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Miniscale Identification and Characterization of Subtilisins from *Bacillus* sp. Strains

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Abstract Subtilisin (EC 3.4.21.14) is the major extracellular alkaline serine protease of *Bacillus* species. Previously, we found that subtilisins did not migrate in the electrophoretic field in the Laemmili buffer system due to their high p*I* values (over 8.8); however, it formed a "binding mode" at the top of the separating gel [5]. Utilizing this characteristic, four subtilisins from *Bacillus* sp. strains (e.g., *B. subtilis* 168, *B. subtilis* KCTC 1021, *B. amyloliquefaciens* KCTC 3002, and *Bacillus* sp. DJ-1 and DJ-4) were easily and quickly identified by an over-running electrophoretic technique with a miniscale culture supernatant (less than 20 ml) without any column chromatographic steps. Two subtilisins (DJ-1 and a recombinant version) from *Bacillus* sp. DJ-1 were characterized, and the enzymatic properties were determined by SDS-fibrin zymography and densitometric analysis. Based on this observation, the recombinant pro-subtilisin DJ-1 showed the same "binding mode," similar to native subtilisin DJ-1. On the other hand, mature subtilisin DJ-1 without pro-peptide showed no enzymatic activity.

Key words: Subtilisin, binding mode, *Bacillus*, over-running electrophoresis, zymography

Gram-positive, spore-forming *Bacillus* sp. strains produce and secrete various proteases in the supernatant of the culture. The two best-characterized extracellular proteases are the neutral (metallo-) protease and the alkaline serine protease (subtilisin) [28, 31]. Several subtilisins (EC 3.4.21.14) have been produced by various *Bacilli*; subtilisin Carlsberg [32] from *B. licheniformis*, subtilisins BPN' [15] and DJ-4 [6, 19] from *B. amyloliquefaciens*, subtilisin NAT [26], E [35], and J [13] from *B. subtilis*, and subtilisin Amylosacchariticus [22] from *B. subtilis* var. *amylosacchariticus*. These subtilisins have been used as industrially important proteases. Many efforts have been made in the field of protein engineering to increase the enzymatic properties that include substrate specificities, enhanced catalytic activity, and cold adaptation and thermostability [33]. In particular, subtilisin BPN from *B. amyloliquefaciens* and subtilisin Carlsberg from *B. licheniformis* have been widely studied for industrial uses [34].

Subtilisins are produced from pre-pro-subtilisin, with the 29 residues pre-sequence serving as a signal peptide for protein secretion from the cell membrane [12]. Upon deletion of the pre-sequence, the pro-subtilisin, consisting of 352 residues, undergoes autolysis, resulting in the removal of the N-terminal 77 residues (pro-sequence). Finally, the active subtilisin consisting of 275 residues is produced [37]. Many active subtilisins have been purified from the culture supernatants of *Bacillus* sp. strains from the fermented foods of Japan, Korea, and China (e.g., *Natto*, *Chungkook-Jang*, *Doen-Jang*, and *Douchi*) by a combination of various chromatographic steps [3, 19, 20, 27].

Under Laemmili buffer system [23], some proteins that form a "binding mode" only showed enzyme activity at the top of the separating gel. Wold and colleagues [2] and Lantz and Ciborowski [24] demonstrated that the enzyme binds tightly to the fixed substrate in the separating gel and hydrolyzes it. Recently, we found that recombinant subtilisins (subtilisin Carlsberg and subtilisin BPN') also formed a "binding mode," because of their high p*I* values. This problem was resolved by using an isoelectric focusing-fibrin zymography (IEF-FZ) gel with a pH range from 3 to 10 [5]. Here, we applied the "binding mode" to identify subtilisins of various *Bacillus* sp. strains by using a new method, named as over-running electrophoretic technique. In this study, a rapid and reliable identification method for

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subtilisins is described, and we also recommend this new technique in identifying the proteins with higher p*I* values (≥8.8) than that of the Laemmili buffer system (pH 8.8), resulting in "binding mode."

