J. Microbiol. Biotechnol. (2000), **10**(4), 507–513

JOURNAL OF
MICROBIOLOGY
AND
BIOTECHNOLOGY

Characterization of a Bacteriocin Produced by *Enterococcus* sp. T7 Isolated from Humans

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Received: April 12, 2000
Accepted: July 5, 2000

Abstract A bacteriocin-producing organism, *Enterococcus* sp. T7, was isolated from human fecal samples. Bacteriocin T7, named tentatively as the bacteriocin, was produced by *Enterococcus* sp. T7 and it inhibited some strains of *Lactobacillus*, *Staphylococcus*, *Enterococcus*, and *Streptococcus*, but not all the lactococci and gram-negative bacteria tested. Bacteriocin T7 inhibited the growth of *Listeria monocytogenes* Scott A, but the degree of inhibition was less than those for other sensitive gram-positive bacteria. Bacteriocin T7 in MRS broth started to produce at the middle of the exponential growth phase and the inhibitory activity reached its maximum level during the stationary growth phase. Bacteriocin T7 was stable against heat treatments, pH variations (pH 2–10), and exposure to organic solvents. The molecular weight of bacteriocin T7 was estimated to be 6,500 Da by SDS-PAGE. All these facts, including physico-chemical stabilities, small molecular size, and inhibition of *Listeria monocytogenes*, indicate that bacteriocin T7 is likely to be a member of the class IIa bacteriocins.

Key words: Bacteriocin, *Enterococcus* sp. T7, human intestines, bacteriocin purification

Many lactic acid bacteria (LAB) produce bacteriocins which are supposedly advantageous for the producers when in the competition for foods and niches [17]. Extensive efforts to find bacteriocins from LAB with a broad inhibition spectrum and superior stabilities against heat treatments and pH variations have been made during the last decade [12, 21]. Since many fermented foods containing LAB have been consumed for thousands of years, bacteriocins from LAB or LAB-producing bacteriocins are generally regarded as safe for human consumption. Therefore, bacteriocins produced by LAB are currently the prime candidates for developing safe food-preservatives which can replace chemical preservatives [4]. LAB-producing bacteriocins also have advantages over non-producers as probiotic strains, since bacteriocins can enhance the abilities of probiotic strains to suppress the growth of undesirable bacteria in the human GI (gastrointestinal) tract [7, 14]. Various functional foods containing probiotic strains are in the market around the world [22]. In human GI tracts, more than 400 bacterial species constitute a normal flora and bacteriocins-producing LAB are one of the important members [9, 19]. LAB with strong bacteriocin activities are very useful for designing and selecting commercial probiotic strains. Kabuki *et al.* [11] purified and characterized a bacteriocin (Reutericin 6) produced by *Lactobacillus reuteri* LA6, an isolate from humans. The molecular weight of Reutericin 6 was determined to be 2.7 kDa by SDS-PAGE and ESI-MS. Maeng *et al.* [16] isolated 56 *Lactobacillus* strains from volunteers' feces by using differential MRS-BPB plates. Among 56 isolates, 6 bacteriocin-producing strains were confirmed. Two isolates, HU-1 and H22-3, showed the most outstanding antimicrobial activities. Bacteriocins produced by both strains inhibited the growth of various Gram (+) bacteria including *Listeria monocytogenes*, *Micrococcus luteus*, and *Staphylococcus aureus*, in addition to many species of LAB. This paper describes the isolation and identification of bacteriocin-producing *Enterococcus* sp. T7 from human fecal samples and characterization of the bacteriocin T7 produced.

Materials and Methods

Isolation of Bacteriocin-Producing LAB from Human Fecal Samples

Fecal samples from volunteers were aseptically diluted and spread on selective plates. Different selective media were employed to recover diverse microorganisms aerobically

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and anaerobically. For isolates, tentative assignments of Genus were carried out based on the colony morphologies and biochemical properties. Bacteriocin production was examined by the spot-on-lawn test [5]. Three microliters of overnight culture in MRS broth was spotted on the MRS plate overlaid with 3 ml of MRS soft agar (0.7%, w/v) containing the indicator strain (1×10^8 CFU). After overnight incubation at 37°C, the presence of an inhibition zone was examined. For tentatively identified bacteriocin producers, culture supernatants were obtained and the above procedures were repeated. The bacteriocin activity, expressed as AU (activity unit) per milliliter, was defined as the reciprocal of the highest two-fold dilution showing inhibitory action towards the indicator organism.

