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Purification and Characterization of a Bacteriocin, BacBS2, Produced by *Bacillus velezensis* BS2 Isolated from Meongge Jeotgal

Venkatesh Perumal[1], Zhuang Yao[2], Jeong A Kim[2], Hyun-Jin Kim[1,2], and Jeong Hwan Kim[1,2*]

[1]*Institute of Agriculture and Life Science, Gyeongsang National University, Jinju 52828, Republic of Korea*
[2]*Division of Applied Life Science (BK21 Plus), Graduate School, Gyeongsang National University, Jinju 52828, Republic of Korea*

Bacillus velezensis BS2 was isolated from meongge (common sea squirt) jeotgal, a Korean fermented seafood, and produces a bacteriocin, BacBS2, which strongly inhibits *Listeria monocytogenes* and *Bacillus cereus*. BacBS2 was partially purified by Q-Sepharose column chromatography after ammonium sulfate precipitation of the culture supernatant, then further purified by Sephadex G-50 column chromatography. Partially purified BacBS2 was estimated to be 6.5 kDa in size by Tricine-SDS PAGE and activity detection by gel-overlay. Enzyme treatment and FT-IR spectrum of partially purified BacBS2 confirmed its proteinaceous nature. BacBS2 was fully stable at pH 4-9, and half of activity was retained at pH 1-3. Full activity was retained after exposure to 80ºC for 15 min, but half of the activity was retained upon exposure to 90ºC for 15 min or 100ºC for 10 min. BacBS2 inhibited *L. monocytogenes* by bactericidal mode of action. *B. velezensis* BS2 and its BacBS2 seem useful as biopreservatives for fermented foods such as jeotgal.

Keywords: Bacteriocin, *Bacillus velezensis*, antibacterial activity, jeotgal

Introduction

Genus *Bacillus* are Gram-positive, aerobic, rod-shaped bacteria including more than 300 species, with many being industrially important [1]. Many *Bacillus* species are well-known producers for diverse antimicrobial compounds such as bacteriocins and lipopeptides [2]. Well known antimicrobial compounds synthesized by *Bacillus* spp. are bacteriocins or bacteriocin-like substances (BLIS) [3]. Bacteriocins are peptides or proteins with antimicrobial activity against closely related species of the producer. But bacteriocins differ in properties such as molecular weight, modification after production, biochemical properties, antimicrobial spectrum, and mode of action.

Bacteriocins produced by lactic acid bacteria (LAB) and *Bacillus* sp. are potential bio-preservatives as alternatives to chemical preservatives because of their GRAS (generally recognized as safe) status [4]. However, to date, only a few bacteriocins are commercially available such as Nisin and Pediocin PA-1, which are produced by *Lactococcus lactis*

and *Pediococcus acidilactici*, respectively. But Nisin and Pediocin PA-1 alone do not fulfill all the desired requirements (broad antimicrobial spectrum and active at extreme pHs) to be used as food preservatives. Hence, worldwide research is ongoing to find novel bacteriocin-producing microorganisms from various sources. Bacteriocins produced by *Bacillus* sp. are reported to have a wide antimicrobial spectrum and be active at extreme pH and temperature [5, 6].

In recent years, non-dairy fermented foods have become popular as sources to isolate potential probiotic LAB and *Bacillus* sp. In this study, a bacteriocin produced by *Bacillus velezensis* BS2 was characterized and partially purified. *B. velezensis* BS2 was isolated from meongge (common sea squirt, *Halocynthia roretzi*) jeotgal, a Korean salted and fermented seafood [7]. *B. velezensis* is a novel species that was recently taxonomically classified to differentiate it from a closely related species, *B. amyloliquefaciens* [8]. *B. velezensis* strains are broadly distributed in different environmental niches including fermented foods, soil, and

water [8, 9]. *B. velezensis* can be used as a starter culture for fermented foods, and also as a probiotic for animal feed as an alternative to antibiotics [8, 10]. Some soil isolates have very good potential as plant growth promoters as well as biocontrol agents against fungal infection in plants [11]. A Brazillian Amazon basin isolate, *B. velezensis* P11, showed strong keratinolytic, proteolytic, and dehairing activities, which could be used for some industrial applications [12]. Apart from the production of various antimicrobial substances such as lipopeptides and bacteriocins, *B. velezensis* strains have been known to be harmless to hosts and environments with strong stress tolerance, making this species able to grow at different ranges of temperature (15°C to 45°C) and pH (5 to 10) [13]. *B. velezensis* strains with antimicrobial activities have been reported, and the genes responsible for antimicrobial activities were identified on the chromosome by genome sequencing [14–16]. But few studies on bacteriocins have been conducted. As far as we know, this is the first report on the purification of a bacteriocin from *B. velezensis* strain originated from marine environments. A *B. velezensis* strain producing bacteriocin could be useful as a food preservative or biocontrol agent.

Materials and Methods

Bacterial Cultures and Media

B. velezensis BS2 was isolated from sea squirt jeotgal, a Korean fermented seafood, and identified by 16S rRNA and *recA* gene sequencing [7]. Indicator strains (Table 1) were either obtained from culture collection centers or our lab culture stocks. All *Bacillus* strains used in this study were grown at 37°C in one of the following media: Luria Bertani (LB, MB cell, Korea); brain heart infusion (BHI, MB cell); nutrient broth (NB, Acumedia, USA); tryptic soy broth (TSB, Becton, Dickinson and Company, Sparks, USA); and Mueller Hinton agar (MH, MB cell). Lactic acid bacteria (LAB) were grown at 37°C in de Man, Rogosa, and Sharpe medium (lactobacilli MRS, Becton, Dickinson and Company).

