

Received: March 03, 2020 Accepted: May 05, 2020 Published: May 15, 2020

Citation: Yuliana Tandi Rubak, Lilis Nuraida, Dyah Iswantini and Endang Prangdimurti, 2020. Proteolytic activity of indigenous lactic acid bacteria and angiotensin-I-converting enzyme (ACE) inhibitory activity in fermented soy milk. Pak. J. Nutr., 19: 295-302.

Corresponding Author: Lilis Nuraida, Department of Food Science and Technology, IPB University (Bogor Agricultural University), IPB Dramaga Campus, Bogor 16680, Indonesia
Southeast Asian Food and Agricultural Science and Technology (SEAFAST) Center, IPB University (Bogor Agricultural University), IPB Dramaga Campus, Bogor 16680, Indonesia

Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Lactic acid bacteria (LAB) are widely involved in a variety of food fermentation. Its ability to produce distinctive characteristics and improve digestibility and provide health effects increases the use of LAB to produce certain physiological functions. LAB is proven to be able to produce a number of peptides and amino acids in fermented milk that have functions such as antidiabetic, anti inflammatory, antibacterial, anticancer and antihypertensive[1,2]. One of the peptides that has received much attention is antihypertensive peptides due to increasing hypertension sufferers and side effects arising from the use of synthetic drugs such as captopril, lisinopryl and ala cepryl[3] in the medication of hypertension. Antihypertensive peptides from fermented foods is a safe and considered as natural source that can be an alternative source of hypertension medication. Angiotensin-I-converting enzyme (ACE, EC 3.4.15.1) plays an important role in the blood pressure regulation system by converting angiotensin I becomes angiotensin II and activates bradykinin, which raises blood pressure[4], therefore ACE inhibition becomes an effective treatment for lowering blood pressure.

Proteolytic activity is one of the keys to enable LAB hydrolyzing proteins and releasing a number of bioactive peptides from the primary structure of the protein during fermentation[5,6]. Some LAB species with high proteolytic activity including *Lactobacillus plantarum*, *Lactobacillus pentosus*, *Lactobacillus sakei*, *Pediococcus acidilactici*, *Pediococcus pentosaceus*, *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Lactococcus lactis*[7,8] are able to produce ACE inhibitory activity in fermented foods. *Lactobacillus helveticus* because of its high proteolytic activity being one of the most widely studied isolate and has been used for the production of antihypertensive bioactive peptides[9].

ACE inhibitory activity can be produced in various food sources that are rich in protein content, one of which is soybeans. Soybeans with a protein content of 36-56% can be used as a source of production for ACE inhibitory activity[10]. Fermented soybeans are a source of antihypertensive peptides[11,12]. Fermentation has become the most efficient and effective method for producing antihypertensive peptides as a results of LAB proteolytic activity. LAB proteolytic activity plays an important role in producing antihypertensive peptides. This research focused on screening of indigenous LAB based on proteolytic activity to obtain LAB isolates that can be used as starter cultures for the production of ACE inhibitory activity in fermented soy milk.

MATERIALS AND METHODS

Lactic acid bacteria: A total of 108 LAB isolated from tempe, kefir grains and breast milk were selected for proteolytic activity. The LAB isolates were confirmed as belonging to the genus *Lactobacillus*, *Pediococcus*, *Lactococcus* and *Enterococcus.* The isolates were obtained from Food Microbiology Laboratory of Southeast Asian Food and Agricultural Science and Technology (SEAFAST) Center, IPB University (Bogor Agricultural University).

Methods: LAB screening based on proteolytic activity:

- **Semi-qualitative proteolytic activity testing[13]:** Qualitative proteolytic activity testing was performed using skim milk agar with composition: skim milk powder (2.8%, Difco), yeast extract (0.25%), dextrose (0.1%), casein (0.5%) and bacteriology agar (1.5%) in 100 mL volume of suspension. Reconstituted skim milk was sterilized separately and mixed with other ingredients prior to the use of the agar. A loop full of colony of 24 h isolates on MRS agar was spotted on to the surface of skim milk agar. Proteolytic activity was observed based on the formation of clear zones surround the colony after incubation at 37°C for 48 h.
- **Peptide formation in skim milk:** Thirty LAB isolates that showed proteolytic activity on skim milk agar were grown on reconstituted skim milk (11%, ZMP Ltd, New Zealand) that has been pasteurized at 95°C for 10 min. The starter culture (2%) was inoculated into reconstituted skim milk and then incubated at 37°C to reach pH 4.6[14] (measured using pH meter 700 Eutech). When the pH has been reached, the LAB was enumerated on De Man Rogosa and Sharpe Agar (Oxoid) and titratable acidity was determined by the titration. The fermentation process was stopped by heating at 75°C for 1 min and then was centrifuged (Hettich, Zentrifugen, Mikro 22R) at 6000 g×10 min at 4°C. The supernatant was collected and analyzed for the soluble protein and peptide content representing proteolytic activity.

