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Bacteriocin Produced by *Lactobacillus curvatus* SE1 Isolated from Kimchi

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Abstract Lactic acid bacteria were isolated from Kimchi and screened for bacteriocin production. Strain SE1, identified as *Lactobacillus curvatus* sp., showed the strongest inhibitory activity against *Lactobacillus delbrueckii* subsp. *delbrueckii*. The bacteriocin was inactivated by amyloglucosidase, trypsin, or protease K treatment. However, it maintained its activity under heat treatment at 100°C for 60 min. The production of the bacteriocin had a growth-related mode and decreased around the early-stationary phase. The optimum temperature for the growth of *L. curvatus* SE1 was 37°C; however, the optimum temperature for bacteriocin production was 30°C. The bacteriocin activity was decreased by treatment with methanol, butanol, acetone, or chloroform, however, it was not affected by treatment with ethanol, iso-propanol, or cyclohexane. The inhibitory activity of bacteriocin was stable over a wide range of pHs (2 to 11). The bacteriocin from *L. curvatus* SE1 killed the indicator strain by a bactericidal mode of action. The bacteriocin from *L. curvatus* SE1 was partially purified by ethanol precipitation and ion exchange chromatography. SDS-polyacrylamide gel electrophoresis was used to determine the molecular weight of the bacteriocin by the bacteriocin activity test. The apparent molecular mass of the bacteriocin produced by *L. curvatus* SE1 was about 14 kDa.

Key words: Bacteriocin, *L. curvatus* SE1, Kimchi

Lactic acid fermentation is an old discovery. Many different cultures in various parts of the world have used it to improve the storage quality, palatability, and nutritive value of perishable foods such as milk, vegetables, meat, fish, and cereals [21]. Lactic acid bacteria such as *Lactococcus, Lactobacillus, Pediococcus*, and *Leuconostoc* contribute to flavor and aroma development, as well as the preservation of foods.

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Bacteriocins are proteinaceous compounds with bactericidal activity against strains of the same or closely related to the bacteriocin producing bacterium. These substances are of particular interest as they are proteinaceous and may thus be degraded during digestion by humans and other animals. Many of the lactic acid bacteria produce bacteriocins [12]. Bacteriocin has great potential as a food preservative due to the occurrence of antibiotic resistant pathogenic bacteria. Bacteriocin is produced by microorganisms isolated from foods such as cheese, sausage, and kimchi, therefore, they can be used as a safe food preservative [3]. Bacteriocin and its immune system can be used in the genetic engineering field as a selective marker. Currently, many antibiotic markers have been used in various plasmids. However, in the production stage of heterologous therapeutics or proteins, it is difficult to remove antibiotics which are added to maintain plasmid stability. Therefore, a bacteriocin and its immune system can be used as new cloning markers for genetic engineering [7].

Many bacteriocins are produced by gram-positive bacteria, especially from lactic acid bacteria [1], and bacteriocins from various food sources have been isolated and their structures identified [17].

The bacteriocin-producing strains usually have been screened from yogurt, the fermented milk, vegetables, and cheese in Europe and America. Recently, studies on bacteriocin and lactic acid bacteria isolated from Korean traditional foods, especially Kimchi, have been reported [2, 8, 9]. Kimchi fermentation occurs mainly due to the microorganisms naturally present in the raw materials, which contain numerous and various microflora, including lactic acid bacteria. Fermentation may be initiated by various microorganisms (*Lactobacillus, Leuconostoc, Pediococcus* etc.), however, the lactic acid bacteria gradually dominate with organic acid formation.

Leuconostoc mesenteroides is the predominant lactic acid bacteria in the early fermentation stage of Kimchi [10, 11]. As the pH drops to 4.6-4.9 with organic acid

accumulation, the growth of *L. mesenteroides* is relatively inhibited, but the fermentation continues with other lactic acid bacteria such as *Enterococcus faecalis, Lactobacillus brevis, Pediococcus cerevisiae*, and *Lactobacillus plantarum* to go into the ripening stage. At the later fermentation stage, aerobic yeasts and molds appear on the surface of the upper layer. The main microorganism responsible for the ripening of Kimchi is *L. plantarum* which is responsible for excessive acidification in the later stage of fermentation [23].

