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Purification and Characterization of Lacticin NK34 Produced by *Lactococcus lactis* NK34 against Bovine Mastitis

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Lactococcus lactis NK34에 의해 생산된 소 유방염 원인균에 효과가 있는 lacticin NK34의 정제 및 특성

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Abstract

Lactococcus lactis NK34, isolated from *jeotgal* (Korean traditional fermented fish), produces bacteriocin against bovine mastitis pathogens such as *Staphylococcus aureus* 7, *S. aureus* 8, *Staphylococcus chromogenes* 10, *S. chromogenes* 19, *Staphylococcus hominis* 9, *Streptococcus uberis* E290, *Enterococcus faecium* E372, *Streptococcus agalactiae* ATCC 13813, *Pseudonocardia autotrophia* KCTC 9455, and *Staphylococcus simulans* 78. Lacticin NK34 was inactivated by protease XIV but not by protease IX, protease XIII, proteinase K, á-chymotrypsin, trypsin, and pepsin. Also, lacticin NK34 was stable over a pH range of 2 to 9 for 4 hr and withstood exposure to temperatures of 30-100°C for 30 min. Lacticin NK34 showed bactericidal effects against *S. simulans* 78. This bacteriocin was purified using ammonium sulfate precipitation, ion exchange chromatography, ultrafiltration, and hydrophobic chromatography. Tricin-SDS-PAGE of purified bacteriocin gave the same molecular weight (3.5 kDa) as nisin. The gene encoding this bacteriocin was amplified by PCR using nisin gene-specific primers. It showed similar sequences to this nisin Z gene. These results indicate that lacticin NK34 is a nisin-like bacteriocin, and could be used as an antimicrobial alternative for livestock.

Key words : *Lactococcus lactis* NK34, lacticin NK34, bovine mastitis, purification, characterization, nisin

Introduction

Bacteriocins are proteinaceous bacterial products which have bactericidal activity. They are produced by various lactic acid bacteria (LAB) including lactococci, lactobacilli, leuconostoc, and pediococci (Klaenhammer, 1988; Nes *et al.*, 2007). Many bacteriocins produced by LAB inhibited not only species closely related to the producer strain, but also the growth of food-borne pathogens such as *Listeria monocytogenes, Clostridium botulinum*, etc. Bacteriocin was applied various foods such as dairy products (Weinbrenner *et al.*, 1997; Oh *et al.*, 2006), *sous vide* product (Kim *et al.*, 2008), meat (Lee *et al.*, 2008), canned foods, etc.

The bacteriocins of LAB are classified into four classes (Klaenhammer, 1998). Class I bacteriocins or lantibiotics, are small (< 5 kDa) membrane active peptides, which contain post-translationally modified amino acid residues like lanthionine. Representative class I bacteriocin is known as nisin. Class II bacteriocins are small, heat-stable, non-lanthionine-containing peptides, including class IIa, *Listeria* active peptides; class IIb, small cationic peptide; and class IIc, sec-dependent secreted bacteriocin.

Bovine mastitis is a disease caused by infection of cow

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udders and is one of the most significant causes of economic losses to the dairy industry due to rejected milk, degraded expenses, and increased labor costs (Green *et al.*, 2002). *Staphylococcus aureus* and *Streptococcus* spp. are main bacterial agents in this disease (Bradley, 2002). Treatment of bovine mastitis have generally used with antibiotics, however, antibiotics may leave harmful residues in raw milk. Bacteriocin may be an alternative to conventional antibiotics.

Nisin has been the most extensively studied bacteriocin. Nisin is permitted as a food additive in at least 46 countries, on dairy products and canned foods (Delves-Broughton, 1990). Also, nisin is inhibitory to many mastitis strains. Lacticin 3147 was reported as a bacteriocin having inhibitory activity against mastitis pathogens using teat seals (Ryan *et al.*, 1998; Twomey *et al.*, 2000). The bacteriocin produced by *Lactobacillus bulgaricus* showed antibacterial activity against antibiotic resistant strain, *S. aureus* ATCC 6538 (Kim *et al.*, 2004). Also, bacteriocin produced by *S. aureus* isolated from cows having bovine mastitis was studied (Nascimento *et al.*, 2002; Coelho *et al.*, 2007).