LAB application in fermentation of soy milk: A total of 10 selected isolates with high proteolytic activity were grown in reconstituted soybean milk (11%, Metabolis, Indonesia) and incubated at 37°C to reach pH 4.6. The LAB count and titratable acidity were analyzed when the pH of 4.6 has been reached. The fermentation process was then stopped by heating at 75°C for 1 min. The fermented soy milk was then

centrifuged at 6000 g×10 min at 4°C. The supernatant was analyzed for soluble protein and peptide content and ACE inhibitory activity.

Determination of peptide content: Peptide content was determined using the o-phthaldialdehyde (OPA) method[15]. A total of 50 µL samples were mixed with 2 mL of OPA reagents [consisting of 25 mL of 100 mM of sodium tetraborate, 2.5 mL of 20% (w/w) of sodium dodecyl sulfate and 1.1 mL of OPA solution, mixed with 21.4 mL of dH2O]. The OPA solution was prepared by dissolving 40 mg of OPA (Sigma, USA) dissolved in 1 mL of methanol +100 mL of ß-mercaptoethanol (Sigma, USA). The sample and OPA reagent were quickly mixed with and then incubated for 2 min, followed by measuring the absorbance at 340 nm (UV-VIS-1240, Shimadzu, Japan). The peptide content was quantified based on tryptone casein (Merck) standard curve.

Determination of soluble protein content: Protein content was determined using the Bradford method[16] and bovine serum albumin (Sigma, USA) as a standard. A total of 10 µL samples with 250 µL Bradford reagents were mixed and then incubated for 5 min. Sample absorbance was measured at 595 nm (Biorad, iMark, Microplate Reader, Japan).

Determination of ACE inhibitory activity in fermented soy milk: Determination of ACE inhibitory activity was carried out *in vitro* according to the methods of Chusman and Cheung[17]. Hippuryl-L-Histidyl-L-Leucine (HHL, Sigma, USA) was used as the enzyme-substrate. A 50 µL of the substrate (50 mM HHL in 0.1 M sodium borate buffer containing 0.3 M NaCl at pH 8.3) was added into 50 µL sample and incubated at 37°C for 5 min. To initiate the reaction, 50 µL of 0.1 U mL^{-1} ACE (Sigma, USA) solution was added and the mixture was incubated at 37°C for 5 min. The reaction was stopped by adding 250 µL 1 M HCl 1 M. The resulted Hippuric Acid (HA) was extracted with 1.5 mL ethyl acetate and centrifuged at 2000×g for 5 min. An aliquot (0.8 mL) of the ethyl acetate layer was transferred to clean tube and evaporated at 85°C for 60 min. Distilled water (4 mL) was then added to dissolve the HA in the tube and the amount of HA formed was measured by measuring optical density at 228 nm (UV-2800, Hitachi, Japan) The extent of inhibition was calculated as 100% [(B-A)/B] where A is the optical density in the presence of ACE and ACE inhibitory component, B is the optical density without ACE inhibitory component.

Statistical analysis: All analysis was carried out with three replications and expressed as Mean±standard deviation. The data obtained were analyzed by Analysis of Variance (ANOVA). The significant difference was further analyzed using Duncan test at a level of 5% using software of SPSS version 22.

RESULTS

LAB proteolytic activity on skim milk agar: Among 108 LAB isolates, 103 isolates (95.6%) showed proteolytic activity with the formation of clear zones around the colony. The clear zone formed showed different size and appearance among LAB isolates. Based on the result, the clear zones formed are then categorized into five categories (Table 1).

Based on this category, the results obtained are 13.8% included in category I (strong proteolytic activity) and 13.8% in category II (moderate proteolytic activity), 55.5% and 12.03% respectively included in categories III and IV(weak proteolytic activity) and 4.6% included in category V (no visible activity). Based on the results, thirty isolates in categories I and II with distinct clear zones (Fig. 1) were selected for further screening based on the formation of peptides content in skim milk. The 30 selected LAB isolates were dominated by *Lactobacillus*, others were *Lactococcus*, *Pediococcus* and *Enterococcus*.

Growth of LAB and peptide formation in skim milk: Fermentation time, LAB population, titratable acidity, protein and peptide contents in fermented skim milk are presented in Table 2. The fermentation time required by 30 LAB cultures to reach pH 4.6 was 24-48 h. Overall LAB was able to grow well with a population in the range of 8.79±0.19 to 9.89±0.19 log CFU mL^{-1}. The highest LAB population was obtained in fermented skim milk of *Lactobacillus fermentum* 1 YK16.

