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Antimicrobial peptide from *Bacillus subtilis* CSB138: characterization, killing kinetics, and synergistic potency

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Summary. We studied the prospect of synergy between the antimicrobial peptide p138c and non-peptide antibiotics for increasing the potency and bacterial killing kinetics of these agents. The production of p138c was maximized in the late exponential growth phase of *Bacillus subtilis* CSB138. Purification of p138c resulted in a total of 4800 arbitrary units (AU) with 19.15-fold and 3.2% recovery. Peptide p138c was thermo-tolerant up to 50 °C and stable at pH 5.8 to 11. The biochemical nature of p138c was determined by a bioassay, similar to tricine-SDS-PAGE, indicating inhibition at 3 kDa. The amino acid sequence of p138c was Gly-Leu-Glu-Glu-Thr-Val-Tyr-Ile-Tyr-Gly-Ala-Asn-Met-X-Ser. Potency and killing kinetics against vancomycin-resistant *Staphylococcus aureus* improved considerably when p138c was synergized with oxacillin, ampicillin, and penicillin G. The minimal inhibitory concentration (MIC) of p138c showed a 4-, 8-, and 16-fold improvement when p138c was combined with oxacillin, ampicillin, and penicillin G, respectively. The fractional inhibitory concentration index for the combination of p138c and oxacillin, ampicillin, and penicillin G was 0.3125, 0.25, and 0.09, respectively. Synergy with non-peptide antibiotics resulted in enhanced killing kinetics of p138c. Hence, the synergy between antimicrobial peptide and non-peptide antibiotics may enhance the potency and bacterial killing kinetics, providing more potent and rapidly acting agents for therapeutic use. [**Int Microbiol** 20(1):43-53 (2017)]

Keywords: *Bacillus subtilis* · antimicrobial peptides · killing kinetics

Introduction

The wide use of antibiotics, in the past and recent decades, has caused the rapid emergence of antibiotic-resistant bacteria, indicating the need for the development of new antimicrobial agents. Antimicrobial peptides (AMPs), present in the host immune system, may be a new, anti-infective alternative to conventional antibiotics. Because of their broad-spectrum antimicrobial activity and exclusive membrane action mechanism, they may replace or accompany conventional antibiotics [26]. Therefore, AMPs can be used to develop future antibiotics. To date, approximately 1000 naturally occurring antibacterial peptides have been isolated. Despite this large number of iso-

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lated peptides, only a few have clinical applications because most of them display poor potency, specificity, and in vivo stability [5]. These drawbacks of AMPs need to be addressed in order to achieve effective development and application of new antimicrobial agents for clinical use. Hence, efforts have been placed into enhancing the killing kinetics, potency, and specificity of antibacterial peptides.

The activity and selectivity of AMPs are driven by their charge, amphipathicity, and hydrophobicity [4,25]. To address these factors, short AMP derivatives, with cell selective toxicity, have been developed to improve the activity and specificity based on the amino acid composition, charge, and hydrophobicity of natural peptides [10,13,15,21,27]. Toxicity has been reduced by minor sequence modifications [2,6,14]; however, such approaches are time consuming and costly. The use of combination therapy is effective in overcoming antibiotic resistance. Employing β-lactamase inhibitors as co-drugs with conventional antibiotics is well known [11,16]. The synergistic effect of antibiotic activity depends on the ability of the two molecules to exert a deleterious effect on the target microorganism higher than the sum of the effects of each drug alone. Synergy reduces the MIC of the combined drugs and helps prevent the development of resistance in microorganisms [16,24].

The widespread use of antibiotics plays a major role in the emergence of drug-resistant bacteria. Unhealthy practices in the pharmaceutical and manufacturing industries are also likely sources of the emergence of antibiotic-resistant strains [9]. *Staphylococcus aureus* and *Enterococcus faecium* are the major antibiotic-resistant pathogens found in hospitals. Methicillin-resistant *Staphylococcus aureus* (MRSA), detected for the first time in 1961 in Britain, is now common in hospitals. The first strain with complete resistance (>16 μg/ml) to vancomycin, termed vancomycin-resistant *Staphylococcus aureus* (VRSA), appeared in the United States in 2002. Vancomycin-resistant *Enterococcus* (VRE) was reported in 1987. A report in 2004 showed that 9% of nosocomial bloodstream infections were caused by enterococci and 2% by the *E. faecalis* strain [23]. This rapid emergence of resistant bacteria highlights the need to find alternatives for conventional antibiotics.

In this study, we isolated, identified, and characterized a bacterial isolate from fermented food using gene sequencing. Further, we studied the possible synergy between the antimicrobial peptide and non-peptide antibiotics. We have identified isolates with promising antimicrobial profiles and evaluated their biological potency. The combination of antimicrobial peptide and non-peptide antibiotics showed effective synergy, which we evaluated using fractional inhibitory concentration (FIC) indices. Our antimicrobial peptide was moderately potent and synergistic with commonly used antibiotics; it also showed robust potency and killing kinetics.

Materials and methods

Materials. This The chemicals and solvents used in our study were of extra pure grade. Sepharose CL-6B and Sephadex G-50 were obtained from Pharmacia (Uppsala, Sweden). All other reagents were of the highest analytical grade available.

Bacterial strain and production of the antimicrobial peptide. More than 100 fermented food samples were used to isolate the bacterial strain. Isolation was performed according to the method described previously [16]. Briefly, 1g of the fermented food sample was mixed with 0.85% NaCl and incubated for 24 h at 37 °C. Following incubation, the sample was serially diluted up to 10^{-7} in Mueller-Hinton broth. The appropriate colony-forming units (CFU/ml) were estimated by streaking the dilutions on Mueller-Hinton agar media. The dilution with the required CFU/ml was considered working stock and maintained in 20% glycerol. The bacterial strain CSB138, with potent antimicrobial activity, was selected for further study. The identification of the bacterial strain, based on morphological characteristics, was performed according to Bergey's Manual of Systematic Bacteriology. Sequencing analysis of the 16S ribosomal RNA (rRNA) gene was conducted for further identification. Antimicrobial peptide production was performed using a bacterial culture grown in optimized medium for 28 h at 37 °C in a rotating incubator at 150 rpm.

