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Characterization of Subtilein, a Bacteriocin from *Bacillus subtilis* CAU131 (KCCM 10257)

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Abstract *Bacillus subtilis* CAU131 (KCCM 10257) isolated from a fermented shrimp product produces subtilein, tentatively named as a bacteriocin, which exhibited a bactericidal effect against closely related species such as *Bacillus subtilis* ATCC 6633, *Bacillus cereus* ATCC 11778, and several other strains of *Bacillus* sp. The purification of the subtilein was achieved by applying a mono-Q anion exchange chromatography on FPLC and C_{18} reverse-phase chromatography on HPLC. After purification, specific activity of subtilein was increased about 3,000-fold compared with culture broth and its molecular mass was about 5,000 Da on SDS-PAGE. The antimicrobial activity of subtilein was well maintained at acidic and neutral pHs between 3 and 8. Subtilein was relatively heat stable, and its antimicrobial activity remained for 2 h at 80°C. However, the activity was reduced after heating at 100°C, and about 80% of the activity was found after 1 h incubation at 100°C. The treatment of *Bacillus subtilis* ATCC 6633 with subtilein led to morphological changes in stationary-phase cells and most cells appeared to be lysed.

Key words: Bacteriocin, *Bacillus subtilis*, subtilein

The bacteriocins are a group of antimicrobial peptides produced by various microorganisms toward closely related strains of their producers [18]. They are divided into four types [16]: membrane-active and heat-stable peptides, including lantibiotics (class I) and nonlantibiotics (class II), and high molecular weight polypeptides, including proteins (class III), and lipo- and glycoproteins (class IV). Since many of these microorganisms are related with food components of fermented food, they can be used in food preservation and for the inhibition of food-borne pathogens [6, 17, 26]. Nisin and pediocin are used as food additives to reduce bacterial contamination in dairy products and meats [4, 12, 15, 23, 29, 32].

The spore forming *Bacillus* sp. are useful in industry for the production of many enzymes, antibiotics, and insecticides. Furthermore, various bacteriocins produced by *Bacillus* sp. have been reported, such as coagulin, tochicin, subtilin, and megasin [2, 13, 14, 19, 27, 33]. *B. subtilis*, a non-pathogenic soil bacterium, is an important microorganism in foods, because it is capable of growth under aerobic and anaerobic conditions on various substrates [25]. It is also known to produce bacteriocins such as lantibiotic type subtilin and subtilosin, undergoing unusual chemical modification during maturation which are unlike those of lantibiotics [1, 2, 31]. In this report, new bacteriocin producer, *B. subtilis* CAU131 (KCCM 10257) isolated from fermented shrimp products, was identified and its bacteriocin, tentatively named as subtilein, was partially characterized. The characteristics of subtilein may lead to a potential use of this bacteriocin as a natural antimicrobial agent for controlling *B. cereus* and other food-borne *Bacillus* sp. pathogens in industrial application.

Materials and Methods

Bacterial Strains and Culture Conditions

Assignments of subtilein producer were carried out based on several morphological characteristics and biochemical traits such as cell morphology, Gram staining, growth condition, catalase test, Voges-Proskauer test (V-P test), acid and gas production from glucose, hydrolysis of casein, along with growth temperature range, according to *Bergey's Manual of Determinative Bacteriology* [10]. *B. subtilis* CAU131 (KCCM 10257), a subtilein producer, was isolated from salted shrimps (Kankyung, Chon-Buk, Korea) and maintained at −70°C in LB (Luria-Bertani, Difco, U.S.A.) broth with 50% glycerol. The strain was

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grown in LB or BHI (Brain Heart Infusion, Difco, U.S.A.) broth at 37°C with shaking at 200 rpm for 18–24 h. The indicator strains obtained from different culture collections were cultured and maintained in appropriate media.

Assay of Bacteriocin Activity

The antimicrobial activity of subtilein was measured by applying the spot-on-lawn method as follows [25]: Brain heart infusion (BHI) plate was overlaid with BHI soft agar (0.7%) seeded with 10^7 cfu/ml of indicator strain, and 10 µl of subtilein produced by *B. subtilis* CAU131 (KCCM 10257) was spotted on this plate. The plate was incubated overnight at 37°C, and the visible clear zone was observed.