The isolation of a bacteriocin producing strain which has a broad inhibitory spectrum and the evaluation of its characteristics to secure a superior strain and develop new natural preservatives. In particular, the bacteriocin can solve the difficulty of the industrialization of Kimchi using the bacteriocin as the preservative. Therefore, a bacteriocin-producing strain was isolated from Kimchi and the species was identified in the study. The inhibitory spectrum of the isolated strain and characteristics of bacteriocin produced by the strain were evaluated.

MATERIALS AND METHODS

Isolation of the *Lactobacillus* from Kimchi

Household Kimchis were collected from various sources. The broth of Kimchi was diluted at $1/10^6$ to $1/10^8$. To isolate lactic acid bacteria, the diluted sample was spread onto 2% $CaCO_3$ added Man-Rogasa-Sharpe (MRS) medium. The plates were incubated at 30°C and 37°C for 2~3 days and clear zone forming colonies were isolated. The isolated strain was transferred onto modified *Lactobacillus* selection (LBS) medium containing acetic acid and sodium acetate at 30°C and 37°C for 3 days in order to isolate *Lactobacillus* sp. from lactic acid bacteria. Isolated strains were stored at -70°C in MRS broth containing 50% glycerol before use.

Screening of the Strain Producing Bacteriocin from the Isolated *Lactobacillus* sp.

Screening for inhibitory substances and sensitivity analysis were performed by the modified agar-well diffusion method of Tagg and McGiven [22]. The isolated *Lactobacillus* sp. strains were grown in MRS broth at 30°C and 37°C for 15 h. Fifty μl of supernatant of culture broth was placed into 0.5 cm diameter wells of a plate overlaid with indicator strain (*Lactobacillus delbrueckii* subsp. *delbrueckii* IFO 3534) and then incubated at 37°C for 12 h. Formation of clear zones around the wells was checked and the largest clear zone forming strain was selected.

Production of Bacteriocin

The effect of incubation time on the production of bacteriocin and cell growth was evaluated. *L. curvatus* SE1 was incubated in MRS broth at 30°C for 20 h, and the growth and bacteriocin activity were measured at 1 h intervals. The optimal temperature for cell growth and bacteriocin activity was determined. *L. curvatus* SE1 was incubated in MRS broth at 25°C, 30°C, 37°C and 45°C for 15 h. Culture broth was spectrophotometrically measured at 600 nm and bacteriocin activity was determined by the agar-well diffusion method.

Determination of Bacteriocin Activity

Bacteriocin activity was assayed by the modified agar-well diffusion method of Tagg and McGiven [22]. Fifty μl of serial two-fold diluted culture supernatant of *L. curvatus* SE1 were placed into each 0.5 cm diameter well of a plate overlaid with *L. delbrueckii* subsp. *delbrueckii* (O.D. 600 vnm = 0.35~0.4) then incubated at 30°C for 12 h.

The activity was defined as the reciprocal of the highest dilution showing definite inhibition of the indicator lawn and was expressed in activity units (AU) per ml.

Characterization of the Bacteriocin

The culture supernatant was treated with various enzymes, including protease K, trypsin, α-amylase, and glucoamylase. Each enzyme was dissolved in proper buffer and the solution was added to culture supernatant to obtain a final concentration of 0.5 mg/ml. The reaction mixture was incubated at 37°C for 1 h to check the effect of the enzyme treatment. The heat stability test (100°C, 30 min) was carried out in the same pattern. The activity remaining in samples was determined by the agar-well diffusion method.