Media optimization. Optimization of the culture media for strain CSB138 was conducted using various carbon and nitrogen sources, and metal ions. The carbon sources included mannitol, starch, fructose, sorbitol, sucrose, glucose, maltose, and lactose. The nitrogen sources included yeast and beef extract, malt, tryptone, peptone, oatmeal, and soymeal. The metal ions were Na_2HPO_4, NaH_2PO_4, $MgSO_4$, $ZnSO_4$, $MgCl_2$, KH_2PO_4, $FeSO_4$, NaCl, and $CaCl_2$. The influence of carbon sources on the production of the antimicrobial compound was assessed using media supplemented with 1% yeast extract as a nitrogen source and various carbon sources. Fermentation was carried out in 250-ml Erlenmeyer flasks at 150 rpm and 37 °C. The influence of nitrogen sources on the production of the antimicrobial compound was assessed using a medium supplemented with 1% sorbitol as a carbon source and various nitrogen sources. The influence of metal ions on the production of the antimicrobial compound was determined using 1% sorbitol, 1% yeast extract, and various metal ions. The influence of sorbitol, yeast extract, and metal ions on the production of the antimicrobial compound by strain CSB138 was determined using different proportions of these supplemented compounds. The bacterial strain CSB138, cultured in fully optimized media containing 1.25% sorbitol and 1% yeast extract (S-YE), was optimal for the production of the antimicrobial compound. The antimicrobial compound, produced by strain CSB138, was designated as p138c.

Kinetics of bacterial growth. The S-YE fully optimized medium-was inoculated with freshly grown *Bacillus* strain CSB138 and incubated at 37 °C. Samples (2 ml) were collected at the interval of 4 h and centrifuged

at 10,000 ×g for 15 min. The obtained supernatant was assayed for antimicrobial activity. Optical density was recorded spectrophotometrically at 620 nm and pH was measured.

Purification of p138c. The seed culture, with the bacterial suspension turbidity equivalent to that of 0.5 McFarland standard solutions, was transferred into the main culture in a 2-liter baffled flask, containing 400 ml S-YE media, and incubated at 37 °C and 150 rpm for 28 h. Then, the fermented broth was centrifuged (Hanil Science Industrial, Supra 22K, Korea) at 10,000 × *g* at 6 °C for 15 min. The obtained cell-free supernatant was treated with diammonium sulfate at the saturation of 20–100%. The active precipitation fraction was recovered using centrifugation at 10,000 × g at 6 °C for 45 min, dialyzed with 10 mM/l Tris-HCl (pH 7.5) buffer, and subjected to ultrafiltration (Millipore). After ultrafiltration through the molecular weight cut-off membranes, the biologically active fraction was loaded onto a Sepharose CL-6B column (2.5 × 85 cm) (Pharmacia, Uppsala, Sweden) using 10 mM/l Tris-HCl (pH 7.5) buffer. The active fractions, obtained from Sepharose CL-6B gel permeation column chromatography, were pooled, concentrated, further purified with a Sephadex G-50 column (1.7 × 120 cm) (Pharmacia, Uppsala, Sweden) using the same buffering system, and subjected to purity analysis.

Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis and bioassay. The concentration of p138c was determined by the Bradford method [1] using bovine serum albumin (BSA) as the standard. Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Tricine-SDS-PAGE) [17] was used to analyze the purity of the antimicrobial peptide. The electrophoresed gel was stained with Coomassie brilliant blue R-250 and destained with methanol/glacial acetic acid/distilled water (1:1:8, v/v/v). The inhibitory activity was assayed according to our previously described method [16]. Briefly, the electrophoresed gel was washed several times with 50 mM/liter Tris-HCl buffer (pH 7.5) containing 2.5% Triton X-100. Afterward, the processed gel was overlaid with 0.6% agar on Mueller-Hinton medium (Difco, USA) containing the indicator organisms (1.5×10^8 CFU/ml) and incubated at 37 °C for 12 h.

The N-terminal amino acid sequence. The N-terminal amino acid sequence of p138c was determined by Edman degradation using the automatic protein sequencing system model 492cLC (Applied Biosystems, Foster, CA, USA).

The effects of pH, temperature, proteases, and surfactant on p138c. The effect of temperature on the activity of p138c was evaluated by incubating this compound at the temperature of 0, 4, 10, 20, 37, 50, 60, 70, 80, and 121 °C for 24 h and assessing antimicrobial activity using the disc diffusion method. The effect of pH on the activity of p138c was tested by adjusting the pH 4.2 to 12.5 using 1 M HCl or 1 M NaOH, respectively, and assessing antimicrobial activity. In a separate procedure, 10 ml of cell-free supernatant were placed into a sterile Petri dish and exposed to UV light in a laminar airflow hood for 5 min; then, antimicrobial activity was examined using the disc diffusion method.

The effect of various proteases on the activity of p138c was examined using 50 µl of cell-free supernatant incubated for 150 min in the presence of 1 mg/ml or 0.1 mg/ml of trypsin, proteinase K, and catalase. Antimicrobial activity was determined using the disc diffusion method. In a separate experiment, Triton X-100, Tween 20, Tween 80, SDS, NaCl, and urea were added to p138c to yield the final concentrations of 1.0, 2.0, and 5.0 mM. Untreated p138c and the tested reagents, at their respective concentrations in distilled water, were used as controls. All the treated and untreated samples were incubated at 37 °C for 6 h, after which the antimicrobial activity was examined using the disc diffusion method.

