J Food Sci Nutr
Vol 15, p 74~77 (2010)
DOI: 10.3746/jfn.2010.15.1.074

J Food Science and Nutrition

Anti-staphylococcal Bacteriocin from *Enterococcus faecium*

– Research Note –

Kyung-Suk Kim, Ung-Soo Lee, and Gi-Seong Moon†

Division of Food and Biotechnology, Chungju National University, Chungbuk 368-701, Korea

Abstract

Antibiotic-resistant *Staphylococcus aureus* is beginning to pose a social issue. Thus, there is an urgent need for the development of effective anti-staphylococcal agents to eradicate antibiotic-resistant *S. aureus* in food systems and to treat the pathogen in clinical areas. To address this need, lactic acid bacteria (LAB) from *kimchi* were screened for the production of anti-staphylococcal bacteriocin. From this screening, a bacteriocin generated by the MK3 strain, which was identified by 16S rRNA gene sequence analysis as *Enterococcus faecium*, demonstrated antimicrobial activity against an *S. aureus* strain, and was designated enterocin MK3. Enterocin MK3 also demonstrated activity against other gram-positive bacteria, including several LAB and *Listeria monocytogenes*, but not gram-negative *Escherichia coli*. The molecular mass of enterocin MK3 was estimated as approximately 6.5 kDa on an SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) gel.

Key words: *Staphylococcus aureus*, bacteriocin, *Enterococcus faecium*, enterocin

INTRODUCTION

Staphylococcus aureus is a clinically important pathogen and has been identified as a food-borne pathogen that generates a heat-stable toxin (1). *S. aureus* has been previously associated with a variety of fatal diseases, including bacteremia, endocarditis, metastatic infection, sepsis, and toxic shock syndrome (2). Furthermore, methicillin-resistant *S. aureus* (MRSA) has been frequently detected in clinical areas (3) and can be spread from hospitals. Thus, there is a clear need for the development of alternative antimicrobial agents against *S. aureus*.

Bacteriocins are ribosomally-synthesized antimicrobial peptides produced and exported by both gram-positive and gram-negative bacteria (4). In particular, bacteriocins from lactic acid bacteria (LAB) have been the focus of many studies, owing to their broad antimicrobial spectra and their general safety (5). The bacteriocins generated by LAB have been principally categorized into two major classes: Class I, called lantibiotics, contain post-translational modifications to form 2,3-dehydroalanine and 2,3-dehydrobutyrine, and Class II, which are small, heat-stable, and unmodified. Class II bacteriocins are grouped into four subclasses, in which Class IIa, pediocin-like bacteriocin, is the most abundant group (6).

An increasing number of basic and clinical studies have been conducted (4,7-9) to develop alternative LAB-derived bacteriocins into antimicrobial agents effective against antibiotic-resistant bacterial pathogens such as MRSA, VISA (vancomycin-insensitive *S. aureus*), and VRE (vancomycin-resistant enterococci). In this study, we screened a bacteriocin-producing enterococcal strain that demonstrated antimicrobial activity against *S. aureus*. We analyzed the antimicrobial spectrum, action mode, and molecular mass of the bacteriocin to obtain preliminary information.

MATERIALS AND METHODS

Bacterial strains and culture conditions

All the bacteria used as indicators including *Escherichia coli* DH5α, *Lactobacillus bulgaricus* CH2, *Lactobacillus plantarum* KFRI 812, *Lactococcus lactis* MG 1363, *Leuconostoc mesenteroides* KFRI 820, *Listeria monocytogenes* KCTC 3569, *Pediococcus acidilactici* K10, and *S. aureus* ATCC 14458 have been stored as stock cultures in our laboratory. ATCC, KCTC, and KFRI strains were previously purchased from American Type Culture Collection, Korean Collection for Type Cultures, and Korea Food Research Institute, respectively. Particularly, *S. aureus* ATCC 14458 was isolated from feces of a child with acute, non-specific diarrhea (http://www.atcc.org/ATCCAdvancedCatalogSearch/ProductDetails/tabid/452/Default.aspx) and used as a target bacterium for this study.

LAB, *Lis. monocytogenes*, and *S. aureus* were cultivated in MRS broth (Merck, Darmstadt, Germany) at 37°C without agitation, and *E. coli* was grown in LB (Luria-Bertani) broth (10 g/L tryptone, 10 g/L NaCl, and 5 g/L yeast extract, pH 7.0) at 37°C with vigorous

†Corresponding author. E-mail: gsmoon@cjnu.ac.kr
Phone: +82-43-820-5251, Fax: +82-43-820-5272

shaking.