We describe here the characterization and purification of lacticin NK34 isolated from *jeotgal* having antimicrobial activity against bovine mastitis-related microorganisms.

Materials and Methods

Bacterial strains and culture media

Lactococcus lactis NK34 was isolated from *jeotgal*, cultured in lactobacilli MRS medium (Difco Laboratories, Detroit, MI, USA) at 35°C. *Staphylococcus simulans* 78 was used as indicator bacterium for bacteriocin activity. *S. simulans* 78 was cultured in tryptic soy broth (TSB, Difco) at 35°C. Other strains listed in Table 2 were obtained from National Veterinary Research & Quarantine Service and Seoul National University. These strains were cultured in TSB agar at 35°C and were stored at -70°C in medium with 20% (v/v) glycerol.

Determination of bacteriocin activity

Lacticin NK34 activity was determined by spot-on-lawn method (Lee and Paik, 2001). Soft agar seeded (1%, v/v) with the indicator organisms was overlayed on the plate, and was allowed to solidify. Concentrated lacticin NK34 solution was diluted serially using two-fold dilution, and 5 μL of each dilution was spotted on the plate. The plates were incubated at 35°C overnight. The bacteriocin activity was determined in arbitrary unit (AU) as follows: Bacteriocin activity (AU/mL) = $2^N \times 200$, where N = dilution number with the smallest zone of inhibition.

Production and ammonium sulfate precipitation of the bacteriocin

L. lactis NK34 was grown to stationary phase in 2 L flask containing 1.5 L of lactobacilli MRS medium at 35°C. The cells were removed by centrifugation at 10,000×g for 20 min at 4°C. The culture supernatant was then precipitated with 60% ammonium sulfate. The precipitate was collected by centrifugation at 10,000×g for 30 min at 4°C, resuspended in a 100 mM phosphate buffer (pH 7.0), and dialyzed against 2 L of 10 mM phosphate buffer (pH 7.0) for 12-18 hr in Spectra-Por no. 3 dialysis tubing (molecular weight cutoff, 3,500; Spectrum Medical Industries, Gardena, CA, USA). The dialyzed samples were stored at -70°C.

Antimicrobial spectrum of lacticin NK34

The antimicrobial spectrum was determined by the well diffusion assay. The supernatant (100 μL) was placed in wells on TSB agar plate. The plate was turn upside down. Soft agar seeded (1%, v/v) with the indicator organisms was the overlaid on the plate. The plates were incubated at 35°C overnight.

Sensitivity of enzyme, pH, and heat

The sensitivity to protease IX, protease XIII, protease XIV, proteinase K, α-chymotrypsin, trypsin, and pepsin was tested at a final concentration of 1 mg/mL for 1 hr. Effect of pH was tested a range of pH 3-9 at 4°C for 4 hr. To test for heat sensitivity, lacticin NK34 was heated to 100°C for 30 min.

Mode of inhibition of lacticin NK34

0, 2,560 and 5,120 AU/mL of partially purified lacticin NK34 were used to determine the mode of inhibition. One milliliter of growing culture of *S. simulans* 78 in 9 mL of 0.1 M potassium phosphate buffer (pH 7.0) containing lacticin NK34 were incubated at 35°C. Samples were collected on interval of 1 hr. Viable cells were counted by general plate counting on TSB agar.