Antibacterial Spectrum of *B. velezensis* BS2

Antibacterial spectrum of *B. velezensis* BS2 was examined using agar well diffusion assay [16]. In brief, MH agar plates were overlaid with 9 ml of MH, LB, MRS or BHI soft agar inoculated with 50 μl of each indicator strain, which was previously grown until the OD_{600} value reached 0.4-0.8. Wells were made on the seeded plates using a sterile Pasteur pipette and culture supernatant (50 μl, pH 7.0) from *B. velezensis* BS2 was added into each well. The plates were incubated overnight at 37°C or 30°C and then inhibition zones were examined. The activity of bacteriocin was determined by the two-fold serial dilution method. For this, cell-free supernatant (CFS) was 2-fold serially diluted, and each dilution was examined. Bacteriocin activity was

Table 1. Inhibition spectrum of BacBS2 against different bacterial indicator strains.

Indicator strains	BS2	EMD4
Bacillus cereus ATCC14579	+++	+++
Bacillus licheniformis ATCC21415	-	-
Bacillus subtilis	-	-
Bacillus thuringiensis ATCC33679	+++	+++
Escherichia coli O157: H7	-	-
Escherichia coli v517	-	-
Enterococcus faecalis ATCC29212	+++	+
Enterococcus faecium ATCC19953	+++	+
Lactobacillus caesei spp. *casei* ATCC4646	-	-
Lactobacillus delbrueckii spp. *lactis* ATCC4797	+++	-
Lactobacillus pentosus ATCC8041	-	-
Leuconostoc mesenteroides ATCC9135	+++	-
Listeria monocytogenes ATCC19111	+++	++
Pediococcus pentosaceus NRRL B-14009	-	+
Salmonella Typhimurium TA98	-	-
Salmonella Typhimurium TA100	-	-
Streptococcus thermophilus	+	+
Staphylococcus aureus	-	-

+ , 0.5 to 2 mm (moderate inhibitory activity); ++, 2 to 4 mm (strong inhibitory activity); +++ , more than 4 mm (very strong inhibitory activity). All the strains were revived two times from glycerol stock before being used as indicators.

defined as the reciprocal of the highest dilution that still gave a definite zone of inhibition and expressed as activity units (AU) per ml after being multiplied by a conversion factor.

Effect of Culture Medium on the Growth and Bacteriocin Activity of *B. velezensis* BS2

For this, overnightly grown *B. velezensis* BS2 was 1% (v/v) inoculated into five different media (LB, NB, TSB, BHI, and MH), and then cultivated for 96 h at 37°C with aeration. The OD_{600} values and bacteriocin activity of each culture were measured at 6 h intervals during the first 24 h and then 12 h intervals after 24 h. Agar well diffusion method was used for the antimicrobial activity measurements and *Listeria monocytogenes* ATCC19111 was used as an indicator.

Stability of Bacteriocin Activity against pH, Heat, and Enzyme Treatments

Activity of BacBS2 (bacteriocin produced by *B. velezensis* BS2) was measured at different pH, temperature, and enzyme treatments (Table 2). CFS of *B. velezensis* BS2 was used as the crude bacteriocin sample for the experiments. For testing pH stability, crude BacBS2 sample was adjusted to pH 1-12 using 1 N HCl or 1 N NaOH. The pH-adjusted CFSs were incubated for 3 h at 37°C and then the pH again adjusted to 7, and the bacteriocin

Table 2. Stability of BacBS2 upon treatment of different pH, heat and enzymes.

pH	Remaining activity (AU/ml)	Heat treatment (15 min)	Remaining activity (AU/ml)	Enzyme treatment	Remaining activity (AU/ml)
Control[1]	320	Control	320	Control	320
1	160	50℃	320	Protease	160
2	160	60℃	320	Pepsin	320
3	160	70℃	320	Trypsin	320
4	320	80℃	320	Proteinase-K	0
5	320	90℃	160		
6	320	100℃[2]	160		
7	320				
8	320				
9	320				
10	160				
11	0				
12	0				

[1]Control was a bacteriocin sample (CFS) that was not pH adjusted, heat-treated, or enzyme treated.

[2]10 min exposure.

activities were checked. CFS without pH adjustment was used as a control.

For testing heat stability, crude BacBS2 samples were heated for 15 min at 50°C, 60°C, 70°C, 80°C, and 90°C, and for 10 min at 100°C. After incubation, samples were allowed to cool at room temperature before checking the residual activities. A sample without heat treatment was used as a control. For proteolytic enzyme treatments, a 1 mg/ml concentration of pepsin, trypsin, protease, and proteinase-K (Sigma-Aldrich, USA) was added to a crude BacBS2 sample and then incubated at 37℃ for 2 h before checking the residual activities. A bacteriocin sample without any enzyme treatment was used as a control. The residual activities of all the samples along with control were measured by agar well diffusion assay using *L. monocytogenes* ATCC19111 as an indicator. After overnight incubation, the activity units were calculated.