Bacteriocin production assay

Bacteriocin production was analyzed via a deferred antagonism assay (10) for cells, and spot on the lawn test (11) for culture supernatants. Specifically, 1 μL of overnight culture (~10^9 CFU/mL) was spotted on an MRS agar plate and incubated for 12 hr at 37°C. Soft agar (0.7%, w/v) inoculated with *S. aureus* was poured onto the plate and incubated for an additional 12 hr at 37°C, and the inhibition zone was evaluated (deferred antagonism assay). In the spot on the lawn test, the pH of an overnight culture was neutralized, and the culture was centrifuged and filtered with a 0.2 μm syringe filter (Millipore Co., Billerica, MA, USA). The filtrate (2 μL) was loaded onto a plate overlaid with soft agar inoculated with *S. aureus*, and incubated for 12 hr at 37°C, after which the inhibition zone was examined.

Identification of bacteriocin-producing strain

The 16S rRNA gene sequence of the strain was used for identification. The bacteriocin-generated strain was cultured in MRS broth and sent to a biotech company (Macrogen Co., Seoul, Korea) for the analysis of the 16S rRNA gene sequence. The DNA sequence homology search was conducted using the BLAST (Basic Local Alignment Search Tool) program (blastn) from the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/).

Preparation of crude bacteriocin

Overnight culture (200 mL) of the bacteriocin-producing strain was centrifuged for 30 min at 8,000 rpm, and the culture supernatant was filtered through 0.2 μm membrane filters (Millipore Co.). Ammonium sulfate (Sigma-Aldrich Co., St. Louis, MO, USA) was added to the supernatant and stirred until a 50% saturation rate was reached. The mixture was centrifuged for 1 hr at 12,000 rpm and the precipitate was resolved with ultrapure water and dialyzed with Spectra/Por® DispoDialyzer® (MWCO 1,000; Spectrum Co., Rancho Dominguez, CA, USA) against ultrapure water. The dialysate was freeze-dried and stored at -75°C until use.

Bacteriocin activity and total protein concentration

The bacteriocin activity, expressed as arbitrary units (AU)/mL, was defined as the reciprocal of the highest two-fold dilution generating an inhibition zone (12). The concentration of crude bacteriocin protein was analyzed using a Bio-Rad protein assay kit (Hercules, CA, USA).

Action mode of the bacteriocin

Overnight culture of *S. aureus* was inoculated into 5 mL of 10-fold diluted MRS broth, and 400 AU of the bacteriocin was added to the inoculum. The inoculum was then incubated for 6 hr at 37°C. Every 2 hr, the viable cell count of *S. aureus* in the inoculum was analyzed and compared with a control sample into which only *S. aureus* was inoculated.

SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) and bioassay

The molecular mass of the bacteriocin was estimated via SDS-PAGE using 12% tris-glycine gel. The gel was assembled in a Mini-PROTEAN® Tetra Cell electrophoresis unit (Bio-Rad Co.), running buffer (25 mM tris, 192 mM glycine, 0.1% SDS, pH 8.3) was poured into the buffer tank, and the samples were loaded onto wells. The unit was run for 60 min at 150 V and the gel was fixed for 30 min in a fixation solution [40% (v/v) MeOH, 10% (v/v) acetic acid]. The fixed gel was stained with Bio-Safe™ Coomassie (Bio-Rad Co.) and washed with ultrapure water, and the protein bands were evaluated. A bioassay was also conducted to confirm the bacteriocin band. Half of the gel run in the same unit was washed with ultrapure water to eradicate SDS and loaded onto an MRS agar plate. Soft agar (0.7%, w/v) inoculated with *S. aureus* was overlaid on the plate. The plate was incubated for 12 hr at 37°C and the inhibition zone was examined.