Antimicrobial inhibitory spectrum. Antimicrobial activity was determined by the disc diffusion method. A disc of filter paper (8 mm, Toyo Roshi Kaisha, Japan), saturated with 40 µl of p138c, was placed on the surface of a Mueller-Hinton agar plate, overlaid with indicator microorganisms, and incubated at 37 °C for 24 h. Following incubation, the clear zone of inhibition around the paper disc was measured. Antimicrobial activity, with respect to minimal inhibitory concentration (MIC), was determined according to our previous report [16]. Various microorganisms, including *Bacillus subtilis* ATCC 6633, *Enterococcus faecalis* ATCC 29212, *Mycobacterium smegmatis* ATCC 9341, methicillin-resistant *Staphylococcus aureus* 5–3, methicillin-resistant *S. aureus* 4–5, vancomycin-resistant *S. aureus*, vancomycin-resistant Enterococci 4, vancomycin-resistant Enterococci 98, vancomycin-resistant Enterococci 89, *S. aureus* KCTC 1928, *Micrococcus luteus* ATCC 9341, *Escherichia coli* KCTC 1923, *Salmonella typhimurium* KCTC 1925, *Pseudomonas aeruginosa* KCTC 1637, extended-spectrum beta-lactamase V4 (*E. coli*), and *Alcaligenes faecalis* ATCC 1004 were used as test microorganisms. Vancomycin and bacitracin were used as reference antibiotics. After the inoculation of test organisms (1.5×10^8 CFU/mL), the plates were incubated at 37 °C overnight. Minimum bactericidal concentration (MBC) was determined using the broth dilution method. Antimicrobial activity was expressed in terms of arbitrary units (AU). An AU is defined as the highest dilution of the sample that produces a zone of inhibition. The reciprocal of the dilution was regarded as the titer of antimicrobial activity (AU/ml).

Assessment of antibiotic susceptibility. Susceptibility testing of the activity of p138c and various antibiotics (oxacillin [Sigma, USA], ampicillin [Sigma], penicillin G [USB, UK], ciprofloxacin [Sigma], bacitracin [USB] and vancomycin [Sigma]) against vancomycin-resistant *Staphylococcus aureus* (VRSA), vancomycin-resistant Enterococci 4, vancomycin-resistant Enterococci 89, and vancomycin-resistant Enterococci 98 was conducted using the broth dilution method for the determination of MIC [22]. The bacterial culture (1.5×10^8 CFU/ml), grown overnight, was suspended in Mueller-Hinton broth; 100 µl of this suspension was aliquoted into a 96-well tissue culture plate (TCP). Various concentrations (0.156-80 µg/ml) of the antimicrobial peptide in 10 mM/l Tris-HCl (pH 7.5) buffer, and antibiotics in distilled water, were added to the suspension. The negative control was maintained without the addition of any antibiotics. The TCP was incubated at 37 °C in a shaking incubator at 150 rpm. After 12 h of incubation, optical density was measured at 620 nm. MIC is defined as the lowest concentration of any drug that inhibits the measurable growth of the indicator organism after a given incubation. Each experiment was performed in triplicate and repeated three times. Minimum bactericidal concentration (MBC) was measured by plating the content of the clear well, used in the MIC assessment, onto Mueller-Hinton agar plates. After the incubation, MBC was determined as the lowest concentration that inhibited bacterial growth (99.9%) on the surface of the agar.

Bacterial killing kinetics. The cultures (1.5×10^8 CFU/ml) of vancomycin-resistant *Staphylococcus aureus* (VRSA), vancomycin-resistant Enterococci 4 (VRE 4), vancomycin-resistant Enterococci 89 (VRE 89), and vancomycin-resistant Enterococci 98 (VRE 98), grown overnight, were diluted in Mueller-Hinton broth; antibiotics, along with the antimicrobial peptide p138c, were added to 100 µl of the diluted culture. The mixture was incubated at 37 °C and 150 rpm. The samples were collected at regular intervals, diluted, and plated on Mueller-Hinton agar plates. The plates were incubated at 37 °C for 18 h and the colonies were counted. For synergy assessment, MICs were determined by the addition of 0.5 MIC.