Titratable acidity measured as lactic acid is a LAB metabolite derived from milk lactose. After the fermentation process, titratable acidity was found in the range of 0.72±0.02 to 0.96±0.02%. The soluble protein content of fermented skim milk was obtained in the range of 0.117±0.00 to 0.438±0.02 mg mL^{-1}. The peptide content describing the proteolytic activity of LAB culture during fermentation was obtained in the range of 0.59±0.07 to 8.73±4.13 mg mL^{-1}.

Table 1: Categories of LAB isolates based on the formation of clear zones on skim milk agar after 48 h incubation at 37°C

Categories based on the formation of clear zones	Total isolates
Category I: Large colony, surrounded by wide clear zone	15
Category II: Small colony, surrounded by clear zone	15
Category III: Large colony, clear zone only under the colony	60
Category IV: Small colony, clear zone only under the colony	13
Category V: No clear zone	5

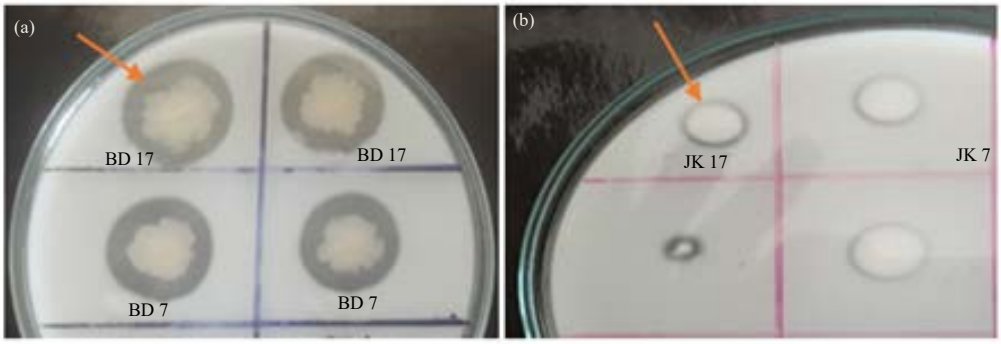

Fig. 1(a-b): Clear zone formed by LAB isolates on skim milk agar medium after 48 h incubation at 37°C: (a) *Lactobacillus delbrueckii* BD7 and *Lactococcus lactis* ssp. *lactis* 1 BD17 (category I), (b) *Lactobacillus kefiri* YK7 and *Lactobacillus kefiri* JK17 (category II)

Table 2: Fermentation time to reach pH 4.6, viable count of LAB, titratable acidity, soluble protein content and peptide content in fermented skim milk

Starter culture	Fermentation time to reach pH 4.6 (h)	Viable LAB (log CFU mL^{-1})	Titratable acidity (%)	Soluble protein content (mg mL^{-1})	Peptide content (mg mL^{-1})
Lactobacillus fermentum R6	24	9.49±0.26	0.80±0.06	0.331±0.00	7.66±0.00
Pediococcus pentasaceus 1 W2SR04	24	9.45±0.20	0.79±0.06	0.389±0.00	4.30±0.87
Lactobacillus kefiri YK4	32	9.08±0.19	0.91±0.06	0.153±0.00	8.73±4.13
Lactobacillus rhamnosus R2	32	9.46±0.36	0.77±0.06	0.137±0.00	6.78±2.10
Lactobacillus plantarum 1 W22408	32	9.21±0.41	0.79±0.04	0.277±0.01	4.33±0.14
Lactobacillus kefiri JK17	32	8.87±0.21	0.84±0.05	0.438±0.02	4.01±0.00
Lactobacillus kefiri JK5	32	8.93±0.20	0.77±0.04	0.411±0.00	2.05±0.41
Enterococcus durans R9	36	9.64±0.19	0.84±0.03	0.153±0.00	0.76±0.04
Lactococcus lactis ssp. *lactis* 1 BGM172	40	9.31±0,24	0.91±0,03	0.152±0,00	4.74±0,63
Lactobacillus R7F	40	9.11±0,22	0.84±0,02	0.382±0,00	4.54±0.00
Lactococcus lactis ssp. *lactis* 1 BD17	40	9.25±0.42	0.82±0.05	0.173±0.01	3.41±0.60
Lactobacillus fermentum S206	48	9.33±0.37	0.87±0.05	0.163±0.00	8.63±2.28
Lactobacillus kefiri BD4	48	9.70±0.24	0.87±0.05	0.333±0.00	4.21±0.23
Lactobacillus R3	48	9.68±0.15	0.92±0.04	0.438±0.02	4.17±1.12
Lactobacillus rhamnosus R19	48	8.80±0.16	0.86±0.03	0.035±0.00	3.79±2.99
Lactobacillus fermentum 1 BGR11	48	8.97±0.17	0.96±0.02	0.266±0.00	3.64±1.40
Lactobacillus rhamnosus YK12	48	9.60±0.42	0.89±0.04	0.177±0.00	3.35±0.07
Lactobacillus delbrueckii BD7	48	9.41±0.25	0.83±0.04	0.254±0.01	3.33±0.75
Lactobacillus R8F	48	8.83±0.58	0.81±0.05	0.258±0.00	3.20±0.70
Lactococcus lactis ssp *lactis* 1 BD16	48	9.16±0.37	0.95±0.04	0.188±0.00	2.85±0.61
Lactobacillus kefiri JK6	48	8.99±0.05	0.88±0.05	0.330±0.00	2.67±1.62
Lactococcus lactis ssp. *lactis* 1 BD3	48	9.62±0.40	0.91±0.04	0.153±0.00	2.59±1.27
Lactobacillus kefiri YK7	48	9.62±0.28	0.86±0.05	0.216±0.00	2.58±0.01
Lactobacillus rhamnosus R18	48	9.17±0.64	0.91±0.02	0.341±0.00	2.30±0.87
Lactobacillus delbrueckii W24802	48	8.79±0.19	0.72±0.02	0.340±0.00	2.05±0.09
Lactobacillus fermentum 1 YK16	48	9.89±0.19	0.86±0.05	0.392±0.03	2.00±0.48
Lactobacillus fermentum 1 BG14	48	9.69±0.28	0.94±0.05	0.270±0.00	1.75±1.24
Lactobacillus fermentum 1 BD 6	48	9.51±0.38	0.94±0.05	0.244±0.00	1.19±1.02
Lactobacillus fermentum 1 YK8	48	9.70±0.12	0.94±0.03	0.117±0.00	0.94±0.19
Lactobacillus fermentum 1 YK2	48	9.36±0.42	0.94±0.04	0.159±0.00	0.59±0.07