MATERIALS AND METHODS

Bacterial Protease Preparation

Bacterial strains used in this study were isolated from *Doen-Jang* (a traditional Korean fermented food) and were identified as *Bacillus* sp. strains from the Korean Collection for Type Cultures (KCTC). Other *Bacillus* strains were obtained from KCTC (Table 2). Bacterial cells were grown at 37°C in a tryptic soy broth (TSB, Difco, Sparks, MD, U.S.A.) for 2 days. Then, the cells were precipitated by centrifugation at 10,000×*g* for 10 min. The supernatant was used for the enzyme assay. The concentration of the protein was determined according to the Bradford method [1], using bovine serum albumin (BSA) as a standard.

SDS-PAGE and Over-Running Electrophoresis

SDS-PAGE was done by the Laemmli method [23]. Samples (10 μg) were diluted 5 times with SDS sample buffer of 0.5 M Tris, pH 6.8, 10% SDS, 20% glycerol, and 0.03% bromophenol blue. In the case of over-running electrophoresis, SDS gel and fibrin zymogram gel were over-run for 24 h (at 10 mA constantly).

Fibrin Zymography

Fibrin zymogram gel was carried out as described previously [4, 7, 17, 18]. Separating gel solution (12%, w/v) was prepared in the presence of fibrinogen (0.12%, w/v; Sigma F-8630) and 100 μl of thrombin (10 NIH units/ml; Sigma T-7513). Samples (10 μg) were diluted 5 times with the SDS sample buffer. After electrophoresis was done in a cold room (at 10 mA constantly), the gel was incubated for 30 min at room temperature on a rotary shaker in 50 mM Tris (pH 7.4), which contained 2.5% Triton X-100. The gel was washed with distilled water for 30 min to remove Triton X-100, and it was then incubated in the zymogram reaction buffer (30 mM Tris, pH 7.4, and NaN_3) at 37°C for 12 h. The gel was stained with Coomassie blue for 1 h and then destained. The active bands were visualized as nonstained regions of the zymogram gel.

Enzymatic Digestion of Protein In-Gel and MALDI-TOF Mass Spectrometric Analysis and Database Search

Enzymatic digestion was performed basically as previously described [11]. The spots on the gel were excised with a razor, placed in an Eppendorf tube, and washed three times with water to remove methanol and acetic acid. Next, the gel was mashed with a glass spatula and then dried in a speed Vac vacuum centrifuge apparatus for 30 min at room temperature. The dried samples were reconstituted in 20 μl of 25 mM ammonium bicarbonate (pH 8.5) containing 0.0125 μg/μl of trypsin and incubated overnight at 37°C. After enzymatic digestion, the supernatant was removed, and the resultant peptides were extracted by adding 20 μl of 0.1% trifluoroacetic acid (TFA)-50% acetonitrile (ACN) (Merck, Darmstadt, Germany). The extraction was repeated twice with the same solution. In the final extraction, 30 μl of 100% ACN were added to completely dehydrate the gel (which turned white). Subsequently, the extracts were pooled and dried in a speed Vac vacuum. Finally, the dried samples were dissolved in 20 μl of a solution of 0.1% TFA-ACN (2:1, v/v). The trypsin-digested sample was then analyzed by mass-spectrometric analysis with a PerSeptive Biosystems (Framingham, MA, U.S.A.) matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) Voyager DE-RP mass spectrometer, which was operated in delayed extraction and reflector mode. Peptide mixtures were analyzed by using a saturated solution of α-cyano-4-hydroxycinnamic acid in 50% acetonitrile-0.1% trifluoroacetic acid [10]. The PEPTIDENT program of ExPASY was used for database searching.