RESULTS AND DISCUSSION

From the screening for bacteriocin-producing LAB against *S. aureus* ATCC 14458, the MK3 strain, which was previously isolated from *kimchi*, was selected (Fig. 1). As anticipated, the strain cells inhibited several gram-positive bacteria, including other LAB such as *L. lactis* MG 1363, *L. plantarum* KFRI 812, *L. bulgaricus* CH2, *P. acidilactici* K10, and *Leu. mesenteroides* KFRI 820 and food-borne pathogens such as *Lis. monocytogenes* KCTC 3569 as well as *S. aureus*, but not gram-negative *E. coli* DH5α. However, the pH-neutral-

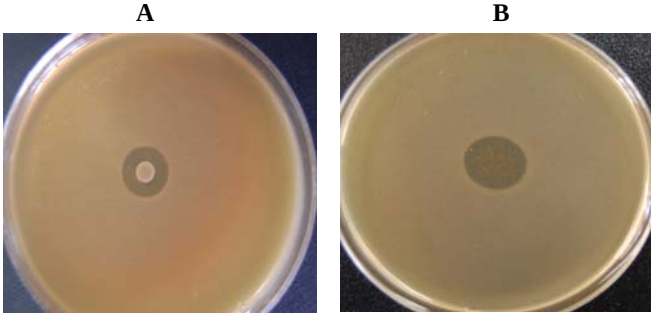

Fig. 1. Anti-staphylococcal activities of cells (A) and the culture supernatant (B) of MK3 strain. A, deferred antagonism assay; B, spot on the lawn test.

ized culture supernatant did not inhibit *L. plantarum* and *Leu. mesenteroides*. In general, the bacteriocins generated by LAB do not inhibit gram-negative bacteria such as *E. coli* and *Salmonella* sp. The majority of the bacteriocins can inhibit strains closely related to the producers (11,13,14).

The MK3 strain was identified by 16S rRNA gene sequence homology search. The MK3 sequence best matched (99% identity) that of an *Enterococcus faecium* strain (GenBank accession number, EU003447.1) and thus the strain was designated *E. faecium* MK3. The bacteriocin generated by *E. faecium* MK3 was designated enterocin MK3. The enterococci are the predominant LAB in human and animal intestines (6). These bacteria have often been detected in fermented foods, such as *kimchi* and cheese (13,15). The enterococcal species producing bacteriocins were primarily identified as *E. faecium* and *E. faecalis*. These bacteriocins belonged to either Class I or Class II. The molecular masses of the bacteriocins ranged from 2,032 to 7,998 Da (6). In particular, bacteriocin AS-48, a 70-residue cyclic peptide generated by *E. faecalis*, evidenced bactericidal activity against some gram-negative as well as gram-positive bacteria, thereby indicating that this bacteriocin might be a promising candidate for a biopreservative or a next-generation antimicrobial agent (16).

The molecular mass of enterocin MK3 was estimated to be 6.5 kDa on the basis of the SDS-PAGE gel staining and bioassay described in the Materials and Methods (Fig. 2). We observed that a protein band at 6.5 kDa became correspondingly thicker as the concentration of the crude bacteriocin increased (Fig. 2A) and inhibition zones corresponding to the protein bands (lane 1～3) were shown in the bioassay (Fig. 2B), which indicate the protein bands represent enterocin MK3. We also attempted to obtain more detailed information, such as the accurate molecular mass and peptide fingerprint of enterocin MK3 via MALDI-TOF (matrix assisted laser desorption ionization-time of flight) mass spectrometry using gel-eluted bacteriocin peptides, however, we were unable to obtain accurate data due to protein contamination (data not shown).

Finally, we tested the action mode of enterocin MK3 against *S. aureus* (Fig. 3). From this test, the viable cell count of *S. aureus* was shown to decrease dramatically when it was cultured with 400 AU of crude enterocin MK3. In detail, the viable cell count was 7.3 log CFU/mL at time point 0, and changed to 5.7 log CFU/mL after 6 hr, whereas the count reached 8.4 log CFU/mL in the control into which only *S. aureus* was inoculated. This result shows that enterocin MK3 evidences a bactericidal action mode against *S. aureus*, which is a good characteristic for the effective inhibition of the pathogen.