Purification of BacBS2

CFS was obtained from *B. velezensis* BS2 culture grown for 48 h in LB by centrifuging at 6,000 ×*g* for 1 h at 4°C. Ammonium sulfate precipitation (80% saturation) was performed with the obtained CFS for overnight at 4°C. Then bacteriocin pellet was collected by centrifuging at 6,000 ×*g* for 1 h at 4°C, and dissolved in minimum volume of buffer A (20 mM Tris-HCl, pH 7.0), and dialyzed against the same buffer using a 1 kDa cut-off dialysis bag (Sigma-Aldrich) for 24 h with 4 buffer changes.

Dialyzed bacteriocin was lyophilized and dissolved in 1 ml of buffer A. Ion-exchange chromatography was done using a Q-Sepharose fast flow column (Amersham Pharmacia Biotech., Sweden) (2.5 × 11 cm). Buffer A was the eluent with 0.2 M NaCl gradient from 0-1 M. A total of 120 fractions were collected with 10 ml each and the absorbance of each fraction was measured at 280 nm. Antimicrobial activity of each fraction was checked by agar well diffusion method against *L. monocytogenes* ATCC19111. Fractions showing antimicrobial activity were pooled, dialyzed, and concentrated by lyophilization. The lyophilized sample was dissolved into 1 ml of buffer A and further purified by size exclusion column chromatography using a Sephadex G-50 (Amersham Pharmacia Biotech.) (1.5 × 90 cm). The eluent was collected into 60 fractions containing 5 ml each. Fractions showing antimicrobial activity were pooled, dialyzed and lyophilized. The active fractions were checked by SDS-PAGE and the protein concentration was analyzed by the Bradford method using a kit (BioRad, USA) and BSA (bovine serum albumin) as a standard [17].

Size of BacBS2 and FT-IR Analysis

The size of the partially purified BacBS2 was determined by Tricine SDS-PAGE [18]. Fifty μg of BacBS2 sample in 1× SDS-PAGE loading buffer was loaded into 16% polyacrylamide gel, and the electrophoresis was done at 110 V constantly. After the run, the gel was cut into two halves, each containing the sample. One half was washed with buffer A for at least 4 h with buffer changes at every 30 min. Then the gel was overlaid with soft agar containing the indicator strain, *L. monocytogenes* ATCC19111 (OD$_{600}$ 0.4−0.8). After overnight incubation at 37℃, the zone of inhibition was observed. The other half of the gel was stained with Coomassie brilliant blue R-250 as previously described [19].

Functional groups in partially purified BacBS2 were analyzed by FTIR spectrometry (Vertex 80v, Bruker, USA) in transmission mode for the range between 4,000–400 cm^{-1} with a resolution of 4 cm^{-1}.

Table 3. Primers used for the amplification of selected antimicrobial genes from *B. velezensis* BS2.

Lipopeptides	Gene	Primers	Sequence (5′ to 3′)	Product size (bp)	+/-	Reference
Surfactin	*srfA*	SRFA-F	TCGGGACAGAAGACATCAT	201	++	[21]
		SRFA-R	CCACTCAAACGGATAATCCTGA			
	Srf/lch	As1-F	CGCGGMTACCGVATYGAGC	424	++	[22]
		Ts2-R	ATVCCTTTBTWDGAATGTCCGCC			
	sfP	SFP-F1	ATGAAGATTTACGGAATTTA	675	--	[21]
		SFP-R1	TTATAAAAGCTCTTCGTACG			
Fengycin	*fen*	Af2-F	GAATAYMTCGGMCGTMTKGA	443,452	--	[22]
		Tf1-R	GCTTTWADKGAATSBCCGCC			
Iturin	*ituD*	ITUD-F1	TTGAAYGTCAGYGCSCCTTT	482	++	[21]
		ITUD-R1	TGCGMAAATAATGGSGTCGT.			
Iturin A	*ituA*	ITUD1F	GATGCGATCTCCTTGGATGT	647	++	[23]
		ITUD1R	ATCGTCATGTGCTGCTTGAG			
Bacteriocins						
Subtilin	*spaS*	SpaS-Fwd	CAAAGTTCGATCATTTCGATTTGGATGT	152	--	[24]
		SpaS-Rev	GCAGTTACAAGTTAGTGTTTGAAGGAA			
Subtilosin A	*sboA*	sboA-F	GTACAACATAGATCTGCTAG	400	--	[20]
		sboA-R	GCTGGTGAACTCTTACAC			
Amylocyclicin	*acnA*	*amycy*-F	CTGTTGAGTTGAGGAATGCCC	702	++	This study
		amycy-R	TATGCTGCCGCAGGAAAACT			

++, amplified and the product showed the expected size; --, not amplified; +-, amplified but the product showed different size.

PCR Detection of Antimicrobial Genes

PCR was carried out to detect genes responsible for the synthesis of antimicrobial substances such as surfactin, fengycin, iturin, iturin A, subtilin, subtilosin A, and amylocyclicin. For this, total genomic DNA was extracted from overnightly grown *B. velezensis* BS2 culture using a G-spin Genomic DNA Extraction Kit (iNtRON, Korea). Primers used are shown in Table 3 and an MJ Mini PCR machine (BioRad, USA) was used. The reaction mixture (50 μl) contained 2 μl of template DNA, 2 μl of each primer (10 μM), 5 μl of dNTPs (0.25 mM), and 1 μl of *ExTaq* DNA polymerase (Takara, Japan). The cycling conditions were as follows: 95°C for 4 min, 40 cycles of 94°C for 30 sec, 43–59°C for 30 sec, 72°C for 30 sec, and a final extension at 72°C for 2 min [20]. After amplification, PCR products were run and visualized on an agarose gel.

