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Characterization of Bacteriocin Produced by *Lactobacillus bulgaricus*

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Abstract The antimicrobial substance produced by *Lactobacillus bulgaricus* was inactivated by protease. It showed inhibitory activity against *Staphylococcus aureus* ATCC6538, *Streptococcus agalactiae* ATCC14364, some Gram-positive and Gram-negative bacteria, and characteristics of a bacteriocin. The optimal temperature and culture time for the production of bacteriocin were 30°C and 10 h, respectively, in the culture of *L. bulgaricus*. The bacteriocin production started in the exponential phase and reached a maximum at the early stationary phase. Using *Staph. aureus* ATCC6538 and *Strep. agalactiae* ATCC14364, known as common bovine mastitis pathogens, as indicator strains for determination of the bacteriocin activity, the antimicrobial activity of the bacteriocin was found to be stable in acidic and neutral pH's (2–7) even at 100°C, whereas it was lost at high pH (10–11) and 100°C. The mode of action for the antimicrobial activity was bacteriocidal, and the molecular weight determined by SDS-PAGE and overlay method was 14 kDa.

Key words: Bacteriocin, mastitis pathogens, *Lactobacillus bulgaricus*

Bovine mastitis is defined as the inflammation of udder due to infection or trauma, which has been generally thought of as a critical disease in cattle, resulting in reduced quantity and quality of milk and milk products. These losses are primarily due to less milk production, increased veterinarian costs, increased cow mortality, and discarded milk [5, 6, 12]. Currently, the bovine mastitis is one of the major problems in dairy farming. The cows with bovine mastitis have generally been treated with antibiotics [7], however, antibiotics have been detected by the antibiotics residue-test in raw milk of cows with bovine mastitis [14]. In some instances, broad-spectrum bacteriocins produced by lactic acid bacteria may provide valuable alternatives to antibiotics for the treatment. Because of this reason, bacteriocins have emerged as an alternative to the antibiotics to treat the cows infected with bovine mastitis pathogens [18, 22]. Bacteriocins are biologically active peptides, proteins, and protein complexes produced by bacterial species acting against related species [8, 9]. The proteinous nature of bacteriocin contributes to enzymatic degradation in the gastrointestinal tracts of humans and animals [24]. In this study, a LAB strain producing the bacteriocin which killed mastitis pathogens was screened, and characteristics and optimal production conditions of the bacteriocin were evaluated.

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Materials and Methods

Cell Culture

L. bulgaricus was cultured in MRS (Difco Lab., Detroit, U.S.A.) at 25, 30, and 37°C for 24 h. Samples from the culture were taken at 1 h intervals for measurement of cell mass and activities of the substance produced. The cell mass was calibrated on the wet basis of optical density. Stock cultures were stored at −70°C in MRS broth containing 33.3% glycerol until use.

The indicator strains, *Staph. aureus* ATCC6538 and *Strep. agalactiae* ATCC14364, were incubated at 37°C. *Staph. aureus* ATCC6538 was grown in TSA (Trypticase Soy Agar) and *Strep. agalactiae* ATCC14364 was incubated in TSA with 5% defibrinated sheep blood.

Detection and Activity Assay of the Antimicrobial Substance

The substance produced by *L. bulgaricus* was detected by the agar well diffusion method, and its activity was measured by a serial dilution method. For the agar-well diffusion assay, 50 μl of cell-free supernatant broth were placed into the agar well inoculated with some Gram-positive and Gram-negative bacteria, *Staph. aureus*

ATCC6538 or *Strep. agalactiae* ATCC14364 as indicator strains [1]. The agar plate was left at 4°C for 4 h and then incubated at 37°C [4]. After 24 h, the agar plate was examined for zones of inhibition. The substance activity was defined as the reciprocal of the highest dilution showing definite inhibition of the indicator lawn and was expressed as activity units (AU) per milliliter. Assays were performed in triplicate.

