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The Two-synergistic Peptide Bacteriocin Produced by *Enterococcus faecium* NKR-5-3 Isolated from Thai Fermented Fish (Pla-ra)

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ABSTRACT: Bacteriocin produced by *Enterococcus faecium* NKR-5-3 isolated from Thai fermented fish was characterized and purified. According to its physical and chemical properties, *E. faecium* NKR-5-3 produced heat tolerant bacteriocin with broad spectrum activity against indicator strains. Treatment of bacteriocin in cell-free neutralized supernatant (CFNS) with some proteases eliminated or reduced its activity, suggesting it is proteinaceous. Furthermore, it was stable in 0 to 24% NaCl, heat tolerant at pH 2 to 10 and bactericidal toward *Enterococcus faecalis* ATCC 19433^T. Two antibacterial peptides, named enterocin NKR-5-3A and enterocin NKR-5-3B, were purified from the culture supernatant by four steps of column chromatography. Enterocin NKR-5-3A had the same mass, MW=5,241, and N-terminal amino acid sequence as brochocin A produced by *Brochothrix campestris* ATCC 43754, suggesting it may be identical to brochocin A. Enterocin NKR-5-3B exhibited MW=6,320, but its N-terminal amino acid sequence could not be determined. When these two purified peptides were mixed in a 1:1 ratio, the bacteriocin activity reached a maximum, which corresponded to 64 times the total calculated bacteriocin activity in the mixture, suggesting their synergism.

KEYWORDS: two-synergistic peptide bacteriocin, *Enterococcus faecium*, enterocin, Thai fermented fish.

INTRODUCTION

Lactic acid bacteria (LAB) are ubiquitous in fermented and non-fermented foods and are common components of the human commensal microflora.[1] Owning to their typical association with food fermentation and also their long tradition as food-grade bacteria, LAB are therefore generally recognized as safe (GRAS). LAB can exert a biopreservative or inhibitory effect against other microorganisms as a result of competition for nutrients and/or of the production of bacteriocins and other antagonistic compounds, such as organic acids and hydrogen peroxide.[2]

Bacteriocins are antimicrobial, proteinaceous compounds with a bactericidal mode of action against bacteria closely related to the producer strain.[3] In addition, some bacteriocins are active against food-borne pathogens such as *Listeria monocytogenes, Clostridium perfringens, Bacillus cereus, Staphylococcus aureus* and spoilage LAB.[2,4] Because of its specificity to sensitive bacteria without any effect on useful normal flora residing in the human body, use of either the bacteriocins or the bacteriocin-producing LAB starter cultures for food preservation has received much interest.[2,4] Therefore, many research groups have been studying bacteriocin production by LAB isolated from various natural sources.

Among LAB, *Enterococcus* was widely distributed and generally associated with food substrates. Although some strains have been implicated as causal agents of antibiotic resistance and human infection, non-pathogenic enterococci have promising commercial potential.[5] Therefore, enterococci are already being used in food fermentation and some strains are routinely employed as starter culture in the manufacture of food products.[6] Strains of enterococci, including *Enterococcus faecium* and *Enterococcus faecalis* are known to produce bacteriocin. Their bacteriocins are called enterocins and generally belong to class II bacteriocins with heat stability and anti-*Listeria* activity.[5,7] In recent years, there have been numerous reports on bacteriocin production by *E. faecium* isolated from various sources.[4-5,7-9] However, no report has

been published on bacteriocin-producing *E. faecium* isolated from Thai fermented fish (Pla-ra) and its bacteriocin characteristics.

This study is the first report on the characterization, purification and synergistic activity of two-peptide bacteriocins produced by *E. faecium* isolated from Thai fermented fish (Pla-ra).

Materials and Methods

Bacterial Strains and Culture Conditions

Previously, the bacteriocin producing strain, *Enterococcus faecium* NKR-5-3, was isolated from Thai fermented fish (Pla-ra) (Wilaipun et al, unpublished). *E. faecium* NKR-5-3 and all indicator strains (Table 1) were maintained as frozen stocks at –80°C in TSBYE (tryptic soy broth supplemented with 6 g l^{-1} yeast extract) containing 15% (v/v) of glycerol. Throughout the experiments, they were subcultured every 2 weeks on TSAYE (TSBYE plus 12 g l^{-1} agar) slants and kept at 4°C. The cultures were propagated twice in TSBYE at 30°C for 18 h before use. Unless otherwise noted, *E. faecium* NKR-5-3 was cultured in M 17 broth at 30°C for 22 h for bacteriocin production and *Enterococcus faecalis* ATCC 19433 was used as the indicator strain for bacteriocin activity determination.

