Properties of a Bacteriocin Produced by *Bacillus subtilis* EMD4 Isolated from Ganjang (Soy Sauce)

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A *Bacillus* species, EMD4, with strong antibacterial activity was isolated from *ganjang* (soy sauce) and identified as *B. subtilis*. *B. subtilis* EMD4 strongly inhibited the growth of *B. cereus* ATCC14579 and *B. thuringiensis* ATCC33679. The antibacterial activity was stable at pH 3–9 but inactive at pH 10 and above. The activity was fully retained after 15 min at 80°C but reduced by 50% after 15 min at 90°C. The activity was completely destroyed by protease treatment, indicating its proteinaceous nature. The bacteriocin (BacEMD4) was partially purified from culture supernatant by ammonium sulfate precipitation, and Q-Sepharose and Sephadex G-50 column chromatographies. The specific activity was increased from 769.2 AU/mg protein to 8,347.8 AU/mg protein and the final yield was 12.6%. The size of BacEMD4 was determined to be 3.5 kDa by Tricine SDS-PAGE. The N-terminal amino acid sequence was similar with that of Subtilosin A. Nucleotide sequencing of the cloned gene confirmed that BacEMD4 was Subtilosin A. BacEMD4 showed bactericidal activity against *B. cereus* ATCC14579.

**Keywords:** Bacteriocin, *Bacillus subtilis*, antimicrobial activity, *ganjang*

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**Introduction**

Bacteriocins are antimicrobial proteins or peptides that are ribosomally produced and secreted [16]. They usually inhibit the growth of microorganisms of the same or closely related species [16, 28]. Bacteriocins inhibit the growth of sensitive cells or kill them by interfering with the synthesis of cell wall or by forming pores in the cell membrane [24]. Many different microorganisms are known to produce bacteriocins, and among them, lactic acid bacteria (LAB) and *Bacillus* species are the most well-known producers. LAB and bacilli are GRAS (generally recognized as safe) organisms and their bacteriocins are also of GRAS class because bacteriocins are degraded by proteases in the intestines of human beings [1, 18]. For these reasons, bacteriocins from LAB and bacilli have a great potential as natural food preservatives, replacing chemical preservatives [7, 19, 25]. So far, a few bacteriocins are commercially available. Nisin, produced by *Lactococcus lactis* strains, and Pediocin PA-1, produced by *Pediococcus acidilactici*, are the only commercially available bacteriocins on the market [3, 8, 24]. Bacteriocins have some drawbacks that hinder the more extensive use of bacteriocins as food preservatives. For example, Nisin becomes unstable under neutral and basic environments [9] and the antimicrobial spectrum is not wide enough. The inhibition activity of Nisin against *Listeria monocytogenes* is comparatively weak and both Nisin and Pediocin PA-1 cannot inhibit the growth of *B. cereus* [6].

*Bacillus* species have been known to produce a variety of different antimicrobial compounds, including bacteriocins [5, 26]. Many *Bacillus* species, including *B. subtilis*,...
**B. coagulans**, *B. cereus*, *B. thuringiensis*, and *B. megaterium*, produce bacteriocins [26]. Their bacteriocin or bacteriocin-like substances include subtilin [26], subtilosin A [2], surfactin [20], coagulin [11], thericin [10], and cremen [3].

Fermented soy foods such as *cheonggukjang*, *doenjang*, and *ganjang* (soy sauce) are rich sources for *B. subtilis* and closely related species producing bacteriocins. In previous studies, we isolated two *B. subtilis* strains from *cheonggukjang* and characterized their bacteriocins [14, 15]. In this study, we isolated another bacteriocin producer, *B. subtilis* EMD4, from *ganjang* and examined the properties of the bacteriocin, BacEMD4.

**Materials and Methods**

**Bacterial Strains and Media**

*B. subtilis* EMD4 was isolated from *ganjang* fermented in a tank (Mongyo Food, Changwon, Korea) in 2012. For the identification of EMD4, a partial 16S rRNA gene was amplified using the following primers: bac-F (5’-CGGCTGCTAATACATGCAAG-3’) and bac-R (5’-GGCATGTGATCCGCATTACTA-3’). A recA gene fragment was amplified using the primer pairs recA-F (5’-TGA GTGATCGTCAGGCAGCCTTAG-3’) and recA-R (5’-CYTRG ATAAGARTACCAWGMACCGC-3’). Chromosomal DNA of EMD4 cells was prepared from a culture grown in LB broth (Luria-Bertani both; Acumedia, Lansing, MI, USA) for 18 h, using the phenol-chloroform extraction method [14]. PCR was performed as reported previously [15]. Nucleotide sequences of the amplified fragments were determined at Cosmogenetech (Seoul, Korea), and analyzed by BLAST (National Center for Biotechnology Information, Bethesda, MD, USA).