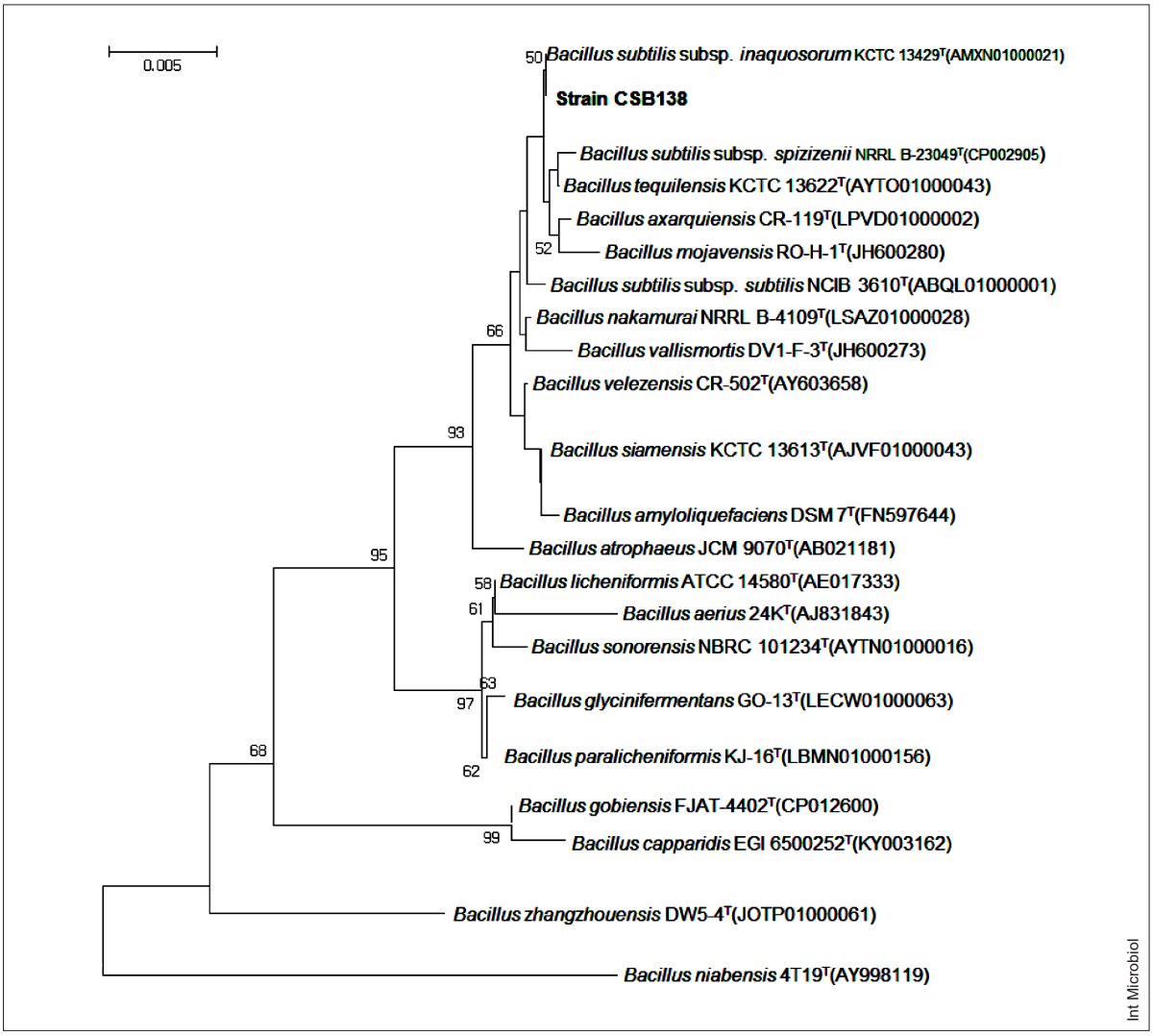

Fig. 1. Phylogenetic tree, based on the complete 16S rRNA gene sequence, showing the relationships between the strain CSB138 and closely related taxa of the genus *Bacillus*. Reference sequences were retrieved from GenBank under the accession number indicated in parentheses after the strain name. Numbers of nodes are percentage bootstrap values based on 1000 replications; only values greater than 65 % are shown. Bar: 0.005 substitutions per nucleotide position.

The checkerboard dilution assay for the assessment of synergy. The assessment of synergy was performed using the checkerboard method [7]. Separate antibiotics, and combinations of two antibiotics, were incubated with bacteria to observe the bactericidal effect. The MIC of combined antibiotics was calculated using the addition of 0.5 MIC of the antimicrobial peptide. The fractional inhibitory concentration (FIC) assay was used as the base for synergism. The fractional inhibitory concentration index (ΣFIC) was estimated according to our previous method [16]. Briefly, $\Sigma FIC = FIC_A + FIC_B = (C_A/MIC_A) + (C_B/MIC_B)$, where MIC_A and MIC_B are the MICs of drugs A and B, and C_A and C_B are the concentrations of the combined drugs, respectively. The interaction was recorded, according to the guidelines provided by the European Committee on Antimicrobial Susceptibility Testing, as follows: FIC index ≤ 0.5 indicated synergy, FIC index > 0.5–1.0 indicated additive action, FIC index > 1.0 to < 2.0 indicated indifference, and FIC index ≥ 2.0 indicated antagonism [3].

Results

Identification of the bacterial strain. Strain CSB138 showed morphological resemblance to *Bacillus*. The gene sequencing analysis, obtained by gene sequencing 16S rRNA, indicated that the isolate showed the closest identity to *Bacillus subtilis* subsp. *inaquosorum* KCTC 13429 (accession no. AMXN01000021) with pairwise similarity of 99.93 %. Based on similarities in morphology and gene sequence, our strain was classified as *Bacillus subtilis* CSB138. A phylogenetic tree, constructed from the 16S rRNA gene sequence, is shown in Fig.1.

Table 1. Minimum inhibitory concentration of peptide p138c

Antimicrobial spectrum of p138c

	MIC (µg/ml)			MBC (µg/ml)
	p138c	Bacitracin	Vancomycin	p138c
Gram-negative bacteria				
Alcaligenes faecalis ATCC 1004	>80	>80	>80	>1280
Escherichia coli KCTC 1923	>80	>80	>80	>1280
Extended-spectrum beta-lactamase V4 *(Escherichia coli)*	>80	>80	>80	>1280
Pseudomonas aeruginosa KCTC 1637	>80	>80	>80	>1280
Salmonella typhimurium KCTC 1925	>80	>80	>80	>1280
Gram-positive bacteria				
Bacillus subtilis ATCC 6633	>80	20	0.312	640
Enterococcus faecalis ATCC 29212	2.5	5	2.5	160
Methicillin-resistant *Staphylococcus aureus* 4-5	0.625	2.5	0.625	640
Methicillin-resistant *Staphylococcus aureus* 5-3	0.625	2.5	1.25	640
Micrococcus luteus ATCC 9341	80	40	2.5	320
Mycobacterium smegmatis ATCC 9341	40	80	2.5	>1280
Staphylococcus aureus KCTC 1928	20	40	1.25	320
Vancomycin-resistant *Staphylococcus aureus*	20	80	>80	640
Vancomycin-resistant Enterococci 4	10	40	>80	160
Vancomycin-resistant Enterococci 89	10	>80	>80	640
Vancomycin-resistant Enterococci 98	10	>80	>80	640

MIC, minimum inhibitory concentration; MBC, minimum bactericidal concentration

Culture media and production of the antimicrobial peptide. Microorganisms require specific biological culture media in order to grow under laboratory conditions. Bacteria, grown in the laboratory culture media, are designed to meet all the requirements necessary for confluent growth. However, the production of specific protein viz. antimicrobial peptide is based on optimized media. Among the carbon and nitrogen sources tested, sorbitol and yeast extract, respectively, were more effective. Metal ions did not significantly affect the production of p138c. Hence, the optimal medium that we used was supplemented with 1.25% sorbitol and 1% yeast extract (S-YE). The *Bacillus* isolate CSB138, producing p138c, was grown on S-YE medium at 37 °C for 28 h and 150 rpm.