Two-Dimensional Gel Electrophoresis

IEF was carried out using 7 cm linear immobiline IPG gels with a pH range of 3–10 (Bio-Rad, Hercules, CA, U.S.A.). The protein was loaded by in-gel rehydration with a reswelling solution containing 8 M urea, 0.3% DTT (w/v), and 2.0% (v/v) pH 3–10 IPG buffer. IEF was carried out for 4,000 Vh at 20°C in a IPGphor Isoelectric Focusing System (Bio-Rad), wherein the voltage was linearly increased from 250 V to 4,000 V over the first 3 h and then was maintained at 4,000 V for the final 10 h. After the IEF, the strips were equilibrated with the SDS equilibration buffer (62.5 mM Tris-HCl, pH 6.8, 2.3% SDS, 30% glycerol, and 0.05% bromophenol blue) for 15 min. The equilibrated strips were loaded into the stacking gel of the SDS gel and fibrin gel, and the 2D gels were also over-run.

N-Terminal Amino Acid Sequence Determination

To determine the N-terminal amino acid sequence, the protein was concentrated by acetone precipitation. After SDS-PAGE was done, the protein on the gel was transferred to a polyvinylidene difluoride (PVDF; Bio-Rad) membrane by electroblotting [30], and the membrane was stained with Coomassie blue. The stained portion was excised and directly used for the N-terminal sequencing by the automated Edman degradation method, using a gas-phase protein sequencer (model Procise 491, ABI, U.S.A.).

Cloning of Subtilisin DJ-1 Gene

The chromosomal DNA from *Bacillus* sp. DJ-1 was prepared according to previously described methods [16,

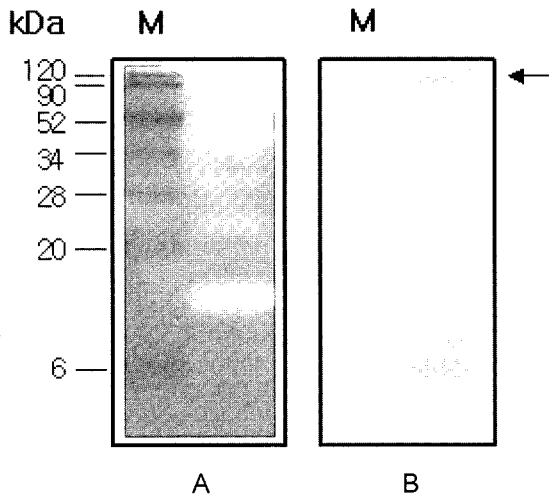

Fig. 1. SDS-fibrin zymography (A) and SDS protein gel (12%) (B) of extracellular proteins of *Bacillus* sp. DJ-1 isolated from *Doen-Jang*.
Proteins were visualized by silver staining on the protein gel, and Coomassie blue was used for the activity staining of the fibrinolytic enzyme in the zymogram gel. M represents protein standard marker.

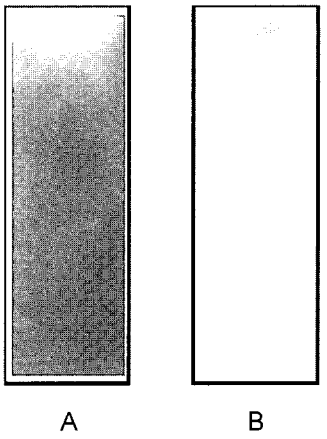

Fig. 2. Over-running SDS-fibrin zymography (A) and SDS protein gel (B) of extracellular proteins of *Bacillus* sp. DJ-1.
After the sample was loaded on the zymogram and protein gels (12%), the gels were over-run for 24 h. Zymogram (A) and the protein gel (B) were visualized by Coomassie blue and silver staining, respectively.