The highest amount of peptide content was obtained from fermented skim milk of *Lactobacillus kefiri* YK4. Based on the peptide content produced in skim milk, 10 isolates i.e. *Pediococcus pentosaceus* 1 W2SR04, *Lactobacillus plantarum* 1 W22408, *Lactobacillus rhamnosus* R2, *Lactobacillus* R7F, *Lactobacillus delbrueckii* BD7, *Lactococcus lactis* ssp. *lactis* 1 BD17, *Lactobacillus fermentum* R6, S206 and *Lactobacillus kefiri* YK4 and JK17 were considered being potential to be applied in soy milk fermentation.

Growth of LAB and peptide formation in fermented soy milk: A total of 10 LAB isolates with high proteolytic activity

Table 3: Fermentation time to reach pH 4.6, viable count of LAB, titratable acidity, soluble protein content and peptide content in fermented soy milk

Starter culture	Fermentation time to reach pH 4.6 (h)	Titratable acidity (%)	Viable LAB (log CFU mL^{-1})	Soluble protein content (mg mL^{-1})	Peptide content (mg mL^{-1})
Lactobacillus plantarum 1W22408	24	0.76±0.04	9.18±0.40	0.416±0.01	4.682±0.18
Lactobacillus kefiri YK4	24	0.73±0.04	9.62±0.23	0.367±0.02	4.292±0.05
Lactobacillus R7F	24	0.71±0.02	8.98±0.11	0.382±0.02	4.183±0.17
Lactobacillus fermentum R6	24	0.72±0.01	9.15±0.59	0.336±0.02	3.367±0.25
Lactobacillus delbrueckii BD7	24	0.86±0.06	9.43±0.33	0.338±0.01	3.262±0.15
Pediococcus pentasaceus 1 W2SR04	24	0.78±0.02	9.22±0.24	0.373±0.00	2.860±0.12
Lactobacillus rhamnosus R2	48	0.77±0.04	9.50±0.04	0.316±0.03	4.470±0.27
Lactobacillus fermentum S206	48	0.66±0.03	9.29±0.56	0.439±0.01	4.435±0.08
Lactobacillus kefiri JK17	48	0.68±0.03	9.23±0.52	0.396±0.02	4.365±0.24
Lactococcus lactis ssp. *lactis* 1 BD17	48	0.71±0.04	9.01±0.48	0.328±0.02	3.710±0.21

Table 4: Angiotensin-I-converting enzyme inhibitory (ACE-I) activity and the value of the inhibitory efficiency ratio (IER) in soy milk fermented by 10 LAB cultures after reaching pH 4.6 incubated at 37°C

