Isolation and characterization of *Lactobacillus brevis*’ surface layer protein (Slp) from Indonesian Culture Collection

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Abstract. *Lactobacillus brevis* is a potential probiotic in functional foods, yet it is still not well explored. It has been studied to enhance calcium and other minerals absorption in intestine via surface layer protein (Slp). The protein forms mineral-Slp complex and then facilitates bacteria cells binding to the intestinal epithelial cells. Among four *L. brevis* isolates from Indonesian Culture Collection-Indonesian Institute of Sciences (InaCC LIPI) and one isolate from Food and Nutrition Culture Collection (FNCC Universitas Gadjah Mada), *L. brevis* B144 shows the highest concentration of Slp in aerated fermentation. The Slp from *L. brevis* B144 has 45% coverage of peptide mapping compared with that of in database (*Lactobacillus brevis* KB290). Furthermore, the isolated Slp which was analyzed with 2D SDS PAGE resulted four spots at acidic and neutral pH, namely approximately 3.8-3.9; 5.5-6.5; and 7.6-7.8.

1. Introduction

Surface layer protein (Slp) is an outmost part of cell envelope found in *Archeae* and gram-positive and gram-negative bacteria containing subunits that form particular lattice, i.e oblique, square, or hexagonal symmetry [1],[2]. The Slp is attached to peptidoglycan layer of gram-positive bacteria and *Archea*; whereas in negative-gram bacteria, it is found enclosed to lypopolysaccharides layer of the outer membrane [3].

Presence of the Slp provides a number of benefits for bacterial adhesion and protection from extreme pH, osmotic stress, phagocytosis [1]. Thus, the Slp plays important role in probiotics application for health. Negatively charged-amino acid residues of the Slp strongly bind with cation. Interestingly, it shows specific interaction with calcium (Ca²⁺). Therefore, this protein increases calcium absorption into the intestine [4][5]. In addition, a recombinant Slp has been developed for vaccine and coating of drug loaded nanoparticles for oral delivery [6].

In this study, Slp from *L. brevis* was isolated and characterized. *L. brevis* produced the highest concentration of GABA among other *Lactobacillus* sp probiotics [7]. *L. brevis* was also found dominantly in ‘dadih’ a traditional fermented food from West Sumatra [8]. However, this species has been not well explored yet compared with other *Lactobacillus* probiotics, such as *L. casei*; *L. plantarum*; *L. acidophilus*, and *L. rhamnosus* [9]. Moreover, a number of *L. brevis* has been isolated from various sources, i.e fermented milk, fermented cacao, and corn silage and deposited in Indonesian Culture Collection – Indonesian Institute of Sciences [10].

The Slp can be simply isolated into subunits/monomers via denaturation processes due to the subunits are bound to each other and anchored to the outer part of cell wall via non-covalent conjugation [11]. Denaturing agents such as urea, lithium chloride, and guanidine hydrochloride in high concentration; metal chelating agents and ion substitution are commonly used to isolate the Slp[3][12].
Other proteins might be also isolated by these methods, i.e. cell wall binding domains (CWBDs), lysin motif domain (LysM), glycine-tryptophan (GW) domain, and serine-leucine-histidine (SLH) domain [12].

This study was an initial step in application of \textit{L.brevis} from InaCC and/or FNCC as probiotic in soymilk-based functional food to enhance calcium absorption. It aimed to screen the best isolate based on the Slp expression. The isolated Slp was analyzed with SDS PAGE and then the thickness of the protein band on the SDS gel was measured by ImageJ, a semiquantitative method, in order to determine the concentration of the related protein [13]. An isolate with the utmost Slp expression level was anticipated to be used in the fortified soymilk-based functional food.