29] and used as the template for a polymerase chain reaction (PCR). Pro-subtilisin and mature subtilisin genes were amplified by PCR using *Nde* I-linked sense primers (5'-GGAATTCCAATGGCCGGAAAAAGCAGTACAGA-3' for pro-subtilisin and 5'-GGAATTCCATATGGCGCA-GTCCGTGCCTTAC-3' for mature subtilisin) and *Bam*HI-linked antisense primer (5'-CGCGGATCCTTACTGAGC-TGCCGCCTGT-3'). PCR amplification was performed under the following conditions: 30 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 1.5 min. The PCR-amplified 1,150 bp (pro-subtilisins) and 825 bp (mature subtilisins) DNA fragments were extracted from the agarose gel and then ligated with a pGEM-T Easy vector (Promega) to generate pT-pre-subtilisin and pT-subtilisin plasmids.

Expression of Subtilisin DJ-1

It is well known that the pro-sequence of the subtilisin plays an essential role in guiding the proper folding of the active conformation [12, 37]. Thus, two proteins, mature and pro-subtilisin DJ-1, were expressed. After digestion of two plasmins, pT-pre-subtilisin DJ-1 and pT-subtilisin DJ-1, with *Nde*I and *Bam*HI, the pro- and mature subtilisin DJ-1 fragments were inserted into the bacterial expression vector pET29a (+). These two fragments expressed in *E. coli* BL21 (DE3) were obtained from inclusion bodies and dissolved in 20 mM Tris-HCl (pH 7.4) buffer containing 6 M urea. The recombinants of pro- and mature subtilisin DJ-1 were dialyzed overnight against the same buffer at 4°C. After dialysis, insoluble materials were removed by centrifugation, and the proteins in the soluble fraction were subjected to the fibrin zymogram gels.

RESULTS AND DISCUSSION

Identification of Extracellular Proteases from *Bacillus* sp. DJ-1

According to the zymogram gel analysis of *Bacillus* sp. DJ-1, five clear bands and one "binding mode," which only showed activity at the top part of the separating gel, were detected (Fig. 1A). After silver staining of the SDS gel (Fig. 1B), the two gels were superimposed to determine the protein bands that coincided with the clear band on the fibrin gel. The protein band (binding mode, arrow marked) on the SDS gel, which was located on the top of the zymogram gel was cut (Fig. 1). The protein was then extracted from the gel and digested with trypsin. The trypsin-digested sample was then analyzed by using MALDI-TOF. The database search revealed that the protein

Table 1. N-terminal amino acid sequence of the isolated protein.

Order	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
First major peak	**A**	A	P	**V**	I	K	S	**I**	T	I	L	H	**A**	W	N	G
Second major peak		**Q**	**S**	S		**Y**	**G**	N	S	Q		**K**		**P**		**L**

Bold: AQSV-YGISQ-KAP-L (subtilisin E).
Underlined: APSIKSNTILH-WNG (α-amylase precursor).

band was a mixture of at least four different proteins, such as the cell wall-associated protease precursor, subtilisin, hypothetical protein yodO, and α-amylase precursor (data not shown). Previously, we found that recombinant subtilisins formed "binding mode" because of their high p*I* values (≥8.8) under Laemmili buffer system (pH 8.8) [5]. To identify the "binding mode," a new electrophoretic method, over-running electrophoresis, was performed.

Over-Running Electrophoresis and N-Terminal Amino Acid Sequence Determination

In order to separate the protein mixture forming the "binding mode" at the top of the separating gel, SDS gel and fibrin zymogram gel were over-run for 24 h. Based on the result presented in Fig. 2, the "binding mode" was also detected. On the other hand, five active bands were taken out from the gel. To determine the N-terminal amino acid sequence, the protein on the gel was transferred to PVDF membrane. The sequence of the first 16 residues revealed that the protein contained two proteins, subtilisin (28–29 kDa) [3, 19, 20, 27] and α-amylase precursor (>45 kDa) [36] (Table 1). In order to separate these two proteins, molecular cutoff with Centricon YM-30 (Millipore, Bedford, MA, U.S.A.) and two-dimensional gel electrophoresis (2DE) were used.