Determination of Bacteriocin Activity

The cell-free neutralized supernatant (CFNS) of *E. faecium* NKR-5-3, grown in culture broth at 30°C for 22 h, was obtained by centrifugation of culture supernatant at 10,000x *g* for 15 min at 4°C. The cell-free supernatant was pH neutralized (pH 6.5) using 5 N sodium hydroxide, hydrogen peroxide neutralized by catalase at a final concentration of 500 unit ml^{-1} (incubate at 25°C for 30 min) and subsequently sterilized by heating at 100°C in a water bath for 5 min. Bacteriocin activity of the CFNS was determined against indicator strains according to the critical dilution method.[10] The CFNS were twofold serially diluted with sterile distilled water and aliquots (10 µl) of each dilution were spotted onto TSAYE plates overlaid with 5 ml of TSAYE soft agar media seeded with 10^7 CFU ml^{-1} of overnight (18 h) cultured indicator strain. Plates were incubated for 24 h at a certain temperature suitable for growth of each indicator strains. The arbitrary activity unit (AU) was defined as the reciprocal of the highest dilution producing a distinct inhibition of the indicator lawn, and was multiplied by a factor of 100 to obtain the AU ml^{-1} of the original sample.

Characterization of Bacteriocin

Determination of antibacterial spectrum

The CFNS of *E. faecium* NKR-5-3 grown in MRS broth at 30°C for 22 h was determined for its bacteriocin activity against various indicator strains (as shown in Table 1) by the critical dilution method.

Sensitivity of bacteriocin to proteolytic enzymes and heat treatment

The CFNS of *E. faecium* NKR-5-3 grown in MRS broth at 30°C for 22 h was treated with the following enzymes: α-chymotrypsin (Sigma Fine Chemicals, St. Louis, MO, USA; pH 7.0), pepsin (Sigma; pH 3.0),

Table 1. Bacteriocin activity in CFNS of *E. faecium* NKR-5-3 against various bacterial strains.

Indicator strain	Bacteriocin activity [a] (AU ml^{-1})
Lactic acid bacteria	
Enterococcus faecalis ATCC 19433[T]	6,400
Enterococcus faecalis JCM 5803[T]	3,200
Enterococcus faecium DMST 4743	1,600
Enterococcus hirae ATCC 10541	6,400
Lactobacillus casei subsp. *casei* JCM 1134[T]	0
Lactobacillus plantarum ATCC 8014	0
Lactobacillus plantarum ATCC 14917[T]	1,600
Lactobacillus sakei subsp. *sakei* JCM 1157[T]	1,600
Lactococcus lactis subsp. *lactis* ATCC 19435[T]	100
Lactococcus lactis subsp. *cremoris* TUA 1344L	800
Leuconostoc mesenteroides subsp. *mesenteroides* JCM 6124[T]	400
Pediococcus pentosaceus JCM 5885	0
Bacteriocin producing bacteria	
Lactococcus lactis subsp. *lactis* JCM 7638 (nisin Z)	3,200
Lactococus lactis NCDO 497 (nisin A)	400
Enterococcus faecium WHE 81 (enterocins A and B)	3,200
Enterococcus mundtii a1 (mundticin)	6,400
Pediococcus pentosaceus TISTR 536 (pediocin AcH/PA-1)	0
Enterococcus faecium NKR-5-3	0
Other indicator bacteria	
Bacillus cereus JCM 2152[T]	1,600
Bacillus circulans JCM 2504[T]	0
Bacillus coagulans JCM 2257[T]	1,600
Bacillus subtilis subsp. *subtilis* JCM 1465[T]	0
Listeria innocua LTH 3096	1,600
Listeria innocua ATCC 33090[T]	1,600
Micrococcus luteus IFO 12708	0
Staphylococcus aureus subsp. *aureus* ATCC 12600[T]	0
Staphylococcus aureus subsp. *aureus* ATCC 25923	0
Staphylococcus piscifermentans JCM 6063	0
Escherichia coli ATCC 25922	0
Salmonella choleraesuis subsp. *choleraesuis* ATCC 13076[T]	0
Salmonella choleraesuis subsp. *choleraesuis* ATCC 14028	0