Spectra of p138c. In the growth kinetics study, the maximal activity of p138c was observed after 28 h in the S-YE broth. The initial pH of the broth was 6.25. The activity of p138c was detected starting at 4 h at pH 6.34, and maximal activity was recorded after 28 h at pH 7.1. The changes in the pH of the culture, from 6.25 to 8.38, were observed for up to 60 h of growth. We recorded increases in the cell density of the cultures at OD 620 nm, in the pH range of 6.34 to 8.36, with maximal density at pH 8.01 and maximal activity at 7.1. In this study, we evaluated the activity of p138c against various microorganisms. The antimicrobial activity of p138c was evaluated along with the standard reference antibiotics viz. vancomycin and bacitracin. The antimicrobial spectrum of p138c was 4 to > 8 times greater than that of bacitracin, and > 4 to > 8 times more effective than that of vancomycin, against vancomycin-resistant *Staphylococcus aureus* and various VREs (Table 1).

Size exclusion chromatography and molecular weight determination of p138c. At the third step of purification, the biologically active sample was loaded onto a

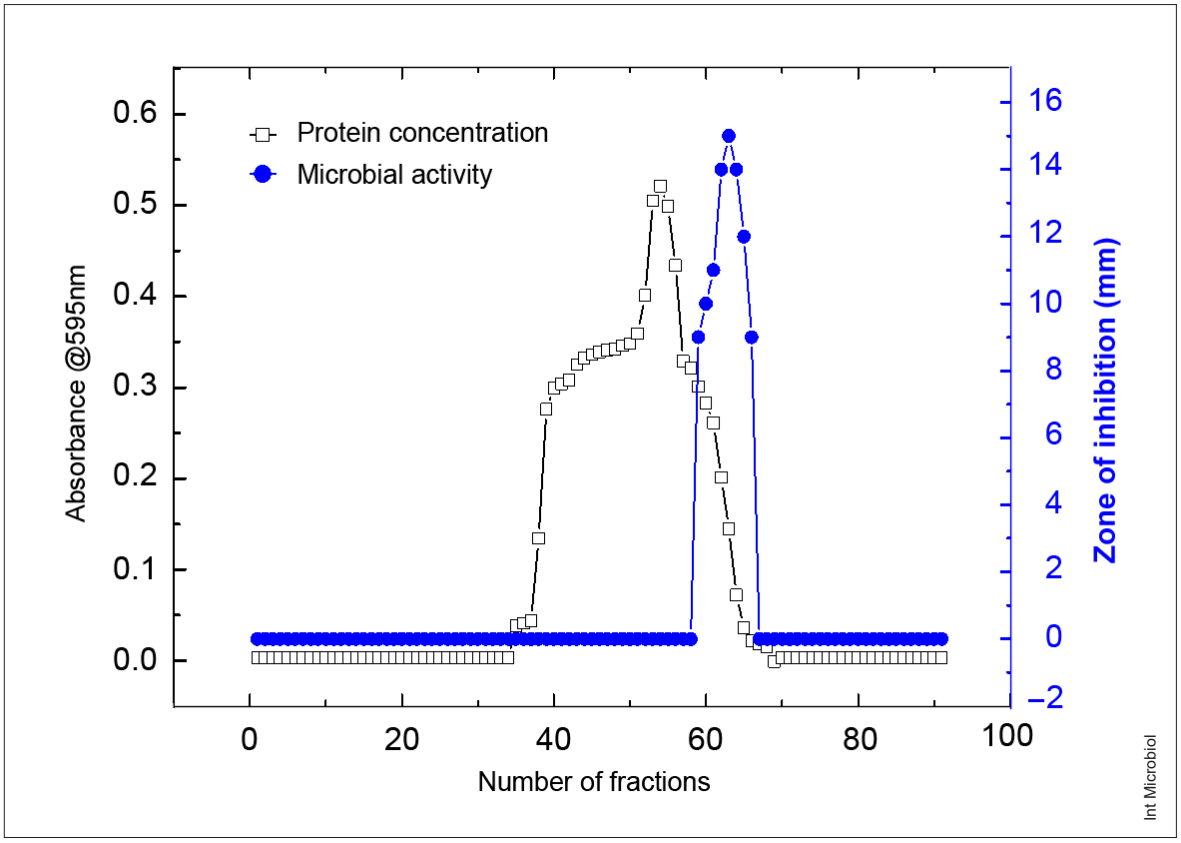

Fig. 2. Elution profile of peptide p138c from the Sepharose-CL-6B column (2.5 × 85 cm). The column was pre-equilibrated with 10 mM/l Tris/HCl buffer (pH 7.5).

Sepharose CL-6B and fractionated at the elution rate of 0.25 ml per min; the fraction eluted between 590 to 660 min showed maximal activity (Fig. 2). The biologically active fractions were collected and loaded onto Sephadex G-50 using the same buffering system. The active fractions were pooled together and desalted using a Sephadex G-25 column. The protein profile was determined using tricine SDS-PAGE, which showed a single protein band near 3 kDa. The inhibitory activity of p138c was examined in situ. Using an indicator microorganism, a clear zone of lysis was observed near 3 kDa. Purification resulted in a total of approximately 4800 AU with 19.15-fold overall purification and recovery of 3.2% (Table 2).

Characterization. Residual activity was determined according to the retained biological activity. The activity of p138c was retained at 100% after heating at temperatures as high as 50 °C. Complete loss of activity occurred after heating at 121 °C and 105 kPa for 15 min. Peptide p138c tolerated a wide range of pH values (5.8 to 11). This ability to retain activity over a wide range of pH values may be the result of the reversible denaturation of the protein. The effects of temperature and pH on the activity of p138c are shown in Fig. 3. The

Table 2. Purification of peptide p138c

Steps	Total protein (mg)	Total activity (AU)	Specific activity (AU/mg)	Purification fold	Recovery %
Crude	308.56	150000	486.12	1	100
Ammonium sulfate pallet	91.59	73600	803.60	1.65	49.1
Sepharose CL-6B	5.79	12000	2071.88	4.26	8
Sephadex G-50	0.52	4800	9310.50	19.15	3.2

antimicrobial activity of p138c was completely inactivated after the cell-free supernatant was treated with proteinase K. The effect of trypsin was not detectable, indicating the proteinaceous nature of our antimicrobial peptide. Generally, antimicrobial peptides have proteinaceous characteristics. Our results show that H_2O_2 was not the factor responsible for the inhibition because no change in activity was recorded when p138c was treated with catalase. Peptide p138c was found to be sensitive to treatment with SDS, Tween 80, and NaCl; however, treatment with Triton X-100, Tween 20, and urea had no effect on the antimicrobial activity of p138c. UV treatment did not affect the antimicrobial activity, possibly because of the proteinaceous nature of p138c. The effects of enzymes, detergents, other reagents, and UV- light on the activity of p138c are shown in Table 3.