Bacteriocin activity was determined by using a modified critical dilution method [30]. It was expressed as arbitrary activity units (AU) per milliliter, which was calculated by the reciprocal of the serial two-fold dilution showing an inhibitory action towards the indicator organism.

Bacteriocin Production

Production of subtilein in LB and BHI liquid media was investigated. Optical density at 600 nm was measured for the measure of cell growth during cultivation. A sample was removed aseptically at different times to determine bacteriocin activity. The effect of growth temperature between 10 and 40°C and salt tolerance up to 10% were investigated in BHI media.

Purification of Subtilein

The supernatant of *B. subtilis* CAU131 culture was obtained by centrifugation at 10,000 ×g for 30 min. Two hundred milliliters of supernatant were loaded onto Q-Sepharose anion-exchange column (1.5×10 cm) on FPLC (Gilson FPLC system, France) equilibrated with 50 mM Tris-HCl (pH 9.0) buffer. After washing the column with the same buffer, bound proteins were eluted by a linear NaCl gradient (0–1 M) for 1 h with a flow rate of 1 ml per min. The elution was monitored at 220 nm and 1.5 ml of each fraction was collected. Active fractions were collected and dialyzed by using membrane (3,500 Da) from Spectrapor (Houston, U.S.A.). Dialyzed solution was loaded onto the same Q-Sepharose column, and bound proteins were eluted by a shorter linear NaCl gradient (0–1 M) for 30 min with a flow rate of 1 ml per min. Bacteriocin fractions were concentrated with a speed vacuum concentrator (RC 10.22, Jouan, France) and purified further by using a RP C_{18} reverse-phase column (0.5×30 cm, Supelco, U.S.A.) on HPLC (Gilson 305 system, France). After equilibration of the column with H_2O at a flow rate of 1 ml/min at 39°C, subtilein was eluted by a linear gradient of acetonitrile [0–60% (v/v)] for 40 min. The elution was monitored spectrophotometrically at 220 nm. Fractions representing all peaks were collected in vials manually and assayed for any bacteriocin activity.

SDS-PAGE of Purified Subtilein

The purity of bacteriocin was examined by sodium dodecyl sulfate polyacrylamide gel electrophoresis with the following standard molecular weight markers (Gibco, U.S.A.) [20]: carbonic anhydrase, 29,000; β-lactoglobulin, 18,400; lysozyme, 14,300; bovine trypsin inhibitor, 6,200; insulin (β-chain), 3,400. After electrophoresis, gels were silver-stained by using the procedure described by Giulian *et al.* [8]

Stability to Enzyme Treatment, Heat, and pH

After subtilein (1,600 AU/ml) was treated for 3 h with various enzymes, including pronase, trypsin, amylase, pullulanase, and lysozyme, the residual activity was measured. All enzymes were dissolved at final concentration of 4 mg/ml in 50 mM Tris-HCl buffer (pH 7), except α-amylase (10 mM sodium phosphate buffer, pH 7) and β-amylase (30 mM sodium acetate buffer, pH 5).

Heat stability of subtilein (1,600 AU/ml) was investigated by treating the enzyme at 50°C, 60°C, 70°C, 80°C, 90°C, and 100°C for 60 min and 121°C for 30 min, and the residual activity was tested every 10 min by following the spot-on-lawn method [24].

The pH stability of subtilein (1,600 AU/ml) was examined with 50 mM citrate (pHs 3, 4, and 5), 50 mM sodium phosphate (pHs 6 and 7), and 50 mM Tris-HCl (pHs 8, 9, and 10) buffers. After being left in an appropriate buffer for 12 h at room temperature, residual antimicrobial activities were measured as previously described.

Morphological Change of Indicator Cell by Treatment with Subtilein

Structural changes of bacteria after the subtilein treatment were observed with a Scanning Electron Microscope (S-2380N, Hitachi, Japan). The indicator cells were suspended in saline and mixed with the same volume of subtilein solution (1,600 AU/ml against *B. cereus*). After incubation at 37°C for 3 h, the cells were freeze-dried and ion sputter (E-1010, Hitachi, Japan)-coated with a gold-palladium. The specimens were observed with a scanning electron microscope, operating at an accelerating voltage of 15 kV.