The bacteriocin present in the supernatant of the culture was precipitated by the addition of 50% ethanol, and the precipitated pellet by centrifugation (6,000×g, for 30 min) was dissolved in cold water and then lyophilized. In order to evaluate the effect of organic solvents, partially purified bacteriocin was treated with 50% organic solvents such as ethanol, methanol, butanol, acetone, isopropanol, cyclohexane, and chloroform. The solvent-treated sample was incubated at 4°C for 6 h. After the reaction, the solvent was removed by an evaporator at 30°C and the residual activity was assayed by the agar-well diffusion method.

To determine the stability of the bacteriocin at various pHs, the partially purified bacteriocin was dissolved with 50 mM sodium citrate (pH 2 to 5), 50 mM sodium phosphate (pH 6 to 8), and 50 mM Tris-HCl (pH 9 to 11) to 12,800 AU/ml, respectively. The bacteriocin solutions were exposed to various pHs for 24 h at 4°C. Then, the residual bacteriocin activity was assayed.

In order to determine the effect of metal ions on bacteriocin activity, the crude bacteriocin was treated with $CoCl_2$, $ZnSO_4$, $MgSO_4$, $BaCl_2$, $CuSO_4$, $FeSO_4$, $CaSO_4$, and $MnSO_4$. Each inorganic salt was dissolved in deionized

water and the solution was added to the culture supernatant to obtain a final concentration of 1 mg/ml. The reaction mixture was incubated at 4°C for 12 h. The activity remaining in samples was determined by the agar-well diffusion method.

Mode of Inhibitory Action

To obtain an indicator strain in log or stationary phases, two batches of *L. delbrueckii* subsp. *delbrueckii* were incubated at 30°C for 5 h and for 18 h, respectively. The cell pellets obtained by centrifugation were resuspended in MRS broth to the optical density of 0.4 at 600 nm. The suspensions were incubated at 30°C for 3 h with and without bacteriocin solution (12,800 AU/ml). At every 1 h, the viable indicator cells were enumerated on MRS plates and the optical density of the cell suspension at 600 nm was measured during incubation.

Purification of Bacteriocin

L. curvatus SE1 was cultured in MRS broth at 30°C for 15 h. The culture broth was centrifuged at 5,000×g for 20 min. The cell-free supernatant was decanted into a beaker in an ice bath and precooled ethanol (-20°C) was added to 50% (v/v). The precipitate collected by centrifuge (6,000×g, for 30 min) was dissolved in cold water and lyophilized. The lyophilized sample was stored at -20°C until use.

The freeze-dried sample was dissolved in 20 mM sodium phosphate buffer (pH 7.0, buffer A) and was applied to an DEAE Sephadex A-25 column which had been equilibrated with buffer A. After the column was washed in buffer A, the bacteriocin was eluted with a linear gradient of 0~1 M NaCl in buffer A.

SDS-polyacrylamide gel electrophoresis (PAGE)

SDS-PAGE was performed as described by Laemmli [13] in a 15% discontinuous gel with a Mini-Protein II electrophoresis system (Biorad, U.S.A.). A molecular mass standard was run with the samples. The gel was stained with Coomassie Brilliant Blue G. For the activity measurement of bacteriocin in an SDS-PAGE gel, the gel was fixed immediately by 2 h treatment in 20% isopropanol and 10% acetic acid and washed for 4 h in distilled water. The gel was placed onto plates overlaid with indicator strain and then incubated at 37°C for 24 h.

RESULTS AND DISCUSSION

Screening of the Strain Producing Bacteriocin from the Isolated *Lactobacillus* from Kimchi

For the detection of bacteriocin production from the isolated *Lactobacillus* subsp., the culture supernatant of *Lactobacillus* subsp. grown overnight in MRS broth was placed into 0.5 cm diameter wells of a plate overlaid with indicator strain (*L. delbrueckii* subsp. *delbrueckii*) and then incubated at 37°C for 12 h. Among 1,782 isolated strains, three strains grown at 30°C showed bacteriocin activity. A strain showing the largest inhibition zone was selected. The strain was identified as *L. curvatus* by the culture characteristics and carbohydrate utilization pattern using the API 50 CHL bacterial identification system and tentatively designated as *L. curvatus* SE1.