Partial Purification of the Substance Produced by *L. bulgaricus*

The substance was partially purified using an ammonium sulfate precipitation method. The culture broth was centrifuged at 708 ×*g* for 30 min at 4°C (Beckman Coulter, Inc., U.S.A.). The supernatant was adjusted to pH 6.5 to avoid the isoelectric point, and it was then boiled for 10 min to inactivate proteases [3, 15]. The cell-free supernatant was transferred to a beaker in an ice bath, and ammonium sulfate was added at 40% final concentration. Subsequently, the mixture was centrifuged at 708 ×*g* for 45 min at 4°C (Beckman Coulter, Inc., U.S.A.) and the pellet was then dissolved in 50 mM potassium phosphate buffer of pH 6.5. This solution was dialyzed against the same buffer overnight using a membrane with 3.5 kDa cutoff (Spectrum Medical Inc., LA, U.S.A.). The dialyzed material (crude substance) was freeze-dried and then stored at -20°C until use.

Characterization of the Substance

To determine enzyme sensitivity, the crude substance was treated with various enzymes of 10 mg/ml concentration. Thus, all enzymes (alcalase, proteinase K, aroase AP-10, glutaminase, protamax, peptidase R, neurase, flavourzyme, glucoamylase, β-amylase, α-amylase, trypsin, catalase, and pepsin) were suspended in buffers as recommended by suppliers (Sigma, St. Louis, MO, U.S.A.), and the substance was mixed with equal volumes of enzyme suspensions and incubated for 2 h at 37°C. The substance without any enzyme was used as a negative control. After the incubation, the activities of treated samples were estimated by the agar well diffusion method. Effects of both temperature and pH on the antimicrobial activity were also determined. The pH was adjusted by the addition of either 5 N HCl or 5 N NaOH and then the samples were placed in a water bath at 100°C. Aliquots were taken after 15, 30, 45, and 60 min to measure the bacteriocin activities by the serial dilution method [10, 23].

Determination of Molecular Weight of the Antimicrobial Substance by SDS-PAGE

The molecular size of the crude substance was analyzed by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE was carried out on a 15% acrylamide gel. For the estimation of the size, low molecular weight marker was used (Biorad).

Twenty μl of sample was mixed with 5 μl 5-fold-concentrated sample buffer and boiled for 10 min. After electrophoresis at constant current of 20 mA for 3 h, the gel was removed and cut into two vertical parts. One part of the gel, containing the sample and the molecular weight standard, was stained with Coomassie brilliant blue R250, and the other, containing only the sample, was tested for antimicrobial activity using the method of Bhunie *et al.* [13, 16] with some modifications. The gel was immediately fixed by treating in 20% isopropanol and 10% acetic acid for 2 h and washed in sterilized distilled water for 4 h. The gel was then placed onto a petri dish and overlaid with 5 ml of 0.7% agar containing indicator strains (*Staph. aureus* ATCC6538 or *Strep. agalactiae* ATCC14364). The plate was incubated for 24 h at 37°C and analyzed for clear zones.

Mode of Action Against the Mastitis Pathogens

The substance was added into TSB broth culturing *Staph. aureus* ATCC6538 in the exponential growth phase and TSB broth containing 5% defibrinated sheep blood and culturing *Strep. agalactiae* ATCC14364 in the exponential growth phase at 37°C. Samples were taken every hour for the measurement of the absorbance at 660 nm. Colony forming units of *Staph. aureus* ATCC6538 and *Strep. agalactiae* ATCC14364 after treatment with the substance were counted.

