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Characterization of partially extracellular proteases from bekasam-isolated *Lactobacillus plantarum* S31 and its application to hydrolyze skimmed-milk with antibacterial property

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Abstract

The extracellular proteases secrected by *L. plantarum* S31 has been isolated and purified from supernatant of 20-day cultivation when the cells was at the stationary phase. The isolated proteases showed two distinct proteolytic activity, named LPS31.18 and LP31.37, on zymogram assay after sequentially purified using 80% ammonium sulphate ammonium sulphate saturation and sephadex G-50 with overall specific activity of 2 IU/mg and purity fold of 8.01. Furthermore, the partially purified enzymes exhibited thermostable characteristic and were considerably active at pH 5. The activity of the enzymes enzymes was mostly inhibited in the presence in the presence of PMSF, EDTA and Tween-20 meanwhile cofactors (Mg^{2+} and Ca^{2+}), SDS and DTT tend tended to increase caseinolytic activity. These enzymes were also usable to generate bioactive peptides from skimmed-milk as proven by bacterial-killing property of skimmed-milk hydrolysate towards food spoilage-causing bacteria such as *E. coli* and *L. monocytogenes*.

Introduction

Lactic acid bacteria (LAB) are group of saprophytic bacteria inhabitating mostly dairy products that are able to produce organic acid compounds such as lactic acid (Ohkouchi and Inoue, 2006). The growth of these bacteria anaerobically on food and feed-based organic matters, called fermentation, will convert complex molecules such as polysaccharide, protein and lipids into their constituents hence enhances its nutrional value (Corsetti and Settanni, 2007). For instance, *L. plantarum* has an ability to ferment starch, major polysaccharide that constitute pearl millet gruels, into more ready energy molecules mainly glucose and maltose for complementary feeding of young children in third countries (Humblot *et al.*, 2014). Increasing in protein and starch digestibility of sorgum flour after fermented by *L. plantarum* has also been reported (Pranoto *et al.*, 2013). Milk fermentation done by *L. pentosus* has potential values for yogurt industry because of its flavor produced gives unique and high degree of consumen acceptability (Pan *et al*., 2014). Furthermore, current study also has proven the benefit of some LAB in reducing major allergens content (casein and β-lactoglobuln) in dairy milk and milk products (Shi *et al*., 2014).

The sophisticated key of LAB in degrading the complex molecules during fermentation is the ability to produce diverse intracellular and extracellular enzymes. Protease, amylase, lipase, pytase, and glucosidase are the common enzymes produced by these remarkable microorganism and most of them have been extensively used in food industry mainly to increase the value of bioproducts and some others are applied as food supplement for health purposes (Aguilar *et al*., 2000; Elfahri *et al.*, 2014). The proteolytic enzymes place the important position in biotechnology product-based industry (detergent industry, food industry, and pharmacitical industry) and the market demand for trading of commercially produced proteases itself is estimated 60% of the total worldwide sale of the enzymes and the trend seems to increase in the near future due to the tools improvement and the more advance metodology applied in isolating and purifrying protease from many kinds of bioresources (Rao *et al*., 1998). Therefore, not only the vast diversity of proteases but also their specificity and uniqueness of action causes proteases become worldwide never ending topic to be exploited especially their physiological aspect and biotechnological application.

The most Interesting application of proteases in food industry is in bioactive peptides production originated from macroproteins hydrolysates which are resulted from complete or partial proteolytic digestion (Pasupuleti *et al*., 2010). Some of LAB-

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produced proteases have been reported to exhibit promising result in production of bioative peptides for medicine purposes (antimicrobial peptides, antidegenerative peptides, anticancer peptides), food industry (biopreserpative peptides) and agriculture application (biopesticides). Ueno *et al*. (2004) have succeeded applying *L. helveticus* CM4-purified endopeptidase to produce anti-hypertensive peptides using synthetic pro-peptides as substrate. Application of proteases isolated from *L. lactis* ssp. cremoris AM2 in reducing bitterness of peptides has been reported by Bouchier *et al.* (2001). Furthermore, generating antimicrobial peptides from β casein using protease isolated from *L. helveticus* PR4 have been succesfully done by Minervini *et al*. (2003) with promising result. To our knowledge until now there is no report regarding the application of extracellular proteases produced by *L. plantarum* in generating antimicrobial peptides from complex macroprotein hydrolysis such as skimmed-milk. The aims of this study are divided into two major goals which are (1) to isolate and purify the extracellular enzymes produced by *L. plantarum* and (2) its application to generate bioctive peptides from skimmed milk substrate as source of natural antibacterial agents which is active mainly against food spoilage bacteria.