RESULTS AND DISCUSSION

Identification and Culture of Subtilein-Producing Strains

A bacteriocin-producing organism was isolated from salted shrimp and named as CAU131. The phenotypic characteristics of strain CAU131 are summarized in Table 1. The isolate was Gram-positive and positive against catalase and V-P reaction. The strain did not grow at 65°C, and hydrolyzed

Table 1. Morphological and biological characteristics of the isolate CAU131.

Characteristics	Result	
	Bergey's manual *Bacillus subtilis*	*Bacillus subtilis* CAU131
Rods 1.0 μm wide or wider	–	–
Parasporal bodies	–	–
Growth in anaerobic agar	–	–
Growth at 50°C	+	+
Growth at 65°C	–	–
Growth in 4% NaCl	+	+
Growth in 7% NaCl	+	+
Catalase	+	+
Voges-Proskauer reaction	+	+
Acid from glucose	+	+
Acid and gas in glucose	–	–
NO_3 reduced to NO_2	+	+
Starch hydrolyzed	+	+
pH in V-P medium <6.0	V	–
Hydrolysis of casein	+	+

+, greater than 85% of strains positive.
–, greater than 85% of strains negative.
V, variable character.

starch and casein. Most characteristics of strain CAU131 shown in Table 1 coincide with the description of *B. subtilis* based on *Bergey's Manual of Determinative Bacteriology* [10]. Therefore, the strain was tentatively designated as *B. subtilis* CAU131 and maintained at the Korea Culture Center of Microorganisms (KCCM) with a number of 10257. Further definitive classification at the species level requires additional experiments such as 16S rRNA sequence analysis.

Antimicrobial Spectrum

The inhibitory spectrum of subtilein from *B. subtilis* CAU131 (KCCM 10257) was evaluated by using various indicator strains, and their susceptibility to subtilein are shown in Table 2. Subtilein exhibited antimicrobial activity on most tested *Bacillus* sp. and on some *Staphylococcus* sp. The growth of *Salmonella typhimurium* KCTC 1925 and some *Escherichia coli* strains were inhibited by subtilein. However, the degree of inhibition was less than those of *Bacillus* sp. Other strains of *Klebsiella*, *Pseudomonas* and some *Shigella* strains were found to be insensitive. Generally, most of the bacteriocins exhibited a narrow inhibitory spectrum toward related producer strains [16]. In agreement with the above, the inhibitory spectra of subtilein exhibited a narrow inhibitory spectrum with strains which belong to the same genus, *Bacillus* sp. Other bacteriocin from *Bacillus* sp. such as cerein from *B. cereus* is similar to subtlein compared with nisin, which inhibits the growth of most of the Gram-positive bacteria and staphylococcin BacR1 with a broad spectrum [5, 12, 19, 32]. However, cerein, a recently found new bacteriocin from *B. subtilis* cx1, exhibited a broad antimicrobial spectrum towards both Gram-positive and Gram-negative strains [14].

Table 2. Inhibition spectrum of subtilein produced by *Bacillus subtilis* CAU131 (KCCM 10257).

Microorganism tested	Activity (AU/ml)
Bacillus subtilis ATCC 6633	1,600
Bacillus cereus ATCC 11778	1,600
Bacillus megaterium ATCC 9885	800
Streptococcus lactis KCTC 2013	200
Streptococcus pyogenes	0
Streptococcus faecium	0
Staphylococcus aureus ATCC 65389	400
Staphylococcus gallinarum ATCC 35539	0
Staphylococcus saprophytious KCTC 3345	0
Staphylococcus epidermidis KCTC 35494	200
Escherichia coli ATCC 43984 O157:H7	200
Escherichia coli DH5α	200
Escherichia coli KCCM11750	200
Enterobactor cloacae	0
Klebsiella oxytosa	0
Klebsiella aerogenes	0
Pseudomonas aeruginosa ATCC15422	0
Pseudomonas fluorescense ATCC 2344	0
Shigella dysenteriae ATCC 133	0
Shigella dysenteriae ATCC 27345	0
Salmonella typhimurium KCTC 1925	200
Salmonella enteritidis ATCC 137	0

*Activity was expressed as arbitrary inhibitory activity units (AU) per milliliter against the indicator organisms.