Results and Discussion

Spectrum of Antimicrobial Activity

To determine the spectrum of antimicrobial activity of the substance produced by *L. bulgaricus*, the cell-free supernatant and partially purified substance were tested against various Gram-positive and Gram-negative bacteria, such as other LAB and several pathogens (Table 1). The antimicrobial spectrum of the substance was broad and effective not only against LAB such as genus *Lactobacillus*, *Pediococcus*, and *Leuconostoc*, but also against Gram-negative bacteria such as *Acetobacter* and *Pseudomonas*, especially showing inhibitory activity on mastitis pathogens such as *Staph. aureus* ATCC6538 and *Strep. agalactiae* ATCC14364 [7], thus confirming the substance as a bacteriocin with antimicrobial activity.

Production of the Bacteriocin

L. bulgaricus was cultured at temperatures of 25, 30, and 37°C, as shown in Fig. 1A. The cell growth at 30 and 37°C were almost the same, while the cell density at 25°C was higher than those at 30 and 37°C. The maximum cell density reached was 1.1 g/l at 25°C. However, the cell growth patterns at 25, 30, and 37°C were almost the same. The logarithmic phase was observed after 4 h of growth, and the stationary phase was observed after 10 h of the culture.

Table 1. Antimicrobial activity spectrum of the bacteriocin.

Indicator strains	Activity[1)]
G (+)	
Lactobacillus brevis	+++
Lactobacillus fermentum	+
Lactobacillus casei	+
Lactobacillus plantarum	+++
Lactobacillus helveticus CNRZ 1096	+++
Lactobacillus delbrueckii	++
Lactococcus sp. JC3	-
Leuconostoc mensenteroides	+
Pediococcus acidilactis	+
Streptococcus mutans	+
Corynebacterium	++
Listeria monocytogenes	-
G (-)	
Acetobacter aceti	+++
E. coli DH5α	-
Pseudomonas synsantha	+++
Staphylococcus aureus ATCC6538 (Mastitis pathogen)	+++
Streptococcus agalactiae ATCC14364 (Mastitis pathogen)	+++

[1)]The activity was expressed as the diameter of inhibition zone against each sensitive indicator.
Degree of clarity of clear zone due to growth inhibition: +, Inhibitory zone; -, no inhibition zone, +++: 1.0 cm>clear zone, ++: 0.5<clear zone<0.9 cm +: 0.1<clear zone<0.5 cm.

The *L. bulgaricus* started to produce the bacteriocin from 6 h of the cultivation. Figures 1B and 1C show the activity of the bacteriocin during the cultivation of *L. bulgaricus* at different temperatures. The maximum activity of the bacteriocin with the indicator *Strep. agalactiae* ATCC14364 reached 5,120 AU/ml for 5 h (from 10 h to 15 h) in the culture at 30°C (Fig. 1B). The activities of bacteriocin reached 2,560 AU/ml for 2 h (from 11 h to 13 h) in the culture at 37°C, and about 160 AU/ml for 3 h (from 10 h to 13 h) in the culture at 25°C. The highest bacteriocin activities were shown at the stationary phase of the *L. bulgaricus* cultures, the results being similar to previous reports [2, 16]. Therefore, the optimal condition for the production of the bacteriocin was determined to be 30°C and early stationary phase from 10 h to 15 h. Figure 1C shows the activity of the bacteriocin obtained at various culture temperatures with *Staph. aureus* ATCC6538 as the indicator: The maximum activity of the bacteriocin reached 320 AU/ml during the culture time from 10 h to 15 h at 30°C, 160 AU/ml during the culture time from 8 h to 18 h at 25°C, and 160 AU/ml during the culture time from 4 h to 16 h at 37°C. The optimal condition for the production of the bacteriocin with *Staph. aureus* ATCC6538 was exactly same as with *Strep. agalactiae* ATCC14364.

As shown in Figs. 1B and 1C, the bacteriocin was more effective toward *Strep. agalactiae* ATCC14364 than *Staph. aureus* ATCC6538. In the culture of *L. bulgaricus*, the maximum activity of the bacteriocin was obtained at 30°C and 10 h in early stationary phase as a typical secondary metabolite production [15, 20]. The maximum activities of bacteriocin were maintained for 5 h at 30°C, as shown in Figs. 1B and 1C. After 15 h of the culture, the activities of bacteriocin were rapidly decreased due to degradation of the bacteriocin by extracellular proteolytic enzymes, similar to trends in the previous report [19].