Purification of lacticin NK34

Lacticin NK34 was purified by means of chromatography and ultrafiltration. Anion exchange chromatography was performed with DEAE-cellulose (Sigma, St. Louis, MO, USA). The column was washed with 20 mM phosphate buffer (pH 7.0) and the absorbed proteins were eluted with a linear salt gradient (0 to 1 M NaCl). Fractions of 6 mL were collected and assayed for bacteriocin activity. The active

fractions were subjected to ultrafiltration (Amicon ultra-4, 10 kDa, Millipore, Billerica, MA, USA). The purified samples were then applied to a C_{18} μBondapak column (3.9 mm×30 cm, Waters, Milford, MA, USA) and subjected to HPLC (Agilent 1100 series, Agilent Technologies Inc., Palo Alto, CA, USA). The buffer used was 10% acetonitrile/0.1% trifluoroacetic acid (TFA), and flow rate was 1 mL/min. One milliliter fractions were collected, and then assayed for bacteriocin activity. Fractions were concentrated by freeze-drying for molecular mass determination. Protein concentration was determined by using the Lowry assay (Daniel *et al.*, 1996).

Molecular mass determination

Tricine-SDS-PAGE was performed to determine the molecular mass of the putative bacteriocin (Schagger and Jagow, 1987). Protein standard and their molecular weights were as follows: triosephosphate isomerase, 26,600 Da; myoglobin, 17,000 Da; α-lactoalbumin, 14,200 Da; aprotinin, 6,500 Da; insulin 3,496 Da; bradykinin, 1,060 Da. The part of the gel containing the sample and molecular mass markers was stained with Coomassie brilliant blue R-250. For direct detection of antimicrobial activity, second part was fixed with fixing buffer (40% MeOH : 10% acetic acid) for 1 hr. And then this part was washed with distilled water, overlayed with soft agar (1%, w/v) with indicator strain and incubated at 35°C.

DNA preparation and PCR detection of lacticin NK34 structural gene

L. lactis NK34 genomic DNA was isolated by lysing protoplasts (Neumann *et al.*, 1992). The DNA primers used in this study are listed in Table 1. DNA was dissolved in 10 mM Tris HCl (pH 8.0)-1 mM EDTA, and approximately 100 ng was subjected to amplification by the polymerase chain reaction (PCR) in a total volume of 100 μL containing 2.5 U of *Taq* polymerase (Solis BioDyne, Tartu, Estonia), 200 μmol/L dNTP (Solis BioDyne), 0.1 volume of 10× buffer (Solis BioDyne), 1 μmol/L primer(s) were set up. After up to 100 μL with distilled water, 1 μL genomic DNA of lactococci was added to the reaction. A DNA segment was amplified by PCR (XP cycler, Bioer, Tokyo, Japan) with synthetic DNA primers, nisin A and nis A/nis Z. DNA was amplified with nisin A primers for 40 cycles (Choi *et al.*, 2000). Samples were denatured at 94°C for 5 min and subjected to amplification cycles in a PCR. Each cycle involved 1 min denaturation at 95°C, followed by an annealing step at 55°C for 1 min and an extension step of 72°C for 1 min. Finally, products were extended for 7 min. The nis A/nis Z primers were denatured at 94°C for 5 min and subjected to amplification cycles in a PCR, and consisted of denaturation at 93°C for 2 min, a primer annealing step at 54°C for 1 min, and an extension step at 72°C for 1.5 min (de Vos *et al.*, 1993). Finally, products were extended for 7 min at the completion of 30 amplification cycles. Five microliters of the reaction mixture was analyzed on 1.2% (w/v) agarose gels with ethidium bromide staining.

Results and Discussion

Antimicrobial spectrum against bovine mastitis-related microorganisms

L. lactis NK34 and lacticin NK34 have demonstrated antimicrobial effects against a broad range of Gram-positive bacteria including *S. aureus* (Lee *et al.*, 2000; Lee *et al.*, 2008). *S. aureus*, *S. chromogenes*, *S. hominis*, *S. simulans*, *Streptococcus uberis*, *Enterococcus gallinarum*, and *Enterococcus faecium* are known to cause bovine mastitis (Table 2). Among these strains, 12 strains were inhibited by lacticin NK34. Lacticin NK34, in particular, showed antimicrobial effects against two strains of *S. aureus*. Also, lacticin NK34 showed antimicrobial effects against a wide range of CNS strains.