*B. subtilis* EMD4 was cultivated at 37°C with aeration in one of the following media: LB, BHI (brain heart infusion; Becton, Dickinson and Company, Sparks, MD, USA), NB (nutrient broth; Becton, Dickinson and Company), and TSB (Bacto tryptic soy broth; MB Cell, Los Angeles, CA, USA). LAB were grown at 30°C into each well. The plates were incubated overnight at 37°C or 30°C and inhibition zones were examined.

**Inhibition Spectrum of *B. subtilis* EMD4**

Inhibition spectrum of *B. subtilis* EMD4 was examined by agar well diffusion assay [13]. LB plates were overlaid with 10 ml of LB, MR, or BHI soft agar inoculated with 50 µl of each indicator organism, which was grown until the OD₆₀₀ value reached 0.4–0.8. Wells were made on the seeded plates using a Pasteur pipette and 50 µl of EMD4 culture supernatant (pH adjusted to 7.0) was added into each well. The plates were incubated overnight at 37°C or 30°C and inhibition zones were examined.

The activity of bacteriocin was determined by the serial 2-fold dilution method as previously described [10]. Culture supernatant was serially 2-fold diluted, and each dilution was tested for the inhibiting activity. Bacteriocin activity was defined as the reciprocal of the highest dilution that still gave a definite zone of inhibition and was expressed as activity units (AU) per milliliter after multiplied by a conversion factor.

*B. cereus* ATCC14579 was used as the indicator strain. All measurements were repeated three times and the means are shown with standard deviations.

**Growth and Bacteriocin Activity of *B. subtilis* EMD4 in Different Culture Media**

*B. subtilis* EMD4 was grown overnight in LB and used to inoculate four different media (LB, TSB, NB, and BHI, 1% (v/v)). The inoculated media were cultivated for 96 h at 37°C with shaking. The optical density (at 600 nm) and bacteriocin activity of each culture were measured at 6 h intervals during the first 36 h and then at 12 h intervals after 36 h. The agar well diffusion method was used for the antimicrobial activity measurements and *B. cereus* ATCC14579 was used as the indicator. All measurements were done in triplicates and the means are shown as the results.

**Effects of pH, Heat, and Enzyme Treatments on the Bacteriocin Activity**

*B. subtilis* EMD4 was inoculated into TSB broth (1% (v/v)) and incubated at 37°C with shaking (130 rpm). After 48 h, the culture was centrifuged at 6,000 x g for 60 min (4°C) and the supernatant was filtered using a 0.45 µm filter (Advantec, Saitama, Japan). The filtered supernatant was named crude bacteriocin sample (CBS). CBS was used for the stability tests for bacteriocin.

**pH tolerance.** CBS was adjusted to pH 1–12 using HCl and NaOH. The pH-adjusted CBSs were incubated for 3 h at 37°C and then the remaining activities were measured by the agar well diffusion method using *B. cereus* as the indicator. CBS without pH adjustment was used as a control.

**Heat stability.** CBSs 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 being cooled to room temperature, the remaining activities were measured by the agar well diffusion method using *B. cereus* as the indicator. CBS without heat treatment was used as a control.

**Resistance to protease treatments.** CBSs were treated with four kinds of proteases (1 mg/ml concentration) for 2 h at 37°C: protease (P5147; Sigma, St. Louis, MO, USA; in 5 mM phosphate buffer, pH 7.0), pepsin (P-6887; Sigma; in 0.02 N HCl), trypsin (T-8918; Sigma; in 10 mM phosphate buffer, pH 7.0), and proteinase K (P2308; Sigma; in 10 mM phosphate buffer, pH 7.0). After incubation, the residual activities were examined as shown above. CBS without enzyme was used as a control.