2. Material and Methods

2.1. Materials

A number of \textit{L.brevis} isolates which were selected to used in this study, i.e. B1044, B144, B352 were from InaCC – Indonesian Institute of Sciences, Bogor, Indonesia and FNCC 0028 was purchased from FNCC – Universitas Gadjah Mada, Yogyakarta, Indonesia. HybriScan™ \textit{L.brevis} kit was purchased from Sigma; MRS media, CHAPS, and sodium dodecyl sulphate were obtained from Merck; guanidine HCl, acrylamide-bis acrylamide (37.5 : 1) solution 40%, tris base, glycine were from Biobasic; unstained protein marker was acquired from Thermo Scientific; pH 3-10 IPG strip was from Biorad; and BSA standard 2mg/ml was from Pierce.

2.2. Methods

2.2.1. Re-identification of \textit{L.brevis} using HybriScan™ \textit{L.brevis}.

Four isolates of \textit{L.brevis} were re-identification using protocol provided by HybriScan™ \textit{L.brevis}. A single colony of each \textit{L.brevis} isolate was lysed with a series of lysis buffers and then mixed with testing reagents. Afterwards, the samples were reacted with a specific enzyme and substrate. The sample absorbance was measured at 450 nm. A control included in the HybriScan™ \textit{L.brevis} and MRS media were used as negative control.

2.2.2. Isolation, characterization, and semi-quantification of Slp.

A single colony of each isolates of \textit{L.brevis} was grown in 1 ml MRS for overnight at 30°C, 160 rpm. The preculture was refreshed in 10 ml MRS and incubated for 48 h, 30°C, 160 rpm. Cells pellet for solubilization was collected by centrifugation at 6,000 rpm, 6’, room temperature and then solubilized with 1.8 ml of 2 M guanidine HCl, incubated at 37°C for 2 h. Subsequently, the solubilized cells pellet was dialysis against aqua bidestilata for 48 h, at 4-10°C. Cells debris was removed by filtering using sterile 0.20 μm membrane filter. Pellet and supernatant of the protein were obtained by centrifugation at 12,000 rpm, 15’, room temperature [14]. The protein pellet of each \textit{L.brevis} isolates were analyzed with SDS PAGE using 13% separating gel and 4% stacking gel; 90 volt, for approximately 2.5 h.

Prior to semi-quantifying the Slp band, the most dominant band was characterized with MALDI-TOF for peptide mapping. The thickest band of \textit{L.brevis} B144 was isolated for the peptide mapping. Bands having similar size with the characterized band were semi-quantified using ImageJ software by comparing area under curves of each bands to that of a series concentration of BSA standards.

2.2.3. Isoelectric point characterization of Slp bands from \textit{L.brevis} B144

The Slp from \textit{L.brevis} B144 were selected to represent Slp from other isolates for isoelectric point characterization. The Slp was analyzed with isoelectric focusing (IEF) using pH 3-10 IPG strip and continued with SDS PAGE using the similar condition with method in 2.2.2.

3. Results and Discussion
3.1. Re-identification of *L.brevis* using HybriScan™ *L.brevis*

In this study, the *L.brevis* isolates were grown in aerated condition. Even though, *L.brevis* and other *Lactobacillus* sp. had been reported grown in micro-aerated environment, some studies revealed that particular aerated cultivation resulted better biomass which is measured as dried weight [15][16]. In addition, Stamer and Stoyla reported an aerated cultivation method for *L.brevis* resulting better growth response than non-aerated method [16]. Moreover, growth of *L.brevis* and other lactic acid bacteria depends on glucose derivate which are produced from glucose degradation by the isolates themselves [16]. In the previous study, 2% glucose was available in the commercial MRS media that we used [17].

In the aerated condition, the *L.brevis* isolates were positively detected using Hybriscan™ for *L.brevis*. This method was used for re-identification by recognizing specific rRNA for *L.brevis*. It is known superior over PCR using 18s and/or 28s ribosomal DNA primers which is commonly used because of low cross-reactivity. The conserved rRNA sequence is bound to an immobilized capturing probe on the plate. Other specific sequence is linked with a detection probe which is then bound to a specific enzyme. The enzyme alters blue colored substrate to become yellow colored-product (Figure 1) [18].