Sensitivity of enzyme to pH changes and heat treatment

Lacticin NK34 was inactivated by protease XIV but not by protease IX, protease XIII, proteinase K,α-chymotrypsin, trypsin, and pepsin (Table 3). Activity loss due to protease XIV treatment confirmed the proteinaceous nature of lacticin NK34. Also, lacticin NK34 was stable over a pH range of 2 to 9 for 4 hr and when heated to 30-100°C for 30 min (data not shown). Nisin's known sensitivity to α-chymotrypsin, heat stability at low pH and non-toxic nature has pro-

Table 1. The primer sequence used in this study

		Sequence (5'→ 3')
nisA/nisZ	Forward	CGCGAGCATAATAAACGGCT
	Reverse	GGATAGTATCCATGTCTGAAC
nisA	Forward	ATGAGTACAAAGATTTTAACTTGGATTGGT
	Reverse	ATAAACGAATGCACTTATGATGTTACTGTT

Table 2. Antimicrobial spectrum of lacticin NK34 against bovine mastitis-related microorganisms

Microorganism	Lacticin NK34
Staphylococcus aureus 1573	-
Staphylococcus aureus 1572	-
Staphylococcus aureus 1512	-
Staphylococcus aureus 1504	-
Staphylococcus aureus 7	+
Staphylococcus aureus 8	+
Staphylococcus chromogenes 10	+
Staphylococcus chromogenes 19	+
Staphylococcus hominis 9	+
Staphylococcus simulans 78	+
Streptococcus uberis E290	+
Enterococcus gallinarum E362	+
Enterococcus faecium E363	+
Enterococcus avium E369	-
Enterococcus faecium E374	+
Streptococcus agalactiae ATCC 13813	+
Pseudonocardia autotrophica KCTC 9455	+

+, positive; -, negative.

Table 3. Enzyme sensitivity of lacticin NK34

Enzyme	Residual activity (AU/mL)
Control	12,800
Protease IX	12,800
Protease XIII	12,800
Protease XIV	1,600
Proteinase K	12,800
α-Chymotrypsin	12,800
Trypsin	12,800
Pepsin	12,800

moted its widespread use (Choi *et al.*, 2000). These experiments show that lacticin NK34 possesses useful properties that may contribute to a potential commercial application in treating bovine mastitis.

Mode of inhibition of lacticin NK34 against *Staphylococcus simulans* 78

The addition of lacticin NK34 to *S. simulans* 78 caused a loss in their viability when compared to control cultures

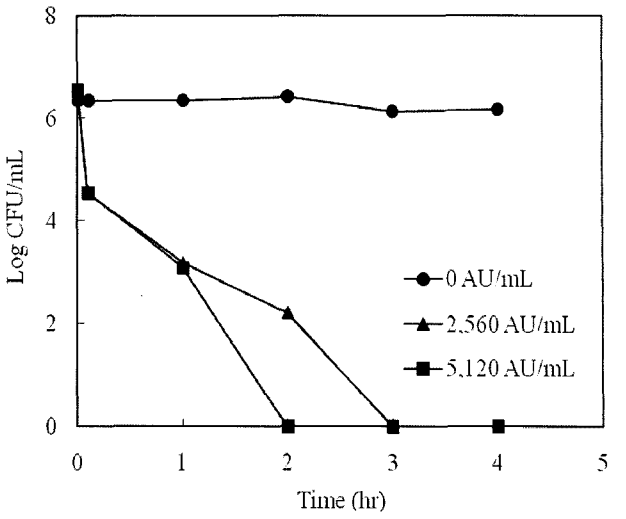

Fig. 1. Mode of inhibition of lacticin NK34 against *S. simulans* 78.