**Purification of BacEMD4**

The bacteriocin secreted by *B. subtilis* EMD4, BacEMD4, was purified from 2 liter of culture supernatant. After 48 h of cultivation in TSB, the culture was centrifuged (6,000 x g, 1 h, 4°C) and the supernatant was filtered. Ammonium sulfate precipitation (80% saturation) was done for the filtered supernatant overnight at 4°C. The ammonium sulfate pellet was obtained by centrifugation (6,000 x g, 1 h), resuspended in 10 ml of buffer A

Cloning of the Gene Encoding BacEMD4

Size of BacEMD4 and N-Terminal Amino Acid Sequencing

Elution was done by applying a NaCl gradient (0-1 M in buffer A), with stepwise increase in 0.2 M increment [14]. Fractions showing antibacterial activity were pooled, dialyzed, freeze-dried, resuspended in buffer A (1 ml), and loaded onto a Sephadex G-50 (Amersham Pharmacia Biotech.) column (1.5 x 90 cm). Elution was done using buffer A, which was also used to wash the column previously. Fractions showing antibacterial activity were pooled and freeze-dried. The Q-Sepharose Fast Flow column was used again for the final purification. After each step, the samples were checked by SDS-PAGE and the protein concentration was determined by the Bradford method using bovine serum albumin (BSA) as the standard [4].

Size of BacEMD4 and N-Terminal Amino Acid Sequencing

Tricine-SDS-PAGE was done for partially purified BacEMD4 according to the method of Schägger and von Jagow [22]. A purified sample (50 µg in 1 x SDS-PAGE loading buffer) was loaded onto a 16% polyacrylamide gel in duplicates and electrophoresis was done at a constant voltage of 110 V. After electrophoresis, half of the gel was washed with buffer A for 4 h, with buffer change every half hour. The washed gel was overlaid with soft agar (0.7% (w/v)) containing every half hour. The washed gel was overlaid with soft agar (0.7% (w/v)) containing B. cereus ATCC14579 cells, which were grown until the OD600 value reached 0.4–0.8. The overnight incubation at 37°C and the inhibition zone was examined. Another half gel was stained with Coomassie brilliant blue R-250 in methanol-water-acetic acid (7:40:53 (v/v)), followed by overnight destaining in methanol-water-acetic acid (5:17:78 (v/v)).

For N-terminal amino acid sequencing, BacEMD4 was transferred from a Tricine SDS acrylamide gel to a PVDF membrane (Immobilon-P transfer membrane; Merck Millipore, Darmstadt, Germany) by using a Trans-Blot Turbo Transfer System (Bio-Rad). The N-terminal amino acid sequencing was done at the Korea Basic Science Institute (Seoul, Korea).

Cloning of the Gene Encoding BacEMD4

PCR, and cloning and sequencing of the gene were done according to standard methods [21]. Primers for the PCR were designed based on the published Subtilosin A gene sequence [27]: Primer F (5'-GTACAACATAGATCTGCTAG-3') and primer R (5'-GCTGGTAGCTTACAC-3'). The PCR mixture (50 µl) included 1 µl of each primer, 1 µl of template DNA (EMD4 chromosomal DNA), 5 µl of dNTP (0.25 mM each), 0.5 µl of ExTag DNA polymerase (Takara, Shiga, Japan), and 5 µl of Taq DNA polymerase buffer. The PCR was performed using a MJ Mini gradient thermal cycler (Bio-Rad, Hercules, CA, USA). The amplification conditions were as follows: 95°C for 5 min; 30 cycles of 95°C for 30 sec, 52°C for 30 sec, 72°C for 30 sec; and a final extension at 72°C for 5 min. The amplified fragment was ligated into a pGEM-T Easy vector (Promega, Madison, WI, USA) and the ligation mixture was introduced into E. coli DH5α by electroporation. White colonies on LB plates with X-Gal (40 µg/ml), IPTG (0.5 mM), and ampicillin (100 µg/ml) were examined for the presence of the recombinant plasmid. The DNA sequence of the insert was determined at Cosmogenetech and analyzed by BLAST.