ATCC=American Type Culture collection, Rockville, MD, USA.
DMST=Department of Medical Sciences, National Institute of Health, Nonthaburi, Thailand.
IFO=Institute for Fermentation, Osaka, Japan.
JCM=Japan Collection of Microorganisms, Saitama, Japan.
LTH=Institut fur Lebensmitteltechnologie, Universitat Hohenheim, Hohenheim, Germany.
NCDO=National Collection of Dairy Organisms, Reading, United Kingdom.
TISTR=Thailand Institute of Scientific and Technological Research, Bangkok, Thailand.
TUA=Tokyo University of Agriculture, Tokyo, Japan.
[a] bacteriocin activity was determined by the critical dilution method.
[T] = type strain.
E. faecium NKR-5-3 was cultured in MRS broth at 30°C for 22 h.

proteinase K (Sigma; pH 7.0), trypsin (Sigma; pH 7.0), bromelain (Novo Nordisk, Copenhagen, Denmark; pH 5.0), flavourzyme (Novo Nordisk; pH 7.0), kojizyme (Novo Nordisk; pH 6.5) at a final concentration of 1 mg ml^{-1}. After incubation at 37°C for 3 h at the above-mentioned pH, enzyme was inactivated by heating at 100°C for 5 min. The sensitivity of bacteriocin to heat treatment was tested by heating CFNS of *E. faecium* NKR-5-3 at 100°C for the indicated periods and at 121°C for 15 min. Untreated sample was used as control. After enzyme and heat treatment, the residual bacteriocin activity was then determined against the indicator strain.

Combined effects of pH and heat treatment on bacteriocin activity

The effect of heat treatment at different pH values on bacteriocin activity was studied. The CFNS of *E. faecium* NKR-5-3 grown in M 17 broth at 30°C for 22 h, with initial bacteriocin activity of 51,200 AU ml^{-1}, was adjusted to the indicated pH values with sterile 5N HCl or NaOH. Samples were then heated at 100°C for 60 min, cooled in an ice bath and the pH adjusted to 6.5 before determining residual bacteriocin activity.

Effect of NaCl on bacteriocin activity

The effect of NaCl on bacteriocin activity was determined by adding NaCl to CFNS of *E. faecium* NKR-5-3 to a final concentration of 0 to 24% (at 3% intervals). The samples were incubated at 4°C and bacteriocin activity was determined at selected time intervals. Sterile NaCl solution at each concentration was used as a negative control.

Mode of action

The bactericidal or bacteriostatic mode of action of bacteriocin against the indicator strain was determined. A diluted overnight culture of the indicator strain (1 ml) was added into 44 ml MRS broth with 5 ml optimum diluted CFNS of *E. faecium* NKR-5-3, to yield a final concentration of indicator strain ca 10^7 CFU ml^{-1}. MRS broth (49 ml) and 1 ml of diluted indicator strain without CFNS was used as control. Cultures were incubated at 30°C. OD_{600} and total viable count (CFU ml^{-1}) of indicator strain after plating onto MRS agar with 0.5% $CaCO_3$ and 0.004% bromocresol purple were determined at selected time intervals. The total viable count was measured by counting the number of colonies on a plate after incubation at 30°C for 24 h.

Bacteriocin Purification

Purification of bacteriocin

All purification steps were performed at room temperature. *E. faecium* NKR-5-3 was cultured in 1 liter of M 17 broth at 30°C for 22 h. Bacterial cells were removed by centrifugation at 10,000x *g* for 15 min at 4°C, and 20 g of Amberlite XAD-16 (Sigma) was added to the supernatant. The sample was gently agitated at 4°C for 2 h. The matrix was washed with 100 ml of distilled water and 200 ml of 40% (v/v) ethanol in distilled water, and the bacteriocin activity was eluted with 150 ml of 70% (v/v) 2-propanol in distilled water containing 0.1% trifluoroacetic acid (TFA) (pH 2.0) (fraction I). The elutate was evaporated to 5-10 ml by vacuum centrifuge (Savant Instruments Inc., Holbrook, NY, USA). The sample was diluted to 100 ml with distilled water and loaded onto a 7-ml SP-Sepharose Fast Flow cation-exchanger (Sigma) column equilibrated with 20 mM sodium phosphate buffer pH 5.7 (buffer A). After subsequently washing the column with 20 ml of buffer A, the bacteriocin was eluted from the column with 40 ml of 1 M NaCl in buffer A (fraction II). Ammonium sulfate was added to fraction II to obtain a final concentration of 10% (w/v), after which the fraction was applied at a flow rate of about 4 ml min^{-1} to a 2-ml Octyl-Sepharose CL-4B (Sigma) column equilibrated with 10% (w/v) ammonium sulfate in buffer A. The column was washed with 8 ml of buffer A. The bacteriocin was eluted from the column with 10 ml of 70% (v/v) ethanol and 30% buffer A (fraction III). Fraction III was diluted to 50 ml with distilled water containing 0.1% TFA and subsequently applied to C_2/C_{18} reverse-phase column (PepRPC HR 5/5, Amersham Pharmacia Biotech, Little Chalfont, UK) equilibrated with distilled water containing 0.1% TFA. The bacteriocin was eluted from the column with a gradient of 15-70% 2-propanol containing 0.1% TFA at a flow rate of 1 ml min^{-1} (fraction IV). Gradients were carried out using an LC-10AD solvent delivery module (Shimadzu, Tokyo, Japan) and detection was performed at 280 nm using an SPD-N10A photodiode array detector (Shimadzu). Fractions (1 ml) eluting from the C_2/C_{18} reverse-phase column were collected. Fractions showing antibacterial activity were diluted four to five fold with distilled water containing 0.1% TFA and rechromatographed on the reverse-phase column. Purified bacteriocin peptides, enterocins NKR-5-3A and NKR-5-3B, were stored in 30-50% 2-propanol containing 0.1% TFA at –20°C.

