Short communication: Bacteriocin KC24 produced by *Lactococcus lactis* KC24 from kimchi and its antilisterial effect in UHT milk

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ABSTRACT

The severity of *Listeria monocytogenes* infections emphasizes the need for prevention or elimination of the pathogen from dairy products. *Lactococcus lactis* KC24, isolated from kimchi, exhibited an antimicrobial effect against food pathogens, including *L. monocytogenes* ATCC 15313. *Lactococcus lactis* KC24 was cultured in a 5-L jar fermenter at 35°C, and bacteriocin activity was maximal at 4 h of incubation and persisted for 20 h. Bacteriocin KC24 was inactivated by protease XIV, indicating that it has a proteinaceous nature. Bacteriocin activity was maintained at pH 3.0 to 9.0 and at temperatures of 50 to 121°C. The mode of inhibition against *L. monocytogenes* ATCC 15313 was shown to involve a bactericidal effect by treatment with 100 and 200 arbitrary units (AU)/mL of bacteriocin KC24. To test the activity of bacteriocin KC24 in a food product, bacteriocin KC24 and nisin (100 and 200 AU/mL) with 4 log cfu/mL of a mixed culture of *L. monocytogenes* (ATCC 15313, ScottA, H7962, and H7762) were applied to UHT milk. Compared with the control, treatment with bacteriocin KC24 completely inhibited the growth of *L. monocytogenes* and resulted in no detectable *L. monocytogenes* after 14 d at 4°C, whereas nisin moderately inhibited *L. monocytogenes*, resulting in a final concentration after 14 d at 4°C higher than the initial inoculum. Bacteriocin KC24 may prove useful in improving the safety of dairy products.

Key words: bacteriocin, *Listeria monocytogenes*, UHT milk, biopreservative

Short Communication

A variety of fermented foods has been screened for lactic acid bacteria with antimicrobial effects. In particular, kimchi is a traditional Korean fermented vegetable food, and has been most frequently studied in screening studies for bacteriocin producers and ap-

plications in Korean fermented foods (Kim et al., 2009; Chang and Chang, 2011). *Leuconostoc citreum* GJ7 was isolated from kimchi and applied to kimchi as a food preservative (Chang and Chang, 2011). Nisin has also been applied to control the ripening of kimchi (Ko et al., 2005).

Bacteriocins are commonly referred to as antibacterial peptides or ribosomally synthesized compounds produced by bacteria, and they are antagonistic to phylogenetically closely related microorganisms (Klaenhammer, 1988). Food safety is an important international concern, and the investigation of novel bacteriocins that target food pathogens has received a great deal of attention. Bacteriocin and bacteriocin-producing strains have been used in dairy products to control *Bacillus cereus*, *Staphylococcus aureus*, and *Listeria monocytogenes* (Gálvez et al., 2007; Bizani et al., 2008; Edalatian et al., 2012).

Listeria monocytogenes is a psychrotrophic foodborne pathogen that has caused outbreaks and sporadic cases of listeriosis worldwide (Reij et al., 2004). Contamination by this microorganism can result in listeriosis in immunocompromised individuals, pregnant woman, children, the elderly, and neonates. *Listeria monocytogenes*, which is widely distributed in nature, is frequently found in food processing environments, can grow at temperatures ranging from 1 to 45°C (Sorrells et al., 1989; Walker et al., 1990), and is highly salt-tolerant (Farber and Peterkin, 1991). These characteristics make *L. monocytogenes* difficult to control in the food industry. Therefore, bacteriocins have been investigated in milk, cottage cheese, and meat products to solve this problem (Nieto-Lozano et al., 2006; Chanos and Williams, 2011).

The principal objective of this study was to isolate an antilisterial bacteriocin-producing strain from kimchi and characterize the inherent bacteriocins. Additionally, the obtained bacteriocin was studied as a possible natural preservative against *L. monocytogenes* in UHT milk.

One hundred twenty-five kimchi samples were obtained from homes, local groceries, and restaurants in Seoul and Kyunggi-do, Korea. The isolation method

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used was the triple agar layer method (Lee and Paik, 2001). Two hundred lactic acid bacteria were isolated from kimchi and tested for antimicrobial effects. Only isolate KC24 showed antimicrobial effects when the supernatant was controlled at pH 6.5, indicating that the effect was not due to organic acids. Isolate KC24 was a gram-positive coccus, identified as *Lactococcus lactis* at an identity of 99.8% by 16S ribosomal DNA sequence and API 50 CHL kit (bioMérieux, Lyon, France). Therefore, the bacteriocin generated by *Lactococcus lactis* KC24 was temporarily named bacteriocin KC24.