Table 1. Inhibitory spectrum of bacteriocin produced by *L. curvatus* SE1 isolated from Kimchi.

Test organism	Susceptibility
Acetobacter aceti IFO 3281	-
Corynebacterium glutamicum ATCC 13058	-
Lactobacillus brevis IFO 13109	-
Lactobacillus bulgaricus IFO 13953	-
Lactobacillus casei ATCC 4646	-
Lactobacillus delbrueckii IFO 3534	+
Lactobacillus fermentum ATCC 11739	+
Lactobacillus helveticus CNRZ 1096	+
Lactobacillus helveticus CNRZ 1094	+
Lactobacillus plantarum ATCC 8014	+
Leuconoxtoc mesenteroides ATCC 10830	-
Listeria monocytogenes ATCC 33090	+
Pediococcus acidilactici ATCC 8042	+
Streptococcus mutans ATCC 25175	-

The inhibitory spectrum of bacteriocin produced from *L. curvatus* SE1 was evaluated using various indicator strains as shown in Table 1. The bacteriocin from *L. curvatus* SE1 was active for gram-positive microorganisms such as *Lactobacillus, Pediococcus*, and *Listeria* and showed the strongest inhibitory activity against *L. delbrueckii* subsp. *delbrueckii*. However, gram-negative bacteria, such as *Acetobacter, Pseudomonas, E. coli*, were not sensitive to the bacteriocin. *Listeria monocytogenes* has been recognized as a major food-born pathogen with the ability to survive in severe environmental conditions, such as refrigeration [6], pHs as low as 3.6 in foods [19], in salt concentrations of up to 10% [15], in the presence of surfactant sanitizers [5], and at a high temperature [4, 14]. These facts indicate that traditional methods of preservation are not sufficient to prevent the growth of *L. monocytogenes* in foods. Therefore, bacteriocin from *L. curvatus* SE1 can be used to protect foods from the pathogenic bacteria, such as *Listeria monocytogenes*.

Effect of Incubation Time on the Production of Bacteriocin and Cell Growth

L. curvatus SE1 was incubated in MRS broth at 30°C for 20 h, and the cell growth and bacteriocin activity was measured at 1 h intervals as shown in Fig. 1. The logarithmic phase was observed after 8 h of growth and the stationary phase was observed after 17 h of culture.

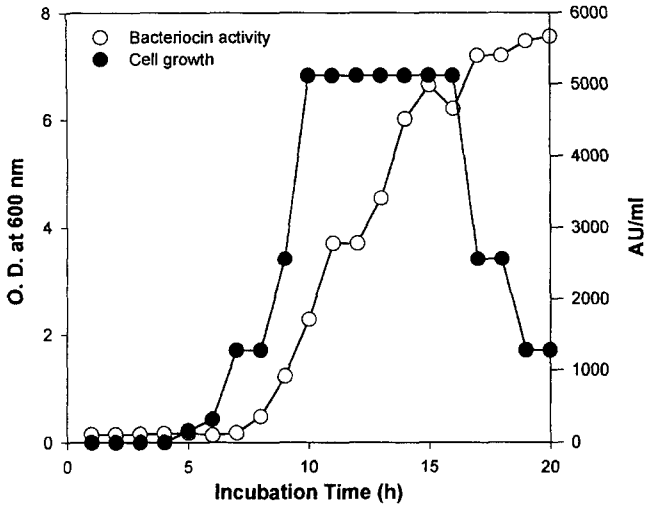

Fig. 1. Growth of *L. curvatus* SE1 isolated from Kimchi and bacteriocin production in MRS medium at 30°C.
Bacteriocin activity was measured by dilution analysis on *L. delbrueckii* subsp. *delbrueckii* indicator agar plate.