Mass spectrometric and amino acid sequence analyses of purified bacteriocin

The molecular mass of the purified bacteriocin peptides was determined using a Voyager-RP (PerSeptive Biosystems , Framingham, MA, USA) matrix-assisted laser desorption and ionization time-of-flight mass spectrometer (MALDI-TOF MS). In this analysis, a saturated *a*–cyano-4-hydoxycinnamic acid solution was used as matrix. The amino acid sequence of the bacteriocin was determined by Edman degradation on a gas-phase automatic sequence analyzer (PSQ-1, Shimadzu) with an on line LC-6A HPLC system (Shimadzu).

Synergistic Antimicrobial Activity of Two Purified

Table 2. Effects of proteolytic enzyme and heat treatment on bacteriocin activity in CFNS of *E. faecium* NKR-5-3.

Treatment	Residual activity [a] (AU ml^{-1})
Untreated (control)	6,400
Proteolytic Enzyme	
Bromelain	0
α -chymotrypsin	100
Flavourzyme	100
Kojizyme	0
Pepsin	100
Proteinase K	0
Trypsin	400
Heat	
5 min at 100°C	6,400
20 min at 100°C	3,200
40 min at 100°C	1,600
60 min at 100°C	1,600
80 min at 100°C	800
100 min at 100°C	800
120 min at 100°C	800
15 min at 121°C	400

E. faecium NKR-5-3 was cultured in MRS broth at 30°C for 22 h.
[a] Residual activity was determined by the critical dilution method and *E. faecalis* ATCC 19433^T was used as the indicator strain.

Bacteriocin

Each purified peptide of enterocin NKR-5-3A and enterocin NKR-5-3B from *E. faecium* NKR-5-3 was diluted 2 times with sterile distilled water, to yield a final bacteriocin activity of each one at 3,200 AU ml^{-1}. Each diluted purified bacteriocin was mixed together at indicated ratios (v/v) in microtiter plates at the final volume of 100 µl and bacteriocin activity (AU ml^{-1}) in each mixture was determined.

Results

Characterization of Bacteriocin

Determination of antibacterial spectrum

The antibacterial spectrum of NKR-5-3 CFNS against various indicator strains was shown in Table 1. It showed strong activity against almost all LAB strains, including strains known as bacteriocin producer, except for *Lactobacillus casei* subsp. *casei* JCM 1134, *L. plantarum* ATCC 8014, *Pediococcus pentosaceus* JCM 5885, *P. pentosaceus* TISTR 536 and its own producing strain. Furthermore, it showed activity against some of other Gram-positive bacteria, such as *B. cereus* JCM 2152 and *Listeria innocua* ATCC 33090, but no activity against any of the Gram-negative bacteria tested was detected.

Sensitivity of bacteriocin to proteolytic enzymes and heat treatment

Bacteriocin activity in CFNS of *E. faecium* NKR-5-3 was completely inhibited by treatment with bromelain, kojizyme, proteinase K and was decreased by α-chymotrypsin, flavourzyme, pepsin and trypsin (Table 2). In heat treatment study, bacteriocin activity was quite stable when heat at 100°C for 5 min. At the increasing time of heat treatment, the relative bacteriocin activity decreased 50, 75 and 87.5% when heated at 100°C for 20, 40 and 80 min, respectively. In addition, the relative bacteriocin activity decreased 93.75% when heated at 121°C for 15 min (Table 2).