Identification of a Bacteriocin-Producing Strain

Biochemical traits such as carbohydrate utilization patterns, enzyme activities, growth temperature range, salt tolerance, and gas production were examined according to *Bergey's Manual of Determinative Bacteriology* [8]. Fatty acids composition of the cellular membrane of *Enterococcus* sp. T7 was determined by using the Sherlock version 2.11 (MIDI Lab).

Bacteriocin Production

The effect of growth temperature and initial pH on the bacteriocin production was investigated. The optical density and bacteriocin activities of *Enterococcus* sp. T7 were determined at 25, 30, and 37°C, in MRS broth. Initial pHs of the MRS broths were adjusted to 5.5, 6.0, 6.5, 7.0, and 7.5 using 6 N HCl and 10 N NaOH, respectively, and the medium was sterilized by filtration using a syringe-type filter (0.22 μm pore size). Filtered medium was inoculated with overnight grown *Enterococcus* sp. T7 culture (2%, v/v) and incubated at 37°C for 24 h. At intervals, samples were taken for measuring the growth and bacteriocin activities.

Sensitivity of Bacteriocin T7 to Heat, Organic Solvents, and Enzymes

Stabilities of bacteriocin T7 against heat, organic solvents, and enzyme treatments were examined. Partially purified bacteriocin (lyophilized sample after dialysis of 50% ammonium sulfate pellet) was resuspended in 50 mM Tris-HCl buffer (pH 8.3) to the concentration of 16,000 AU/ml and held at constant temperatures of 60, 80, 100, and 121°C for up to 60 min. After the heat treatments, remaining bacteriocin activities were examined by employing the spot-on-lawn method. Stabilities against organic solvents were assessed by measuring the remaining bacteriocin activities after partially purified bacteriocin sample in 50 mM Tris-HCl was mixed with an equal volume of organic solvent and the mixture was kept for 1 h at 25°C. Partially purified bacteriocin was treated with hydrolyzing enzymes including proteases. Bacteriocin sample (16,000 AU/ml) was digested for 1 h at 37°C with each enzyme at a concentration level of 1 mg/ml.

Purification of Bacteriocin T7

The supernatant of overnight cultured *Enterococcus* sp. T7 was obtained by centrifugation at 10,000 ×g for 15 min, and filtered through 0.22 μm-pore size membrane filters. Two-liter aliquots of culture supernatants were brought to 50% saturation by adding solid ammonium sulfate while stirring at 4°C. The samples were stirred at 4°C for 4 h and then centrifuged at 17,000 ×g for 1 h. The pellets were dissolved in distilled water and dialyzed in Spectrapor CE membrane (Spectrum Medical Industries, Inc., Houston, U.S.A.; MW cutoff, 1,000) against distilled water for 12 h at 4°C. Dialysate was freeze-dried and stored at −76°C for later experiments. For ion-exchange chromatography, freeze-dried dialysate was resuspended in 50 mM Tris-HCl (pH 8.3) and loaded onto a DEAE Sephacel column (2.5× 30 cm) equilibrated with the same buffer. After washing the column with the same buffer, a linear NaCl gradient (0–1.0 M) was applied to elute bound proteins. The flow rate was adjusted to 0.5 ml/min and 10 ml of each fraction was collected. Fractions with bacteriocin activity were pooled and freeze-dried. The next purification step was gel filtration chromatography on Sephadex G-25, using 50 mM Tris-HCl buffer (pH 8.3) as eluent. Protein concentrations were measured according to the Bradford's method [2] using bovine serum albumin as a standard.