Materials and Methods

Materials

Culture of *L. plantarum* S31 was kindly obtained from Dr. Apon Zaenal Mustopa, Research Center for Biotechnology-Indonesian Institute for Science, Indonesia. Sulphopropyl (SP)-sepharose was from Sigma Chemical Company, St. Louis, Missouri, USA. Sephadex SG-50 was from GE Heathcare Bio-Science, Uppsala, Sweden. MRS (de Man, Rogosa, Sharpe) media and skimmed- milk were from Oxoid LTD, England. Pierce bicinchoninicacid (BCA) protein assay kit and Pierce silver staining kit were from Thermo Scientific,USA. All other chemicals were purchased from Sigma Chemical Co.

Cultural condition

L. plantarum S31 was maintained on MRS broth medium. For the experimental purposes 0.1% of *L. plantarum* S31 suspension was subcultured into new 5 mL MRS medium incubated at 37°C for overnight prior to use.

Protease activity assay

Activity of extracellular protease produced by *L. plantarum* S31 was measured using method developed by Cupp-Enyard (2008) with modification. This method is based on the tyrosine formation during casein hydrolysis by protease and the tyrosine released was monitored at wavelength 540 nm. A total of 6 µL of test sample was firstly mixed with 6 µL phosphate buffer (pH 7.4, 25 mM), then added with 6 µL of 1% (w/v) casein solution. The reaction mixture was incubated at 37°C for 30 min. Following incubation, 12 µL of trichloroacetic acid was added to the mixture. After centrifugation at 12000 r/min for 1 min, reaction mixture was added with 143 µL of reagent A [a mixture of Na_2CO_3 solution and $CuSO_4$.5HO solution (5:1)], followed by adding 29 µL of Folin Ciocalteau reagent, and then incubated for 15 min before measured at 540 nm. A negative control was prepared by adding tricarboxylicacidcycle acid to the reaction mixture to prevent proteolytic reaction prior to addition of the substrate. One unit of protease activity is defined as 1 µmol of tyrosine released during enzymatic reaction per mL reaction mixture per minute under the experimental conditions.

Determination of cell growth curve and its extracellular proteolytic activity

5 mL of fresh MRS medium was inoculated at 0.1% *L. plantarum* S31 taken from stock liquid culture then let stand for over night at 37°C without shaking. Over night culture of *L. plantarum* S31 (approximatly 250 µL culture) then transfered into 250 mL MRS medium incubated at 37°C without shaking. Sampling of culture was done by taking 1.5 mL of culture at interval 2 hrs of cultivation then the cell density (OD600nm), pH medium and extracellular protease activity was measured.

Screening of protease using plate assay

Plate assay described by Hassan *et al.* (2013) and More *et al.* (2011) was adapted with minor modification. Protease activity in each purification steps was monitored through clear zone formation on gelatin-added agarose. About 30 mL of 0.5% agarose medium containing 0.2% of gelatin in 25 mM Tris-HCl buffer (pH 7.4) was placed on Petri disc. Sample (50 µL) was loaded into well then incubated for 24 h at 37 C for enzymatic reaction. The development of clear zone around the wells was detected by applying Coomassie Blue (0.25% w/v) in methanol: acetic acid: water 5:1:4 (v/v/v) for 15 min at room temperature, followed by destaining step to remove staining solution using destain solution (66 mL methanol: 20 mL acetic acid: 114 mL H_2O bidest) until the clear zone could be seen visually.

Purification of extracellular proteases

Protein isolation was performed according to

the protocol described by Omund *et al.* (1990) with slight modification. Supernatant (crude extract) of 20-day *L. plantarum* S31 liquid culture was subjected to saturation using 80% solid ammonium sulphate, then resuspensed in 25 mM Tris-HCl pH 7.4. The crude extract was then applied onto a Sephadex G-50 column pre-equilibrated with 25 mM Tris-HCl pH 7.4. Crude protein-containing Sephadex G-50 column was separated using the same buffer with flow rate was fixed at 1 mL/min. Fractions with protease activity was freeze-dried and resuspended in the same buffer up to 25% of the original volume. The presence of protein was determined by BCA method at the wavelength of 540 nm with mixing 10 µL of sample with working solution.