Combined effects of pH and heat treatment on bacteriocin activity

After heating bacteriocin in CFNS of *E. faecium* NKR-5-3 at 100°C for 60 min at different pH values, the bacteriocin activity was quite stable at pH 2.0 to 3.0. At higher pH values, the relative activity decreased 50% at pH 4.0 to 9.0 and 87.5% at pH 10.0 (Table 3). This indicated that bacteriocin activity of *E. faecium* NKR-5-3 was more tolerant against heat treatment at acid pH than at neutral and alkaline pH.

The effect of NaCl on bacteriocin activity was studied and bacteriocin activity in CFNS of *E. faecium* NKR-5-3 did not change under different NaCl concentrations from 0 to 24% at 4°C for 28 days (data not shown). This indicated the stability of bacteriocin to high salt.

Mode of action

As CFNS of *E. faecium* NKR-5-3 was added to the culture of *E. faecalis* ATCC 19433^T at a final concentration of 256 AU ml^{-1}, the viable count decreased from 7.18 log CFU ml^{-1} to 5.72 log CFU ml^{-1} at 12 h. In addition, when CFNS was added at a final concentration of 2,560 AU ml^{-1}, the viable count decreased from 7.17 log CFU ml^{-1} to less than 10 CFU ml^{-1} within 3 h. Meanwhile, viable count of control without CFNS increased from 7.19 log CFU ml^{-1} to 9.47 log CFU ml^{-1} in 12 h (Fig 1). This indicated that bacteriocin in CFNS of *E. faecium* NKR-5-3 had a

Table 3. Bacteriocin activity in CFNS of *E. faecium* NKR-5-3 after heat treatment at different pH values.

pH	Residual bacteriocin activity [a] (AU ml^{-1})
2.0	51,200
3.0	51,200
4.0	25,600
5.0	25,600
6.0	25,600
7.0	25,600
8.0	25,600
9.0	25,600
10.0	6,400

E. faecium NKR-5-3 was cultured in M 17 broth at 30°C for 22 h.
[a] Bacteriocin activity was determined by the critical dilution method and *E. faecalis* ATCC 19433^T was used as the indicator strain.

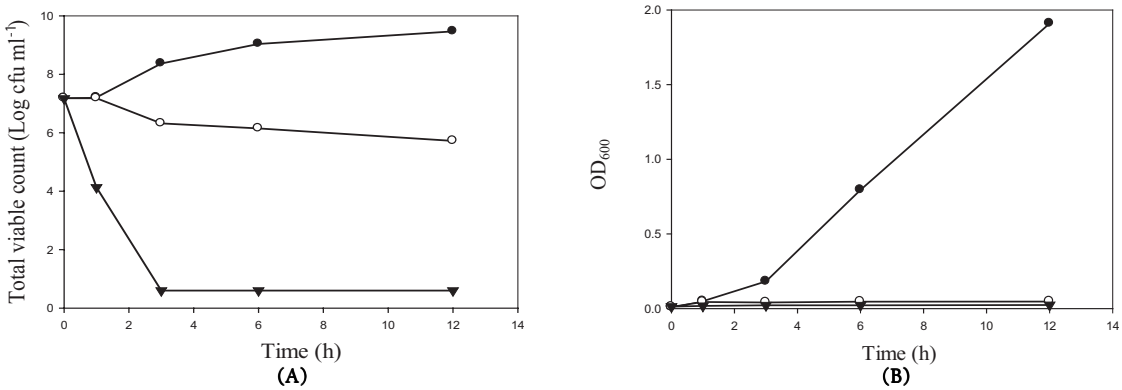

Fig 1. Effect of bacteriocin in *E. faecium* NKR-5-3 CFNS on viability (A) and OD_{600} (B) of *E. faecalis* ATCC 19433^T. CFNS was added to growing cells of *E. faecalis* ATCC 19433^T in MRS broth at a final concentration of 0 (closed circles), 256 (open circles) and 2,560 (closed triangles) AU ml^{-1}, incubated at 30°C, and the total viable count and OD_{600} were determined at selected time intervals.