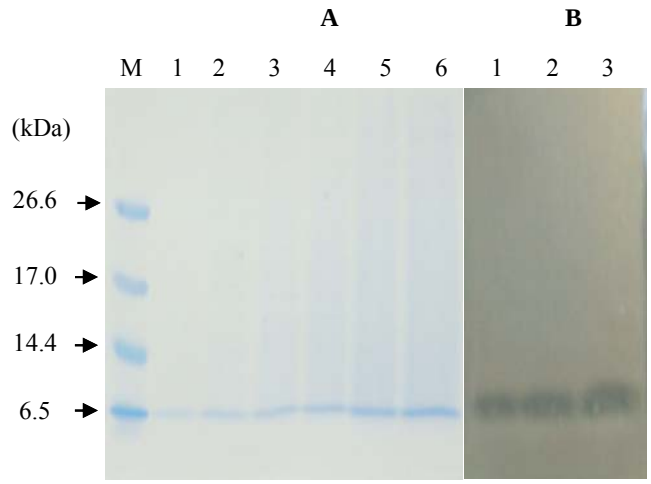

Fig. 2. Staining (A) and bioassay (B) after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of crude enterocin MK3. A, The gel stained with Bio-Safe™ Coomassie (Bio-Rad Co.). M, Polypeptide SDS-PAGE MW standards (Bio-Rad Co.); lane 1, 250 ng; lane 2, 500 ng; lane 3, 750 ng; lane 4, 1 μg; lane 5, 2 μg; lane 6, 3 μg loading of crude enterocin MK3. B, *Staphylococcus aureus* strain was used as the indicator in the bioassay. Lane 1, 250 ng; lane 2, 500 ng; lane 3, 750 ng loading of crude enterocin MK3.

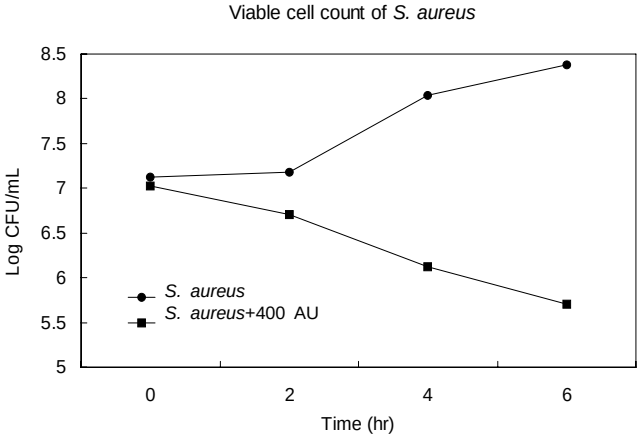

Fig. 3. Action mode of enterocin MK3 against *Staphylococcus aureus*. *S. aureus* means only *S. aureus* was inoculated in 10-fold diluted MRS broth; *S. aureus*+400 AU means *S. aureus* was inoculated with 400 AU (arbitrary unit) of crude enterocin MK3.

In the mechanistic model experiments, bacteriocins function at the cell membranes of target cells, forming pores, destroying membrane potentials, and introducing the cells to death (17). The Class IIa bacteriocins consist of highly conserved N- and C-termini (18), where the N-terminal regions are hydrophilic and charged for the binding of the target cell membrane and the C-terminal regions are hydrophobic and/or amphiphilic for the in-

tegration of cell membrane lipids.

In order to confirm whether or not *E. faecium* MK3 harbors a plasmid, a plasmid preparation method (19) was used. According to the results, *E. faecium* MK3 harbored no plasmids (data not shown), thereby indicating that the structural genes for enterocin MK3 production reside within the chromosome. The absence of plasmids in *E. faecium* MK3 is promising in terms of safety, since plasmids harboring antibiotic-resistance genes could be transferred into pathogenic bacteria, thereby resulting in the occurrence of antibiotic-resistant pathogens (20). In general, enterococci tend to contain plasmids that harbor antibiotic-resistance genes, and thus the enterococci have been reluctantly used for commercial purposes only because of safety concerns, even though they are members of the LAB. In accordance with the results of this study, we have selected a bacteriocin-producing *E. faecium* strain, and this bacteriocin efficiently inhibited *S. aureus* with a bactericidal action mode.

Antibiotic-resistant *S. aureus* is expected to become widely prevalent in foods as well as clinical areas. Thus, there is an urgent need to develop effective antimicrobial agents that can effectively control *S. aureus*. In this regard, bacteriocins from LAB might be considered promising alternative candidates for antibiotics, and could eventually be used as food biopreservatives or therapeutic agents. In the future, the amino acid sequence of enterocin MK3 and the structural genes for bacteriocin production will be analyzed for mass production and further studies.