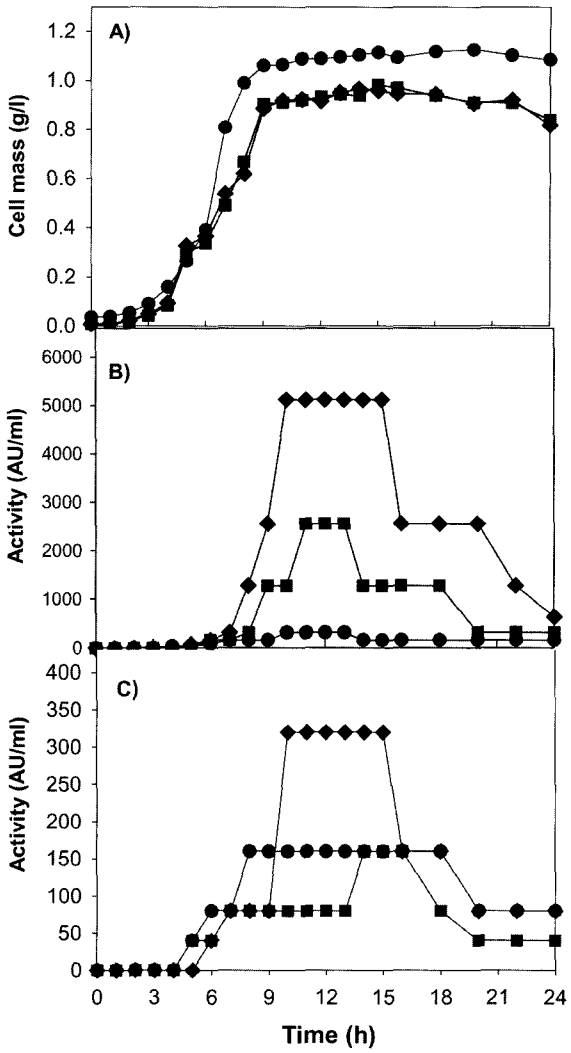

Fig. 1. Time course on the cell mass and production of the bacteriocin against *Strep. agalactiae* ATCC14364 and *Staph. aureus* ATCC6538.
The cell density (A) and the change of bacteriocin activity were monitored every hour against *Strep. agalactiae* ATCC14364 (B) and *Staph. aureus* ATCC6538 (C). Symbols: ●, 25°C; ◆, 30°C; ■, 37°C.

Activity of the Bacteriocin Exposed to Enzymes and Heat Ttreatment

As shown in Table 2, the activities of the bacteriocin treated with various enzymes were measured. Proteolytic

Table 2. Sensitivity of the bacteriocin to various enzymes.

Enzyme	*Staph. aureus* ATCC6538[1)]	*Strep. agalactiae* ATCC14364[2)]
Alcalase	-	-
Proteinase K	-	-
Aroase AP-10	-	-
Glutaminase	-	+
Protamax	-	-
Peptidase R	-	-
Neurase	-	-
Flavourzyme	-	+
Glucoamylase	-	+
β-Amylase	-	-
α-Amylase	-	+
Trypsin	-	+
Catalase	+	+
Pepsin	-	-

Staph. aureus ATCC6538 and *Strep. agalactiae* ATCC14364 were used as indicators. +, Detection of inhibitory zone observed; -, no inhibition zone.

enzymes (aroase AP-10, protamax, proteinase K, peptidase, neurase, alcase, and pepsin) completely inactivated the bacteriocin activity, indicating that the bateriocin was proteinous nature. In addition, when the bacteriocin was treated with α-amylase, β-amylase, and glucoamylase, the bacteriocin activity against the indicator *Staph. aureus* ATCC6538 was lost. Also, when the bacteriocin was treated with β-amylase, the bacteriocin activity against *Strep. agalactiae* ATCC14364 was lost. Therefore, the loss of an antimicrobial activity of the bacteriocin by α-amylase, β-amylase, and glucoamylase indicates that the carbohydrate composition in the bacteriocin is associated with the antimicrobial activity of the bacteriocin. Accordingly, the bacteriocin was considered to be a class IV bacteriocin [1, 13, 20].