Starter culture	inhibition of ACE (%)	IER (% per mg mL^{-1})
Lactobacillus plantarum 1W22408	21.73±2.33^{a}	4.63±0.32^{b}
Lactobacillus rhamnosus R2	12.98±1.33^{c}	2.93±0.50^{c}
Lactobacillus fermentum S206	16.91±1.13^{b}	3.82±0.28^{b}
Lactobacillus kefiri JK17	11.04±1.00^{cd}	2.53±0.13^{c}
Lactobacillus kefiri YK4	9.60±1.12^{d}	2.24±0.26^{cd}
Lactobacillus R7F	18.50±1.52^{b}	4.42±0.20^{b}
Lactococcus lactis ssp. *lactis* 1 BD17	9.06±0.84^{d}	2.44±0.15^{c}
Lactobacillus fermentum R6	23.93±0.87^{a}	7.17±0.84^{a}
Lactobacillus delbrueckii BD7	4.99±0.38^{e}	1.53±0.17^{d}
Pediococcus pentasaceus 1 W2SR04	13.26±0.96^{c}	4.64±0.34^{b}

Different superscripts in same column indicates significant ($p<0.05$) between samples

(based on formation clear zones on skim milk agar and peptide content in skim milk) were used as starter cultures for soymilk fermentation. Fermentation time, titratable acidity, LAB population, protein and peptide contents in fermented soy milk after reaching pH 4.6 were evaluated and the results are presented in Table 3.

The results showed that 10 isolates used were able to grow well in soy milk with a population of LAB varied between 8.98±0.11 to 9.62±0.23 log CFU mL^{-1}. Six isolates required 24 h incubation time to reach pH 4.6 while 4 other isolates required longer time, which was 48 h. The content of lactic acid was obtained in the range of 0.66±0.03 to 0.86±0.06% and soluble protein content was found in the range of 0.316±0.03 to 0.439±0.01 mg mL^{-1} and peptide content was obtained in the range of 2.86±0.12 to 4.68±0.18 mg mL^{-1}.

ACE inhibitory activity in fermented soy milk: The percentage of ACE inhibitory activity and the value of the inhibitory efficiency ratio (IER) in soy milk fermented by 10 LAB cultures are presented in Table 4. ACE inhibitory activity was obtained in the range of 4.99±0.38 to 23.93±0.87%. The highest ACE-I activity was produced in soy milk fermented by *L. fermentum* R6 and *L. plantarum* 1W22408. IER values from 10 samples were obtained in the range of 1.53±0.17 to 7.17±0.84. The value of IER is obtained by dividing the percentage of ACE-inhibitory activity by peptide content. High IER values indicate inhibitory efficiency against ACE. The highest IER value was obtained in soy milk fermented by *L. fermentum* R6.

DISCUSSION

Proteolytic activity plays the important role in the formation of antihypertensive peptides by LAB in protein-rich fermented foods[18]. LAB with its proteolytic system hydrolyzes proteins to produce bioactive peptides and amino acids. Initial screening of proteolytic activity was carried out by growing LAB on the skim milk agar. A total of 108 indigenous LAB isolates used, 103 (95%) isolates showed proteolytic activity in different extend indicated by the formation of clear zones around the colony after incubation at 37°C for 48 h. Proteolytic activity of LAB was strain specific[5]. Piyu *et al.*[19] also showed variations of 13 LAB isolates on skim milk agar, 5 isolates showed the highest clear zone after incubation at 37°C for 48-72 h. Likewise, Tulini *et al.*[20] reported that 815 LAB isolates screened on skim milk agar, 123 LAB isolates with high clear zone formation were obtained. The present research showed that of 108 isolates, 15 isolates indicates strong proteolytic activity and 15 isolates indicates moderate activity as shown by the clear zone formed surround the colony.

LAB isolates with high proteolytic activity on skim milk agar grew well during fermentation of skim milk with population of 9.89 log CFU mL^{-1} and not significantly different between LAB (>0.05). Gonzalez-Gonzalez *et al.*[21] reported that *L. helveticus* MF20/5 grews well in skim milk with a population reaching10.30±0.24 log CFU mL^{-1} after 48 h of incubation. Although no difference in the number of LAB, however, the present research showed that the peptide content in skim milk fermented by 30 selected LAB varied. The highest proteolytic activity was successively found in fermented milk of *L. kefiri* YK4, *L. fermentum* S206, *L. fermentum* R6 and *L. rhamnosus* R2. *Lactobacillus* species in several studies have been reported to have high proteolytic activity[22,23] such as *L. delbrueckii* ssp. *bulgaricus*, *L. lactis* ssp. *diacetylactis*, *L. lactis* ssp. *cremoris* and *Streptococcus salivarius* ssp. *thermophylus*[24]. This has led to the widespread use of *Lactobacillus* species in the fermented milk industry, such as sour milk and yogurt[25]. With a high proteolytic activity, *Lactobacillus* release bioactive peptide during the fermentation process. *L. helveticus* has been used for the production of a bioactive peptide in fermented milk, especially for the antihypertensive peptide[26].

