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 autotrophica* 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 monocyogenes*, *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 Ia, *Listeria* active peptides; class Iib, small cationic peptide; and class IIc, see-dependent secreted bacteriocin.

Bovine mastitis is a disease caused by infection of cow
udder 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×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 turned 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