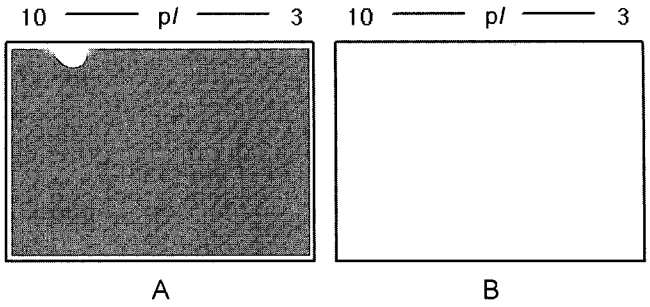

Fig. 3. 2D zymography (A) and protein (B) patterns of subtilisin DJ-1.
After molecular fractionation with the Centricon YM-30, the protein that was taken out from the Centricon YM-30 was precipitated by acetone. The sample was then applied to the 2D system, as described in Materials and Methods.

Two-Dimensional Gel Electrophoresis

The protein, which was taken out from the Centricon YM-30, was precipitated with acetone. IEF was carried out using 7 cm linear immobiline IPG gels with a pH range of 3–10. After the IEF, the equilibrated strips were loaded into the stacking gel of the SDS gel and fibrin gel, and the 2D gels were also over-run. As shown in the 1D zymogram gel (Figs. 1 and 2), the "binding mode" was also detected in the

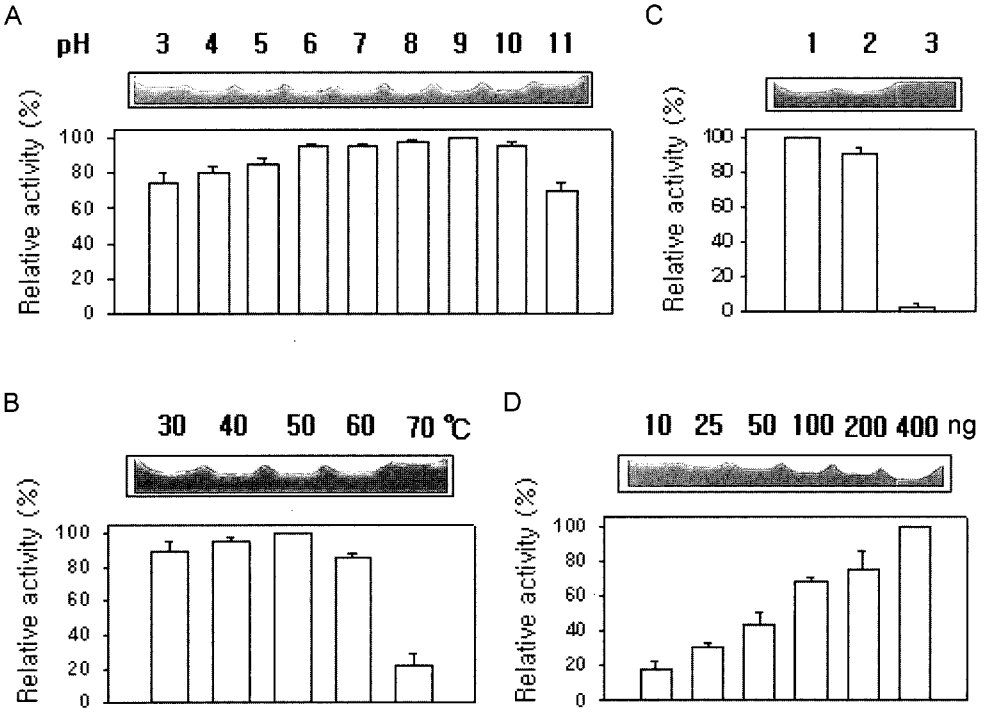

Fig. 4. Effects of pH, temperature, and inhibitors on the activity of subtilisin DJ-1 with SDS-fibrin zymography.
(A) The effect of pH on stability was measured over a range from 3.0 to 11.0 by using different buffer systems. (B) To establish thermostability, the residual activity was assayed after incubation at the indicated temperatures for 30 min. (C) Inhibition test of subtilisin DJ-1 was performed by incubation of subtilisin DJ-1 and protease inhibitors, EDTA (2) and PMSF (3). Lane 1, control (without any protease inhibitor). (D) SDS-fibrin zymography of various amounts (10–400 ng) of subtilisin DJ-1. Every value represents the mean of four independent experiments.