Inhibition Activity of BacEMD4

B. cereus ATCC14579 was inoculated into TSB broth (10 ml, 1% (v/v)) and incubated at 37°C until the OD600 value reached 0.6–0.8. Two milliliters of bacteriocin sample (6,400 AU/ml) was added to one tube and viable cells were counted at 3 h intervals for the next 12 h. Viable cell counting was repeated in triplicates and the mean values were calculated. The bacteriocin sample was prepared by concentrating the supernatant (48 h culture in TSB) with an Amicon ultra-15 centrifugal filter device (3K device; Merck Millipore Ltd., Cork, Ireland).

Results and Discussion

Identification of EMD4

EMD4 was isolated from soy sauce under fermentation at a commercial plant. It grew as a typical bacilli colony on LB plates. To identify the strain at the species level, the 16S rRNA and recA genes were amplified by PCR using the primer pairs described in the Methods section. The amplified 16S rRNA gene fragment (1,149 bp) was sequenced. BLAST search indicated that the 16S rRNA gene of EMD4 showed 99% homology with those of B. subtilis strains (data not shown). To confirm the result, the recA gene was amplified and sequenced (697 bp). BLAST analysis showed 99% homology with those of B. subtilis strains, too. EMD4 was identified as a B. subtilis strain and named accordingly as B. subtilis EMD4. The GenBank accession number for the partial 16S rRNA and recA genes are KJ028111.1 and KJ028113.1, respectively.

Effect of Medium on the Inhibition Activity

The antibacterial activity of B. subtilis EMD4 showed the highest value when grown in TSB (Fig. 1). In TSB broth, the highest activity (320 AU/ml) was observed at 36 h and remained until 60 h. Then, the activity decreased to 160 AU/ml at 72 h and remained until 96 h. In BHI medium, the highest activity (320 AU/ml) was observed at 48 h and then the activity decreased to half (160 AU/ml) at 60 h, and then remained at the same level until 96 h. In LB medium, the activity reached the highest point (160 AU/ml) at 30 h and then decreased gradually, and was not detected at
96 h. NB was the worst medium for the activity. The highest activity was just 40 AU/ml at around 18 h and then decreased gradually. Although different media showed different antibacterial activities, all four media supported growth of *B. subtilis* EMD4 and the OD<sub>600</sub> values were in the range of 1.6–1.8. The medium for the highest antibacterial activity varies depending upon a specific strain. For example, the best medium was BHI for the bacteriocin (BacH27) production in *B. subtilis* H27 [14]. BHI and TSB were equally good for the production of BacW42 in *B. subtilis* W42 [15]. The best medium and the optimum culture conditions should be decided for each bacteriocin producer individually.

Effects of Enzyme, pH, and Heat Treatments on the Bacteriocin Activity
When CBS was exposed to different pH values (1 to 12), the activity was not decreased at pH 3–9. However, the activity reduced to half at pH 1 and 2 and was completely destroyed at pH 10 and above (Table 1). It can be concluded that BacEMD4 is stable at neutral and acidic pHs but unstable at pH 10 and above. The heat stability of a bacteriocin is an important property if the bacteriocin is used as a preservative for foods. BacEMD4 maintained full activity after 15 min at 80°C. However, the activity decreased to half (160 AU/ml) after 15 min at 90°C. At 100°C, some activity remained after 4 min but the activity was completely destroyed after 10 min (Table 1). BacEMD4 has moderate heat stability, suitable for applications for foods that are not subjected to extensive heat treatments. BacEMD4 has better heat resistance than BacW42 and BacH27. For BacW42, the activity reduced to half after 15 min at 60°C [15] and for BacH27, the activity reduced to half after 15 min at 80°C [14]. The activity of BacEMD4 was completely destroyed by proteinase K and protease treatments (Table 1). However, the activity was reduced to

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**Fig. 1.** Growth and bacteriocin activity of *B. subtilis* EMD4.
Four different culture media were compared; Tryptic soy broth (A), brain heart infusion broth (B), Luria-Bertani broth (C), and nutrient broth (D). ○—○—, growth (OD<sub>600</sub>); ●—●—, bacteriocin activity (AU/ml).
half by pepsin and trypsin treatments. The results indicated the proteinous nature of BacEMD4. Proteinase K and protease are nonspecific proteases, hydrolyzing the peptide bonds of target proteins at various sites. On the contrary, pepsin and trypsin are enzymes with narrow substrate specificities. Because of the small size of BacEMD4 (see below), pepsin and trypsin did not hydrolyze BacEMD4 or hydrolyzed it limitedly.