Mode of Action of BacBS2

Partially purified BacBS2 was used in this assay. LB broth (20 ml) was 1% inoculated with overnight culture of *L. monocytogenes* ATCC19111. Inoculated broth was grown until it reached the exponential phase of growth (OD600 = 0.68). Then 2 ml (50 μg/ml) of BacBS2 sample was added. Two ml of LB broth was added to another tube as a negative control. Culture tubes were shaken at 37°C and the viable cells were counted at every 3 h for the next 12 h period.

Results and Discussion

Antibacterial Spectrum of *B. velezensis* BS2

The antibacterial spectrum of *B. velezensis* BS2 was studied against various Gram-positive and negative bacterial species (Table 1). Bacteriocin-producing *B. subtilis* EMD4 was used as a positive control along with *B. velezensis* BS2 [19]. Based on the diameter of the inhibition zone observed from the agar plates, the antibacterial activities were described as moderate (less than 2 mm), strong (2 to 4 mm) and very strong (more than 4 mm). At pH 7, BacBS2 showed similar inhibition spectrum of BacEMD4 against the used indicator strains. BacBS2 showed very strong inhibition against *L. monocytogenes* ATCC19111 and *B. cereus* ATCC14579. BacEMD4 also inhibited *B. cereus* ATCC14579 very strongly but inhibited *L. monocytogenes* ATCC19111 at a reduced degree.

BacBS2 showed very strong activity against LAB strains, *Enterococcus faecalis* ATCC29212, *Enterococcus faecium* ATCC19953, *Lactobacillus delbrueckii* spp. *lactis* ATCC4797, and *Leuconostoc mesenteroides* ATCC9135. On the contrary, BacEMD4 did not show any strong activity against the aforementioned LAB indicator strains. Commercially
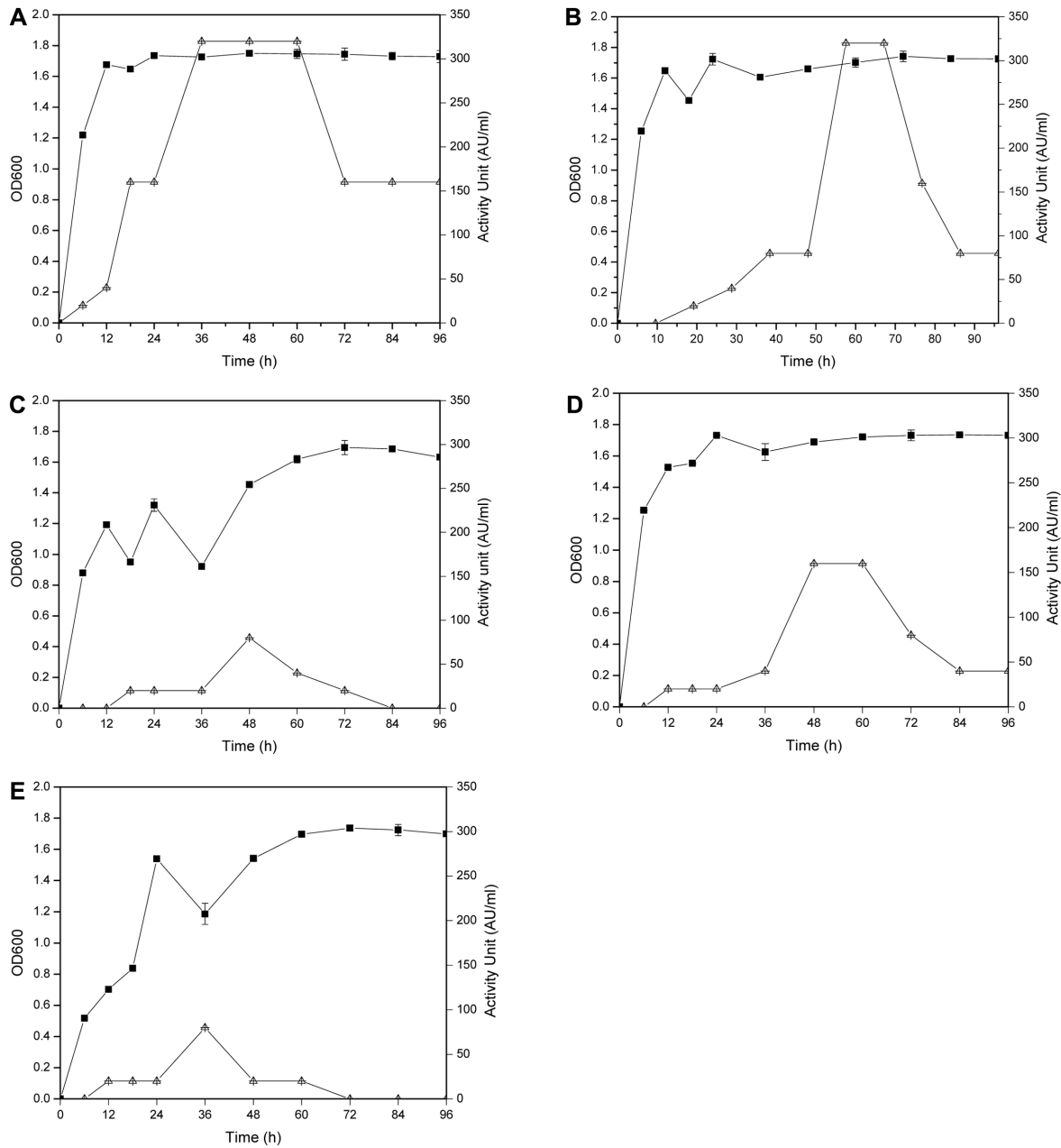
Fig. 1. Growth and antimicrobial activity of *B. velezensis* BS2 on different growth media.
Luria Bertani broth (**A**); tryptic soy broth (**B**); nutrient broth (**C**); brain heart infusion broth (**D**); and Mueller Hinton broth (**E**). ▬■▬, growth (OD$_{600}$); ▬△▬, bacteriocin activity (AU/ml).