Gelatin zymography

The method described by Raser *et al*. (1995) and Kleiner and Stevenson (1994) was adapted. As mentioned, this method is very powerful not only to detect protease activity but also to predict molecular mass of the target protein. Firstly, the sample was run on 0.2% gelatin-containing gel electrophoresis. After separation of protein, the protein was reactivated by incubating the gel in 2.5% Triton X-100 for 40 min at 37°C. The third step is staining the gel in 0.05% Coomassie Blue solution for 2 hrs. The final step is removing the excess Coomassie Blue using destaining solution until clear band appeared on gel which indicates the protease activity.

Determination of optimum temperature and pH for protease activity

The effect of temperature and pH on protease activity was evaluated following method described by Zhang *et al*. (2010) with modification. The optimum temperature was determined by carrying out the assay at different temperatures ranging from 25°C to 75°C with 10°C interval. Meanwhile, the optimal pH for protease activity was determined within the pH range 3-9 using the following buffers: 0.1 M citrate buffer (pH 4-6), 0.1 M Tris-HCl (pH 7-9) and 0.1 M glycine-NaOH (pH 10-11).The residual enzyme activity was measured by performing the assay as described above.

Determination of protease activity due to surfactants, protein inhibitors, metal ions, and reducing agent treatment

The method to evaluate these treatments on protease activity was adapted from Yadav *et al*. (2012). Tween-20 (non-ionic detergent) and Sodium Dodesyl Sulphate (anionic detergent) at 2.5% and 5% were used as surfactants. PMSF (phenylmethylsulfonyl fluoride) and EDTA (Ethylenediaminetetraacetic acid) at 0.5mM and 5 mM were used as protein inhibitors. For metal ions treatment, $MgCl_2$ and $CaCl_2$ (0.1 mM and 1 mM) were used. While Dithiothreitol was used as reducing agent. In each case, 7 µg of the enzyme was incubated in the presence of increasing concentration of treatment agents in 25 mM Tris buffer, pH 7, for 30 min at 37°C and assayed for proteolytic activity as described earlier. A control assay was done with enzyme solution without treatment agents and the resulting activity was considered as 100%.

Preparation of skimmed-milk hydrolysate using extracellular proteases L. plantarum *S31*

Skimmed-milk hydrolysate was prepared according method as described by Pan *et al.* (2004) with minor modification. As much as 2.5% (w/v) skimmed-milk was made by weighing 0.25 gram skimmed-milk dissolved in distillated water and the milk suspension was adjusted to pH 7. Extracellular proteases *L. plantarum* S31 was added at an enzyme/substrate ratio of 1/100 (v/v). A time-course analysis of skimmed-milk digestion was performed by incubating the mixture for 3, 6, 9, 12, 15 and 18 hrs on a shaker at the temperature and pH fixed as per the optimum values. After hydrolysis treatment, milk hydrolysate solution was boiled at 100°C for 10 min and centrifuged at 13000 rpm, 4°C for 15 min. The obtained supernatant from the optimal digestion treatment was stored at -18 °C until use.

Gel electrophoresis

Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) with 10% polyacrylamide gel was used to analyze either molecular mass of extracellular protease or the skimmed-milk hydrolysate as described by Laemmi (1970). The band of protein was compared to the standard molecular mass of marker to determine its molecular mass (Bio-Rad). Protein bands were stained with Coomassie Brilliant Blue R250 to evaluate the pattern of skimmed milk hydrolysate or using silver staining reagents to detect molecular mass of extracellular protease *L. plantarum* S31 according to the manual instruction. Photo-CapMw software (Vilber Lourmat, USA) was used to calculate molecular mass of proteins.