SDS-PAGE

SDS-PAGE was carried out according to the method of Laemmli [13]. Fifteen percent of acrylamide gel and a Mini-Protein II (Bio-Rad) electrophoresis system were employed. After gel electrophoresis, the gel was stained with Coomassie Brilliant Blue R-250. The bioassay detection of antimicrobial peptides in polyacrylamide gel was performed as described by Bhunia *et al.* [3]. The slab gels were first washed with sterile ultrapure water for 4 h. After sodium dodecyl sulfate (SDS) concentrations had decreased to nontoxic levels, the gels were placed on MRS plates and covered with a lawn of soft agar (0.7%, w/v) containing an exponentially growing culture of the indicator strain (1×10^8 CFU), *Lactobacillus delbrueckii* subsp. *lactis*. After 16 h of incubation at 37°C, the plate was examined for a growth inhibition zone.

Mode of Action of Bacteriocin T7

Cells of a log-phase culture of *Enterococcus faecalis* (indicator) were washed and suspended in 3 ml of 50 mM Tris-HCl buffer (pH 8.3) to a final concentration of 3×10^7 CFU/ml. After adding partially purified bacteriocin (1,600 AU), the cells were incubated at 37°C and taken at appropriate time intervals to determine the number of viable cells by plating them onto an MRS agar plate.

RESULTS AND DISCUSSION

Isolation of a Bacteriocin-Producing *Enterococcus* Strain from Human Fecal Samples

A bacteriocin-producing organism was isolated from volunteers' feces. The isolate named T7 was recovered from the TATAC plate [9], a selective media for streptococci, and effectively inhibited the growth of the indicator strain, *Lactobacillus delbrueckii* subsp. *lactis*. Bacteriocin production by isolate T7 was confirmed via a well diffusion assay [24] by using a pH-neutralized, catalase-treated supernatant to eliminate a possibility of either hydrogen peroxide or lactic acid inhibition. The growth inhibition was not affected by the catalase treatment or pH adjustment of culture supernatant of T7, however, it was abolished by proteinase K treatment (results not shown). The isolate was tentatively designated as *Streptococcus* T7 and later identified as a strain belonging to the genus of *Enterococcus* (see below).

Identification of T7

Morphological and biochemical properties of T7 were examined according to the Bergey's Manual of Determinative Bacteriology [8]. The results are summarizied in Table 1. This organism was a gram-positive, catalase-negative, facultatively anaerobic coccus. The organism grew at both 10°C and 45°C. It was also able to grow at pH 9.6 and in the presence of 6.5% NaCl. These properties are in agreement with the reported characteristics of enterococci [6]. Fatty acids composition analysis of the cellular membrane of T7 also confirmed that *Streptococcus* T7 was a strain belonging to the genus of *Enterococcus* (*E. solitarius*, 49%; *E. faecalis*, 25% probability). However, further definitive classification at the species level requires additional experiments such as 16S rRNA sequence analysis. Thus, the isolate was designated as *Enterococcus* sp. T7. A few reports had dealt with bacteriocin activity in enterococci including *E. faecalis* and *E. faecium* [15, 20, 23]. Enterocin A produced by *E. faecium* CTC492 is the most thoroughly characterized bacteriocin so far in terms of protein and gene levels. The N-terminal amino acid sequences of enterocin A were determined, and the structural gene was PCR-cloned using PCR-primers designed based on the amino acid sequences. The cloned gene was sequenced and the nucleotide sequences were compared with those of other bacteriocin genes in the databank [1]. Enterocin A shared a significant amount of amino acid sequence homology with pediocin PA-1 (54.6%), a class IIa bacteriocin. The N-terminal amino acid sequence of bacteriocin T7 should also be determined to define whether bacteriocin T7 could also be a member of class IIa bacteriocins.

Table 1. Characteristics of *Enterococcus* sp. T7 isolated from human fecal samples.

Test items	Result
Gram staining	+
Cell form	Cocci
Colony form	Circular
Colony color	White
Mobility	-
Catalase	-
Growth at 10°C	+
Growth at 45°C	+
NaCl tolerance	≤6.5%
Growth pH range	4.5-9.6
Carbohydrate utilization pattern	
D(+) Cellobiose	+
α-L-Rhamnose	-
Salicin	+
Melibiose	+
D(+) Trehalose	+
D(-) Ribose	+
D(+) Raffinose	+
D(-) Arabinose	-
D(+) Mannose	+
Starch	-
Maltose	+
D(+) Xylose	-
D(-) Fructose	+
D(+) Galactose	-
α-Lactose	+
D-Mannitol	+
Sucrose	+

Symbols. +, positive; -, negative.