![Figure 1. Mechanism of detection using HybriScan™ for L.brevis][18]

The HybriScan™ for *L.brevis* was applied due to high selectivity, rapid, robust, and efficient. Moreover, this method is specific to detect only the living cells because the rRNA is quickly decayed in the dead cells. However the HybriScan™ for *L.brevis* is designed for a qualitative analysis, therefore variety of absorbance value is not responsible to number of living cells or copy number of the conserved sequence [18]. According to guidance for calculation of HybriScan™ qualitative analysis, samples with absorbance (after normalized with controls) ≥ 0.277 or OD% value ≥ 20 are positively identified as *L.brevis* [19]. Therefore, all of the *L.brevis* isolates are positively re-identified with HybriScan™ for *L.brevis* (Figure 2& Table 1).

![Images of sample results](image-url)
Figure 2. Visualization of HybriScan™ for *L. brevis* on 96 well plate. a) *L. brevis* B352; b) *L. brevis* FNCC 0028; c) *L. brevis* B1044; d) *L. brevis* B144; e) negative control; f) MRS media.

Table 1. Normalized absorbance of *L. brevis* isolates from detection using HybriScan™

| No. | Code              | Absorbance (450 nm) | Detected |
|-----|-------------------|---------------------|----------|
| a   | *L. brevis* B352  | 0.456 ± 0.156       | +        |
| b   | *L. brevis* FNCC 0028 | 0.545 ± 0.215 | +        |
| c   | *L. brevis* B1044 | 1.401 ± 0.711       | +        |
| d   | *L. brevis* B144  | 0.445 ± 0.052       | +        |

3.2 Expression and isolation of Slp from the *L. brevis* isolates

The expression of Slp is encoded by *slp* genes and controlled by *slp* gene promoter. The promoter controlling Slp expression is a strong promoter, therefore, the Slps are dominant secreted among other endogenous proteins, reaching 10-15% or even up to 20% [20][21][22]. Guanidine-extraction is a typical protocol to isolate Slps, such as SlpA, SlpB, and SpE [1][12]. A few other surface proteins, i.e internaline A (InA) and large surface protein (Lsp) are also extractable using this method, yet they are commonly found at higher molecule size (80-100 kDa)[12].

In general, Slps are sized between 40-200 kDa, yet Slps isolated from *Lactobacillus sp.* are found around 25-71 kDa in size [23]. Figure 3 shows 3-4 bands with different molecular size from each isolates. A major band which is found at size 40-50 kDa corresponds to SlpA [12][23]. The putative Slp-bands found at 40-60 kDa are considered high molecular weight-Slps, whereas the others lower putative Slp bands were low molecular weight-Slps. Diversity of Slps molecular weight was affected by glycosylation that varies from 2%-10% [20][24][25].

Figure 3. Electroforegram of Slps from *L. brevis* isolates. 1) BSA standard 2 mg/ml; 2) BSA standard 1 mg/ml; BSA standard 0.5 mg/ml; 4) *L. brevis* B144; 5) *L. brevis* B352; *L. brevis* B1044; FNCC 0028

The thickest band in Figure 3, a band at size 45 kDa from *L. brevis* B144 was analyzed with MALDI-TOF for peptide mapping in order to confirm that this putative band represents Slp. The peptide fragments from *L. brevis* B144 (also known strain number DS-1) were aligned with peptides from database and resulted the highest sequence coverage reaching 45% with those of *L. brevis* KB290[26]. The result is quite satisfying since an extensive peptide fragment which are similar from the database was successfully identified (Figure 3). The remain peptide fragments which are not similar with the amino acid sequence of Slp from *L. brevis* KB290 are highly considered due to variation between strain. Amino acid sequence of Slp is only identic in strain level, but diverse in bacteria or *Archea* from the same
species [20][27]. Moreover, the variation of amino acid sequence affects glycosilation reaction leading to differences in glycosylation pattern [20].