Growth of *B. subtilis* CAU131 and Subtilein Production

Figure 1 shows the cell growth and bacteriocin production of *B. subtilis* CAU131 in LB and BHI broths at 37°C.

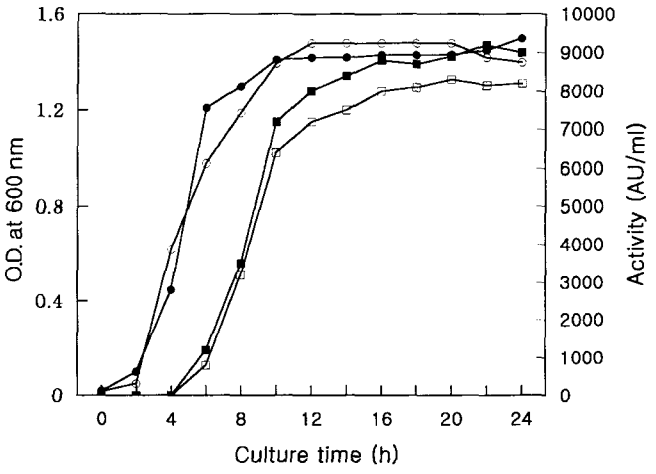

Fig. 1. Growth of *Bacillus subtilis* CAU131 (KCCM 10257) and production of subtilein.
(-○-), cell growth in LB medium; (-●-), cell growth in BHI medium; (-□-), subtilein production in LB medium; (-■-), subtilein production in BHI medium.

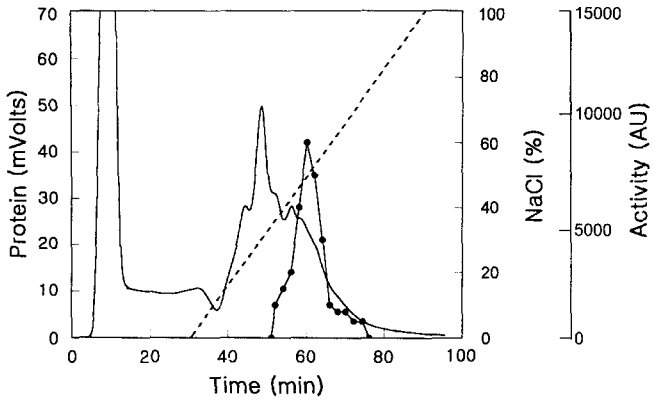

Fig. 2. First Q-Sepharose anion-exchange chromatography on FPLC.
Cell-free supernatant (200 ml) was applied to Q-Sepharose anion-exchange column (1.5×10 cm) on FPLC equilibrated with 50 mM Tris-HCl (pH 9.0) buffer. Subtilein was eluted by a linear NaCl gradient (0–1 M) for 1 h with the flow rate of 1 ml per min. (—), protein profile; (---), NaCl gradient; (●), antimicrobial activity of subtilein.

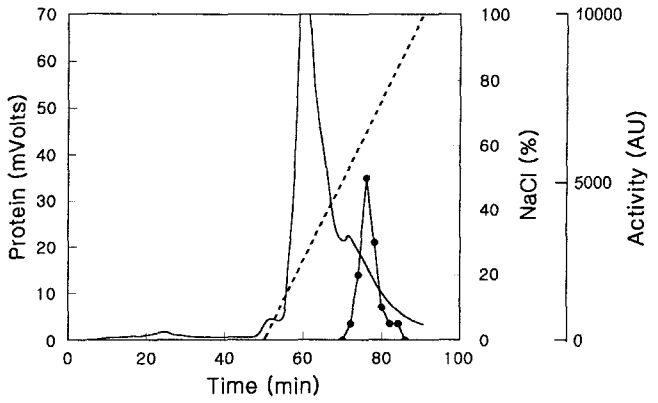

Fig. 3. Second Q-Sepharose anion-exchange chromatography on FPLC.
Dialyzed active fractions from the 1st step were loaded onto the same Q-Sepharose column. Subtilein was eluted by a shorter linear NaCl gradient (0–1 M) for 30 min with the flow rate of 1 ml per min. (—), protein profile; (---), NaCl gradient; (●), antimicrobial activity of subtilein.