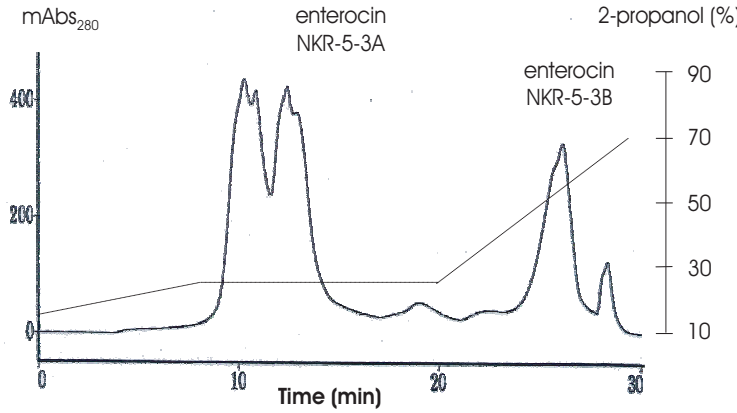

Fig 2. Reverse-phase chromatography of enterocin NKR-5-3A and enterocin NKR-5-3B. The amount applied to the C_2/C_{18} reverse-phase column was obtained from 1-liter of culture supernatant and active fractions were eluted at 28 % (enterocin NKR-5-3A) and 50-60 % (enterocin NKR-5-3B) 2-propanol containing 0.1% TFA at a flow rate of 1 ml min^{-1}.

bactericidal mode of action against *E. faecalis* ATCC 19433^T.

Bacteriocin Purification

Purification of the bacteriocin was performed by 4 steps from 1-liter of cultured supernatant of *E. faecium* NKR-5-3. The result of each purification step is shown in Table 4. In the reverse-phase HPLC step, the antibacterial

Table 4. Purification of enterocin NKR-5-3A and NKR-5-3B.

Purification step	Volume (ml)	Total protein [a] (mg)	Total activity [b] (AU)	Specific activity [c] (AU mg^{-1})	Increase in specific activity (fold)	Yield(%)
Culture supernatant	1,000	11,850	$5.1x10^7$	$4.3x10^3$	1	100
Amberlite XAD-16	150	1,516	$3.1x10^7$	$2.0x10^4$	4.6	60.7
SP-Sepharose	40	273	$8.2x10^6$	$3.0x10^4$	7.0	16
Octyl-Sepharose CL-4B	10	12.3	$2.0x10^6$	$1.6x10^5$	37.2	3.9
Reverse-phase HPLC						
- fraction A (enterocin NKR-5-3A)	1	0.18	$6.4x10^3$	$3.5x10^4$	8.1	0.00012
- fraction B (enterocin NKR-5-3B)	1	0.13	$6.4x10^3$	$4.9x10^4$	11.3	0.00012
- fraction A+B (1:1/v:v)	2	0.31	$8.2x10^5$	$2.6x10^6$	604	1.6

[a] Protein concentration (mg ml^{-1}) in solution was determined by GeneQuant *pro* RNA/DNA calculator (Amersham Pharmacia Biotech) and total protein is the concentration of protein in solution 1 ml multiply by the volume in milliliters.
[b] Bacteriocin activity was determined by the critical dilution method and *E. faecalis* ATCC 19433^T was used as the indicator strain and total activity is the bacteriocin activity in solution 1 ml multiplied by the volume in millilitres.
[c] Specific activity is total activity divided by total protein.

activity was detected in two different fractions which eluted at different 2-propanol concentrations, 28% and 50-60% respectively. In order to isolate purified bacteriocin peptides, each active fraction was separately rechromatographed on the same reverse-phase column, which resulted in two single peaks, coinciding with the antimicrobial activity (Fig 2).

MALDI-TOF MS and N-terminal amino acid sequence analyses were done on the purified fractions. The first fraction contained a peptide named enterocin NKR-5-3A with a MW=5,241 (Fig 3A) and its first 24 N-terminal amino acid residues were Tyr-Ser-Ser-Lys-Asp-Cys-Leu-Lys-Asp-Ile-Gly-Lys-Gly-Ile-Gly-Ala-Gly-Thr-Val-Ala-Gly-Ala-Ala-Gly. This mass and sequence are identical to those of brochocin A produced by *Brochotrix campestris* ATCC 43754.[11] The other fraction contained a peptide named enterocin NKR-5-3B with a MW=6,320 (Fig 3B). Edman degradation of enterocin