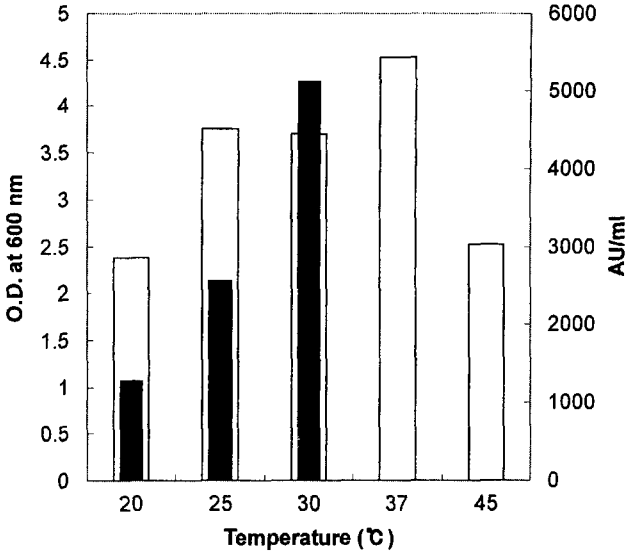

Fig. 2. Effect of temperature on the cell growth and bacteriocin production of *L. curvatus* SE1.
□, O.D. at 600 nm; ■, AU/ml.

According to the literature [16, 20], bacteriocin has been produced at the late-logarithmic or stationary phase as a typical secondary metabolite. However, the bacteriocin from *L. curvatus* SE1 showed a growth-related trend. The productivity of bacteriocin started to decrease when the growth of the cells was in the middle of the logarithmic phase and became zero at the early stationary phase, according to cumulative bacteriocin activity measurement. The cumulative bacteriocin activity decreased at the stationary phase probably due to released protease from cell autolysis. The reason why the pattern of bacteriocin production is closely related to the cell growth is that *Lactobacillus* species are dominant flora in the early Kimchi fermentation stage and provide acids and flavors to Kimchi. In order to become a dominant species, the *Lactobacillus* produces a proteinaceous growth inhibitory substance, the bacteriocin, to prevent the growth of other microorganisms.

Effect of Temperature on the Production of Bacteriocin and Cell Growth

The temperature effect on cell growth and bacteriocin productivity was determined as shown in Fig. 2. *L. curvatus* SE1 showed the maximum growth at 37°C, however, the maximum bacteriocin activity was obtained at 30°C. The cell growth at 30°C and 25°C were almost the same while the bacteriocin production at 30°C was about five times higher than that at 25°C. Therefore, the optimum temperature for bacteriocin production was 30°C. Usually, bacteriocin producing strains from Kimchi tend to a maximum production of bacteriocin below 30°C. This was probably due to flavor development of Kimchi at ambient temperatures below 30°C.

Characterization of the Bacteriocin from *L. curvatus* SE1

Treatment of culture supernatant of *L. curvatus* SE1 with protease K and trypsin and amyloglucosidase showed the inactivation of bactriocin activity (Table 2, Fig. 3). The loss of the activity by protease K and trypsin suggested that the bacteriocin produced by *L. curvatus* SE1 was a proteinaceous substance. α-Amylase did not inactivate the bacteriocin. However, the amyloglucosidase treatment inactivated the bacteriocin. Therefore, the carbohydrate compound on the bacteriocin was considered to play a part of the inhibitory action. This indicated that the bacteriocin produced by *L. curvatus* SE1 was a Class IV type bacteriocin which contains essential lipid or carbohydrate moieties in addition to the protein [24].

The effect of organic solvents on bacteriocin activity was determined and shown in Fig. 4. The bacteriocin activity was decreased by treatment with methanol, butanol, acetone, and chloroform, however, it was not affected by treatment with ethanol, iso-propanol, and cyclohexane. Some organic solvents which did not have any effect on bacteriocin activity could be used to remove other proteins except bacteriocin for the reduction of purification steps.