(Fig. 1). After 3 hr, the addition of 2,560 AU/mL of lacticin NK34 killed *S. simulans* 78 below 1 log CFU/mL. And the addition of 5,120 AU/mL of lacticin NK34 killed below 1 log CFU/mL after 2 hr. These data indicate that lacticin NK34 showed a bactericidal mode of inhibition against *S. simulans* 78. Bactericidal mode of action is characteristics of the bacteriocin of LAB (Davey *et al.*, 1981).

Purification of lacticin NK34

The purification of lacticin NK34 was achieved by the four steps (60% ammonium sulfate precipitation and anion-exchange chromatography (AEC), ultrafiltration, and HPLC) (Table 4). Bacteriocin has generally hydrophobic and cationic antimicrobial compounds (Kaenhammer 1998; O'Sullivan *et al.*, 2002). But, lacticin NK34 has the higher yield in AEC. In this step, compound with bacteriocin activity were eluted between 0.15-0.33 M of NaCl. In the second step, collected active fractions from AEC were subjected to ultrafiltraiton. The recovery of bacteriocin activity was 70% after the ultrafiltration step. The final step of purification was used RP-HPLC, where one peak was eluted at retention time of 4.2 min (Fig. 2). This peak was active against *S. simulans* 78. Analysis of the activity fraction isolated after AEC by tricin-SDS-PAGE, and direct detection of antimicrobial

Table 4. Purification of lacticin NK34

Purification step	Total protein (mg)	Total activity (AU)	Specific activity (AU/mg)	Recovery (%)
Culture supernatant	2,520	144,000	57.14	100
Ammonium sulfate precipitation	348	307,200	882.76	213
Anion-exchange chromatography	36	100,800	2,800	70
Ultrafiltration	28.8	100,800	3,500	70
HPLC	11.35	64,000	5,639	44

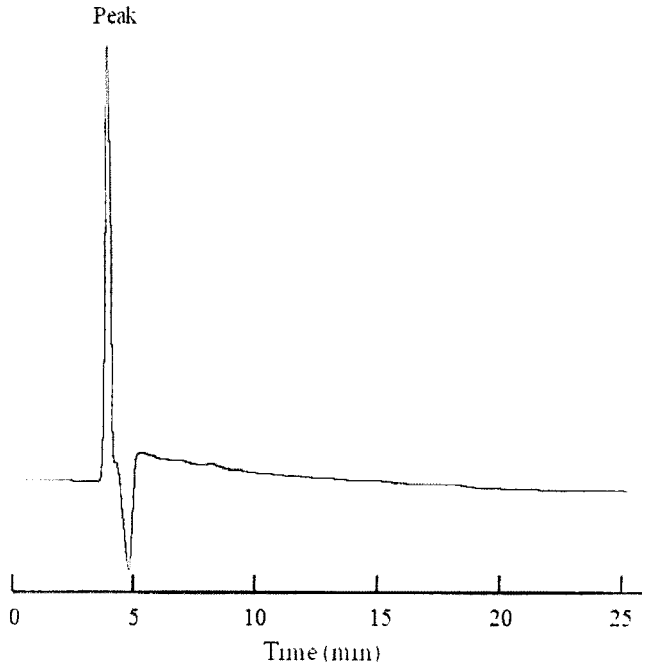

Fig. 2. Reverse-phase HPLC chromatogram of ultrafiltered bacteriocin monitored by absorbance at 220 nm. The peak with 4.2 min retention time exhibited bacteriocin activity.