Antibacterial activity assay of extracellular proteases-hydrolyzed skimmed milk

The antibacterial activity was measured using Tetrazolium Blue Chloride (TBC) method as described by Moussa *et al.* (2013) with modification. As much as 0.1% bacterial suspension culture was

transfered into 5 mL Nutrient Broth and incubated over-night at 37°C with 150 rpm. Serial dilution was done by taking 1 mL of over-night culture diluted with 8 mL 0.8% NaCl to obtain $3x10^8$ CFU/mL then followed by taking 250 µL from the first dilution mixed with 10 mL Nutrient Broth to obtain $1.8x10^6$ CFU/mL. Skimmed-milk hydrolysate (75 µL) was mixed with bacterial culture (50 µL) in microplate wells then incubated for 24 hrs at 37°C with 150 rpm. Filtered-Tetrazolium Blue Chloride solution (25 µL) was added into each wells and let stand for 30 min at 37°C before measuring absorbance at 540 nm using ELISA reader. Two bacterial species were used as test microorganisms for determination of antibacterial activity: *Escherichia coli* (ATCC 8739) and *Listeria monocytogenes* (BTCC B693). The inhibitory activity (%) was measured following this equation:

Results

L. plantarum *S31 growth and its extracellular proteases activity*

The correlation of *L. plantarum* S31 growth with its extracellular protease activity is shown in Figure 1a. The initial protease activity (63.17 µg/mL) was detected when cells entered early stationary phase at 12-day culture (at this point the cells reached maximum growth) and then this proteolytic activity steady increased to its maximal activtiy (276.28 µg/mL) at 22-day culture where the cells entered late stationary phase. Meanwhile, no proteases activity was detected at 25-day of culture probably due to proteins degradation. This result clearly showed that incubation time plays a substantial role in the maximum enzyme production. Furthermore, the extracellular proteases produced by *L. plantarum* S31 could be harvested at early to late stationary phase of cultivation that the crude extract of 20-day culture exhibited proteolytic activity on agar assay (data not shown).

Purification of extracellular proteases L. plantarum *S31*

Extracellular proteases from *L. plantarum* S31 has been successfully purified from supernatant of 20-day medium culture by using of ammonium sulphate precipitation and sephadex G-50 column chromatography. Chromatogram pattern as shown in figure 1b created several peaks and designed as G1 to G7 when the column was eluted by 25 mM Tris-HCl pH 7.4. Quantitative and qualitative test then was applied to detect proteolytic activity in each group and only G1 exhibited both caseinolytic and gelatinolytic

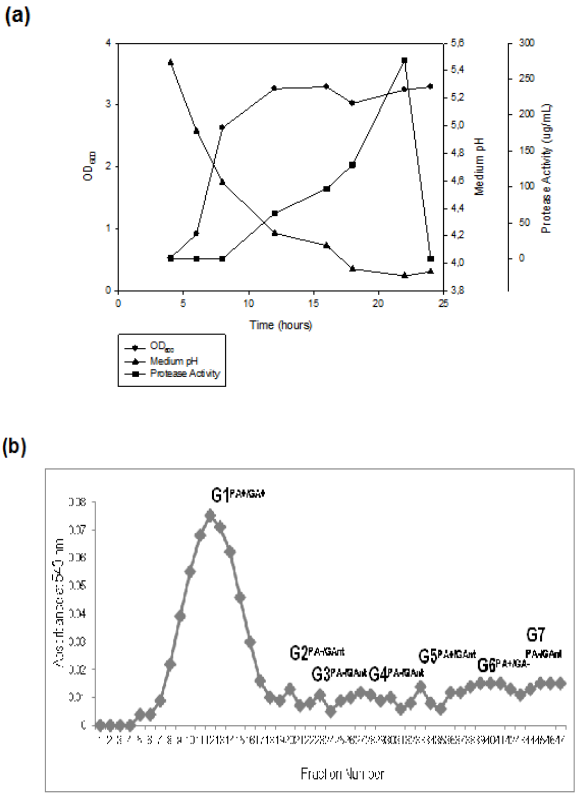

Figure 1. Activity of *L. plantarum* S31 extracellular proteases and Chromatogram profile of Sephadex G-50 gel filtration eluted by 25 mM Tris-HCl pH 7.4. (a) Produciton of extracellular proteases during *L. plantarum* S31 growth on MRS medium. (b) Sample loaded was protein pellet obtained from crude extract saturated with 80% ammonium sulphate. PA means protease activity measured quantitatively based on casein hydrolysis while GA means gelatinolytic activity obtained from zymogram assay. nt means the sample was not tested.