Table 2. Effect of enzymes and heat treatment on the bacteriocin activity.

Treatment	Bacteriocin Activity
Protease K	-
Trypsin	-
α-Amylase	+
Glucoamylase	-
Heat treatment (100°C, 60 min)	+

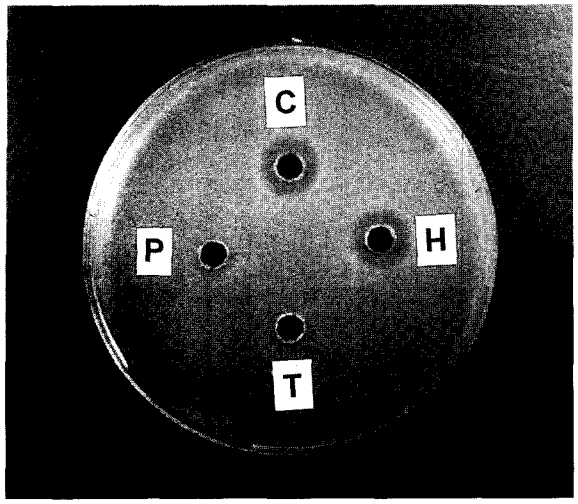

Fig. 3. Antagonistic activity of the bacteriocin from *L. curvatus* SE1 overlaid with *L. delbrueckii* subsp. *delbrueckii*.

C: Culture supernatant, H: Heat treatment, P: Protease K treatment, T: Trypsin treatment.

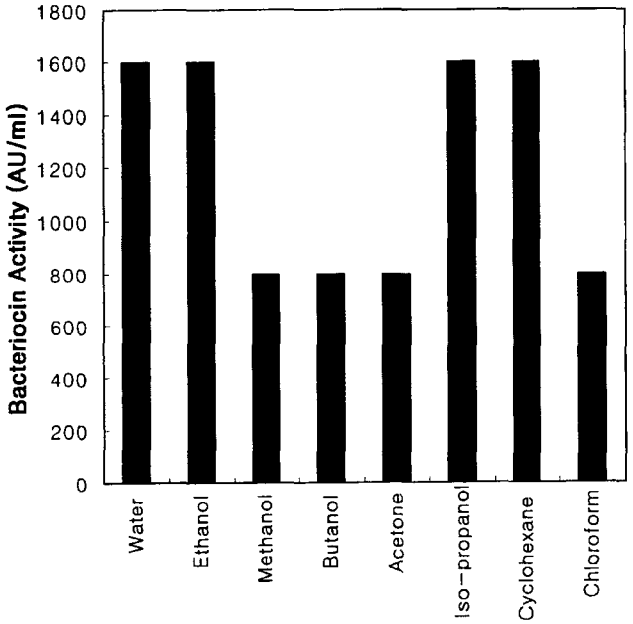

Fig. 4. Effect of organic solvents on bacteriocin activity.

The effect of pH on the bacteriocin activity was evaluated with partially purified bacteriocin. The dried bacteriocin was dissolved in 50 mM sodium citrate, sodium phosphate, and Tris-HCl at the bacteriocin activity of 12,800 AU/ml. The bacteriocin activity was stable over the wide range of pHs (2~11) tested in this experiment (data not shown). In the case of nisin, the activity was stable at acidic pH ranges and was rapidly decreased at neutral and alkaline pH ranges [3]. Thus nisin is applicable to food systems in acidic conditions. However, the bacteriocin from *L. curvatus* SE1 can be used in food systems in wider pH ranges.

Table 3. Effect of metal ions on the bacteriocin stability.