Table 3. Effect of proteases, detergents, UV, and other reagents on the activity of p138c

Proteases	Trypsin	ND
	Proteinase K	+
	Catalase	−
Detergents	SDS	+
	Triton X-100	−
	Tween 20	−
	Tween 80	+
NaCl and urea	NaCl	+
	Urea	−
UV	UV-light	−

ND: non-detectable; + inactivation by treatment; − resistant to treatment

Amino acid sequence. The N-terminal sequence of the last 15 amino acids of p138c was determined to be Gly-Leu-Glu-Glu-Thr-Val-Tyr-Ile-Tyr-Gly-Ala-Asn-Met-X-Ser. This sequence did not show significant homology with those of peptides of similar origin. No full query is covered during the search. Searching the National Centre for Biotechnology Information (NCBI) protein database using BLAST showed some similarity with other protein sequences from the *Bacillus* sp. (Table 4).

Synergy between p138c and conventional antibiotics. The antimicrobial activity of p138c, determined in terms of MIC, was moderately active against gram-positive bacteria (MIC of 0.625–80 µg/ml). The antimicrobial spectrum of p138c possessed more potency than those of the commonly used bacitracin and vancomycin. Peptide p138c showed higher antibacterial activity against vancomycin-resistant *Staphylococcus aureus* (MIC of 20 µg/ml, 4-fold greater than bacitracin and > 4-fold greater than vancomycin, and MBC of 640 µg/ml), vancomycin-resistant Enterococci 4 (MIC of 10 µg/ml, 4-fold greater than bacitracin and > 8-fold greater than vancomycin, and MBC of 160 µg/ml), vancomycin-resistant Enterococci 89 (MIC of 10 µg/ml, > 8-fold greater than bacitracin and vancomycin, and MBC of 640 µg/ml), and vancomycin-resistant Enterococci 98 (MIC of 10 µg/ml, > 8-fold greater than bacitracin and vancomycin, and MBC of 640 µg/ml). The synergy between p138c and six conventional antibiotics was tested in vitro by the checkerboard dilution assay against VRSA and three vancomycin-resistant isolates of Enterococci (Table 5). Each combination of the drugs was assigned a ΣFIC. FIC indices

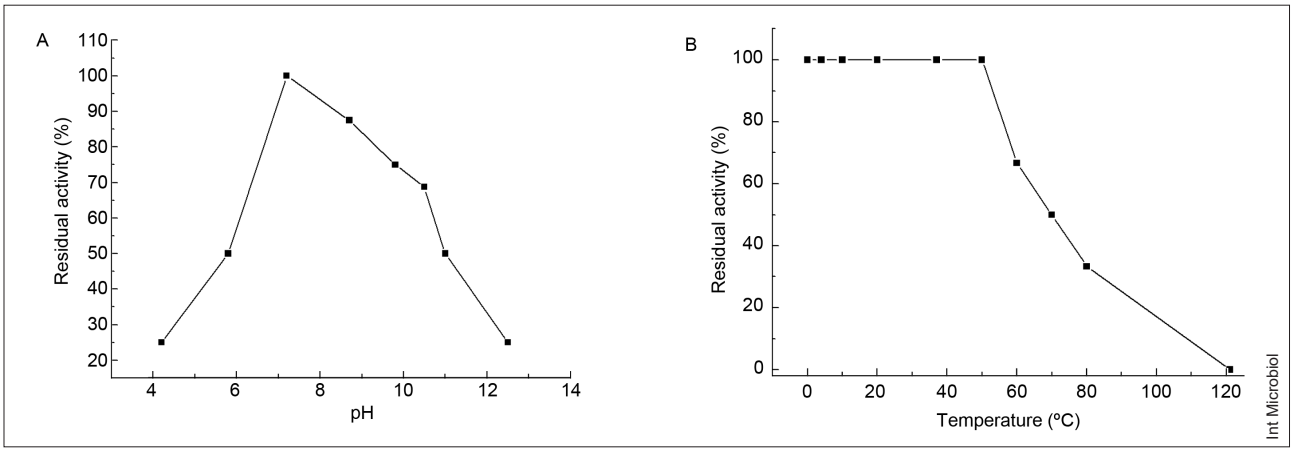

Fig. 3. Effect of pH (A) and temperature (B) on the activity of peptide p138c.

Table 4. Comparison of N-terminal amino acid sequence of peptide p138c with other related proteins from the *Bacillus* sp.

SN	Alignment	Identity	Peptide or protein	References
1	GLEETVYIYGANMXS	100 %	p138c [*Bacillus subtilis*]	Current study
2	EET+Y YGA EETIYFYGA	78 %	peptidase M16 [*Bacillus subtilis*]	NCBI Reference Sequence: WP_060399736.1
3	+ET YIY GA DETRYIYTGA	70 %	peptidase M20 [*Bacillus subtilis*]	NCBI Reference Sequence: WP_060399755.1
4	+ET YIY GA DETRYIYTGA	70 %	protein RocB [*Bacillus subtilis*]	NCBI Reference Sequence: WP_003235954.1

≤ 0.5 were regarded as synergistic. Peptide p138c showed synergy with the three β-lactam antibiotics (oxacillin, ampicillin, and penicillin G) used for inhibiting bacterial growth (VRSA). p138c, combined with oxacillin or ampicillin, or with penicillin G, substantially enhanced the potency of the combinatorial effect. The antibacterial activity of combined treatment with oxacillin and p138c against VRSA was 4-fold greater than that of p138c alone. Similarly, combining p138c with ampicillin produced antimicrobial activity that was 8-fold greater than that of p138c alone; the antimicrobial activity of p138c, combined with penicillin G, was 16-fold greater than that of p138c alone. These results show that synergizing p138c with β-lactam antibiotics is an effective strategy for improving antibacterial potency and cost effectiveness of antimicrobial peptides.

