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Identification of Recombinant Subtilisins

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Abstract To identify the activity of recombinant subtilisins (subtilisin BPN' and subtilisin Carlsberg), three different zymography methods, SDS-fibrin zymography (SDS-FZ), reverse fibrin zymography (RFZ), and isoelectric focusing-fibrin zymography (IEF-FZ), were used. The recombinant subtilisins BPN' and Carlsberg did not migrate into the electrophoretic field based on a Laemmli buffer system, instead forming a "binding mode" at the top part of the separating gels with the SDS-FZ and RFZ techniques. Yet, this problem was resolved when using IEF-FZ with a pH range from 3 to 10. In addition, all these methods enabled the activity of a recombinant pro-subtilisin DJ-4 to be detected without a refolding pathway.

Key words: *Bacillus*, binding mode, isoelectric focusing, fibrin zymography, recombinant subtilisin

Subtilisin (EC 3.4.21.14), an alkaline serine protease, is the major extracellular protease in *Bacillus* species [21, 24]. Several subtilisins are known to be produced by various *Bacilli*, including the subtilisin Carlsberg [25] from *B. licheniformis*, subtilisins BPN' [9] and DJ-4 [13] from *B. amyloliquefaciens*, subtilisin NAT [19], E [7], and J [8] from *B. subtilis*, and subtilisin Amylosacchariticus [15] from *B. amylosaccharitícus*. Plus, these subtilisins are all produced from a pre-pro-subtilisin with a 29 residue pre-sequence that serves as a signal peptide for protein secretion from the cell membrane [6]. Upon deletion of the pre-sequence, the resulting pro-subtilisin consisting of 352 residues undergoes autolysis resulting in the removal of the N-terminal 77 residues (pro-sequence), which are known to play an essential role in guiding the proper folding of the active conformation [7, 28].

Many active subtilisins consisting of 275 residues have been isolated from *Bacillus* sp., and exhibited activity in relation to the hydrolysis of fibrin [3, 13, 14, 20, 27]. Recently, the current authors purified and characterized the subtilisin DJ-4 from *Bacillus* sp. strain DJ-4 (*Doen-Jang*, a traditional fermented food in Korea), and the activity of subtilisin DJ-4 was found to be 2.2 and 4.3 times higher than that of the subtilisins BPN' and Carlsberg, respectively [13]. The enzyme activity of subtilisin DJ-4 was easily characterized using the SDS-FZ method [4, 11, 12], which is able to detect proteolytic enzymes following electrophoretic separation on an SDS-polyacrylamide gel copolymerized with a protein substrate that is degraded by the proteases restored during an incubation period in an enzyme reaction buffer. Activity in the zymogram gel is visualized as a clear band, indicating that the substrate has been proteolytically degraded in the gel. As such, zymography is one of the most important tools for protein identification at nanogram quantities.

Under the Laemmli buffer system [16], certain proteins that form a "binding mode" only exhibit activity at the top part of the separating gel. Yet, Brown *et al.* [2] and Ciborowski *et al.* [17] demonstrated that the enzyme binds tightly to the fixed substrate in the separating gel and hydrolyzes it. Thus, to overcome this problem, various buffer systems have been introduced [2, 18], including the zymogram system developed by the current authors [5], where an IEF-FZ gel with a pH range from 3 to 10 is used to identify the "binding mode" based on separating the proteins according to their pI values. As such, the current study uses three different zymography methods to identify recombinant subtilisins and, as a new methodology, directly applies a recombinant pro-subtilisin DJ-4 to three zymogram gels without a renaturation (refolding) step.

Materials and Methods

Reagents

The recombinant subtilisins (subtilisin BPN' and subtilisin Carlsberg), bovine fibrinogen, thrombin, glycerol, Triton

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X-100, and ultra-pure urea were all purchased from Sigma (USA). The ampholytes (pH 3 to 10) and prestained IEF standard marker were purchased from Bio-Rad (U.S.A.). pGEM-T Easy (Promega) and pET29a (+) plasmid (Novagen) were used as the cloning vector for the PCR-amplified DNA fragments and bacterial expression vector, respectively. All other chemicals were of analytical grade.

SDS-PAGE and IEF Gels

The SDS-PAGE was performed using Laemmli's method [16]. The samples (10 μg) were diluted 5 times with an SDS sample buffer of 0.5 M Tris, pH 6.8, 10% SDS, 20% glycerol, and 0.03% bromophenol blue. An IEF gel containing 4 M urea was prepared based on the methods of Choi *et al.* [5] and Robertson *et al.* [22], with minor modifications. The samples (10 μg) were diluted twice with an IEF sample buffer of 6 M urea, 2.4% 3-10 ampholyte, 30% glycerol, and 0.01% bromphenol blue, and then centrifuged at 10,000 ×*g* for 10 min to remove any insoluble materials. The electrode buffer (cathode and anode solutions) and running conditions are described in Table 1. After the SDS-PAGE and IEF gel electrophoresis were completed, the gels were stained with Coomassie blue.