Inhibitory Spectrum of Bacteriocin T7

Various gram-positive and gram-negative bacteria were tested for their susceptibilities to bacteriocin T7 and the results are shown in Table 2. As shown in this table, bacteriocin T7 inhibits some strains of *Lactobacillus*, *Staphylococcus*, *Enterococcus*, and *Streptococcus*. Sensitive strains include *L. acidophilus* KFRI 161 and ACID, and *L. helveticus* KFRI 347. Tested single strains of *Enterococcus faecalis*, *Staphylococcus epidermidis*, and *Streptococcus bovis* were also inhibited. However, other strains of *L. acidophilus*, *L. casei*, *L. bulgaricus*, and *L. pentosus* along with all strains of *Lactococcus lactis* were found to be insensitive. *Listeria monocytogenes* Scott A was inhibited by bacteriocin T7 but the degree of inhibition was less compared to those of other organisms. Bacteriocins belonging to Class IIa, also known as pediocin-like bacteriocins, inhibited the growth of listeriae, therefore, the sensitivities of various *Listeria* strains against bacteriocin T7 need to be closely checked. Similar to enterocin A, bacteriocin T7 did not inhibit the growth of the lactococci and gram-negative bacteria such as *Escherichia coli* and *Salmonella typhimurium*. The results indicate that bacteriocin T7 has a medium-range inhibition spectrum compared with the broad-range bacteriocins such as pediocin PA-1 [15] and narrow-range bacteriocins such as helveticin J [10].

Table 2. Inhibition spectrum of the bacteriocin T7.

Indicator strains	Inhibition
Lactobacillus acidophilus KFRI 161	++[1]
Lactobacillus acidophilus KFRI 217	-
Lactobacillus acidophilus ACID	+++
Lactobacillus acidophilus IAM 1084	-
Lactobacillus helveticus KFRI 347	++
Lactobacillus casei YIT 9018	-
Lactobacillus bulgaricus CH2	-
Lactobacillus pentosus KFRI 481	-
Lactobacillus brevis 2014	-
Lactobacillus delbrueckii subsp. *lactis* ATCC 4797	++
Lactobacillus delbrueckii subsp. *lactis* ATCC 7830	++
Bacillus subtilis DB104	-
Bacillus cereus	-
Enterococcus faecalis	+++
Listeria monocytogenes Scott A	++
Staphylococcus aureus	-
Staphylococcus epidermidis	++
Staphylococcus carnosum	-
Streptococcus bovis	+++
Streptococcus thermophilus	+
Salmonella typhimurium TA98	-
Escherichia coli K-12	-
Lactococcus lactis IL1403	-
Lactococcus lactis MG1363	-
Lactococcus lactis MG1614	-
Lactococcus lactis LM0230	-

[1]Inhibition zone size: +, 0.5[2] to 2 mm, ++, 2 to 4 mm; +++, more than 4 mm.
[2]The value equals to (diameter of an inhibition zone in mm-diameter of a colony)/2.

Table 3. Effect of enzymes, organic solvents, pH, and heat treatments on the activity of bacteriocin T7.

Treatments	Residual activity (AU/ml)
Control	16,000
Enzymes[1]	
Lysozyme	16,000
Proteinase K	0
Pepsin	16,000
RNase A	16,000
Trypsin	0
Catalase	16,000
Protease	0
Solvents[2]	
Ethanol	16,000
Methanol	16,000
Acetonitrile	16,000
Acetone	16,000
Chloroform	16,000
pH 2 to 10	16.000
60°C, 60 min	16,000
80°C, 30 min	16.000
80°C, 60 min	8.000
100°C, 10 min	16,000
100°C, 30 min	4.000
100°C, 60 min	250
121°C, 15 min	250

[1]The final enzyme concentration was 1 mg/ml.
[2]50% (v/v) concentration of solvent was used