The varying proteolytic activity among LAB isolates confirms that proteolytic activity is fully influenced by LAB strains, which are thought to be strongly related to the proteolytic system possessed by each of LAB culture. The proteolytic system of lactic acid bacteria consists of three primary components: (1) Protease that is bound to the cell wall which is known as cell-envelope proteinase (CEP); protease that is bound to the cell wall that initiates the initial hydrolysis of casein into oligopeptide containing 4-30 amino acid residues, (2) Specific transporter, transferring oligopeptide to cytoplasm, (3) intracellular peptidase, which catalyze the hydrolysis of peptide which then transported into cell interior into free amino acid and/peptide with low molecular weight[27,28]. CEP plays an important role in the LAB proteolytic system. The removal of the CEP component causes LAB unable to grow in milk. LAB with high proteolytic activity is thought to have different CEP from the other LAB. LAB generally has one CEP but *L. helveticus* which has high proteolytic activity is thought to have at least 2 CEPs which are PrtH and PrtH2[29]. In this study, the fermentation process was stopped when the pH value reached 4.6. Control of pH needs to be performed to reduce the decrease in proteolytic activity[21]. The release of peptide from the protein matrix by the culture could decrease when the pH value decreases below 4.5, thus, to avoid continuous decrease then the fermentation process needs to be stopped when the pH value reaches 4.5 or pH could be controlled with the addition of an alkaline solution such as sodium hydroxide[30]. A decrease in pH will cause casein coagulation which can inhibit cell diffusion through protein tissue, thus inhibit proteinase accessibility to milk protein so that the casein hydrolysis process slows down or stops. Pan and Guo[31] reported that the proteolytic activity of *L. helveticus* LB 10 fermented milk experienced a significant decrease at lower pH (<6.5). The same condition was reported by Nielsen *et al.*[32] that the bioactivity and concentration of *L. helveticus* fermented milk peptide decreased significantly when the pH value dropped from 5.3-4.3.

Of 30 isolates LAB that produced the highest peptide content, 10 isolates were used to ferment soy milk. The10 LAB were able to grow well on the soy milk as substrate and the population reached 9.62±0.23 log CFU mL^{-1} at 24 h fermentation. This population was not significantly different (>0.05) with those when grown in skim milk, which indicates that the 10 LAB was able to utilize components from soybean to support their growth. Soybean contains oligosaccharide complexes such as stachyose and raffinose which are not easily hydrolyzed by LAB so, that they can be a limiting factor for the growth of LAB[33].

The fermentation time required by the 10 LAB isolates to reach pH 4.6 was 24-48 h, similar to the growth in skim milk. *L. plantarum* 1 W22408, *Lactobacillus* R7F, *L. delbrueckii* BD7 and *L. kefiri* YK4 required a shorter time to reach pH 4.6 when grown in soy milk, than in skim milk and LAB cultures of *L. rhamnosus* R2, *L. lactis* ssp. *lactis* 1 BD17 and *L. kefiri* JK17 required a longer time when grown in soy milk than in skim milk.

During fermentation, hydrolysis of soy protein (glycinin and β-conglycinin) occurs which produces a number of peptides and amino acids[2,8,34]. The highest peptide content was produced from soy milk fermented by *L. plantarum* 1W22408 but it was not significantly different (>0.05) from soy milk fermented by *L. rhamnosus* R2, *L. fermentum* S206, *L. kefiri* JK17 and *L. kefiri* YK4.The results of peptide formation in soy milk confirm that peptide formation by LAB depends on strain and the substrate. *L. kefiri* YK4, *L. fermentum* S206, *L. rhamnosus* R2, *Lactobacillus* R7F, *L. fermentum* R6 and *P. pentasaceus* 1 W2SR04 produced higher peptide content in fermented skim milk than in soy milk, meanwhile 4 other isolates produced higher peptide content in fermented soy milk.

The ACE inhibitory activity of 10 fermented soy milk varies and the highest ACE inhibitory activity was produced in fermented soy milk of *L. fermentum* R6 and *L. plantarum* 1W22408. There was a correlation between proteolytic activity and ACE inhibitory activity produced in soy milk fermented by *L. plantarum* 1W22408. However, other samples show a poor

correlation between proteolytic activity and ACE inhibitory activity. Several LAB with the same species produced different ACE inhibitory activities (Table 4).

In present research, the highest ACE inhibitory activity in fermented soy milk was produced by *L. plantarum* 1W22408 and *L. fermentum* R6.However the activities were still lower than that produced by *Pediococcus pentasaceus* SDL1409 and *L. plantarum* stain C2 in similar substrate[35].

CONCLUSION

Proteolytic activity is strain dependent, which has an impact on the peptide content that varies in skim milk and soy milk. The ACE inhibitory activity in fermented soy milk varies and the highest ACE inhibitory activity produced in fermented soy milk of *L. plantarum* 1W22408 and *L. fermentum* R6. These isolates were potential to be used as starter cultures to produce peptide with ACE inhibitory activity in fermented soy milk.