Effect of synergy on bactericidal activity. Drugs are categorized into bacteriostatic and bactericidal agents based on their effectiveness against infectious microorganisms. Bacteriostatic drugs inhibit the growth of bacteria, whereas bactericidal drugs kill them. We evaluated the minimum bactericidal concentration of the synergistic combination of p138c (MBC, 640 μg/ml) and oxacillin (MBC, 80 μg/ml), p138c (MBC, 640 μg/ml) and ampicillin (MBC, 20 μg/ml), and p138c (MBC, 640 μg/ml) and penicillin G (MBC, 40 μg/ml) against vancomycin-resistant *Staphylococcus aureus*. The combination of p138c (combined MBC, 160 μg/ml) and oxacillin (combined MBC, 5 μg/ml), p138c (combined MBC, 80 μg/ml) and ampicillin (combined MBC, 2.5 μg/ml), and p138c (combined MBC, 80 μg/ml) and penicillin G (combined MBC, 1.25 μg/ml) were bactericidal. The fractional inhibitory concentration (FIC) index for the combination of p138c and oxacillin was low (0.3125). Similarly, the FIC index for the combination of p138c and ampicillin was 0.25 and that of p138c and penicillin G was 0.09. FIC indices < 0.5 indicate a strong synergy with respect to bactericidal activity. Thus, oxacillin, ampicillin, or penicillin G potentiates bactericidal activity, demonstrating 4 to 8-fold improvement in the bactericidal action of p138c. Our results indicate that synergy with conventional antibiotics can transform a moderately bacteriostatic agent into a potent bactericidal drug.

The effect of synergy on bacterial killing kinetics. The bacterial load clearance in patients corresponds with how well antibiotics perform in killing the targeted bacterium. We tested the synergistic effect of p138c (MIC of 20 μg/ml) and various commonly used conventional antibiotics (oxacillin, MIC of 80 μg/ml; ampicillin, MIC of 20 μg/ml; and penicillin G, MIC of 40 μg/ml) against vancomycin-resistant *Staphylococcus aureus* to find a synergistic combination of the peptide and non-peptide antibiotics that affected the rate of bacterial killing kinetics. Peptide p138c alone was largely bacteriostatic against VRSA; in synergy with various tested antibiotics, p138c showed improved potency and more rapid killing kinetics (Fig. 4). Synergizing the peptide with commonly used antibiotics improved the potency of the antimicrobial peptide and the rate of bacterial killing kinetics.

Discussion

Peptide p138c, produced by *Bacillus subtilis* CSB138, was isolated from fermented food. According to Selhub et al. [18], the relationship between bioactive compounds in fermented food and fermentation-enriched chemicals affects the profile of the human intestinal microbiota, indicating that the

Table 5. Fractional inhibitory concentration (FIC) of peptide p138c

Tested compounds	Strains	MIC of p138c (µg/ml)	MIC in combination with p138c (µg/ml)	EFIC	Interpretation
Oxacillin (OXA)	VRSA	20	5	0.31	Synergy
	VRE 4	10	5	0.63	
	VRE 89	10	10	<1.125	
	VRE 98	10	5	0.56	
Ampicillin (AMP)	VRSA	20	2.5	0.25	Synergy
	VRE 4	10	2.5	0.50	Synergy
	VRE 89	10	5	0.63	
	VRE 98	10	5	0.75	
Penicillin G (PENG)	VRSA	20	1.25	0.09	Synergy
	VRE 4	10	5	0.75	
	VRE 89	10	10	1.50	
	VRE 98	10	5	0.63	
Ciprofloxacin (CIP)	VRSA	20	5	2.25	
	VRE 4	10	5	1.50	
	VRE 89	10	5	4.50	
	VRE 98	10	2.5	1.25	
Bacitracin (BAC)	VRSA	20	>80	>5	
	VRE 4	10	80	10	
	VRE 89	10	80	<9	
	VRE 98	10	>80	>9	
Vancomycin (VAN)	VRSA	20	>80	>5	
	VRE 4	10	>80	>9	
	VRE 89	10	>80	>9	
	VRE 98	10	>80	>9	

purified product from fermented food may alter the pre-consumption of dietary products. This ultimately amends the action by which fermentation-enriched chemicals act on the human intestinal microbiota. It is known that the antimicrobial peptides have functional activities including antimutagenic, anticancer, immunomodulatory, antiatherosclerotic, and anti-obesity effects. In our study, p138c inhibited a wide spectrum of bacteria and demonstrated antagonistic potential. The lower potency, instability, and high production cost of antimicrobial peptides have caused major hurdles in their clinical development [5]. One of the solutions to these issues is synergizing antimicrobial peptides with conventional antibiotics, thereby lowering the dosage of each molecule in the synergistic combination.

Our study was designed to characterize p138c and study the prospect of synergy between the antimicrobial peptide and non-peptide antibiotics. The strain, isolated from fermented food, was identified via morphological and 16S rRNA gene sequencing analyses. p138c can be produced in inexpensive optimized culture media (S-YE). Generally, bacteriocin-like substances are produced in complex media [12]. The purification of p138c was conducted using sequential gel filtration, resulting in the eluted compound that was free of unwanted proteins and possessing robust antimicrobial activity. The purification of p138c, using precipitation in the presence of a low saturation of diammonium sulfate, and elution at void volume using gel permeation chromatography, indicated that p138c was secreted in the form of large aggregates. This result is consistent with those of other studies examining [8,16]. Peptide p138c was purified 19.15-fold with 3.2% recovery. Bacterial growth kinetics and inhibitory activity indicated that antimicrobial activity occurred during the steady state of the growth curve, from early to late stationary phase, and declined in the very late stationary phase. Antimicrobial activity of the

