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Identification of Total Extracellular Fibrinase from *Bacillus* sp. DJ Using One- or Two-Dimensional Fibrin Zymography for Proteomic Approach

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Abstract An extracellular fibrinolytic-enzyme-producing bacterium was isolated from Doen-Jang, a Korean traditional fermented food, and identified as *Bacillus* sp. DJ based on its morphology and cellular fatty acid composition. The total extracellular fibrinase (EF) from *Bacillus* sp. DJ was analyzed using three fibrin zymographic techniques, SDS-fibrin zymography (SDS-FZ), isoelectrofocucing-fibrin zymography (IEF-FZ), and a two-dimensional SDS-fibrin zymographic analysis (2D SDS-FZ). As a result, the EF map of *Bacillus* sp. DJ was established. The results suggest that the 2D SDS-FZ method will be a useful tool for the proteomic approach for many other bacterial proteases.

Key words: *Bacillus*, fibrinase, proteomics, two-dimensional fibrin zymography

Bacillus sp. strains are Gram-positive bacteria, which are widely used for the production of industrially-useful enzymes. During their growth, these bacteria produce several extracellular proteases, serine protease, neutral metalloprotease, and esterase [9]. Among these, the fibrinolytic enzyme (fibrinase) is secreted to the culture medium. In particular, nattokinase (NK) from *Bacillus natto* [17, 18], CK 11-4 from *Bacillus* sp. strain CK 11-4 [9], and subtilisin DJ-4 from *Bacillus* sp. strain DJ-4 [7] have already been isolated. In addition, to identify the bacterial proteolytic activity, zymographic techniques based on SDS-PAGE copolymerization with protein substrates, such as casein [13], fibrin [2, 3, 6, 8], gelatin [5, 11, 12], and starch [16], have been used.

Zymography is a versatile two-staged technique involving protein separation by electrophoresis followed by the detection of proteolytic activity. This technique is routinely used to identify the protease activity in polyacrylamide gels under nonreducing conditions. Recently, the genomic sequence of *Bacillus subtilis* enabled the establishment of a theoretical two-dimensional (2D) zymographic map. This report introduces a proteomic technique using two-dimensional SDS-fibrin zymography (2D SDS-FZ) combined with 2D SDS-PAGE with fibrin zymography to identify the EF from *Bacillus* sp. DJ. In particular, using a 2D SDS-FZ gel, the EF map of *Bacillus* sp. DJ was established, thereby, confirming the new technique, for identification of the proteins in mixture and for searching a specific protein in proteomics.

The fibrinogen and thrombin from bovine used for the fibrin zymographic gels and ultra-pure urea were purchased from Sigma (St. Louis, MO, U.S.A.). The ampholytes, within pH ranges of 3 to 10 and 5 to 7, were obtained from Bio-Rad (Hercules, CA, U.S.A.). All other chemicals were of analytical grade.

A bacterial strain producing an extracellular fibrinolytic enzyme (EF) was isolated from Doen-Jang, a Korean traditional fermented food [9]. To identify the strain, its morphology and cellular fatty acid composition were analyzed. To observe the cell morphology of *Bacillus* sp. DJ, scanning electron microscopy (SEM) was used. The samples were fixed in a 2.5% paraformaldehyde-glutaraldehyde mixture buffered with 0.1 M sodium phosphate (pH 7.2) for 2 h, postfixed in 1% osmium tetroxide in the same buffer for 1 h, dehydrated in graded ethanol, and substituted by isoamyl acetate. The samples were then dried at the critical point in CO_2, sputtered with gold in a sputter coater (SC502, Polaron), and finally observed using a scanning electron microscope (SEM 515, Philips). Under the SEM, the cell morphology of *Bacillus* sp. DJ was shown to be rod-shaped bacteria (Fig. 1). For the fatty acid composition, whole cell lipids were extracted with a Folch solution and saponified [4]. Fatty acid methyl esters were prepared with 5% HCl in methanol at 80°C for 1 h, extracted with n-hexane, and analyzed by gas chromatography. The fatty

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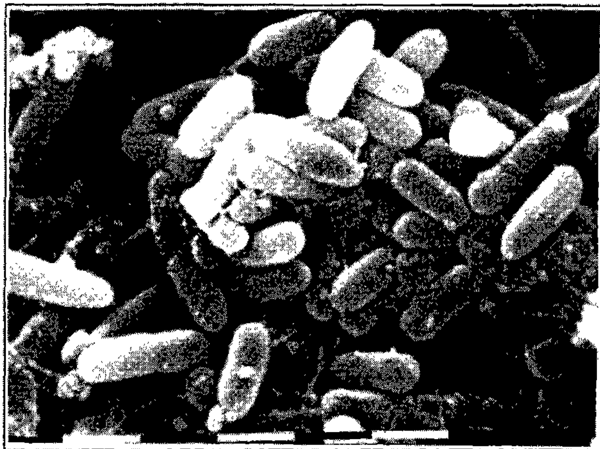