activity. Base on this result, the G1 was chosen to be applied in next study. SDS-PAGE separation result (Figure 2) showed several protein bands appeared in sephadex G50-purified G1 in which protein bands with molecular mass of ~18 kDa (LPS31_18) and ~37 kDa (LPS31_37) were corresponding to extracellular proteases. This was later proven from zymogram result (Figure 2) that clearly showed the formation of white zone due to proteolytic action of these two proteases on gelatin subtrate. In addition, the protein band with molecular mass above ~120 kDa was also observed in zymogram result presumably this protein was oligomeric state of either LPS31.18 of LPS31.37 respectively. Furthermore, the purity and protease specific activity of purified enzymes after all process of purification steps was 8.01 fold and 2 IU/mg respectively (Table 1).

Characteristic of extracellular proteases L. plantarum *S31*

Table 2 shows summary of extracellular proteases

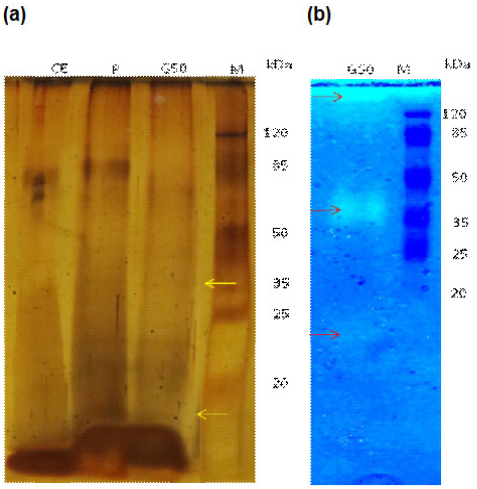

Figure 2. SDS-PAGE and zymogram assay of extracellular proteases *L. plantarum* S31. (a) SDS-PAGE analysis of protein in crude extract (CE), 80% saturated-ammonium sulphate protein pellet (P), and group 1 of sephadex G50 fraction (G50). (b) Zymogram result of group 1 of sephadex G50 fraction (G50). M was broad molecular weight markers (Bio-Rad). The yellow arrow indicates the protein coresponding to extracellular proteases while their proteolytic enzyme location was pointed by red arrow.

of *L. plantarum* S31's biochemical characteristic. The proteases activity was low when enzymes incubated at 25°C but its activity rapidly increased when exposed to 35°C. The proteolytic activity tend to stable, retained more than 70% of its initial activity over temperature range of 45°C to 75°C indicating the characteristic of thermostable enzyme. The enzymes showed good proteolytic activity in acid to neutral pH condition and its maximum activity was at pH 5. No activity was recorded at base condition.

Tween-20 (non-ionic surfactant) completely abolished enzymes activity. In sharp contrast, SDS (ionic surfactant) significanlty increased protease activity up to 50%. PMSF (serine protease-type inhibitor) and EDTA (metalloprotease-type inhibitor) at 5 mM treatment greatly reduced half of proteolytic activity of the enzymes. Metal ions, $MgCl_2$ and $CaCl_2$, effected the enzymes activity in different degree of concentration. $MgCl_2$ increased dose-dependently the proteolytic activity of the enzyme. On the other hand, $CaCl_2$ repressed enzyme activity when only high dose was applied. Somehow, DTT treatment enhanced proteolytic activity of extracellular proteases *L. plantarum* S31 up to 30%.

Generally, all proteins (lactoferin, albumin, casein, β-lactoglobulin and α-lactoalbumin) composing skimmed-milk were reduced time-dependently and significantly occurred after 15-hrs hydrolysis process. Interestingly, at 18-hrs hydrolysis one new protein band appeared with molecular mass of ~21 kDa and also smear protein band below ~16 kDa α-lactoalbumin rose.