There was no remarkable difference of cell growth and subtilein production between LB and BHI liquid culture media. In both cases, the biosynthesis of the subtilein and its excretion into the medium took place during logarithmic growth phase and no increase of production was observed in the stationary phase. A maximal production was reached at the beginning of the stationary phase, suggesting that subtilein was produced during the active growth phase, similar to lantibiotics such as rep 5 [9].

Purification of Subtilein Produced by *B. subtilis* CAU131 (KCCM 10257)

Subtilein produced by *B. subtilis* CAU131 (KCCM 10257) was purified by using Q-Sepharose Fast Flow HR 10/10 anion-exchange chromatography on FPLC (Gilson FPLC system, France) and C_{18} reverse-phase chromatography on HPLC (Gilson 305 system, France).

Subtilein bound to Q-Sepharose could be separated easily from the column by NaCl gradient, as shown in Fig. 2. After the first purification step, the specific activity increased 34-fold compared to that of the culture broth (Table 3). Active fractions obtained from the first column were rechromatographed on the same column with a shorter NaCl gradient (Fig. 3). Eluted subtilein fraction exhibited 190-fold increased specific activity. The final purification step, RP C_{18} reverse-phase column chromatography on HPLC, was the most effective and the inhibitory activity was detected in the peak 2. As shown in Fig. 4, subtilein could be purified after this chromatography, and the overall purification procedure resulted in about 3,000-fold increase in specific activity (Table 3). However, further attempts are required to increase the purification yield in order to obtain a large quantity of pure subtilein.

The relative molecular weight and purity of the purified subtilein were determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). When the purified subtilein was analyzed by SDS-PAGE, a single band was detected, migrating at the region of approximately 5,000 Da (Fig. 4). Some of the bacteriocins from *Bacillus* sp. showed different molecular weights (≤10 kDa): cerein (8.2 kDa) from *B. cereus*, subtilin (3.5 kDa), subtilosin (3.6 kDa) and a recently found bacteriocin from *B. subtilis* (9.5 kDa) [1, 2, 14, 24, 28]. These three bacteriocins from *B. subtilis* exhibited physiological characteristics different from those of subtilosin, having relatively broad antimicrobial spectrum and being relatively more heat labile than subtilein.

Table 3. Purification of subtilein produced by *Bacillus subtilis* CAU131 (KCCM 10257).

Purification step	Total protein (mg)	Total activity (AU)	Specific activity (AU/mg)	Purification fold	Yield
Culture supernatant	54	128,000	237	1	100
1st Q-Sepharose on FPLC	4	32,000	8,000	33.8	25.5
2nd Q-Sepharose on FPLC	0.25	11,400	45,600	192.4	9.1
RP-C_{18} chromatography on HPLC	0.0032	2,280	712,500	3006.3	1.8