Fibrin Zymography

The fibrin zymogram gel procedure was performed as described previously [4, 11, 12]. The separating gel solution (12%, w/v) was prepared in the presence of fibrinogen (0.12%, w/v) and 100 μl of thrombin (10 NIH units/ml). The samples (100 ng) were diluted 5 times with the SDS sample buffer. After conducting the electrophoresis 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) that contained 2.5% Triton X-100. The gel was then washed with distilled water for 30 min to remove the Triton X-100 and incubated in the zymogram reaction buffer (30 mM Tris, pH 7.4, and NaN_3) at 37°C for 12 h. In the case of reverse fibrin zymography, the SDS gel (containing no fibrin) was overlaid on the fibrin plate 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 the nonstained regions of the zymogram gel.

Table 1. Conditions for IEF-FZ gel containing 6 M urea.

Component	Volume (ml)
Gel solution	
Acrylamide-bisacrylamide (30%:0.8%)	1.75
	3.6 g
Glycerol (50%)	2.4
3-10 Ampholyte (40%)	0.6
Distilled water	A
Bovine fibrinogen[a]	0.25
Bovine thrombin (1 unit/ml)	0.05
10% (w/v) ammonium persulfate	0.05
TEMED	0.02
Total volume	10 ml
Electrode buffer	
20 mM NaOH (cathode)	Upper
10 mM H_3PO_4 (anode)	Lower
Running conditions	
Prerunning	100 V, 30 min
	150 V, 30 min
	200 V, 30 min
	250 V, 60 min
Running	250 V, 6 h

A: total volume (10 ml) was adjusted using distilled water.
[a]Bovine fibrinogen (10 mg) was dissolved in acrylamide-bisacrylamide and then centrifuged to remove any insoluble impurities.

Isoelectric Focusing-Fibrin Zymography (IEF-FZ)

An IEF-FZ gel containing 6 M urea was made as described in Table 1. The samples (100 ng) were diluted twice with the IEF sample buffer. The IEF-FZ gel electrophoresis was carried out in a cold room according to the same conditions as described above. After the electrophoresis, the gel was rinsed with distilled water and then incubated in the zymogram reaction buffer at 37°C for 12 h.

Fibrin Plate Assay

The enzyme activity was determined by the fibrin plate method, as previously described [1]. In a petri dish, 5 ml of a 1% (w/v) fibrinogen solution in a 50 mM sodium phosphate buffer (pH 7.4) was mixed with the same volume of a 2% (w/v) agarose solution along with 0.1 ml of a thrombin solution (10 NIH units/ml). The petri dish was then left for 1 h at room temperature to allow a fibrin clot layer to form. Next, 20 microliters (3 μg) of the sample solution were dropped into holes that had been made on a fibrin plate by a capillary glass tube (5-mm diameter), and the plate incubated at 37°C for 12 h. The enzyme activity was estimated by measuring the dimensions of the clear zones.

Recombinant Mature and Pro-Subtilisin DJ-4

The chromosomal DNA from *Bacillus* sp. DJ-4 was prepared according to described methods [10, 23] and used as the template for the PCR. The pro-subtilisin DJ-4 and mature subtilisin DJ-4 genes were amplified by a PCR using *Nde*I-linked sense primers (5'-GGAATTCCATATGAGAGGC-AAAAAAGTATGGATCA-3' for pro-subtilisin DJ-4 and GGAATTCCATATGGCGCAGTCCGTGCCTTAC for mature subtilisin DJ-4) and a *Bam*HI-linked antisense primer (5'-CGCGGATCCTTACTGAGCTGCCGCCTGT-3'). The 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-subtilisin DJ-4) and 825 bp (mature subtilisin DJ-4) DNA fragments

were extracted from the agarose gel, then ligated with a pGEM-T Easy vector (Promega) to generate the pT-pre-subtilisin DJ-4 and pT-subtilisin DJ-4 plasmids, respectively. After digestion with *Nde*I and *Bam*HI, the pro- and mature subtilisin DJ-4 fragments were inserted into the bacterial expression vector pET29a (+). The two fragments expressed in *E. coli* BL21 (DE3) were then obtained from the inclusion bodies and dissolved in a 20 mM Tris-HCl (pH 7.4) buffer containing 4 M urea. Finally, the recombinants of pro- and mature subtilisin DJ-4 were confirmed using a fibrin plate, plus SDS-FZ and IEF-FZ gels.