REFERENCES

1. O'Brien M, Hunt K, McSweeney S, Jordan K. 2009. Occurrence of foodborne pathogens in Irish farmhouse cheese. *Food Microbiol* 26: 910-914.
2. Lowy FD. 1998. *Staphylococcus aureus* infections. *N Engl J Med* 339: 520-532.
3. Sakwinska O, Kuhn G, Balmelli C, Francioli P, Giddey M, Perreten V, Riesen A, Zysset F, Blanc DS, Moreillon P. 2009. Genetic diversity and ecological success of *Staphylococcus aureus* strains colonizing humans. *Appl Environ Microbiol* 75: 175-183.
4. Sit CS, Vederas JC. 2008. Approaches to the discovery of new antibacterial agents based on bacteriocins. *Biochem Cell Biol* 86: 116-123.
5. Gálvez A, Abriouel H, López RL, Ben Omar N. 2007. Bacteriocin-based strategies for food biopreservation. *Int J Food Microbiol* 120: 51-70.
6. Nes IF, Diep DB, Holo H. 2007. Bacteriocin diversity in *Streptococcus* and *Enterococcus*. *J Bacteriol* 189: 1189-1198.
7. Gillor O, Ghazaryan L. 2007. Recent advances in bacteriocin application as antimicrobials. *Recent Pat Antiinfect Drug Discov* 2: 115-122.
8. Sang Y, Blecha F. 2008. Antimicrobial peptides and bacteriocins: alternatives to traditional antibiotics. *Anim Health Res Rev* 9: 227-235.
9. Piper C, Cotter PD, Ross RP, Hill C. 2009. Discovery of medically significant lantibiotics. *Curr Drug Discov Technol* 6: 1-18.
10. Ray B, Daeschel M. 1992. *Food biopreservatives of microbial origin.* CRC Press, Boca Raton, Florida, USA. p 64-70.
11. Moon GS, Jeong JJ, Ji GE, Kim JS, Kim JH. 2000. Characterization of a bacteriocin produced by *Enterococcus* sp. T7 isolated from humans. *J Microbiol Biotechnol* 10: 507-513.
12. Moon GS, Pyun YR, Kim WJ. 2006. Expression and purification of a fusion-typed pediocin PA-1 in *Escherichia coli* and recovery of biologically active pediocin PA-1. *Int J Food Microbiol* 108: 136-140.
13. Moon GS, Pyun YR, Kim WJ. 2005. Characterization of the pediocin operon of *Pediococcus acidilactici* K10 and expression of His-tagged recombinant pediocin PA-1 in *Escherichia coli*. *J Microbiol Biotechnol* 15: 403-411.
14. Shin MS, Han SK, Choi JH, Ji AR, Kim KS, Lee WK. 2009. Characterization of antimicrobial substance produced by *Lactobacillus* sp. HN 235 isolated from pig intestine. *Kor J Microbiol Biotechnol* 37: 125-132.
15. Izquierdo E, Wagner C, Marchioni E, Aoude-Werner D, Ennahar S. 2009. Enterocin 96, a novel class II bacteriocin produced by *Enterococcus faecalis* WHE 96, isolated from Munster cheese. *Appl Environ Microbiol* 75: 4273-4276.
16. Maqueda M, Gálvez A, Bueno MM, Sanchez-Barrena MJ, González C, Albert A, Rico M, Valdivia E. 2004. Peptide AS-48: prototype of a new class of cyclic bacteriocins. *Curr Protien Pept Sci* 5: 399-416.
17. Oscáriz JC, Pisabarro AG. 2001. Classification and mode of action of membrane-active bacteriocins produced by gram-positive bacteria. *Int Microbiol* 4: 13-19.
18. Drider D, Fimland G, Héchard Y, McMullen LM, Prévost H. 2006. The continuing story of class Ⅱa bacteriocins. *Microbiol Mol Biol Rev* 70: 564-582.
19. O'Sullivan DJ, Klaenhammer TR. 1993. Rapid mini-prep isolation of high-quality plasmid DNA from *Lactococcus* and *Lactobacillus* spp. *Appl Environ Microbiol* 59: 2730-2733.
20. Klare I, Konstabel C, Badstübner D, Werner G, Witte W. 2003. Occurrence and spread of antibiotic resistance in *Enterococcus faecium*. *Int J Food Microbiol* 88: 269-290.

(Received December 30, 2009; Accepted March 17, 2010)