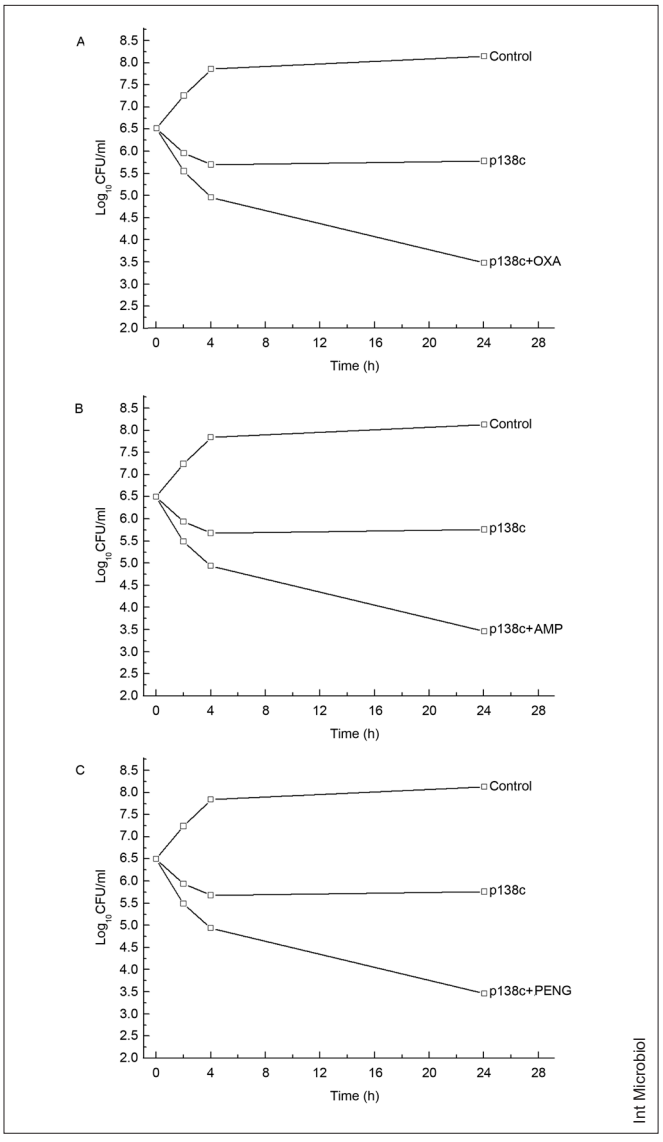

Fig. 4. Synergy improves killing kinetics of p138c. Killing kinetics of p138c, and of p138c combined with oxacillin **(A)**, ampicillin **(B)**, and penicillin G **(C)**, with added 0.5 MIC of p138c, against vancomycin-resistant *Staphylococcus aureus* (VRSA).

test strain was detected in the mid-stationary phase, attaining maximum in the late exponential phase with significant decrease at 44 h of incubation. Our results suggest that sporulation had no effect on the production of the antimicrobial compound.

Peptide p138c was found to be stable at a temperature as high as 50 °C and a wide range of pH values (5.8–11). Our result is comparable to, and shows an even wider pH range than those in other studies [8,16,19]. Antimicrobial activity was retained over a wide range of pH values, which may be due to the reversible denaturation of the protein. Peptide p138c was completely inactivated in presence of proteinase K, but this effect was not detected after treatment with trypsin, indicating the proteinaceous nature of p138c. Neither H_2O_2 nor UV light had any effect on the activity of p138c, again indicating the proteinaceous property of our antimicrobial peptide. The N-terminal amino acid sequence analysis of p138c was performed using automated Edman degradation. Among the last 15 N-terminal amino acids in the sequence, one residue, which could not be identified, is shown as X. During the sequencing cycles of the analysis, the reaction was not blocked. The unknown residue in the amino-acid sequence may be the result of uncleaved amino acids.

Synergizing antimicrobial peptides and non-peptide antibiotics is an efficient approach for lowering the dosage of each molecule in combinatorial therapy. Our study showed an improvement in the MICs of p138c combined with oxacillin, ampicillin, and penicillin G. Synergy was indicated when FIC indices were ≤ 0.5. p138c, along with oxacillin, ampicillin, or penicillin G, caused dramatic decreases in MIC. The observed potentiation of antibacterial activity against VRSA was 4-fold greater when p138c was combined with oxacillin, 8-fold greater when p138c was combined with ampicillin, and 16-fold greater when p138c was combined with penicillin G. The effectiveness of synergy can be evaluated by an effective utilization of a weak or moderately effective antimicrobial peptide, which, if used independently, would be considered a poor antibiotic. These combinations influence the antibiotic potency of the peptide-nonpeptide effect and decrease the dose of each agent in the drug combination, rendering synergy more economical. In our study, the synergy of the peptide with three β-lactam antibiotics (oxacillin, ampicillin, and penicillin G) suggested that the antimicrobial peptide might be synergistic with other antibiotics. Our results indicate that synergy could potentiate the bacteriostatic effect and improve bactericidal potency. In synergy with oxacillin, ampicillin, or penicillin G, the bactericidal potency increased 4 to 8-fold, demonstrating a potent bactericidal combination. According to Stratton, bactericidal drugs are preferable to bacteriostatic drugs because the risk for emergence of a resistant mutant is very low; the development of resistant mutants is prevented by killing the microorganism [20]. Hence, we can reduce the chances of antibiotic resistance by increasing the bactericidal potency of antimicrobial peptides. In addition to high potency, rapid killing kinetics are among the properties of antibiotics. In this study, the synergistic activity of oxacillin (MIC of

80 μg/ml), ampicillin (MIC of 20 μg/ml), and penicillin G (MIC of 40 μg/ml), combined with p138c (MIC of 20 μg/ml), against VRSA showed a robust improvement in potency (Fig. 4). As shown in this figure, p138c alone was largely bacteriostatic against VRSA, but in synergy with the three β-lactam antibiotics, p138c showed improved potency and killing kinetics.

In summary, our study investigated the potency and bacterial killing kinetics of the antimicrobial peptide p138c synergized with conventional antibiotics. The results of our study highlight the use of synergy to enhance the therapeutic potential of antimicrobial peptides. These strategies can be applied to a wide spectrum of antimicrobial peptides to produce a suitable agent for the treatment of MDR infections.

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Competing interests. None declared.

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