### Inhibition Spectrum

The inhibition spectrum of CBS was examined using various gram-positive and gram-negative bacteria as indicators (Table 2). As positive controls, CBSs from *B. subtilis* W42 and *B. subtilis* H27 were tested together. The same volume of each CBS was added into each well and the degree of inhibition was determined by measuring the diameter of the inhibition zone. If the diameter was less than 2 mm, the degree of inhibition was described as moderate. If it was between 2 and 4 mm, the inhibition was strong, and if it was more than 4 mm, the inhibition was described as very strong. The inhibition spectrum of CBS from *B. subtilis* EMD4 was not much broader compared with controls (Table 2). For example, CBS from *B. subtilis* EMD4 failed to inhibit the growth of *Lactobacillus* and *Leuconostoc* species, which were inhibited by controls. All three strains did not inhibit the growth of gram-negative bacteria, which agreed with a known fact that bacteriocins from gram positive bacteria do not inhibit the growth of gram-negative bacteria because of different cell wall structures. The results showed that the *B. subtilis* strains produced bacteriocins with different inhibition spectra.

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**Table 1. Effects of pH, heat, and enzyme treatments on BacEMD4.**

<table>
<thead>
<tr>
<th>pH</th>
<th>Remaining activity (AU/ml)</th>
<th>Heat treatment (15 min)</th>
<th>Remaining activity (AU/ml)</th>
<th>Enzyme treatment</th>
<th>Remaining activity (AU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>320</td>
<td>Control</td>
<td>320</td>
<td>Control</td>
<td>320</td>
</tr>
<tr>
<td>1</td>
<td>160</td>
<td>50°C</td>
<td>320</td>
<td>Pepsin</td>
<td>160</td>
</tr>
<tr>
<td>2</td>
<td>160</td>
<td>60°C</td>
<td>320</td>
<td>Trypsin</td>
<td>160</td>
</tr>
<tr>
<td>3</td>
<td>320</td>
<td>70°C</td>
<td>320</td>
<td>Protease</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>320</td>
<td>80°C</td>
<td>320</td>
<td>Proteinase K</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>320</td>
<td>90°C</td>
<td>160</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>320</td>
<td>100°C ¹</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>320</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>320</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>320</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

¹BacEMD4 was treated for 10 min at 100°C.

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**Table 2. Inhibition spectrum of BacEMD4.**

<table>
<thead>
<tr>
<th>Indicator strains</th>
<th>EMD4</th>
<th>W42</th>
<th>H27</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Bacillus cereus</em> ATCC14579</td>
<td>+++</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td><em>B. subtilis</em> ATCC14593</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><em>B. licheniformis</em> ATCC21415</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><em>B. thuringiensis</em> ATCC33679</td>
<td>+++</td>
<td>+++</td>
<td>–</td>
</tr>
<tr>
<td><em>Enterococcus faecalis</em> ATCC29212</td>
<td>+</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td><em>E. faecium</em> ATCC19953</td>
<td>+</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td><em>Lactobacillus delbrueckii</em> ssp. lactis ATCC4797</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>Lb. casei</em> ssp. casei ATCC4646</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>Lb. pentosus</em> ATCC8041</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><em>Leuconostoc mesenteroides</em> ATCC9135</td>
<td>–</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td><em>Listeria monocytogenes</em> ATCC19111</td>
<td>++</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td><em>Pediococcus pentosaceus</em> NRRL B-14009</td>
<td>+</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td><em>Streptococcus thermophilus</em> KFRI 193</td>
<td>+</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em> ssp. <em>aureus</em> ATCC25923</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Escherichia coli</em> O157:H7</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><em>E. coli</em> V519</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><em>Salmonella Typhimurium</em> TA98</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

Degree of inhibition: +, 0.5 to 2 mm (moderate inhibitory activity); ++, 2 to 4 mm (strong inhibitory activity); ++++, more than 4 mm (very strong inhibitory activity). Inhibitory zone = (diameter of an inhibition zone in mm – diameter of a well)/2.
B. subtilis EMD4 strongly inhibited the growth of B. cereus ATCC14579, which was not inhibited by Nisin and Pediocin PA-1. B. subtilis EMD4 also strongly inhibited the growth of B. thuringiensis ATCC33679 and Listeria monocytogenes ATCC19111, both common pathogens. Moreover, it can moderately inhibit the growth of Enterococcus faecalis ATCC29212 and Enterococcus faecium ATCC19953 as well as Pediococcus pentosaceus NRRL-B-14009 and Streptococcus thermophilus KFRI193. Considering all these results, including the stability tests, it can be concluded that BacEMD4 has potential to be used as a food preservative.