RESULTS AND DISCUSSION

Identification of Recombinant Subtilisins (BPN' and Carlsberg) by SDS-PAGE and SDS-Fibrin Zymography Analyses

First, an attempt was made to identify the recombinant subtilisins BPN' and Carlsberg using SDS-PAGE and SDS-FZ gels based on Laemmli's gel system [16] under nondenaturing conditions. However, the recombinant subtilisins did not migrate into the electrophoretic field on the SDS gel (Fig. 1A) or on the fibrin zymogram gel (Fig. 1B), resulting in only a "binding mode" at the top part of the separating gel, as previously described by Brown *et al.* [2] Ciborowski *et al.* [17], who demonstrated that the protease binds tightly to the copolymerized substrate in the separating gel and hydrolyzes it. Thus, to test whether the substrate in the gel might affect the migration of the recombinant subtilisins, reverse fibrin zymography was performed. Therefore, after the recombinant subtilisins were separated according to their molecular mass under Laemmli's gel system, the gel was overlaid on the fibrin plate. As shown in Fig. 2, a "binding mode" was also detected.

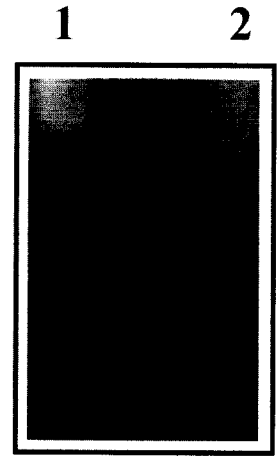

Fig. 2. RFZ of recombinant subtilisin BPN' and Carlsberg.
After SDS-PAGE was done, the gel was overlaid on the fibrin plate and then incubated at 37°C for 12 h. The symbols 1 and 2 represent pro-subtilisin BPN' and Carlsberg, respectively.

Identification of Recombinant Subtilisins (BPN' and Carlsberg) by IEF Gel and IEF-FZ Analyses

To separate the recombinant subtilisins that did not migrate in Laemmli's gel system during electrophoresis (Fig. 1), IEF and IEF-FZ gels with a pH range from 3 to 10 were applied. Based on the results presented in Fig. 3, it was found that the migration of the recombinant subtilisins BPN' and Carlsberg in the IEF and IEF-FZ gels was due to their high p*I* values, and the "binding mode" was not found to be related to the interaction of the enzyme and substrate (Figs. 1, 2, and 3).

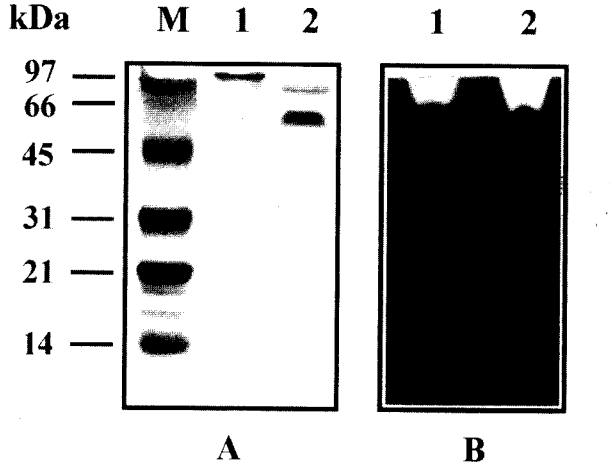

Fig. 1. SDS-PAGE and SDS-FZ analyses of recombinant subtilisin BPN' and Carlsberg.
SDS-PAGE (a) and fibrin zymography (b) were performed under the same conditions (in a cold room and at 10 mA constantly). The symbols 1 and 2 represent pro-subtilisin BPN' and Carlsberg, respectively.

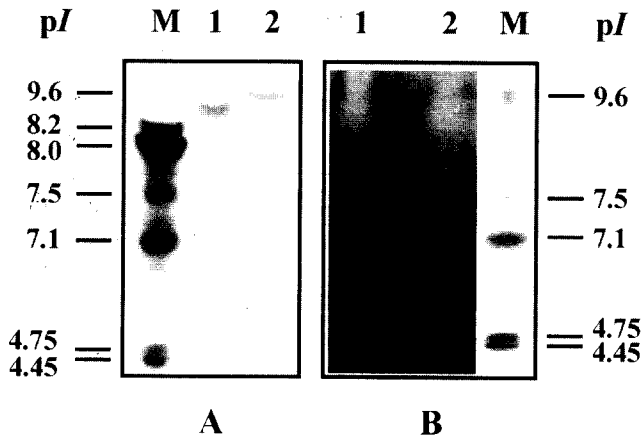

Fig. 3. IEF and IEF-FZ analyses of recombinant subtilisin BPN' and Carlsberg.
IEF (a) and IEF-FZ (b) gels were made as described in Table 1. Standards (M) were stained with Coomassie blue (a) or left unstained (b). The symbols 1 and 2 represent recombinant subtilisin BPN' and Carlsberg, respectively.