Fig. 1. Cell morphology of *Bacillus* sp. DJ isolated from Doen-Jang under scanning electron microscope (SEM).
Bar indicates 1.0 μm.

acid composition of the bacterium is shown in Table 2. The major fatty acids were iso-$C_{15:0}$ (24.49%), anteiso-$C_{15:0}$ (37.63%), iso-$C_{17:0}$ (13.00%), and anteiso-$C_{17:0}$ (12.46%), all of which belong to *Bacillus* sp. strains (by Korean Collection for Type Culture, KCTC), therefore, the strain was named as *Bacillus* sp. DJ [14].

To identify the EF from *Bacillus* sp. DJ, the cells were grown at 37°C in a tryptic soy broth (TSB, Difco, Sparks, MD, U.S.A.) for 1 day. The crude extraction was separated from the cells by centrifugation at 10,000 ×g for 10 min. The EF in the culture supernatant was analyzed using the fibrin zymographic method. The protein concentration was determined according to Bradford's method [1] using bovine serum albumin (BSA) as the standard.

SDS-fibrin zymography gel was performed using the method of Kim and Choi [2, 3, 6, 8], and conducted in 12% polyacrylamide gels containing 0.12% fibrinogen (wt/vol) and 100 μl of thrombin (10 NIH unit/ml). As shown in Fig. 2(A), the EF of the culture supernatant from *Bacillus* sp. DJ was identified by SDS-FZ, and three EFs (26, 22, and 21 kDa) were detected. To determine the pI values of the fibrinases, IEF-FZ containing 4 M urea was carried out. The isoelectrofocusing was performed using the immobilized pH gradient (IPG) technique described by Robertson *et al.* [15], with minor modifications. The IEF-FZ gel was made as described in Table 1. The sample (1.0 μg) was diluted in an IEF sample buffer (2×), which consisted of 4 M urea, 2% 5–7 ampholyte, 0.4% 3–10 ampholyte, 30% glycerol, and 0.01% bromophenol blue. The cathode and anode solutions used were 25 mM NaOH and 10 mM H_3PO_4, respectively. The electrophoresis was carried out at 4°C for 1 h at a constant voltage of 150 V, then increased to 200 V for an additional 2 h. As shown in Fig. 2B, three bands were identified in the gel: The most prominent band was at alkaline pI 9.5,

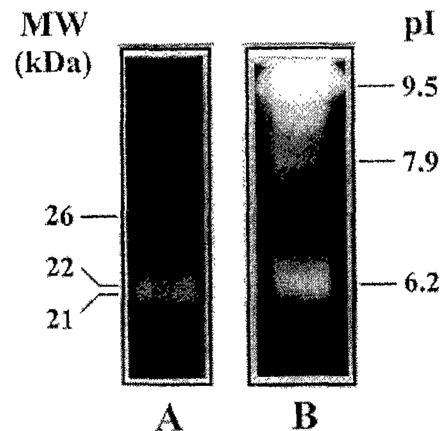

Fig. 2. SDS-FZ (12% gel) (A) and IEF-FZ containing 4 M urea (B) with the culture supernatant of *Bacillus* sp. DJ.
The sample (1.0 μg) was diluted in zymogram sample buffer (5×, 0.5 M Tris-HCl, pH 6.8, 10% SDS, 20% glycerol, and 0.01% bromophenol blue) or IEF sample buffer (2×, 4 M urea, 2% 5–7 ampholyte, 0.4% 3–10 ampholyte, 30% glycerol, and 0.01% bromophenol blue), and then electrophoresed in a cold room (4°C) at a constant current of 12 mA. After the electrophoresis was performed, the SDS-FZ gel was washed in 2.5% Triton X-100 for 30 min and then incubated in a reaction buffer (30 mM Tris, pH 7.4, 200 mM NaCl, and 0.02% NaN_3) at 37°C for 12 h. In the case of IEF-FZ, after the electrophoresis, the gel was rinsed with distilled water and incubated in the same reaction buffer. The gel was stained with Coomassie blue for 1 h and then destained.

the less prominent at alkaline pI 7.9, and one at neutral fibrinase pI 6.2.