available bacteriocins Nisin and Pediocin PA-1 are either weak or not active against *L. monocytogenes* and *B. cereus* [25]. Hence, the strong inhibition activity of BacBS2 against *L. monocytogenes* ATCC19111 and *B. cereus* ATCC 14579 can be considered as a big advantage of BacBS2 to be used as a natural bio-preservative in foods.

Effect of Culture Medium on the Growth and Bacteriocin Activity of *B. velezensis* BS2

The best growth medium and optimum culture conditions will be different for each strain and should be decided individually [19]. *B. velezensis* BS2 was grown in different media to find out the optimum medium for its

bacteriocin activity and growth (Fig. 1). In LB broth, *B. velezensis* BS2 showed higher activity (320 AU/ml) at 36 h and retained this activity until 60 h, then the activity was decreased to 160 AU/ml and retained until 96 h. In TSB, *B. velezensis* BS2 attained 320 AU/ml at 48 h and retained the same activity until 60 h and then gradually decreased. In BHI broth, *B. velezensis* BS2 showed bacteriocin activity from 12 h to all the tested time intervals with a maximum of 160 AU/mL from 48 to 60 h. In NB and MH broth, *B. velezensis* BS2 showed lower activity (maximum, 80 AU/ml) and the activity was not prolonged in all the tested time intervals. Unlike bacteriocin activity, all media successfully supported the growth of *B. velezensis* BS2, reaching maximum growth from 1.69 to 1.75 at OD_{600} in different time intervals. LB broth was the best because *B. velezensis* BS2 showed the maximum activity (320 AU/ml) at 36 h and maintained this activity until 60 h. TSB is the next best medium.

Stability of Bacteriocin Activity against pH, Heat, and Enzyme Treatments

BacBS2 remained fully active after being treated with proteolytic enzymes, pepsin and trypsin but retained half of the activity after treatment with protease, and lost all activity upon the treatment of proteinase-K (Table 2). These observations confirmed the proteinaceous nature of BacBS2. BacBS2 maintained complete activity after exposure to 50°C to 80°C for 15 min, and lost half of the activity upon exposure to 90°C for 15 min, and 100°C for 10 min. These results proved the significant thermal stability of BacBS2. Further, BacBS2 was fully active at pH 4 to pH 9. BacBS2 showed half activity at pH 1−3 and pH 10, and no activity was observed at pH 11 and 12.

Purification of BacBS2

A 1,900-ml CFS from *B. velezensis* BS2 culture (a specific activity 803 AU/mg protein) was the starting material for BacBS2 purification. After ammonium sulphate precipitation, the specific activity of BacBS2 was calculated to be 1,003.9 AU/mg protein with 53.9% yield. Then it was further purified by ion exchange chromatography using Q-Sepharose fast flow resin. Fractions 45 to 54 (98 ml), which were eluted with buffer A containing 0.4 M NaCl (Fig. 2A), showed antibacterial activity against *L. monocytogenes* ATCC19111 by agar well diffusion assay. The collected fractions were pooled, dialyzed, and lyophilized. The specific activity was 3,276.9 AU/mg protein with a 4.07-fold increase. Size exclusion chromatography using a Sephadex G-50 was done, and fraction numbers 30 to 33 showed
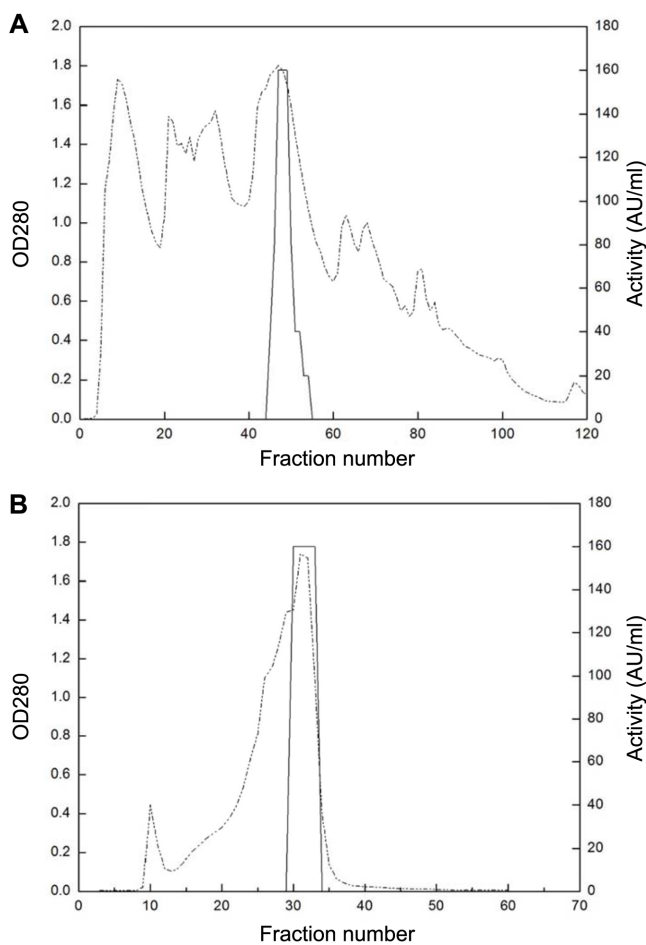
Fig. 2. Elution profiles of BacBS2 from Q-Sepharose column (**A**) and Sephadex G-50 column (**B**).
A dotted line indicates the absorbance values at 280 nm and a solid line denotes the bacteriocin activities (AU/ml).