SIGNIFICANCE STATEMENT

Proteolytic activity of LAB isolated from indigenous fermented food varies between strains. This study discovered that two LAB isolates, *L. plantarum* 1W22408 and *L. fermentum* R6 could be used to produce peptide with ACE inhibitory activity in fermented soy milk.

ACKNOWLEDGMENTS

The author would like to thank the Indonesian Ministry of Research and Higher Education (RISTEKDIKTI) for providing research funding under the scheme of competitive-based research in 2018-2020 for the recipient Lilis Nuraida with the grant number 1609/IT3.11/PN/2018.

REFERENCES

1. Castro, R.J.S.d. and H.H. Sato, 2015. Biologically active peptides: Processes for their generation, purification and identification and applications as natural additives in the food and pharmaceutical industries. Food Res. Int., 74: 185-198.
2. Sanjukta, S. and A.K. Rai, 2016. Production of bioactive peptides during soybean fermentation and their potential health benefits. Trends Food Sci. Technol., 50: 1-10.
3. Zeng, Y., N. Wang and W. Qian, 2013. Production of angiotensin i converting enzyme inhibitory peptides from peanut meal fermented with lactic acid bacteria and facilitated with protease. Adv. J. Food Sci. Technol., 9: 1198-1203.
4. Lin, Y.S., Y.L. Lu, G.J. Wang, H.J. Liang and W.C. Hou, 2014. Vasorelaxing and antihypertensive activities of synthesized peptides derived from computer-aided simulation of pepsin hydrolysis of yam dioscorin. Bot. Stud., Vol. 55, No. 49 10.1186/s40529-014-0049-3
5. Wang, J., C. Li, J. Xue, J. Yang, Q. Zhang, H. Zhang, Y. Chen, 2015. Fermentation characteristics and angiotensin I-converting enzyme–inhibitory activity of *Lactobacillus helveticus* isolate H9 in cow milk, soy milk and mare milk. J. Dairy Sci., 98: 3655-3664.
6. Georgalakia, M., G. Zoumpopouloua, E. Mavrogonatoub, G.V. Driesschec, V. Alexandrakia *et al*., 2017. Evaluation of the antihypertensive angiotensin-converting enzyme inhibitory (ACE-I) activity and other probiotic properties of lactic acid bacteria isolated from traditional Greek dairy products. Int. Dairy J., 75: 10-21.
7. Li, C., L.Y. Kwok, Z. Mi, J. Bala and J. Xue *et al*., 2017. Characterization of the angiotensin-converting enzyme inhibitory activity of fermented milks produced with *Lactobacillus casei*. J. Dairy Sci., 100: 9495-9507.
8. Daliri, E.B.M., B.H. Lee, M.H. Park, J.H. Kim and D.H. Oh, 2018. Novel angiotensin I-converting enzyme inhibitory peptides from soybean protein isolates fermented by *Pediococcus pentosaceus* SDL1409. LWT, 93: 88-93.
9. Chen, Y., C. Li, J. Xue, L.y. Kwok, J. Yang, H. Zhang and B. Menghe, 2015. Characterization of angiotensin-converting enzyme inhibitory activity of fermented milk produced by *Lactobacillus helveticus*. J. Dairy Sci., 98: 5113-5124.
10. Singh, B.P., S. Vij and S. Hati, 2014. Functional significance of bioactive peptides derived from soybean. Peptides, 54: 171-179.
11. Mun, E.G., H.S. Sohn, M.S. Kim and Y.S. Cha, 2017. Antihypertensive effect of ganjang (traditional korean soy sauce) on sprague-dawley rats. Nutr. Res. Pract., 11: 388-395.
12. Hermanto, S., F. Hatiningsih and D.K. Putera, 2018. Antihypertensive bioactive peptides from hydrolysates of soy milk yoghurt (soygurt). J. Phys. Conf. Ser., Vol. 1095 10.1088/1742-6596/1095/1/012034
13. El-Ghaish, S., M. Dalgalarrondo, Y. Choiset, M. Sitohy, I. Ivanova, T. Haertle and J.M. Chobert, 2010. Screening of strains of *Lactococci* isolated from Egyptian dairy products for their proteolytic activity. Food Chem., 120: 758-764.
14. Donkor, O.N., A. Henriksson, T.K. Singh, T. Vasiljevic and N.P. Shah, 2007. ACE-inhibitory activity of probiotic yoghurt. Int. Dairy J., 17: 1321-1331.
15. Church, F.C., H.E. Swaisgood, D.H. Porter and G.L. Catignani, 1983. Spectrophotometric assay using *o*-phthaldialdehyde for determination of proteolysis in milk and isolated milk proteins. J. Dairy Sci., 66: 1219-1227.
16. Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem., 72: 248-254.