As shown in Fig. 2, the activity of the bacteriocin was lost at high pH and 100°C. The 100% activity is referred to the activity at pH 7 and 30°C, as shown in Figs. 1B and 1C. One hundred % of the bacteriocin activity was maintained at pH 2-7 with heat treatment for 30 min at 100°C. However, the activity decreased to 50% after 60 min of heat treatment. In alkali condition (pH 10-11), the activity was decreased by heat treatment for a short period of time. This result indicates that the bacteriocin was stable in acidic and neutral conditions (pH 2-7) with heat treatment for 30 min [13, 17].

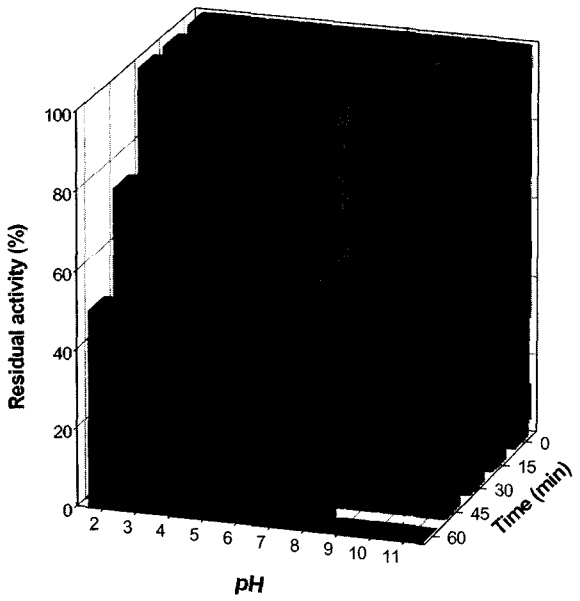

Fig. 2. Effects of pH and heat treatment at 100°C on the bactericidal activity of the bacteriocin against. *Strep. agalactiae* ATCC14364 and *Staph. aureus* ATCC6538.

Determination of Molecular Weight of the Bateriocin by SDS-PAGE

The apparent molecular weight of the bacteriocin was determined by SDS-PAGE (Fig. 3). Lane A shows a standard marker, and lanes B, C, and D show the crude bacteriocin samples. Lanes A and B were stained by Coomassie brilliant blue. Lane B shows the molecular mass of the bacteriocin to be approximately 14 kDa, and clear bands of the bacteriocin were observed on the lanes C and D. Lanes C and D were overlaid with indicator strains, *Strep. agalactiae* ATCC14364 (Lane C) and *Staph. aureus* ATCC6538 (Lane D), and they also show that the molecular mass of the bacteriocin from *L. bulgaricus* was 14 kDa.