Lactococcus lactis KC24 was grown in de Man, Rogosa, and Sharpe (MRS; Becton Dickinson, San Diego, CA) broth for 28 h in a 5-L jar fermenter controlled to pH 6.5, at 35°C and 200 rpm. Cell concentration was assessed as cell number (log cfu/mL) on an MRS plate (Becton Dickinson) and optical density (absorbance) at 660 nm. The supernatant was collected via centrifugation ($15,130 \times g$ for 20 min at 4°C), and filtered through a 0.22- μ m pore size cellulose acetate filter. Bacteriocin activity was determined by the spot-on-lawn assay (Lee and Paik, 2001). Soft agar (0.8% agar) seeded with the indicator strain (*L. monocytogenes* ATCC 15313) was overlaid on the plates and left to solidify. Bacteriocin KC24 samples were serially diluted, 5 μ L of each dilution was spotted on each plate, and the plates were incubated at 35°C. Bacteriocin activity (AU/mL) = $2^n \times 200$, where n is the final dilution number showing inhibition.

The production of bacteriocin KC24 appeared to follow a kinetic pattern typical of primary metabolite synthesis (Figure 1). The activity of bacteriocin KC24 increased as the culture reached the exponential growth phase and reached a maximum (6,400 AU/mL) after 4 h of incubation. Almost all bacteriocins of lactic acid bacteria are generated during the exponential growth phase (Lim, 2010). In addition, the activity of bacteriocin KC24 remained until 20 h after exponential growth phase. Therefore, bacteriocin KC24 can be harvested in the stationary phase. This comparatively early production of bacteriocin KC24 could be exploited as an industrially useful characteristic.

The antimicrobial spectrum of *L. lactis* KC24 supernatant (adjusted to pH 6.5) was determined using the spot-on-lawn assay. *Bacillus cereus* KCCM 11341, *B. cereus* KCCM 40133, *Listeria ivanovii* ssp. *ivanovii* KCTC 3444, *L. monocytogenes* ATCC 15313, *Listeria seeligeri* KCTC 3591, *Micrococcus flavus* ATCC 10240, and *Staphylococcus aureus* KCCM 32395 were sensitive, whereas *Escherichia coli* O157:H7 and *Salmonella* Enteritidis KCCM 12021 were insensitive to the supernatant of *L. lactis* KC24 (data not shown).

Bacteriocin KC24 was partially purified from the culture supernatant by precipitation with 40% ammo-

nium. The effects of enzymes on partial purified bacteriocin KC24 were assessed via reaction with various proteases at a final concentration of 1 mg/mL for 1 h at 35°C. The proteases used were protease XIV, protease IX, proteinase K, trypsin, α -chymotrypsin, and protease XIII. Treatment with protease XIV induced a loss of bacteriocin activity (75% reduction; $P < 0.001$). The loss of bacteriocin activity was not observed when bacteriocin KC24 was treated with the other tested enzymes. These results confirmed the proteinaceous nature of the antimicrobial substance.

The effect of heat was assessed by 30 min of heating at 50, 60, 70, 80, 90, and 100°C and by autoclaving at 121°C for 15 min. Bacteriocin KC24 proved to be heat-stable: it was stable after 30 min of heat treatment at 50, 60, 70, 80, 90, and 100°C, and retained 50% of bacteriocin activity after autoclaving at 121°C for 15 min. This heat stability may be attributable to the formation of small globular structures and the occurrence of strongly hydrophobic regions, stable cross-linkages, and high glycine content (De Vuyst and Vandamme, 1992; Oscáriz and Pisabarro, 2001). Compared with our results, several bacteriocins are reported to remain stable under autoclaving conditions (Luo et al., 2011). Additionally, bacteriocin KC24 was stable in a pH range of 3.0 to 9.0. The stability of bacteriocin KC24 is of particular importance to food industry applications.

To determine whether bacteriocin KC24 has a bactericidal or bacteriostatic effect, bacteriocin KC24 was added to indicator cells suspended in 0.1 M potassium phosphate buffer (pH 7.0). The mode of inhibition was

Figure 1. Production of bacteriocin KC24 in 5-L jar fermenter. ○ = viable cells (log cfu/mL); ● = antimicrobial activity of bacteriocin KC24 [$\times 1,000$ arbitrary units (AU)/mL]; □ = optical density at 660 nm (A_{660}).