Metal ion	Residual activity (%)
$CoCl_2$	100
$ZnSO_4$	100
$MgSO_4$	100
$BaCl_2$	100
$CuSO_4$	100
$FeSO_4$	100
$CaSO_4$	100
$MnSO_4$	0

Bacteriocin was incubated at 4°C for 12 h in 1 mg/ml inorganic salts.

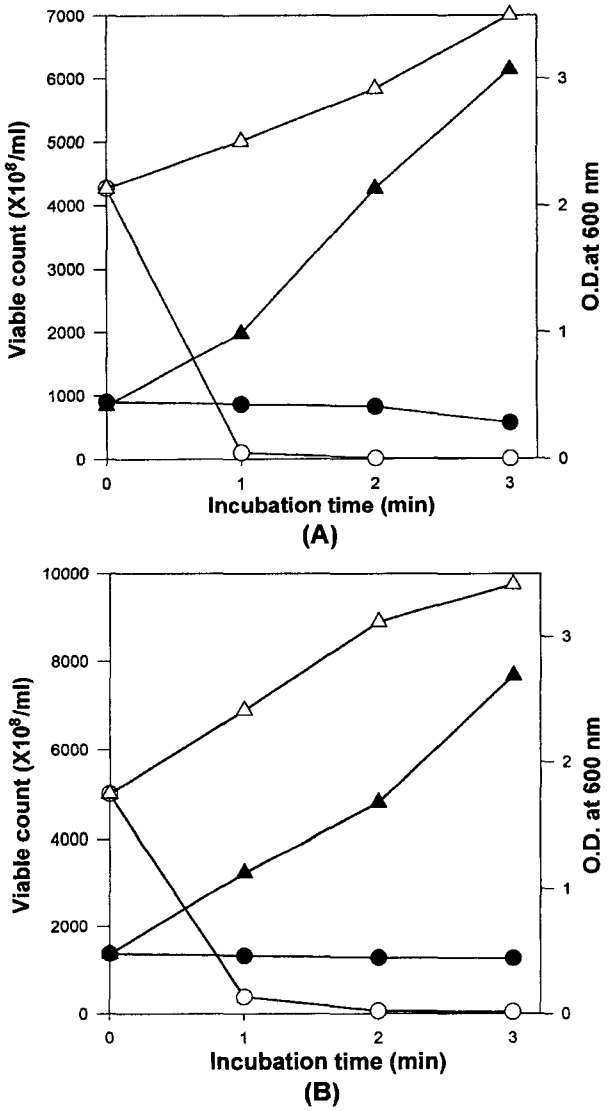

Fig. 5. Bactericidal effect of partially purified bacteriocin against indicator strain from log-phase (A) and stationary phase (B).

○, Viable count on bacteriocin treatment; ●, Optical density on bacteriocin treatment; △, Viable count on control; ▲, Optical density in control.

The effect of metal ions on the bacteriocin stability was characterized with the partially purified bacteriocin (Table 3). The bacteriocin was inactivated by the treatment with $MnSO_4$. However, other metal ions did not affect bacteriocin activity. Parente and Hill [18] reported optimum biomass and bacteriocin production when Tween 80 and Mn^{2+} were added to the growth medium. The MRS medium for growth of *L. curvatus* SE1 contains Mn^{2+} (0.005%). This indicates that Mn^{2+} was required for bacteriocin production, however, the excessive addition of Mn^{2+} in the culture medium could inhibit the bacteriocin activity.

Mode of Inhibitory Action

In order to test the mode of antimicrobial action on the indicator strains in both the log and stationary phases, the viable cell count and optical density of the indicator strain with and without bacteriocin were determined (Fig. 5).

Three hours of incubation of indicator cells with bacteriocin resulted in the death of 99% of the cell population. However, the optical density remained constant throughout the experiment for both indicator cells from the log and stationary phases, indicating that the bacteriocin did not act in a bacteriolytic fashion. These data showed that the bacteriocin induced cell death without detectable lysis and confirmed a bactericidal mode of the bacteriocin action.