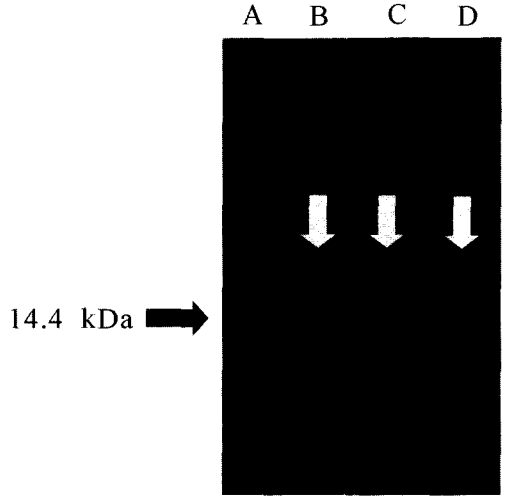

Fig. 3. Polyacrylamide gel (15%) electrophoresis of the bacteriocin.
Lane A: standard marker with 97.4, 66.2, 45, 31, 21.5, 14.4 kDa (Biorad, low molecular weight). Lane B: the bacteriocin sample stained by Coomassie brilliant blue R250. Lanes C and D: the bacteriocin sample overlaid with indicator strains, *Strep. agalactiae* ATCC14364 (lane C) and *Staph. aureus* ATCC6538 (lane D). The arrow indicates the location of bacteriocin.

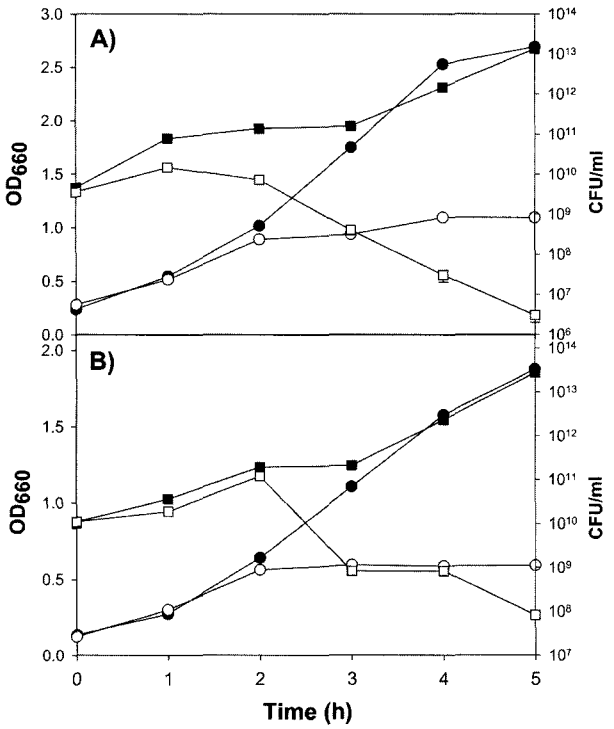

Fig. 4. CFU of the bacteriocin against indicator strains *Strep. agalactiae* ATCC14364 and *Staph. aureus* ATCC6538.
A) The inhibitory effect of the bacteriocin against *Strep. agalactiae* ATCC14364; B) The inhibitory effect of the bacteriocin against *Staph. aureus* ATCC6538. Symbols: ●, Optical density of control; ○, Optical density of culture treated by the bacteriocin; ■, CFU of control; □, CFU of culture treated with the bacteriocin.

Mode of Action Against Mastitis Pathogens

The colony forming units (CFU) of *Strep. agalactiae* ATCC14364 and *Staph. aureus* ATCC6538 with the bacteriocin were measured (Fig. 4). Five-hour incubation of indicator strains with the bacteriocin resulted in the decrease of live cell number. When *Strep. agalactiae* ATCC14364 was treated with the bacteriocin, the cell number decreased from 1.35×10^{13} CFU/ml to 3.8×10^{6} CFU/ml. Similarly, when *Staph. aureus* ATCC6538 was treated with the bacteriocin, the cell number decreased from 2×10^{13} CFU/ml to 9.2×10^{7} CFU/ml. However, as shown in Fig. 4, the optical density increased for 2 h and then remained constant in both cases of the indicator strains. These results indicate that the bacteriocin acted in bactericidal mode [11, 17].

In conclusion, *Staph. aureus* ATCC6538 and *Strep. agalactiae* ATCC14364, known as common mastitis pathogens, were inhibited by the bacteriocin produced by *L. bulgaricus*. The molecular mass of the bacteriocin was about 14 kDa, as determined by SDS-PAGE, and the bacteriocin can be used for treatment of cows infected by mastitis pathogens.

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