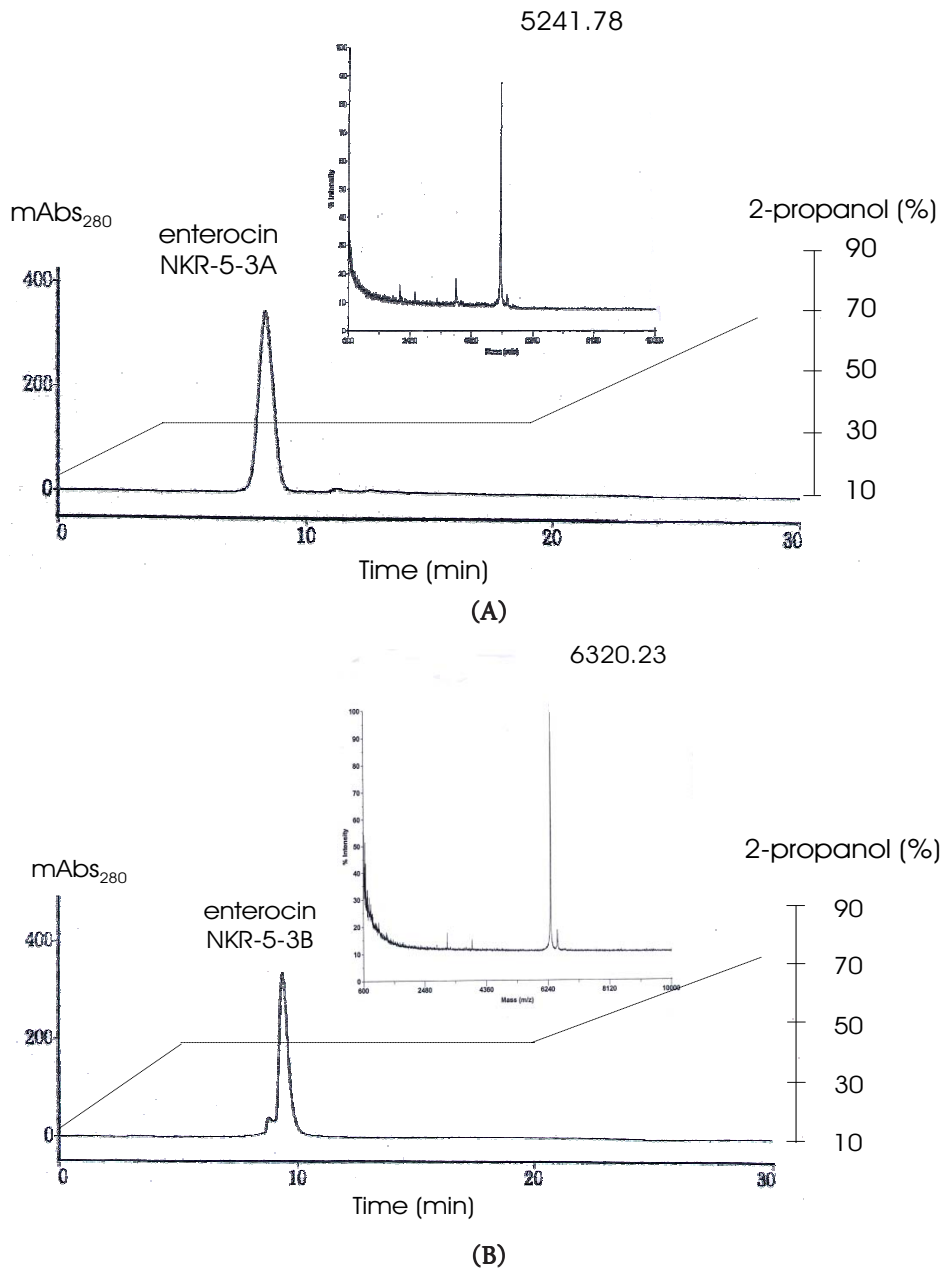

Fig 3. Reverse-phase chromatograms and MALDI-TOF mass spectra of purified enterocin NKR-5-3A (A) and enterocin NKR-5-3B (B). The fractions with high and specific bacteriocin activity related to each active peak were pooled and separately rechromatographed. Purified bacteriocin was eluted from the C_2/C_{18} reverse-phase column by the indicated gradients of 2-propanol containing 0.1% TFA at a flow rate of 1 ml min^{-1}.

Table 5. The synergistic activity of enterocin NKR-5-3A and enterocin NKR-5-3B.

Mixed ratio (v/v) NKR-5-3A	NKR-5-3B	Bacteriocin activity [a] (AU ml^{-1})
0	10	3,200
1	9	12,800
2	8	25,600
3	7	51,200
4	6	102,400
5	5	204,800
6	4	102,400
7	3	51,200
8	2	25,600
9	1	12,800
10	0	3,200

[a] Bacteriocin activity was determined by the critical dilution method.

NKR-5-3B did not yield any amino acid signal, suggesting a blocked N-terminus.

Synergistic Antimicrobial Activity of Two Purified Bacteriocin

Diluted enterocin NKR-5-3A and NKR-5-3B were mixed together at different ratios (v/v) and their activities were determined against *E. faecalis* ATCC 19433^{T}. As shown in Table 5, the combination of the two purified fractions exhibited higher activity than the sum of bacteriocin activity in each fraction and different synergistic activities were observed at different mixed ratios of the two. At 1:1 (v/v), the two purified active fractions exhibited the highest degree of synergism with the synergistic activity corresponding to 64 times of the total calculated bacteriocin activity (AU ml^{-1}) in the bacteriocin mixture.