antibacterial activity against *L. monocytogenes* ATCC19111 (Fig. 2B). After passage through a Sephadex G-50 column, the specific activity of BacBS2 was 7,069.8 AU/mg protein, which was an 8.8-fold increase and an 8% yield from the initial purification step.

Size of BacBS2 and FT-IR Analysis

Partially purified BacBS2 (after Q-Sepharose fast flow column) was used as a sample for SDS-PAGE to determine the molecular size of BacBS2. After electrophoresis was done, half of the gel was washed, and overlaid with soft agar (0.7% MHA) seeded with *L. monocytogenes* ATCC19111 culture grown until OD_{600} value of 0.4. After incubation, a zone of inhibition was observed at 6.5 kDa position (Fig. 3).
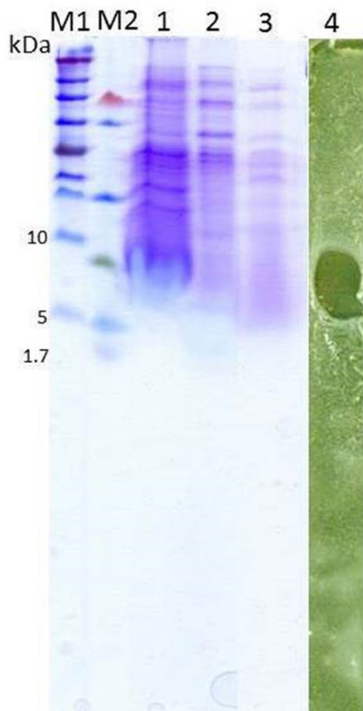
FT-IR spectroscopy is useful to categorize an unknown

Fig. 3. Tricine SDS-PAGE and activity detection of BacBS2. M1, broad range protein markers (SMOBIO Technology, Inc., Hsinchu City, Taiwan); M2, low range protein markers (Cell Signaling Technology, Danvers, MA, USA); 1, dialyzed BacBS2 (50 μg); 2, partially purified BacBS2 (50 μg) after Q-Sepharose column; 3, partially purified sample (50 μg) after Sephadex G-50 column; 4, activity detection of BacBS2 by overlaying an acrylamide gel with soft agar containing *L. monocytogenes* cells.

Fig. 4. FT-IR spectrum of partially purified BacBS2 (50 μg/ml).

compound by unraveling the presence of functional groups and chemical bonds. Intense broad peaks between 3,000 and 3,600 cm^{-1} indicate–OH and NH stretching in BacBS2 (Fig. 4). The observed peaks at 1,522, 1,630 cm^{-1} (Gauzian amide bond), correspond to peaks at 3,418 cm^{-1} (Hydrogen bond of OH group) designating the presence of peptide bonds. The peak at 1,630 cm^{-1} associated with spectrum between 3,500 and 3,183 cm^{-1} revealed the presence of amide group in BacBS2 [26]. The absorption peak at 2,986 results in C–H stretching and designates the existence of an aliphatic chain. The peak at 1,460 and 1,401 cm^{-1} arises from the amide II bond which results from the deformation of N–H bond combined with C–N stretching molecule [27]. The peaks at 1,630 and 1,552 cm^{-1} indicate the existence of amide I and amide II. Peaks of BacBS2 designated only the functional groups and bonds used to present in the protein and not for any lipid moiety, hence this result proved that BacBS2 has a proteinaceous nature.
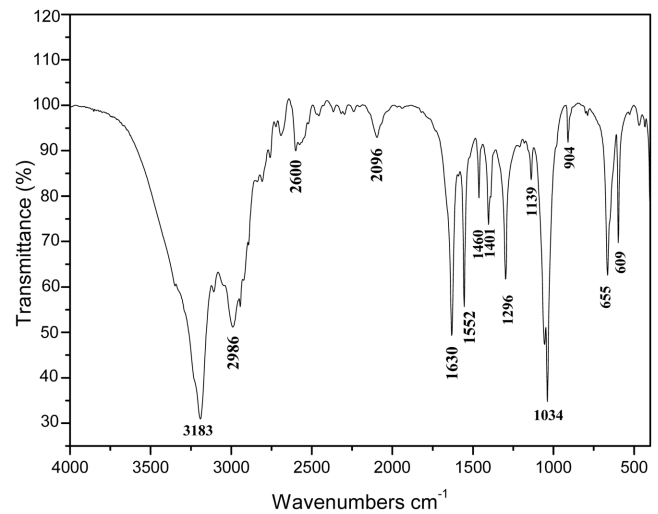
PCR Detection of Antimicrobial Genes from *B. velezensis* BS2