2D gel (Fig. 3). To determine the N-terminal amino acid sequence, the protein was transferred to a PVDF membrane. The first 15 amino acid residues of the N-terminal amino acid sequence of the protein were AQSVPYGISQIKAPA, which is identical to those of subtilisin NAT (P35835) [26] and subtilisin E (SUBSI) [35] of *B. subtilis*, and subtilisin Amylosacchariticus (P00783) [22] of *B. subtilis* var. *amylosacchariticus*, respectively. Therefore, the identified protein was named as subtilisin DJ-1.

Enzymatic Characterization of Subtilisin DJ-1 with Fibrin Zymograpy

The effect of pH on the activity of subtilisin DJ-1 was determined by using fibrin zymography. The enzyme was incubated either in 0.1 M citrate-phosphate buffer (pH 3.0 to 5.0), sodium phosphate buffer (pH 6.0 to 7.0), Tris-HCl buffer (pH 8.0 to 9.0), or glycine-NaOH buffer (pH 10.0 to 11.0) at 4°C for 24 h. The results showed that subtilisin DJ-1 was stable in a wide pH range from 3.0 to 11.0 (Fig. 4A). The influence of temperature on the activity of subtilisin DJ-1 showed that the enzyme activity increased up to 50 or 60°C and disappeared after incubation at 70°C for 30 min (Fig. 4B). Thus, the subtilisin DJ-1 can be considered to be a heat-resistant enzyme. Also, the effects of the protease inhibitors were studied. The subtilisin DJ-1 was strongly inhibited by 5 mM PMSF, but it was not affected by 5 mM EDTA (Fig. 4C), indicating that the subtilisin DJ-1 is a serine protease. Finally, the activity was examined as a function of the amount of subtilisin DJ-1 (10–400 ng). As shown in Fig. 4D, the more the subtilisin DJ-1, the higher the activity.

Recombinant Mature and Pro-Subtilisin DJ-1

Recombinant pro-subtilisin DJ-1 also showed the same binding mode on the 1D and 2D zymogram gels (Fig. 5). On the other hand, the enzyme activity of the recombinant mature subtilisin DJ-1 was not detected (Figs. 5A, lane 1), implying that the pro-sequence is essential for guiding the appropriate folding of the subtilisin to produce activity [12, 37]. Also, the recombinant pro-subtilisin DJ-1 was found to increase the stability toward heat (70–80°C) (Fig. 5C), compared with the native subtilisin DJ-1(Fig. 4B).

Application of Over-Running Electrophoretic Technique to Various *Bacillus* sp. Strains

We applied the over-running electrophoretic method to other *Bacillus* sp. strains (Table 2). First of all, their extracellular proteases were confirmed by using a fibrin zymography. In all strains, "binding mode" was detected (Fig. 6).

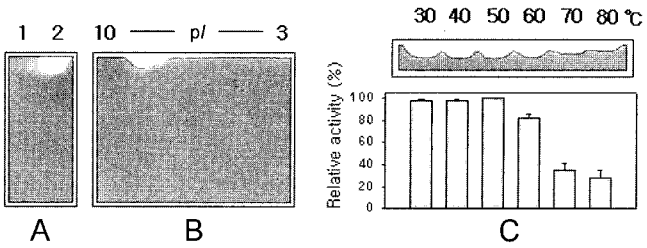

Fig. 5. 1D (A) and 2D (B) zymography of recombinant subtilisin DJ-1.
The symbols 1 and 2 represent the recombinant mature subtilisin DJ-1 and the recombinant pro-subtilisin DJ-1, respectively. (C) To establish thermostability, the residual activity was assayed after incubation at the indicated temperatures for 30 min, as described in Fig. 4B.