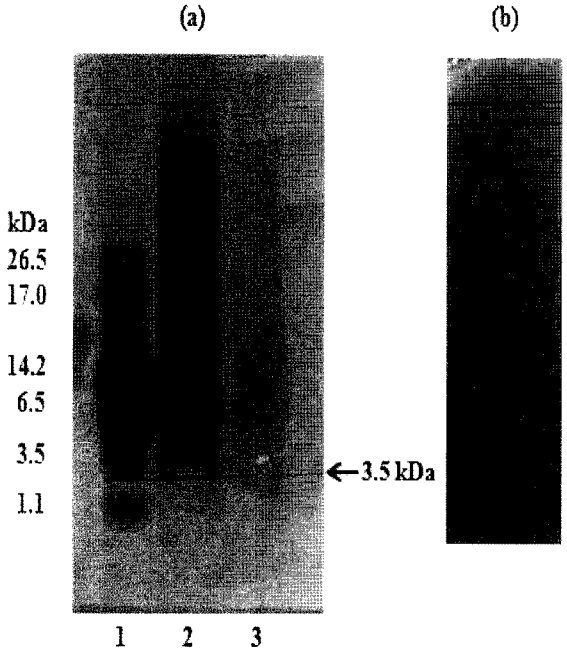

Fig. 3. Tricin-SDS-PAGE of the purified lacticin NK34. Lane 1, size marker (26.5, 17.0, 14.2, 6.5, 3.5, 1.1 kDa); Lane 2, 3, fraction from anion-exchange chromatography; (b) the gel overlaid with *S. simulans* 78.

activity on the electrophoresis gel indicated that molecular mass of the bacteriocin is approximately 3.5 kDa. The overlaid part of the gel shows that this fraction was shown antimicrobial effect against *S. simulans* 78 as about 3.5 kDa (Fig. 3). However, analysis of the activity fraction from HPLC by tricin-SDS-PAGE was not shown as band.

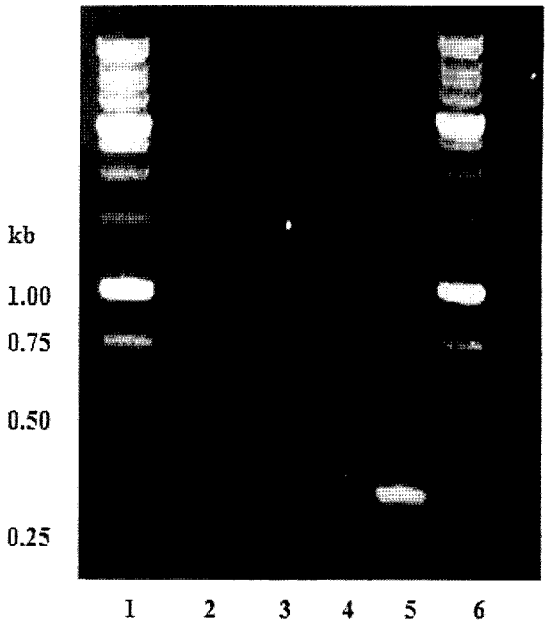

Fig. 4. Agarose gel electrophoresis of PCR products with nisin A and nisin Z primers. Lane 1, 5, DNA leading marker; Lane 2, 3, nisA specific primer and genomic DNA of *L. lactis* NK34; Lane 4, 5, nisA/nisZ specific primer and genomic DNA of *L. lactis* NK34.

Genetic determination by PCR of the presence of nisin A and nisin Z structural genes

Using the published sequence of the nisin structural gene (Dodd *et al.*, 1992), two primers, which are complementary to sequences occurring proximal to the 3' and 5' ends of the nisin A and nisin Z structural gene (nisA), were synthesized. Using the nisA genomic DNA of *L. lactis* NK34, no PCR products were obtained with the nisA-specific primer and this new reverse primer (Fig. 4, lane 2 and 3). But nisA/nisZ primers did amplify a product of 300 bp from template DNA (Fig. 4, lane 4 and 5). Therefore, bacteriocin produced by *L. lactis* NK34 was found to be a nisin-like bacteriocin. Nisin A and nisin Z primers generated 300 bp fragments. This result indicates that *L. lactis* NK34 contain the nisin Z gene.

In conclusion, bovine mastitis pathogens such as several methicillin resistant *Staphylococcus aureus* and coagulase negative *Staphylococcus* were inhibited by lacticin NK34. The molecular mass of lacticin NK34 was about 3.5 kDa, as determined by tricin-SDS-PAGE. These results indicate that lacticin NK34 is a nisin-like bacteriocin, and could be used as an antimicrobial alternative for livestock.

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