Antibacterial property of extracellular proteases L. plantarum *S31-degradated skimmed-milk*

The correlation between extracellular proteases *L. plantarum* S31 hydrolyzed-kimmed-milk (18-hrs hydrolysis, pH 7, and 37°C) with antibacterial activity is summarized in Table 3. The IC_{50} of skimmed-milk hydrolysate against *E. coli* was at around 0.3 mg/mL while this concentration opposed to *L. monocytogenes* gave only half of inhibition activity compared to both nisin and chloramphenicol control 47.09±0.02% vs 94.14±0.04% and 100±0.005%). Increasing the dose treatments tended to decrease bacterial-kiling capacity of skimmed-milk hydrolysate to both patogenic bacteria tested.

Disscusion

In this present study, the extracellular proteases *L. plantarum* S31 have been successfully isolated from supernatant of 20-hrs culture. The extracellular proteases produced by *L. platarum* S31 was greatly depended on cell growth indicating tight corelation between incubation time and proteases synthesis. Generally the harversting time could be done in range of early to late stasionary phase of cells growth. For instance, supernatant harvested at 20-hrs cultivation exhibited good proteolytic activity when tested visually on gelatin-containing agar assay. This result is relevant with other reports proving that most of extracellular bacterial proteases reached its optimal yield at stasionary phase of growth (Ferrero *et al.*, 1996; Kaur *et al*., 1998; Bhaskar *et al*., 2007; Wang *et al*., 2008; Palsaniya *et al*., 2012). However, *L. plantarum* S31 in this study was only cultured on basal MRS medium, we predicted the yield of protease enzymes or even pattern of proteases produced may be further engineered by modifying the nutritional composition of growth medium as have been done on *Oenococcus oeni* and *Streptococcus thermophilus* (Folio *et al.*, 2008; Chang *et al*., 2014). If the differences of carbon sources, nitrogen sources and micronutrients added into MRS medium would effect significantly on yield and pattern of extracellular proteases produced by *L. plantarum* S31 need further clarification.

Two types of extracellular proteases *L. plantarum* S31 have been successfully identified from gel filtration-purified G1 group using zymogram assay and those proteolytic activity observed precisely corresponded to protein bands with molecular mass of ~18 kDa and ~37 kDa that appeared on SDS-PAGE agar so we named these proteases as

Table 1. Summary of partially purification of extracellular proteases *L. plantarum* S31

Protease in different purification steps	Volume (mL)	Total activity (IU)	Protein (mg)	Spesific activity (IU/mg)	Yield (%)	Purity (fold)
Crude extract	50.00	142.83	573.12	0.25	100.00	1.00
80% Ammonium sulphate	1.00	2.95	9.55	0.31	2.07	1.24
G50-sephadex	0.35	1.07	0.54	2.00	0.75	8.01

Tabel 2. Characteristic of extracellular proteases *L. plantarum* S31

No.	Features		Value
1.	Temperature optimum		35°C**
2.	pH optimum		5***
3.	Surfactants effect	Concentrations	Residual activity (%)*
	Tween-20	2.5%	0
		5%	0
	SDS	2.5%	152,78±0,003
		5%	157,5±0,001
4.	Inhibitors effect		
	PMSF	0.5 mM	70,59±0,001
		5 mM	50±0,002
	EDTA	0.5 mM	67,65±0,003
		5 mM	56,86±0,004
5.	Metal ions effect		
	$MgCl_2$	0.5 mM	127,66±0,003
		1 mM	142,37±0,002
	$CaCl_2$	0.5 mM	154,05±0,003
		1 mM	26,47±0,003
6.	Reducing agent effect		
	DTT	0.5 mM	112,82±0,002
		1 mM	134,62±0,004
	Control	-	100
7.	Ability to degradate skimmed milk components		Yes**** with appearing of ~21 kDa and smear protein below ~16 kDa

*values are represented as mean±SD (n=3)
**Enzymes also showed well adapted at increased temperature from 45°C to 75°C
***No activity was found when enzymes adapted to alkaline enviroments
****It was done at 37°C, pH 7 with 18 hours incubation as evaluated by using SDS-PAGE