PCR detection of genes of known bacteriocins and lipopeptides was tried using primers designed from commonly known genes (Table 3). The amplified PCR products were run using 1% agarose gels (Fig. 5). The results showed clear bands with expected sizes for the genes of lipopeptide surfactin (*srfA*, *srf/lch*), iturin (*ituD*) and iturin A (*ituA*). Bands were not found for the genes of surfactin (*sfp*) and fengycin (*fen*). Though lipopeptide genes surfactin and iturin were present in *B. velezensis* BS2, the main antimicrobial compound was suspected to be a bacteriocin since CFS lost antibacterial activity upon proteinase-K treatment. Hence, commonly known genes of bacteriocins in *Bacillus* such as subtilin and subtilosin A were checked for the presence but their genes were not amplified.

A primer set for detection of *acnA* (encoding the precursor of amylocyclicin) was expected to produce a 702 bp fragment. The size of amplified PCR product matched with the expected size. When the product was sequenced after extraction from the gel, the nucleotide sequence (Fig. 5B) showed 100% homology to *acnA* (amylocyclicin precursor gene) from *B. velezensis* FZB42, formly known as *B. amyloliquefaciens* FZB42 [28]. *B. velezensis* FZB42 is a model organism for plant-growth promoting rhizobacteria [28]. In addition to amylocyclicin, *B. velezensis* FZB42 produces non-ribosomally synthesized lipopeptides such as surfactin, fengycin, and bacillomycin D, inhibiting plant pathogenic fungi [28].
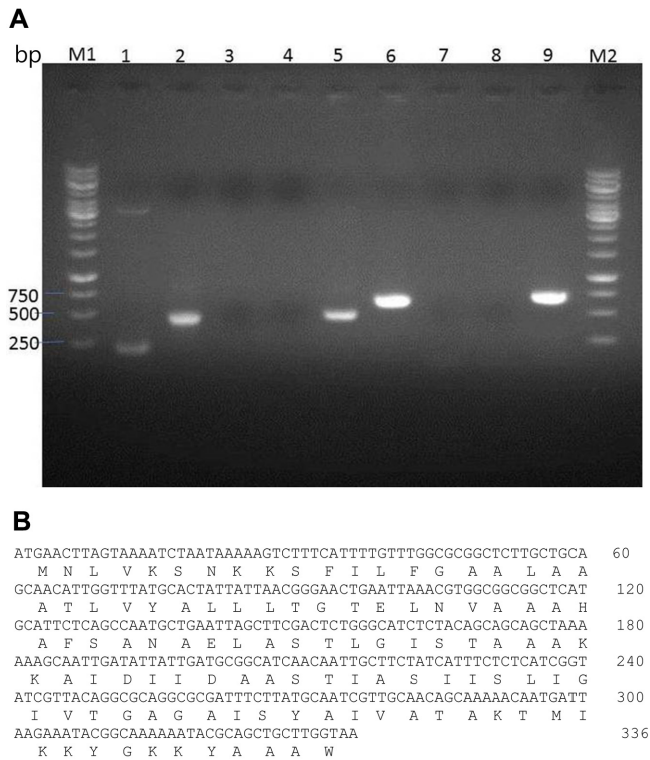
A

B

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ATGAACTTAGTAAAATCTAATAAAAAGTCTTTCATTTTGTTTGGCGCGGCTCTTGCTGCA    60
  M  N  L  V  K  S  N  K  K  S  F  I  L  F  G  A  A  L  A  A
GCAACATTGGTTTATGCACTATTATTAACGGGAACTGAATTAAACGTGGCGGCGGCTCAT   120
  A  T  L  V  Y  A  L  L  L  T  G  T  E  L  N  V  A  A  A  H
GCATTCTCAGCCAATGCTGAATTAGCTTCGACTCTGGGCATCTCTACAGCAGCAGCTAAA   180
  A  F  S  A  N  A  E  L  A  S  T  L  G  I  S  T  A  A  A  K
AAAGCAATTGATATTATTGATGCGGCATCAACAATTGCTTCTATCATTTCTCTCATCGGT   240
  K  A  I  D  I  I  D  A  A  S  T  I  A  S  I  I  S  L  I  G
ATCGTTACAGGCGCAGGCGCGATTTCTTATGCAATCGTTGCAACAGCAAAAACAATGATT   300
  I  V  T  G  A  G  A  I  S  Y  A  I  V  A  T  A  K  T  M  I
AAGAAATACGGCAAAAAATACGCAGCTGCTTGGTAA                           336
  K  K  Y  G  K  K  Y  A  A  A  W
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Fig. 5. PCR detection of antimicrobial genes from *B. velezensis* BS2 genome.

A, M1 and M2, 1 kb DNA ladder (Thermo Scientific, Carlsbad, CA, USA); 1, *srfA*; 2, *srf/lch*; 3, *sfP*; 4, *fen*; 5, *ituA*; 6, *ituD*; 7, *spaS*; 8, *sboA*; 9, *acnA*. **B**, partial nucleotide sequence of the PCR product (A, lane 9) and the translated amino acids.