For further study, a 2D SDS-FZ gel was performed. IPG gel (without fibrin, described in Table 1), carried out on the first gel running, was applied to the SDS-FZ gel. After the IEF electrophoresis was completed, the gel slice was equilibrated with an equilibration buffer (62.5 mM Tris, pH 6.8, 2.3% SDS, and 10% glycerol) for 30 min. The equilibrated gel slice was placed in direct contact with the stacking gel of the SDS-fibrin gel. The electrophoresis was

Table 1. Composition of IEF-FZ (containing 4 M urea) and IEF gels.

Component	IEF-FZ gel	IEF gel
Solution A	1.75	1.75
Urea	2.4 g	2.4 g
Glycerol (50%)	2.4	2.4
5-7 Ampholyte (40%)	0.5	0.5
3-10 Ampholyte (40%)	0.1	0.1
Distilled water	2.5	2.8
Bovine fibrinogen[a]	0.25	
Bovine thrombin (1 U/ml)	0.05	
Ammonium persulfate (10%)	0.05	0.05
TEMED	0.02	0.02
Total	10 ml	10 ml

Solution A: 30% acrylamide, 0.8% bis-acrylamide.
[a]Fibrinogen (6 mg) was dissolved in solution A and then centrifuged to remove any insoluble impurities.

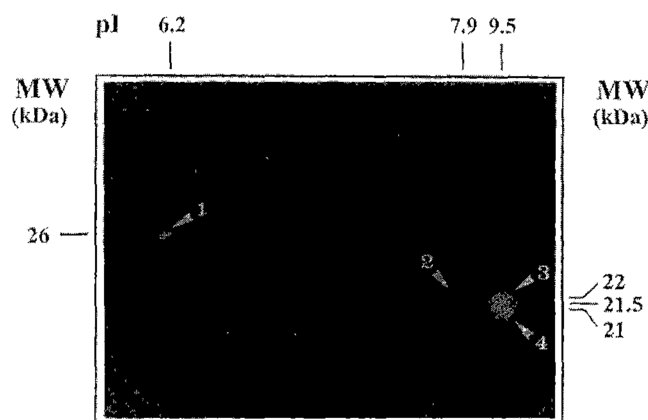

Fig. 3. 2D SDS-FZ analysis of fibrinase from *Bacillus* sp. DJ.
After the IEF electrophoresis was completed, the gel slice was equilibrated with an equilibration buffer (62.5 mM Tris, pH 6.8, 2.3% SDS, and 10% glycerol) for 30 min. Then, the gel slice was loaded on the SDS-FZ gel. The SDS-FZ was performed as described in Fig. 2.

then carried out. As a result of the 2D SDS-FZ gel analysis (Fig. 3), four spots were detected. Two strong alkaline fibrinases, which showed the same isoelectric point (pI 9.5), were determined with molecular sizes of 22 and 21 kDa. In addition, two fibrinases, which exhibited isoelectric points at 6.2 and 7.9, and molecular sizes of 26 and 21.5 kDa, respectively, were detected on the 2D SDS-FZ gel.

The EFs from *Bacillus* sp. DJ were summarized as follows based on the zymographic analyses. *Bacillus* sp. DJ secreted four EFs (26, 22, 21.5, and 21 kDa) into the culture broth and their pI values were determined as 9.5 (22 and 21 kDa), 7.9 (21.5 kDa), and 6.2 (26 kDa). However, the EF of 21.5 kDa was not possible to separate in the SDS-FZ gel (Fig. 2A), and the two EFs of 22 and 21 kDa showed the same pI values (Fig. 3), thus, they were not separated in the IEF-FZ (Fig. 2B).

The present study established three fibrin zymographic techniques, SDS-FZ, IEF-FZ, and 2D SDS-FZ. Many studies on the SDS-FZ to identify the proteolytic enzymes from bacterial strains were reported previously by several investigators [2, 3, 6-8, 12]. We have applied the zymographic method to the 2D SDS gel system to analyze the EF from *Bacillus* sp. DJ. Using this improved 2D SDS-FZ, the EF map of *Bacillus* sp. DJ was established. Further study is required to identify the spots in the 2D SDS-FZ for proteomic analysis.

Table 2. Cellular fatty acid composition of *Bacillus* sp. DJ from Doen-Jang.

Fatty acid	Cellular fatty acid composition
12:0 anteiso	0.30
14:0 iso	1.62
15:0 iso	24.49
15:0 anteiso	37.63
16:0 iso	5.39
16:0	1.93
17:0 iso	13.00
17:0 anteiso	12.46
18:0	0.32

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