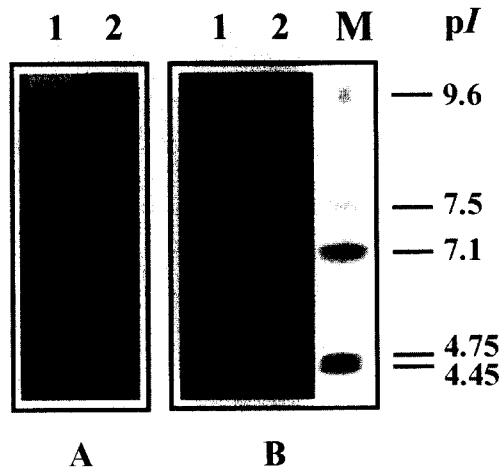

Fig. 4. FZ (a) and IEF-FZ (b) analyses of recombinant pro-subtilisin DJ-4 and mature subtilisin DJ-4.
All procedures were performed as described in Figs. 1 and 2. The symbols 1 and 2 represent recombinant pro-subtilisin DJ-4 and recombinant mature subtilisin DJ-4, respectively.

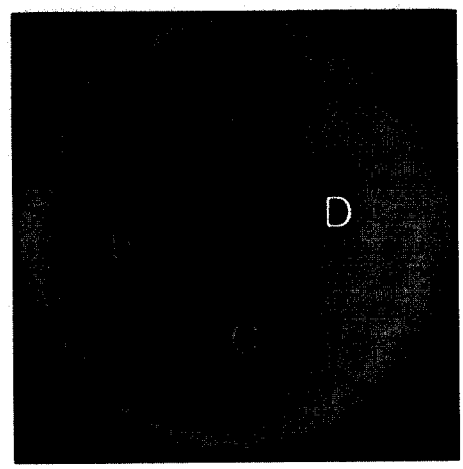

Fig. 5. Fibrin plate assay with recombinant subtilisins (BPN' and Carlsberg), recombinant pro-subtilisin DJ-4, and recombinant mature subtilisin DJ-4.
A, B, C, and D represent recombinant subtilisin BPN', Carlsberg, pro-subtilisin DJ-4, and mature subtilisin DJ-4, respectively.

Identification of Recombinant Pro- and Mature Subtilisin DJ-4 by SDS-Fibrin and IEF-FZ Analyses

To establish active recombinant subtilisins, the pro-subtilisins need to undergo "active processing," which involves denaturation in 6 M urea and renaturation (refolding) based on a dialysis step against a renaturing buffer [26]. This means that the pro-sequence is essential for guiding the appropriate folding of the subtilisin to produce activity [7, 28]. As such, recombinants pro- and mature subtilisin DJ-4 were expressed in *E. coli* BL21 (DE3) using the bacterial expression vector pET29a (+). The two proteins, expressed as inclusion bodies, were dissolved in a 20 mM Tris-HCl (pH 7.4) buffer containing 4 M urea, then directly subjected to SDS-FZ and IEF-FZ gels without a refolding pathway. As shown with the recombinant subtilisins BPN' and Carlsberg (Fig. 1B), the recombinant pro-subtilisin DJ-4 exhibited a "binding mode" at the top part of the separating gel when using the SDS-FZ gel, whereas the IEF-FZ with a pH range from 3 to 10 was able to migrate the "binding mode" (Fig. 4). Meanwhile, no enzyme activity was detected for the recombinant mature subtilisin DJ-4 on either the SDS-FZ or IEF-FZ gel (Fig. 4), confirming that the pro-sequence plays an essential role in guiding the proper folding of the active conformation [7, 28].

Fibrin Plate Assay of Recombinant Pro- and Mature Subtilisin DJ-4

The two expressed proteins, recombinant pro- and mature subtilisin DJ-4, were compared with the recombinant subtilisins BPN' and Carlsberg using a fibrin plate. The recombinant subtilisins BPN', Carlsberg, and recombinant pro-subtilisin DJ-4 formed clear zones on the fibrin plate (Figs. 5A, 5B, and 5C). Meanwhile, the mature subtilisin DJ-4 without a pro-sequence exhibited no enzymatic activity (Fig. 5D), confirming that the pro-sequence is essential for guiding the appropriate folding of the subtilisin to produce activity [7, 28].

In conclusion, it was found that the recombinant subtilisins (BPN', Carlsberg, and DJ-4) formed a "binding mode" at the top part of the electrophoretic gel, due to their high p*I* values. This problem was resolved when using an IEF-FZ gel with a pH range from 3 to 10 (Figs. 3 and 4). More interestingly, the recombinant subtilisin DJ-4 was successfully applied to three zymography systems (SDS-FZ, RFZ, and IEF-FZ) and a fibrin plate assay. However, as shown in Figs. 4 and 5, the pro-sequence was confirmed as an essential factor for producing the enzyme activity of subtilisins.

Acknowledgments

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