**Activity was expressed as arbitrary inhibitory activity units (AU) per milliliter against *B. subtilis* ATCC 6633.

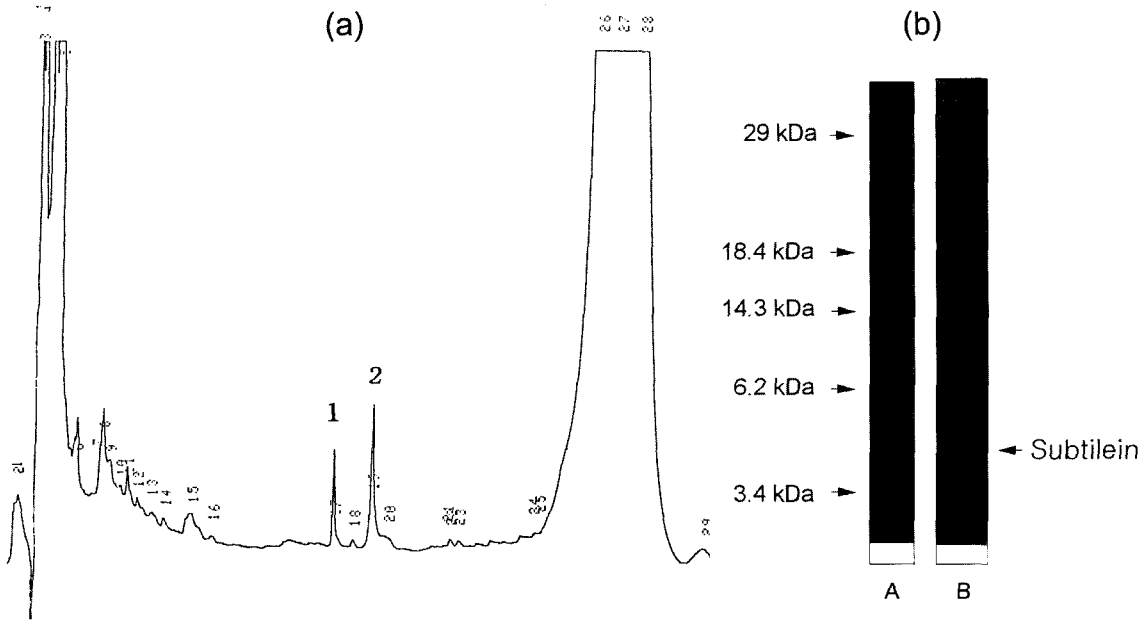

Fig. 4. RP-C_{18} chromatography on HPLC (a) and SDS-PAGE of subtilein (b).
The inhibitory activity was detected in the peak 2.

Effect of Various Enzymes, pH, and Heat

The effects of various enzymes on the purified subtilein are shown in Table 4. The antimicrobial activity of subtilein completely disappeared when treated with pronase, suggesting the proteinous nature of subtilein. Treatment of subtilein with other enzymes including chymotrypsin, α-amylase, β-amylase, lipase, papain, or lysozyme showed no change of antimicrobial activity. Buffers and enzyme solution alone had no effect on the indicator strain.

Subtilein was found to be stable at acidic and neutral pHs between 3 and 8 (Table 4). The stability decreased significantly as the pH increased. The stability at acidic pH of bacteriocins from *Streptococcus faecalis* and *Pediococcus acidolactaici*, etc. has been reported [3, 7], and both exhibited the same tendency in instability at basic pH.

Subtilein produced by *B. subtilis* CAU131 (KCCM 10257) was relatively stable against heat treatment, similar to other heat-stable bacteriocins such as pediocin A (Fig. 5) [3, 4, 11]. Inhibitory activity was well maintained for 2 h during the heat treatment up to 80°C. However, its inhibitory activity was reduced by heating at 90 and 100°C, and about 80% of the residual activity was found after incubation for 1 h at 100°C. Cerein from *B. cereus* and other bacteriocins from *B. subtilis,* such as subtilin (3.5 kDa), subtilosin (3.6 kDa), and a recently found bacteriocin from *B. subtilis* (9.5 kDa) [1, 2, 14] were relatively more heat labile than subtilein [14, 20, 24]. Antimicrobial activity of cerein disappeared only after 15 min heat treatment at 90°C and other new bacteriocin from *B. subtilis* lost 50% of its activity only after 15 min at 50°C [14]. Heat stability

Table 4. The effect of various enzymes and pH on the activity of subtilein.

pH	Activity (AU/ml)	Enzymes	Activity (AU/ml)
3	1,200	α-chymotrypsin	1,600
4	1,600	trypsin	1,600
5	1,600	pronase	0
6	1,600	papain	0
7	1,600	α-amylase	1,600
8	1,600	β-amylase	1,600
9	800	amyloglucosidase	1,600
10	800	pullulanase	1,600
11	400	lipase	1,600

* Activity is expressed as AU against *Bacillus subtilis* ATCC 6633.