17. Cushman, D.W. and H.S. Cheung, 1971. Spectrophotometric assay and properties of the angiotensin-converting enzyme of rabbit lung. Biochem. Pharmacol., 20: 1637-1648.
18. Fuglsang, A., F.P. Rattray, D. Nilsson and N.C. Nyborg, 2003. Lactic acid bacteria: Inhibition of angiotensin converting enzyme *in vitro* and *in vivo*. Antonie van Leeuwenhoek, 83: 27-34.
19. Phyu, H.E., Z.K. OO and K.N. Aye, 2015. Screening on proteolytic activity of lactic acid bacteria from various yogurts and fermented milk. Int. J. Adv. Sci. Eng. Technol., 5: 34-37.
20. Tulini, F.L., N. Hymery, T. Haertlé, G.L. Blay and E.C.P.D. Martinis, 2016. Screening for antimicrobial and proteolytic activities of lactic acid bacteria isolated from cow, buffalo and goat milk and cheeses marketed in the southeast region of Brazil. J. Dairy Res., 83: 115-124.
21. Gonzalez-Gonzalez, C., T. Gibson and P. Jauregi, 2013. Novel probiotic-fermented milk with angiotensin I-converting enzyme inhibitory peptides produced by *Bifidobacterium bifidum* MF 20/5. Int. J. Food Microbiol., 167: 131-137.
22. Mugula, J.K., T. Sørhaug and L. Stepaniak, 2003. Proteolytic activities in *togwa*, a Tanzanian fermented food. Int. J. Food Microbiol., 84: 1-12.
23. Hati, S., N. Patel and S. Mandal, 2018. Comparative growth behaviour and biofunctionality of lactic acid bacteria during fermentation of soy milk and bovine milk. Probiotics Antimicrob. Proteins, 10: 277-283.
24. Quirós, A., B. Hernandez-Ledesma, M. Ramos, L. Amigo and I. Recio, 2005. Angiotensin-converting enzyme inhibitory activity of peptides derived from caprine kefir. J. Dairy Sci., 88: 3480-3487.
25. Hebert, E.M., G.S.D. Giori and R.R. Raya, 2001. Isolation and characterization of a slowly milk-coagulating variant of *Lactobacillus helveticus* deficient in purine biosynthesis. Applied Environ. Microbiol., 67: 1846-1850.
26. Yamamoto, N., A. Akino and T. Takano, 1993. Purification and specificity of a cell-wall-associated proteinase from *Lactobacillus helveticus* CP790. J. Biochem., 114: 740-745.
27. Hafeez, Z., C. Cakir-Kiefer, E. Roux, C. Perrin, L. Miclo and A. Dary-Mourot, 2014. Strategies of producing bioactive peptides from milk proteins to functionalize fermented milk products. Food Res. Int., 63: 71-80.
28. Savijoki, K., H. Ingmer and P. Varmanen, 2006. Proteolytic systems of lactic acid bacteria. Applied Microbiol. Biotechnol., 71: 394-406.
29. Gilbert, C., B. Blanc, J. Frot-Coutaz, R. Portalier and D. Atlan, 1997. Comparison of cell surface proteinase activities within the *Lactobacillus* genus. J. Dairy Res., 64: 561-571.
30. Leclerc, P.L., S.F. Gauthier, H. Bachelard, M. Santure and D. Roy, 2002. Antihypertensive activity of casein-enriched milk fermented by *Lactobacillus helveticus*. Int. Dairy J., 12: 995-1004.
31. Pan, D. and Y. Guo, 2010. Optimization of sour milk fermentation for the production of ACE-inhibitory peptides and purification of a novel peptide from whey protein hydrolysate. Int. Dairy J., 20: 472-479.
32. Nielsen, M.S., T. Martinussen, B. Flambard, K.I. Sørensen and J. Otte, 2009. Peptide profiles and angiotensin-I-converting enzyme inhibitory activity of fermented milk products: Effect of bacterial strain, fermentation pH and storage time. Int. Dairy J., 19: 155-165.
33. Youssef, C.B., G. Goma and A. Olmos-Dichara, 2005. Kinetic modelling of *Lactobacillus casei* ssp. rhamnosus growth and lactic acid production in batch cultures under various medium conditions. Biotechnol. Lett., 27: 1785-1789.
34. Gibbs, B.F., A. Zougman, R. Masse and C. Mulligan, 2004. Production and characterization of bioactive peptides from soy hydrolysate and soy-fermented food. Food Res. Int., 37: 123-131.
35. Singh, B.P. and S. Vij, 2017. Growth and bioactive peptides production potential of *Lactobacillus plantarum* strain C2 in soy milk: A LC-MS/MS based revelation for peptides biofunctionality. LWT, 86: 293-301.