To identify the "binding mode," sample preparations, molecular cutoff with Centricon YM-30, and precipitation with acetone were followed as conducted for subtilisin DJ-1. After 2D over-running electrophoresis and electrotransfer to a PVDF membrane, the N-terminal amino acid sequences of all "binding modes" were determined (Table 2) to be a subtilisin group; subtilisin NAT [26], subtilisin E [35], subtilisin Amylosacchariticus [22], and subtilisin BPN' [25].

Originally, protein complexes have been purified by classical column chromatography methods, and they are the primary protein purification methods in use in most laboratories. However, this process is generally time-consuming, especially for low concentration of complexes, since very large amounts and volumes are needed as starting materials (hundreds of grams and one or two liters of culture supernatant). Moreover, yields are usually low, and the procedure is long so that less stable proteins are not preserved [8]. To date, many subtilisins have been purified by numerous purification steps [3, 9, 14, 19, 20, 21, 27].

In this study, we found that subtilisins formed a "binding mode" under the Laemmili buffer system [23], thus making it possible to identify and characterize the subtilisins from various *Bacillus* sp. strains without any chromatographic steps. To identify subtilisin, only 20 ml of bacterial culture broth were needed, when the new over-running electrophoretic technique was used. Also, using this system, the enzymatic comparison of the native subtilisin DJ-1 and the recombinant subtilisin DJ-1 from *Bacillus* sp.

Table 2. Identification of binding modes of four *Bacillus* sp. strains.

Lane no.	Strains	N-terminal sequence	Protein identity	References
1	*B. subtilis* 168	AQSVPYGISQ	subtilisin E, NAT, Amylosacchariticus	22,26,35
2	*B. subtilis* KCTC 1021	AQSVPYGISQ	subtilisin E, NAT, Amylosacchariticus	22,26,35
3	*Bacillus* sp. DJ-4	AQSVPYGVSQ	subtilisin BPN'	19,25
4	*B. amyloliquefaciens* KCTC 3002	AQSVPYGVSQ	subtilisin BPN'	19,25

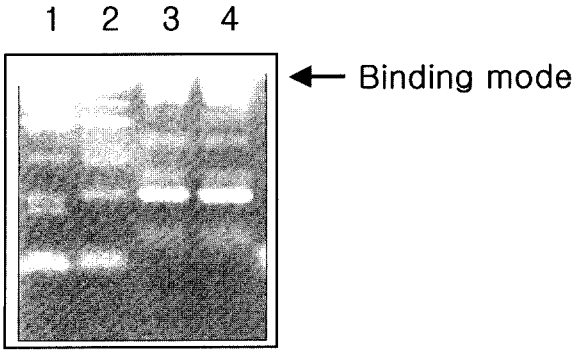

Fig. 6. SDS-fibrin zymography analysis of culture supernatants from four *Bacillus* sp. strains.
Lane 1, *B. subtilis* 168; lane 2, *B. subtilis* KCTC 1021; lane 3, *Bacillus* sp. DJ-4; lane 4, *B. amyloliquefaciens* KCTC 3002.

DJ-1 was made. Furthermore, subtilisins could be migrated into the separating gel part and their molecular weights determined (29 kDa) when they were denatured by strongly acidic condition, such as trichloroacetic acid (TCA) precipitation (data not shown). By using the over-running electrophoretic technique and synthetic chromogenic substrates, future studies, including comparative studies, substrate specificity, and enzymatic kinetics of various subtilisins and their recombinant subtilisins described in Table 2, will be carried out.

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