Figure 2. Mode of inhibition of bacteriocin KC24 on *Listeria monocytogenes* ATCC 15313. ○ = control; ● = tryptic soy broth (TSB) with 50 arbitrary units (AU)/mL of bacteriocin KC24; □ = TSB with 100 AU/mL of bacteriocin KC24.

determined via treatment with 0, 50, and 100 AU/mL of bacteriocin KC24. One milliliter of *L. monocytogenes* ATCC 15313 culture in 9 mL of 0.1 M potassium phosphate buffer (pH 7.0) containing bacteriocin KC24 was incubated at 35°C. Samples were collected at 0, 1, 2, 4, 6, 8, and 10 h. Viable cells were counted via the general plate counting method on tryptic soy broth (TSB, Becton Dickinson). After 4 h, *L. monocytogenes* ATCC 15313 exposed to 50 and 100 AU/mL of bacteriocin KC24 was not detected (Figure 2). In addition, the cell morphology determined by scanning electron microscopy showed damaged cell membranes (data not shown). Therefore, bacteriocin KC24 has a bactericidal effect against *L. monocytogenes* ATCC 15313. The antimicrobial mechanism of most bacteriocins is associated with membrane permeabilization (Sirtori et al., 2008; Zhou et al., 2008).

Listeria monocytogenes is capable of surviving and growing on chilled food at temperatures commonly used for refrigeration (Walker et al., 1990). We purchased UHT milk (Seoul Milk, Seoul, Korea) from a local grocery. A mixed culture of *L. monocytogenes* was used, with combination of strains ATCC 15313, ScottA, H7969, and H7762 in 1 L of UHT milk. Controls contained no bacteriocin, and nisin was used as a reference bacteriocin. The final concentrations of bacteriocin KC24 were 100 and 200 AU/mL, and the same concentrations were used for nisin. Each sample was placed in a 50-mL Falcon tube and stored at 4°C. Each experiment was conducted in triplicate, and sam-

Figure 3. Effect of (A) bacteriocin KC24 and (B) nisin against *Listeria monocytogenes* in UHT milk at 4°C. ○ = control; ● = UHT milk with 100 arbitrary units (AU)/mL of bacteriocin (bacteriocin KC24 or nisin); □ = UHT milk with 200 AU/mL of bacteriocin.

pling times were 0, 1, 2, 4, 6, 8, 10, and 14 d. *Listeria monocytogenes* was enumerated via the general plate counting method using *Listeria* selective medium (BD, Sparks, MD). A significant ($P < 0.01$) decline in the number of viable cells was observed during the storage of UHT milk with 100 and 200 AU/mL of bacteriocin KC24, and *L. monocytogenes* was not detected after 4 d (Figure 3). Cerein 8A, a bacteriocin produced by *Bacillus cereus* 8A, was reported to result in a successive increase in the shelf life of UHT milk, similarly to that observed with bacteriocin KC24 (Bizani et al., 2008). Treatment of UHT milk with 400 AU/mL of cerein 8A

delayed increase of cell concentrations of *L. monocytogenes* compared with the control without bacteriocin. The viable control cells (without bacteriocin) were increased to 7.1 log cfu/mL in the study of Bizani et al. (2008). The UHT milk with nisin showed a decrease of *L. monocytogenes* to 2 log cfu/mL over 2 d. However, *L. monocytogenes* counts increased to 4.9 log cfu/mL after 14 d. The pH of commercial milk is 6.7, which caused the loss of nisin activity. Similar results in other studies indicate that nisin has unstable activity at pH >6.0. *Listeria monocytogenes* ATCC 7644 was not detected in yogurt with added nisin (50 IU/mL) at pH 4.5; however, a reduction in *L. monocytogenes* ATCC 7644 of approximately 4 log cfu/mL was observed at pH 6.8 after 48 h in nonfat dry milk (Benkerroum et al., 2003). These results show a difference in the stability of bacteriocins in food systems.

Bacteriocin KC24 was characterized and evaluated for its antilisterial effect in UHT milk for 14 d at 4°C. It was demonstrated to be a very stable bacteriocin under a broad pH range (3.0 to 9.0) and temperature range (50–121°C). *Listeria monocytogenes* added to UHT milk treated with bacteriocin KC24 was nondetectable after 14 d of refrigerated storage, indicating the stable nature of the bacteriocin. These characteristics indicate that bacteriocin KC24 may be applied as a natural food preservative in dairy products.

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