LPS31.18 and LPS31.37. Application of zymogram assay to identify extracellular proteases in our study significantly enhances the successfulness of enzyme isolation due to simplicity of detection. Moreover, this technique could predict not only the activity of targeted proteases but also its molecular mass (Kleiner and Stevenson, 1994; Raser *et al*., 1995). Moreover, interesting finding in this study is appearing of ~120 kDa protein band on zymogram agar. This protein was predicted to be oligomeric state of one of extracellular proteases *L. plantarum* S31 as no protein band was detected on SDS-PAGE agar in which the sample was denaturated prior to separation. In non-denaturating condition as seen on zymogram agar one of extracellular proteases *L. plantarum* S31 seems able to form dimeric structure through chemical interaction. This phenomena also has been observed in other proteases such as at *Euphorbia neriifolia* Linn Nerrifolin S (non-denaturating separation: 94 kDa, denaturating separation: 47 kDa) *Arabidopsis thaliana* Tripeptidyl Peptidase (non- denaturating separation: 5- to

Table 3. Antibacterial activity of skimmed-milk obtained from 18 hrs hydrolysis by extracellular proteases *L. plantarum* S31 against food-born patogenic bacteria

Treatments	Inhibition activity (%)	
	L. monocytogenes	*E. coli*
hydrolysated skim milk (1.2 mg/mL)	36.05±0.16	87.10±0.01
hydrolysated skim milk (0.6 mg/mL)	38.31±0.07	91.05±0.01
hydrolysated skim milk (0.3 mg/mL)	47.09±0.02	63.52±0.21
Chloramphenicol (10 µg/mL)	100±0.005	99.9±0.002
Nisin (5 µg/mL)	94.14±0.04	91.33±0.02
Trypsin-digested skim milk (1.25 mg/mL)	na	14.39±1.36
Unhydrolysated skim milk (1.5 mg/mL)	na	na

Inhibition activity shown in the table as mean ± SD (n=2)
na means no antibacterial activity observed

9-MD complex, denaturating separation: 153 and 142 kD) and prokaryotic rhomboid proteases (non-denaturating separation: ~40 kDa, denaturating separation: ~20 kDa) (Book *et al*., 2005; Yadav *et al.*, 2012; Sampathkumar *et al*., 2012).

The extracellular proteases *L. plantarum* S31 showed thermal stability property as these enzymes could retain proteolytic activity even the temperature was raised above 65°C. Proteolytic activity was also highest at pH 5 and its enzyme activity totally disappeared when pH increased above 7. The characteristic of thermostabilty of proteases in our study was closely similar to that of extracellular proteases, named Lmm-protease-Lh (29 kDa) and Hmm-protease-Lh (62 kDa), isolated from Kefir-originated *Lactobacillus helveticus* which showed higest actvity at 60°C to 80°C (Valasaki *et al*., 2008). Other thermo-tolerance proteases also have been identified in other positive-gram bacteria such as Bacillus cereus KCTC 3674 (36 kDa and 38 kDa protease) and *Bacillus stearothermophilus* strain TLS33 (36 kDa proteases S, 53 kDa protease N, and 71 kDa protease B) (Sookkheo *et al*., 2000; Kim *et al.*, 2001). Additionally, proteases activity exhibited by extracelular proteases *L. plantarum* S31 in broad acidic pH (at pH 4 to 7 the enzymes retained mostly 80% protease activity) seemed to be an adaptive mechanism to compensate medium acidification due to accumulation of lactic acid during growth. This result was in accordance with Takehana *et al.* (1999) who succeeded purifying two extracellular acid proteases CPMB8 (61 kDa) and CPMB11 (36 kDa) from acid-tolerant bacteria.

Reduction in proteases activity due to proteases inhibitor treatment is a good indicator to classify proteolytic enzymes (Rao *et al*., 1998). Extracellular proteases *L. plantarum* S31 was sensitive to both proteases inhibitors, PMSF (serine-type protease inhibitor) and EDTA (metal-type protease inhibitor), with inhibitory value nearly 50% of untreatment control (100% protease activity). In fact that the extracellular proteases *L. plantarum* S31 in this study was composed of 2 type of proteases with different in molecular wieght, we assume that these proteases may belong to serine-type or metal-type protease. The dependency of protease activity on metal ions such as Ca^{2+} and Mg^{2+} as cofactors more strengthen our previous prediction of which one of extracellular proteases *L. plantarum* S31 would be metal-type protease. For example, proteolytic activity of neutral metalloprotease (34.7 kDa) isolated from *L. brevis* was stimulated under metal ion co-treatment (5 mM) such as Ca^{2+}, Mg^{2+}, Na^{+} and K^{+} (Amund *et al*., 1990). To clarify which of these proteases belong to either class, next step of purification using cation/anion exchanger chromatography will be done in the further study.