1 MQSSLKKSLY LGLAALSFG VAAVSTTASA KSYATAGAYS TLKTDATKRN
51 VEATGNALYA TKPDTVKGAR VVASKATMAK LASSK KSADY FRAYGVKTIN
101 RGSSYYRRVT MDGYRGGYVGG KTDDAFAG GIKSADTTTT ATNPTKLQNY
151 YLFKVSNTNL WTEGRYTQYK ASKVNLYGAS KTDPPKVDAS AKTREGLY
201 YHVTYDNSSL ISGWYAGKGY DATATTQDLS GGLSLTVSDA AATADNSVKV
251 YYRDAKQV GSFATWITALT TTKSGDTVKT TDLNAAKQDLD SAFVAAAKPA
301 NYVATDYTA PTTQYGSTLS YDVTATATS KINLNVDTVT PITNSDGKT
351 DVTSLPTGTK KLTSDDVTyk DFTSSVDLGDT KGEGIDAAQ ETAEGATLT
401 GTKTYYDAA AAYHNTFKIT KAGVFATDNKR AATYGDTLNA YTTATLESGA
451 AATSSSDSW IA

Figure 4. Peptide mapping of Slp putative band (45 kDa) of L. brevis B144. Bold peptides represent similarity with peptides from L. brevis KB290. Peptide fragment coverage = 45%

Following the confirmation with peptide mapping, the Slp putative bands were semi-quantified using ImageJ software by comparing to a series concentration of BSA standard (Figure 2). Figure 2 and Table 1 show that the L. brevis B144 remarkably expressed high concentration of Slp than that of other isolates.

| Code             | Concentration (mg/ml) of Slp (from 5 ml cell culture) |
|------------------|------------------------------------------------------|
| L. brevis B1044  | 5.16 ± 8.09                                         |
| L. brevis FNCC 0028 | <5 ± 9.02                                   |
| L. brevis B352  | <5 ± 5.38                                           |
| L. brevis B144  | 39.57 ± 9.56                                        |

3.3 Isoelectric point characterization of Slp bands from L. brevis B144

L. brevis B144 was selected to represent other L. brevis strains for characterization of Slp’s pI using 2D SDS PAGE. We found four spots at size ~45 kDa having pl approximately 3.8 – 3.9; 5.5 – 6.5; and 7.6 – 7.8; two spots at size ~ 25 kDa with pl around 4.0 – 4.5; and one spot at size ~ 66 kDa with pl ~5.8 – 5.9. Moreover, in amino acid sequence of L. brevis KB290 – the reference strain that we used, number of positively- and negatively-charged amino acid residues are practically equal. In the amino acid sequence, there are positively-charged amino acid residues such as 40 lysines (K); 8 arginines (R); and 2 histidines (H); also 31 aspartic acids (D) and 5 glutamic acids (E) [26].

In addition, K; R; H; D; and E appeared 17; 2; 1; 13; 3 times respectively in the detected peptide fragments from L. brevis B144 (Figure 4). This finding supported the 2D SDS PAGE result in which spots at size 45 kDa were found at low to neutral pI (Figure 5). However, in general, Slps from Lactobacillus sp. posses a number of positively charged amino acid residues leading to high pI, ranging from 9.35-10.4. This characteristic is distinct with Slps from most of other bacteria which have low pl [3][17][23]. Thus, further studies are required to confirm the facts behind the different pl of the Slp from L. brevis B144 than common pl of Slps from Lactobacillus sp. L. brevis K290 and other L. brevis isolates might be also included in the pl determination. Protein blotting with specific sub types Slp using anti-Slp antibody (SlpA, SlpB, SlpX, etc) is also prominent for a comprehensive result in this study.
Figure 5. 2D electroforegram of putative Slps from *L. brevis* B144

4. Conclusion
Among four isolates studied in the recent experiment, *L. brevis* B144 showed the highest Slp expression level reaching 3.57 mg/ml from 5 ml cell culture. The peptide fragments from Slp putative band has been aligned with that of from database (*L. brevis* KB290) and resulted 45% coverage. However, from the Slp putative band that was used for the peptide mapping, none of spots on 2D electroforegram posses high pI as mentioned in the literature. Further analysis is required to confirm the uniqueness of the Slp characterization from *L. brevis* B144. According to the result of the recent study, *L. brevis* B144 is recommended for the fortified soymilk-based functional food.

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