Sensitivity of Bacteriocin T7 to Heat, pH, and Enzymes

Bacteriocin T7 activity was not affected after 30 min treatment at 80°C or after 10 min at 100°C (Table 3). The activity was reduced to half after 60 min at 80°C and to a quarter after 30 min at 100°C. Only 1.6% of the original activity was detected after 60 min at 100°C or after autoclaving at 121°C for 15 min at 15 lb/in^2. It was not affected by pH variation in the range between 2 and 10. The activity was not detected after treatment with proteinase K, trypsin, and protease, while full activity remained after treatment with lysozyme, catalase, RNaseA, and pepsin. It was not affected by exposure to organic solvents of 50% concentrations under the conditions described in Materials and Methods. These results indicate that bacteriocin T7 has a considerable degree of stabilities against heat and organic solvent, a common characteristics of class II bacteriocins [12], and is a desirable property when bacteriocin is incorporated into foods.

Bacteriocin Production by *Enterococcus* sp. T7

The effect of growth temperature and initial pH of the growth medium on the bacteriocin T7 production were investigated (Fig. 1). As shown in Fig. 1, the bacteriocin production started at the middle of the exponential phase and reached its maximum level during the stationary growth phase in all the temperatures tested. The maximum bacteriocin activity (2,000 AU/ml) was the same with cells grown either at 25 or 30°C, but cells grown at 37°C had only half the activity (1,000 AU/ml). The reduction of maximum activity at 37°C might have been due to the increased activities of cellular proteases. The difference in the initial pH of the MRS medium did not cause any significant difference in the maximum activity level (data not shown), except that it took longer to reach the maximum level at lower initial pH values (less than 6.5). Production of bacteriocins in many LAB strains is known to be greatly influenced by the pH of the media [25]. Based on these observations, *Enterococcus* sp. T7 was cultivated at 30°C with an initial pH of 6.5 for bacteriocin production.

Mode of Bacteriocin Action

To determine whether the bacteriocin produced by *Enterococcus* sp. T7 had bactericidal or bacteriostatic effects against sensitive strains, partially purified bacteriocin T7 (1,600 AU) was added to the indicator strain of *Enterococcus faecalis* which was suspended in 3 ml of 50 mM Tris-HCl buffer (pH 8.3). The number of viable cells was determined by standard plate counting at specific times after the addition

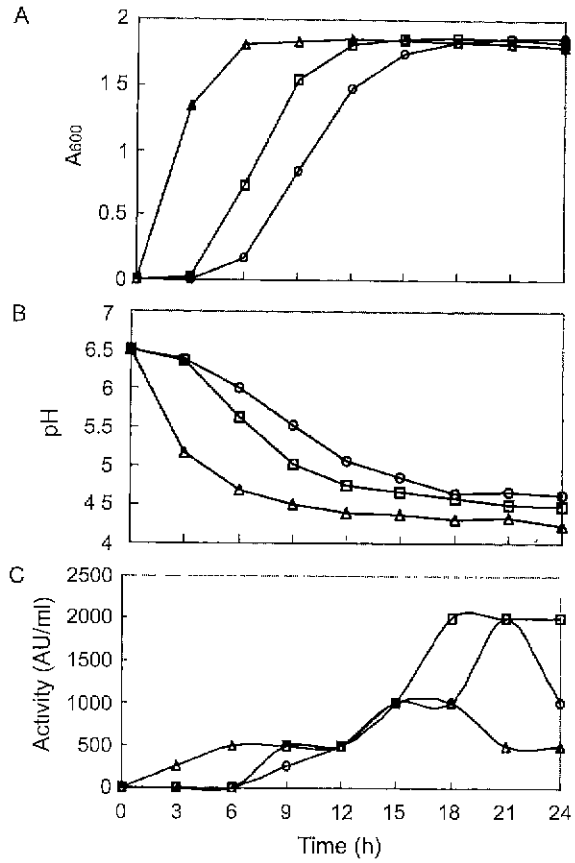

Fig. 1. The cell growth and bacteriocin production of *Enterococcus* sp. T7 in MRS broth.
Growth at 25°C (-○-), 30°C (-□-), and 37°C (-△-). A, Absorbance at 600 nm during growth; B, pH change during growth; C, bacteriocin production