Two bacteriocin genes encoding amylolysin and amylocyclicin were located after the examination of whole genome sequences of *B. velezensis* LS69 [29]. Amylocyclicin is a ribosomally synthesized circular bacteriocin with growth inhibition specificity against Gram-positive bacteria and its molecular weight was calculated to be 6.38−6.40 kD [30]. Moreover, *B. velezensis* strains are known to produce antimicrobial lipopeptides such as surfactin, iturin and fengycin with a molecular weight ranging from 1 to 2 kDa [31, 32]. Lipopeptides of *B. velezensis* BS2 were precipitated by acid precipitation using 6 N HCl and the antibacterial activity was checked by agar well diffusion method. But a negligible or small zone of inhibition was observed by lipopeptide precipitate (data not shown). By considering the inhibition specificity for Gram-positive bacterial strains and molecular weight of active peptide (around 6.5 kDa), and loss of activity upon proteinase-K treatment, bacteriocin BS2 may possibly be amylocyclicin. Successful detection of the amylocyclicin gene from the BS2 genome could be

supporting evidence. BLAST analysis of the translated amino acids (Fig. 5B) showed that amylocyclicin is a member of circular bacteriocins (circularin A/uberolysin family), which are widely present among Gram-positive bacteria such as *Bacillus, Paenibacillus, Gracilibacillus,* and *Staphylococcus.* Putative amylocyclicins translated from genes are highly conservative among *B. amyloliquefaciens* (99%) and *B. subtilis* groups (97%).

However, the possibility can't be ruled out that BacBS2 is a different bacteriocin. Inhibition spectrum of BacBS2 is different from that of amylocyclicin. BacBS2 does not inhibit *B. licheniformis* and *B. subtilis* but amylocyclicin does. BacBS2 inhibits *B. cereus* strongly but amylocyclicin inhibits weakly [26]. As a member of circular bacteriocins, amylocyclicin possesses high thermostability but the thermostability of BacBS2 is not that high. *B. velezensis* FZB42 is an organism originated from soil and plants but *B. velezensis* BS2 was isolated from meongge (common sea squirt) jeotgal, originated from marine environments. Considering these facts, BacBS2 might be a novel bacteriocin not reported yet, but further studies such as N-terminal amino acid sequencing of BacBS2 are necessary before a definite conclusion can be reached. As far as we know, this is the first report on a bacteriocin produced by a *B. velezensis* strain originated from marine environments.

Mode of Action of BacBS2

The viable count of *L. monocytogenes* ATCC19111 was reduced from 7.25 log CFU/ml to 6. 27 log CFU/ml at 3 h after addition of BacBS2 (100 μg), and decreased continuously (Fig. 6). The viable count was further decreased to 3.8 log CFU/ml at 12 h after addition. On the contrary, the viable count of control increased continuously (Fig. 6). The results suggested that BacBS2 killed sensitive *L. monocytogenes* ATCC19111 cells. The bacteriocidal mode of inhibition is often reported for bacteriocins synthesised by Gram-positive bacteria including *Bacillus* species [33].

B. velezensis BS2 was originated from marine environments. It was isolated from meongge (common sea squirt) jeotgal, a Korean fermented seafood prepared from just salt and meongge. *B. velezensis* BS2 grows well at 15% NaCl (w/v) and at 10°C. Thus *B. velezensis* BS2 seems promising as a starter for salted and fermented foods such as fish sauce and jeotgal. *B. velezensis* BS2 produces a bacteriocin, BacBS2, which strongly inhibits important food pathogens such as *L. monocytogenes* and *B. cereus.* This property is a big advantage for *B. velezensis* BS2 as a starter. In addition to bacteriocin production, the strain secretes a protease with strong fibrinolytic activity [7]. Finally, *B. velezensis*
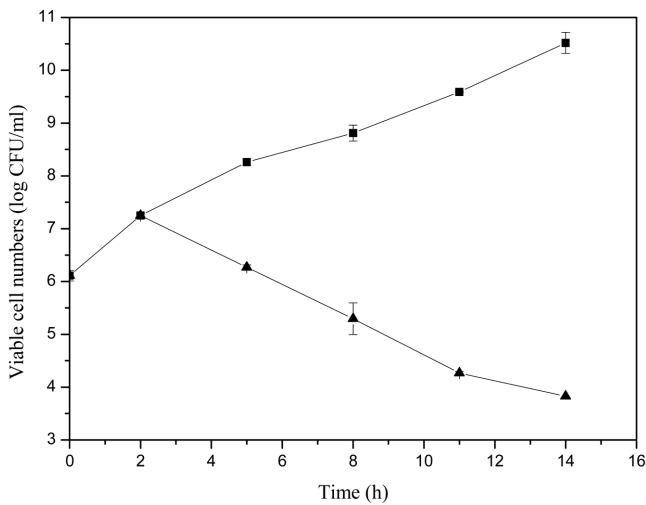
Fig. 6. Mode of inhibition of *L. monocytogenes* ATCC19111 by BacBS2.

━■━, viable counts of *L. monocytogenes* (log CFU/ml) culture without BacBS2 addition; ━▲━, viable counts of *L. monocytogenes* (log CFU/ml) culture treated with BacBS2.

strains can be considered GRAS organisms considering their close relationship with *B. amyloliquefaciens* [34]. Considering all these, *B. velezensis* BS2 and BacBS2 may be used as a natural food preservative for fermented foods replacing chemical preservatives.

Acknowledgments

Conflict of Interest

The authors have no financial conflicts of interest to declare.

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