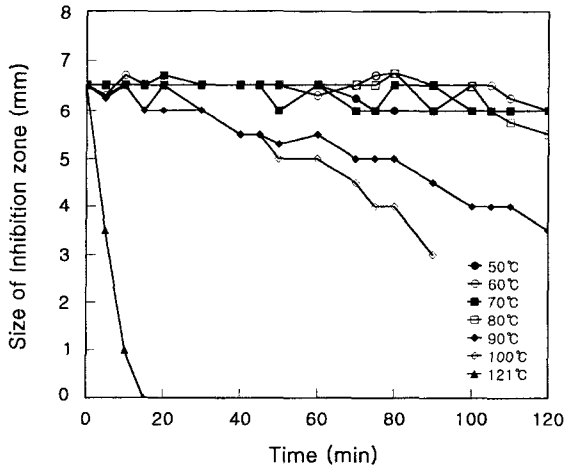

Fig. 5. Heat stability of subtilein produced by *Bacillus subtilis* CAU131 (KCCM 10257).

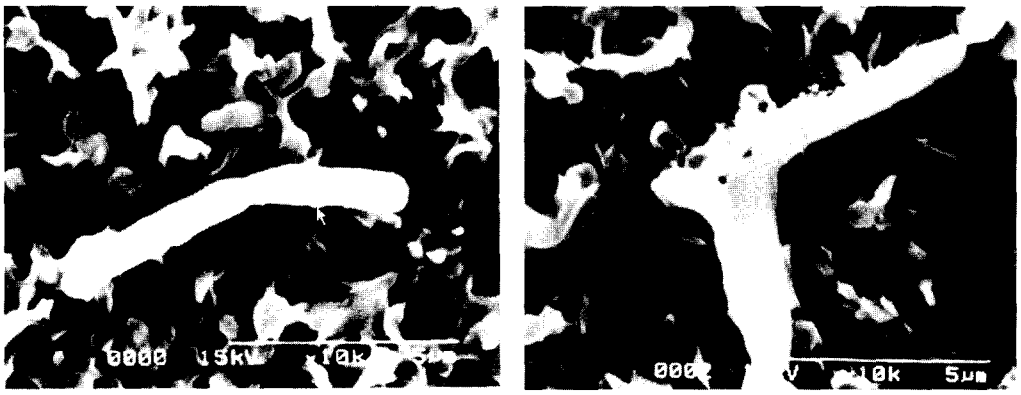

Fig. 6. Scanning electron micrographs of *Bacillus subtilis* ATCC 6633 cells treated with subtilein.
Subtilein concentration: 1,600 AU/ml; treated time: 3 h at 37°C in a BHI medium; (A) untreated, (B) treated cells.

is very important when bacteriocins are to be used as a food preservative, especially for *B. cereus* and other food poisoning *Bacillus* species, for industrial application.

Morphological Change of Indicator Cell by Treatment with Subtilein

The effect of subtilein on the indicator cell was studied by scanning electron microscopy (Fig. 6). Intact cells of the indicator strain, *B. subtilis* ATCC 6633, were seen, as shown in Fig. 6(A). The treatment of subtilein induced morphological changes in stationary-phase cells of *B. subtilis* ATCC 6633 and most of the cells appeared to be lysed [Fig. 6 (B)].

Bacteriocin had three types of inhibition mode against the indicator strains; bacteriostatic action, bactericidal action, and bacteriolytic action. Most of the bacteriocins, such as bavaricin A, pediocin SJ-1, and mensenteriocin 52, have bactericidal action [11, 18, 31]. The bacteriocin produced by *B. subtilis* CAU131 (KCCM 10257) also showed bactericidal action against the indicator strain. So far, only a few studies are available on the mechanism of bactericidal action of bacteriocin. In the case of lactococcin A, it specifically increases permeability of lactococcal cytoplasmic membranes in a voltage-independent, protein-mediated manner [22]. Subtilin produced by *B. subtilis* acts by forming a voltage-dependent multi-state pore in bacteria [31]. This effect, which is related to permeability, may be one of the most important mechanisms of some bacteriocins. Elucidation of the intrinsic nature of cell lysis by subtilein requires further investigation.

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