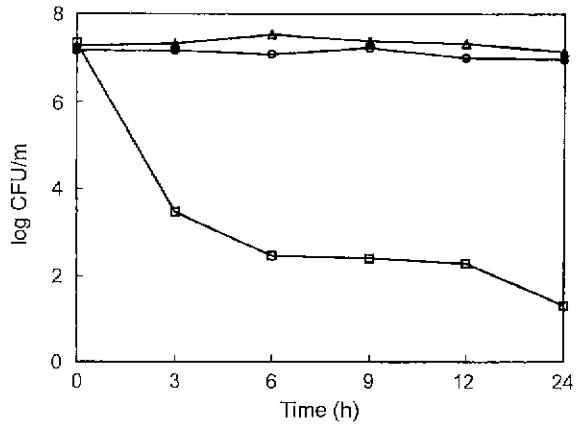

Fig. 2. Mode of inhibitory action of bacteriocin T7.
-○-, No addition of bacteriocin T7 into the suspension of *Enterococcus faecalis* cells (3×10^7 CFU/ml); -□-, partially purified bacteriocin T7 (1,600 AU) was added into the suspension of indicator cells; -△-, simultaneous addition of proteinase K and bacteriocin T7 into the suspension of indicator cells

of bacteriocin. As shown in Fig. 2, the number of viable cells decreased rapidly during the first 3 h and then

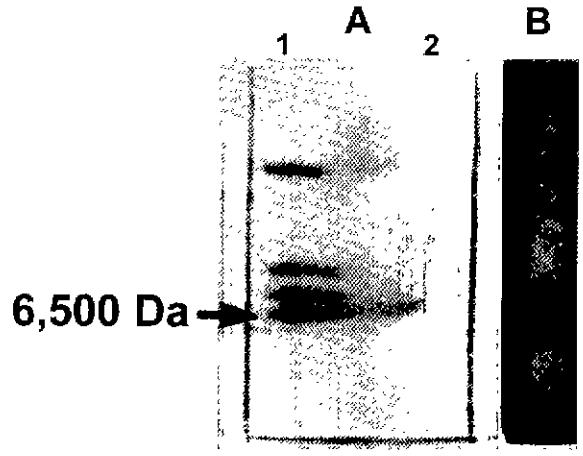

Fig. 3. SDS-PAGE of bacteriocin T7 and detection of antimicrobial activity.
(A) Coomassie Blue-stained gel 1. The size marker (ultra low range, Sigma M3546) and the position of 6.500 Da protein is marked by an arrow; 2 bacteriocin T7. (B) Gel overlaid with MRS agar containing indicator, *Enterococcus faecalis* cells

gradually thereafter. Approximately 5 log-scale reduction in viable cell numbers was observed during a 12 h period, whereas no reduction in viable cell numbers was observed in a sample where proteinase K (18.6 units) was added in addition to bacteriocin. The result again indicated the proteinaceous nature of bacteriocin T7. In the case of Reutericin 6, the inhibition of indicator strains was accompanied by the decrease of viable cell numbers and the leakage of β-galactosidase, a cytoplasmic enzyme [11]. This indicates that Reutericin 6 kills sensitive cells at least partly by disrupting cellular membrane structure. More studies on the exact mode of killing by bacteriocin T7 are necessary.

SDS-PAGE

The approximate molecular weight of bacteriocin T7 was estimated to be 6,500 Da, determined by SDS-PAGE (Fig. 3). When the bioassay of antimicrobial peptide was performed on polyacrylamide gel under the same conditions, a growth inhibition zone of an indicator strain appeared at the same position.