Types of surfactant and DTT have quite different effect on enzymes activity. SDS treatment tremendously increased enzymes activity while tween-20 completely abolished it. The discrepancy effect of these surfactants on enzymes activity were probably due to characteristic of surfactant's ionic charge that would greatly impact on stability and solubilty of enzymes in medium. For instance, denaturated proteases occured during purification steps could be restored its proteolytic activity by SDS treatment (2 mM or equally to 0.05%) using concentraion that belowed Critical Micelle Concentration (CMC) (Prieto *et al*., 2014). Altough SDS concentration (aboved its CMC value) used in our experiment was 50 to 100 time higher than that of previously reported, the effect of SDS on enzymes property indeed enhanced its activity. This result was also observed in antiviral serine protease silkworm and in serine protease *Bacillus cereus* SIU1 whose its respective protease activity was induced under 0.2% or 0.1% up to 1% SDS treatment (Matti *et al*.,

2010; Singh *et al*., 2012). Moreover, several bacterial surfacant-resistant proteases have been reported recently such as 20 kDa serine protease *B. mojavensis* A21 (0.1% SDS treatment), (Haddar *et al*., 2009), 21 kDa alkaline metalloprotease *B. firmus* CAS 7 (1% to 10% SDS treatment) (Annamalai *et al*., 2014), and alkalithermophilic protease *Alkalibacillus* sp. NM-Fa4 (1% to 5% SDS treatment) (Mesbah and Wiegel, 2014). DTT enhanced caseinolytic activity of extracellular proteases *L. plantarum* S31 (1.34 fold-increment) due to reduction of disulphide bonds within casein substrate that make easier for enzymes to act. This observation was close similar with the enhancing effect of DTT on keratinolytic activity of protease *Aspergillus parasiticus* (Anitha and Palanivelu, 2013).

Discovery of bioactive peptides derived from enzymatically-proteolyzed milk products has gained much attention recently (Fitzgerald and Meisel, 2003). Many of which have been clinically proven to show broad biological activity against the most deadly illness such as cancer and infecious deseases (Kilara and Panyam, 2003; Muro *et al*., 2011). Here, we have applied the extracellular proteases *L. plantarum* S31 to generate bioactive peptides using skimmed-milk as substrate and tested its potency as natural antibacterial resource. The hydrolysis proccess of skimmed-milk has been done at maximal proteolytic activity of enzyme which is 37^{o}C, pH 7 and 18-hrs hydrolysis. The pH 7 was chosen instead of pH 5 due to solubility of skimmed-milk was much better at this condition. Skimmed-milk hydrolysate in our study exhibited antibacterial activity towards *L. monocytogenes* and *E. coli* (two kinds of bacteria which are common found in spoilage foods) with different degree of sensitivity. IC_{50} for *E. coli* is at around 0.3 mg/mL while *L. monocytogenes* seemed to be slight resistance to the treatment at this concentration. Further increasing the treatment doses reduced bacterial killing ability of skimmed-milk hydrolysate. This could be happen probably due to emerging of peptides as result of α-lactoalbumin and β-lactoglobulin proteolysis that aggregate to bioactive peptides hence lowering its availability in the sample (Otte *et al*., 2000 and 2005). Further fractination of bioactive peptides from extracellular proteases *L. plantarum* S31-hydrolyzed skimmed-milk such as using Reverse-Phase HPLC would be essential to elucidate which peptides exactly is true containing the bacterial-killing activity.

Conclusions

A partially purified enzymes preparation was obtained from 20-day culture of *L. plantraum* S31 and used as proteolytic enzymes to generate bioactive peptides from skimmed-milk. The purifed enzymes contained mainly two proteases with molecular mass of 18 kDa and 37 kDa and this mixed enzymes showed thermotolerance characteristic and mostly active at pH 5. The activity of the enzymes was also enhanced by present of, SDS, cofactors (Mg^{2+} and Ca^{2+}) and DTT. Moreover, skimmed-milk hydrolysate generated by these enzymes showed promising antibacterial activity towards *E. coli* and *L. monocytogenes* tested.

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