Purification of Bacteriocin

The bacteriocin from *L. curvatus* SE1 was not effectively separated by ammonium sulfate precipitation. The culture supernatant which was treated with ammonium sulfate

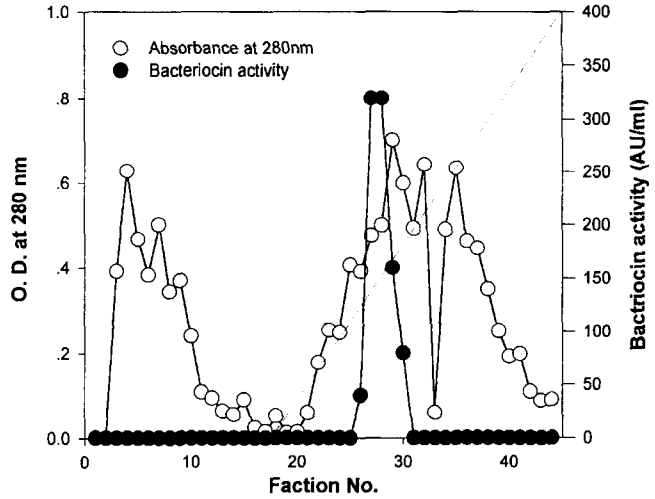

Fig. 6. Elution of bacteriocin from DEAE Sephadex.

Ethanol precipitate containing bacteriocin was applied to DEAE Sephadex A-25 equilibrated with 20 mM sodium phosphate buffer (SPB), pH 7. The bacteriocin was eluted with a linear gradient of 0~1 M NaCl in 20 mM SPB.

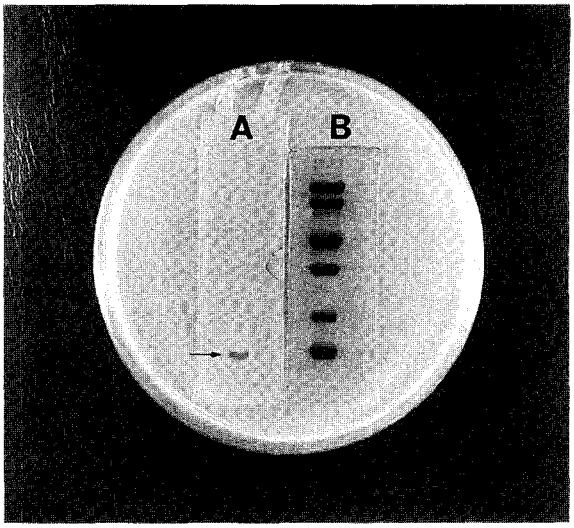

Fig. 7. Sodium dodecyl sulfate polyacrylamide gel electrophoresis showing bacteriocin activity.

Lane A: Arrow indicates purified bacteriocin from *L. curvatus* SE1; B: standard marker.

divided a bottom pellet and the floating pellicle after centrifugation. The recovery of bacteriocin activity of the floating pellicle was unsatisfactory. Therefore, ethanol which did not have any inhibitory effect on bacteriocin activity, was used to precipitate the bacteriocin fraction. The fraction obtained by 50% ethanol precipitation was loaded to a DEAE Sephadex A-25 column which had been equilibrated with 20 mM sodium phosphate buffer, pH 7. The bacteriocin was eluted in a single peak at around 0.45 M NaCl in 20 mM sodium phosphate buffer (Fig. 6). The purified bacteriocin was collected and dialyzed. The dialyzed bacteriocin was lyophilized.

Direct Determination of the Bacteriocin Molecular Weight by SDS-PAGE

SDS-PAGE was used to separate the bacteriocin and estimate its molecular mass from *L. curvatus* SE1. The standard molecular weight markers were phophorylase b (94 kDa), albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.4 kDa). The gel-placed plate overlaid with indicator strain showed an inhibitory zone corresponding to an apparent molecular mass of about 14 kDa (Fig. 7).

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