Purification of Bacteriocin T7

Bacteriocin T7 was purified from a supernatant of broth cultures prepared in the MRS medium. Ammonium sulfate pellet, obtained by 50% ammonium sulfate saturation was dissolved in 10 ml distilled H_2O, and dialyzed against H_2O for more than 12 h. The dialysate was freeze-dried and then solubilized in a 50 mM Tris-HCl buffer (pH 8.3). Partially purified bacteriocin T7 was applied to a DEAE-Sephacel anion-exchange column equilibrated with 50 mM Tris-HCl buffer (pH 8.3). After washing the column with the same buffer, a linear NaCl (0–1.0 M) gradient in the same buffer was applied to elute bound proteins from the

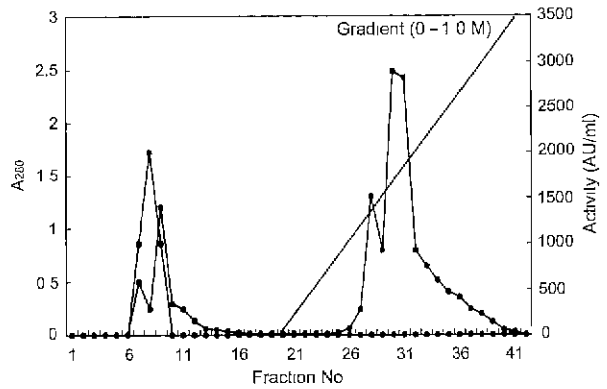

Fig. 4. Elution profile of bacteriocin T7 from DEAE-Sephacel column equilibrated with 50 mM of Tris-HCl buffer (pH 8.3).
Bacteriocin T7 was eluted during the washing step (washing buffer, 50 mM Tris-HCl, pH 8.3). The bacteriocin activity of each fraction (10 ml) was assayed using the spot-on-lawn method -●-, A_{280} (Absorbance at 280 nm); -○-, Bacteriocin activity (AU/ml).

column. As shown in Fig. 4, bacteriocin T7 did not bind to the resin and it eluted from the column during washing (fraction 5–12). The fractions containing bacteriocin T7 were pooled, freeze-dried, and dissolved in 50 mM Tris-HCl buffer (pH 8.3). Gel filtration chromatography using

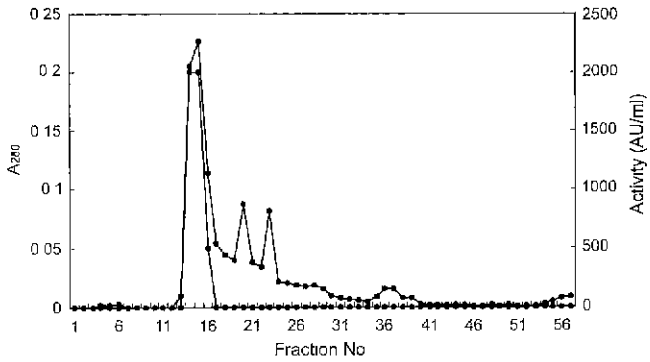

Fig. 5. Elution profile of bacteriocin T7 from a Sephadex G-25 column equilibrated and eluted with 50 mM of Tris-HCl buffer (pH 8.3).
The bacteriocin activity of each fraction (6 ml) was determined using the spot-on-lawn method -●-, A_{280} (Absorbance at 280 nm); -○-, Bacteriocin activity (AU/ml)

Table 4. Purification of bacteriocin T7.

Step	Total activity (AU)	Total protein (mg)	Specific activity (AU/mg)	Fold of purification
Supernatant	682,500	110.00	6,205	1.0
Ammonium sulfate precipitation	288,000	7.74	37,209	6.0
Anion-exchange chromatography	225,600	2.40	94,000	15.2
Gel-filtration chromatography	192,000	1.36	141,176	22.8

Sephadex G-25 (Pharmacia, Uppsala, Sweden) was employed as a next purification step. Tris-HCl buffer (50 mM, pH 8.3) was used for elution and the chromatogram is shown in Fig. 5. An overall purification procedure is summarized in Table 4. These purification steps resulted in a 23-fold increase in the specific activity and a 28% recovery.

Acknowledgments

This work was supported by a research grant received from the Korean Ministry of Health and Welfare (HMP-98-F-5-0020). G.-S. Moon was supported by the Brain Korea 21 project of the Ministry of Education. The authors are grateful for all the support and would like to express their appreciation to Dr. Jae-Won Kim of Dept. of Microbiology, Gyeongsang National University for his advice and assistance during the bacteriocin purification study.

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