**Purification of BacEMD4**

Purification of BacEMD4 in the culture supernatant was done and the results are summarized in Table 3. After the first Q-Sepharose column chromatography, fractions (23–32) showed antibacterial activity and they were eluted with buffer containing 0.2 M NaCl (Fig. 2A). The fractions were pooled (108 ml) and further purified on a Sephadex G-50 column. Fractions numbered 20–30 were the active fractions (total 58 ml, Fig. 2B). Further purification was done on a Q-Sepharose column again. Fractions (29–34, 30 ml) with antibacterial activity were eluted with buffer containing 0.2 M NaCl (Fig. 2C). The specific activity of purified BacEMD4 was 8,347.8 AU/mg protein, a 10.8-fold increase from the specific activity of supernatant (769.2 AU/mg protein). The overall purification yield was 12.6%.

**N-Terminal Amino Acid Sequencing of BacEMD4 and Its Gene Cloning**

The size of partially purified BacEMD4 was estimated to be 3.5 kDa as determined by inhibition activity detection using a Tricine-SDS gel (Fig. 3). A single, clear inhibition zone appeared on a polyacrylamide gel that was previously washed and overlaid with indicator cells, B. cereus ATCC11579. The corresponding band could not be located at the same position on the Coomassie blue stained gel. This might be due to the small size of BacEMD4. The size of

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**Table 3. Summary of purification of BacEMD4.**

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume (ml)</th>
<th>Total activity (AU)</th>
<th>Total protein (mg)</th>
<th>Specific activity (AU/mg protein)</th>
<th>Yield (%)</th>
<th>Fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supernatant</td>
<td>1,900</td>
<td>76,000</td>
<td>98.8</td>
<td>769.2</td>
<td>100</td>
<td>-</td>
</tr>
<tr>
<td>AS</td>
<td>33</td>
<td>42,240</td>
<td>41.9</td>
<td>1,007.8</td>
<td>55.6</td>
<td>1.3</td>
</tr>
<tr>
<td>IEC</td>
<td>108</td>
<td>34,560</td>
<td>10.5</td>
<td>3,298.9</td>
<td>45.5</td>
<td>4.3</td>
</tr>
<tr>
<td>GF</td>
<td>58</td>
<td>18,560</td>
<td>2.75</td>
<td>6,749.1</td>
<td>24.4</td>
<td>8.8</td>
</tr>
<tr>
<td>IEC</td>
<td>30</td>
<td>9,600</td>
<td>1.15</td>
<td>8,347.8</td>
<td>12.6</td>
<td>10.8</td>
</tr>
</tbody>
</table>

AS, ammonium sulfate precipitation; IEC, ion-exchange chromatography using Q-Sepharose resin; GF, gel filtration using Sephadex G-50 resin.

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A and C are elution profiles from a Q-Sepharose column and B is an elution profile from a Sephadex G-50 column. A dotted line indicates the absorbance (OD$_{280}$) values and a solid line represents the bacteriocin activities (AU/ml).
BacEMD4 was smaller than BacH27 (4.9 kDa) and BacW42 (5.4 kDa).