Discussion

Bacteriocin of *E. faecium* NKR-5-3 was proteinaceous in nature, heat tolerant, even at 121°C, and had broad spectrum of activity. These attributes have also been reported in enterocin 900 and brochocin C.[9,11] It had a bactericidal mode of action, quite high activity after heating at neutral to alkaline pH and stability at high NaCl concentrations. Because of its quite stability under the indicated critical environments, this bacteriocin is worth studying for its potential as a natural food preservative in pre-cooked or fermented food with high salt concentration.

Two purified peptides with inherent antibacterial activity were isolated from culture supernatant of *E. faecium* NKR-5-3. The first active peptide, named enterocin NKR-5-3A, exhibited a MW=5,241 and the N-terminal 24 amino acids were identical to those of brochocin A, which is a component of two-peptide bacteriocin (brochocin C) produced by *B. campestris* ATCC 43754.[11] This strongly suggested that enterocin NKR-5-3A may be identical to brochocin A. On the other hand, the second active peptide, named enterocin NKR-5-3B, showed a MW=6,320 that was obviously different from brochocin B, which is the other active component of brochocin C. Edman degradation of NKR-5-3B yielded no amino acid signal, suggesting that its N-terminal amino acid was blocked. The MW of enterocin NKR-5-3B was also different from those of enterocin A and B produced by *E. faecium* WHE 81[12] which have MW of 4,833 and 5,462, respectively, and other known bacteriocins in the nr database of the National Center for Biotechnology Information (NCBI), National Institutes of Health, USA. Some LAB have been reported to produce more than one bacteriocin and these include lantibiotics as well as non-lantibiotics.[11-14] Only a few studies on multi-bacteriocin produced by *E. faecium* have been described.[12,14] Biochemical as well as genetic investigation of enterocin NKR-5-3A and B is now being done in order to identify their amino acid sequences and to elucidate the organization of the genes responsible for their production.

Two-peptide bacteriocins have been isolated from some LAB. In most cases, it has been found that each of two peptides exerts its own bacteriocin activity and its activity is stimulated by the presence of the other peptide.[15-17] On the other hand, in brochocin C and lactococcin, neither of the two peptides had its own bacteriocin activity but bacteriocin activity was observed when both peptides were mixed together.[11,18] However, each purified peptide of enterocin NKR-5-3A, which may be identical to brochocin A, and NKR-5-3B had inherent antibacterial activity and exhibited the maximum synergistic activity at 1:1 (v/v) ratio, corresponding to 64 times of the total calculated bacteriocin activity (AU ml^{-1}) in the bacteriocin mixture.

Although brochocin A and brochocin B had no inherent activity and both of them were required for the complementary activity of brochocin C, antimicrobial activity of purified brochocin A has also been described.[11] The same explanation of this in brochocin A can also be used to explain in the case of enterocin NKR-5-3. It was supposed that at high concentrations of purified enterocin NKR-5-3A, it had antimicrobial activity, as has been shown for high concentrations of ThmA of thermophilin 13 produced by *Streptococcus thermophilus*.[15] This phenomenon was also similar to LafX of the two-component bacteriocin lactacin F from *Lactobacillus johnsonii*.[19] Another hypothesis is that very small amounts of contaminating brochocin B or another enterocin NKR-5-3A enhancing substance, which could not be purified and detected by our purification procedure, are present in the purified enterocin NKR-5-3A sample, perhaps

due to the hydrophobic nature of the peptide. The synergistic phenomenon has been reported on many two-peptide bacteriocins such as brochocin C,[11] enterocin L50A and enterocin L50B,[14] thermophilin 13,[15] plantaricins EF and JK,[16] anti-*Staphylococcus aureus* lantibiotic,[17] plantaricin W [20] and lactococcin G.[21] Although there is a firm understanding of the molecular recognition responsible in some two-peptide bacteriocins, the synergistic mechanism in those two-peptide is far from understood.

In conclusion, *E. faecium* NKR-5-3 isolated from Pla-ra produced two-synergistic bacteriocin peptides showing strong antagonistic activity against various species of Gram-positive bacteria, including food pathogens. Together, its antibacterial spectrum, heat tolerance, and stability in a wide range of pH and salt concentrations suggests this bacteriocin may be of great benefit for use as a biological preservative in food industries.

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