The BacEMD4 band on a Tricine-SDS gel was transferred onto a PVDF membrane and then the amino acid sequence was examined by N-terminal amino acid sequencing. Identified amino acids are as follows: Asp (Thr)-Ile (Pro,Lys)-X-Ala-Ala-X-Leu-Val-Asp(Ile)-Gly-Pro (Ala)-Ile-Pro-Leu-Val. X denotes an unidentified position and amino acid(s) in parenthesis are other possible amino acids at that position. Ten amino acids (AAXLVDGPIP) were selected and protein database searched by BLAST. Matching sequences were found and they corresponded to the internal region of Subtilosin A from \textit{B. subtilis} strains. Subtilosin A is a bacteriocin with modified amino acids and the mature form consists of 35 amino acids [12]. Subtilosin A has an unusually short leader peptide of 8 amino acids in addition to thioether bridges. From the BLAST search result and the reported size of Subtilosin A (3,398.9 Da) [2], BacEMD4 was suspected to be Subtilosin A. To confirm this, cloning of the gene for BacEMD4 was tried by PCR. Primers were designed based on a Subtilosin A gene [12]. It was suspected that if the Subtilosin A gene and the surrounding regions are conserved among \textit{B. subtilis} strains, then the Subtilosin A gene could be amplified from genomic DNA of \textit{B. subtilis} EMD4. A 300 bp fragment was amplified and sequenced. When the sequence was examined, an ORF consisting of 43 amino acids (MKKAVIVENKGCATCIGAACLVDGPIP E1AGATGLFGLWG) was located (the amino acid sequence used for the BLAST search is underlined). BLAST search showed that the amino acid sequence was identical with that of previously reported Subtilosin A [27].

There were some mismatches between the amino acids translated from the cloned gene and those determined by N-terminal sequencing of purified BacEMD4. The last two amino acids determined by N-terminal sequencing were Leu-Val, but the amino acids translated from the cloned gene were Asp-Phe. Moreover, the first amino acid determined was either Asp or Thr, but the amino acid in the gene was Ser (16th amino acid in the ORF). The reason for these discrepancies is not clear but they are probably caused by technical difficulties in N-terminal sequencing. The small amount of BacEMD4 might be the reason for poor identification, in addition to the presence of modified amino acids in Subtilosin A [2, 12]. As far as we know, this is the first report on the characterization of Subtilosin A from \textit{B. subtilis} isolated from fermented soy foods.

Many bacteriocins secreted by bacilli are 3-7 kDa in size. For example, Cerein 7 from \textit{B. cereus} Bc7 is 3,940 Da as determined by mass spectrometry [17]. Another bacteriocin from \textit{B. cereus} NS02, an isolate from buffalo milk, is in the range of 3.5–6.0 kD [23]. Thuricin 17 from \textit{B. thuringiensis} NEB17 was reported to be 3,162 Da [10]. \textit{B. thuringiensis} ssp. entomocidus secreted Entomocin 110 of 4.8 kD in size [5]. \textit{B. thuringiensis} BUPM4 also secreted Bacthuricin F4 and its size was determined to be 3,160.5 Da by mass spectrometry [13].

**Inhibition Activity of BacEMD4**

\textit{B. cereus} ATCC14579 culture was in the exponential phase at 1.5 h after incubation started. BacEMD4 (12,800 AU) was added into one of two cultures. After addition of BacEMD4, the viable cell count decreased sharply from 8.3 to 4.4 log CFU/ml in the following 3 h and then finally decreased further to 1.8 log CFU/ml in the following 12 h (Fig. 4). On the contrary, the number of \textit{B. cereus} cells increased in the control (no BacEMD4 addition). This result indicated that BacEMD4 had a bactericidal mode of inhibition against \textit{B. cereus}. The bactericidal mode of inhibition is often observed among bacteriocins produced by gram-positive bacteria, including bacilli species [15].

It is expected that more bacteriocin-producing bacilli will be isolated from fermented soy foods such as doenjang, cheonggukjang, and ganjang in the future. Bacteriocins from...
BacEMD4 addition (control); —

Fig. 4. Bactericidal effect of partially purified BacEMD4. ——, viable counts of B. cereus ATCC14579 (log CFU/ml) without BacEMD4 addition (control); — ——, viable counts of B. cereus ATCC14579 with the addition of BacEMD4 (12,800 AU).

B. subtilis and closely related species might be useful as safe preservatives for fermented soy foods and other foods, replacing chemical preservatives for which safety issues are raised continually [24]. B. subtilis strains are GRAS organisms and have been used for various food fermentations for a long period of time. Thus, their bacteriocins are very attractive as natural food preservatives. In particular, many of them efficiently inhibit the growth of B. cereus and L. monocytogenes, the most common and serious food pathogens. For fermented soy foods, B. cereus is the most common pathogen and use of B. subtilis EMD4 as a starter can be an effective method to reduce B. cereus contamination. In this respect, further studies are necessary to examine the potential of BacEMD4 as a food preservative. Improvements of the thermostability and inhibition host range are the next step for the utilization of BacEMD4 for the food industry.

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References


