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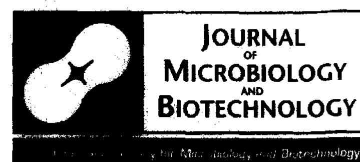

Purification and Characterization of a New Peptidase, Bacillopeptidase DJ-2, Having Fibrinolytic Activity: Produced by *Bacillus* sp. DJ-2 from *Doen-Jang*

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Abstract A new *Bacillus* peptidase, bacillopeptidase DJ-2 (bpDJ-2), with molecular mass of 42 kDa and isoelectric point (p*I*) of 3.5–3.7, was purified to homogeneity from *Bacillus* sp. DJ-2 isolated from *Doen-Jang,* a traditional Korean soybean fermented food. The enzyme was identified as an extracellular serine fibrinolytic protease. The optimal conditions for the reaction were pH 9.0 and 60°C. The first 18 amino acid residues of the N-terminal amino acid sequence of bpDJ-2 were TDGVEWNVDQIDAPKAW, which is identical to that of bacillopeptidase F (bpf). However, based on their N-terminal amino acid sequence, molecular size, and p*I*, it is different from that of bpf and extracellular 90 kDa. The whole (2,541 bp, full-bpDJ-2) and mature (1,956 bp, mature-bpDJ-2) genes were cloned, and its nucleotide sequence and deduced amino acid sequence were determined. The expressed proteins, full-bpDJ-2 and mature-bpDJ-2, were detected on SDS-PAGE at expected sizes of 92 and 68 kDa, respectively.

Key words: Bacillopeptidase, *Bacillus* sp. DJ-2, *Doen-Jang*, fibrinolytic enzyme, serine protease, zymography

Thrombolytic agents, which dissolve fibrin clots and maintain blood flow at vascular injury sites, are important components of normal hemostatic response. The major thrombolytic agents are plasminogen activators, such as urokinase, tPA (tissue type plasminogen activator) and streptokinase, and plasmin-like proteins, such as, nattokinase [24] and lumbrokinase [15].

Sumi *et al.* [24, 25] purified a powerful fibrinolytic enzyme (nattokinase) produced by *Bacillus natto* screened from *natto*, a traditional soybean fermented food in Japan. They reported that *natto* increased the fibrinolytic activity in plasma and the production of tPA [25]. Subsequently, many fibrinolytic enzymes have been identified from different traditional fermented foods [31], such as CK from *Chungkook-Jang* [13] and subtilisin DJ-4 from *Doen-Jang* [2, 10] in Korea, and subtilisin DFE from *douchi* in China [18]. All of these enzymes were discovered in the same bacterial strain, *Bacillus* sp. strains.

Bacillus sp. strains produce several extracellular proteases after the end of exponential growth, paralleling the onset of sporulation. These proteases have been characterized as alkaline serine protease or subtilisin [16, 27], neutral (metallo-) protease [28, 29], and acidic protease, esterase, or bacillopeptidase F [7, 21, 23, 30].

Bacillopeptidase F (bpf), the most poorly characterized of these serine proteases, is an acidic protease that has two forms of molecular mass, 33 (p*I*, 4.4) and 50 kDa (p*I* 5.4), produced by *Bacillus subtilis* 168 [21]. Wu *et al.* [30] demonstrated that bpf is synthesized as a preproenzyme (m=92 kDa), and that it is gradually converted into various forms with molecular masses from 80 to 40 kDa, through processing at both the NH_2 and COOH termini. In addition, several authors have reported more than one active band of bpf, however, no explanation for this multiplicity of forms has been offered [5, 19]. In the present study, we purified a 42 kDa fibrinolytic enzyme (bpDJ-2) with p*I* of 3.5–3.7 from *Bacillus* sp. DJ-2, which was confirmed to be a new form of bpf.

MATERIALS AND METHODS

Materials

Human fibrinogen, thrombin, plasmin, and p-nitroanilide chromogenic substrate (*N*-*p*-tosyl-Gly-Pro-Lys-*p*NA, T-6140)

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for plasmin, ultra-pure urea, PMSF (phenylmethylsulfonyl fluoride) and leupeptin were purchased from Sigma (U.S.A.). Ampholytes (pH range 3 to 10), prestained IEF, and protein molecular mass standards were purchased from Bio-Rad (U.S.A.). Other chemicals were of analytical grade.

Bacterial Strain and Cultural Conditions

The bacterium isolated from *Doen-Jang*, a traditional soybean fermented food in Korea, was confirmed by the Korean Collection for Type Cultures (KCTC) as a *Bacillus* sp. DJ-2 and was grown in tryptic soy broth (TSB, Difco, U.S.A.) in 1-l Erlenmeyer shaking flasks (150 rpm) at 37°C for 2 days.

Enzyme Assay

Fibrinolytic activity was determined by fibrin plate by the method previously described [10]. Twenty microliters of sample solution were applied on a fibrin plate and incubated at 37°C for 12 h. An equal volume of plasmin solution (1 NIH unit/ml) was also incubated on the fibrin plate as a positive control of fibrinolytic protease. The amidolytic activity [10, 13] was measured with a Beckman DU-70 spectrophotometer by using chromogenic substrate T-6140. Assays were carried out in 25 mM Tris-HCl buffer (pH 9.0), 0.2 ml of 0.5 mM substrate, and purified enzyme (0.3 µg/0.2 ml). The mixture was incubated at 37°C for 5 min, and the reaction was stopped by adding 0.1 ml of 50% acetic acid. Activity was determined from absorbance at 405 nm, due to formation of *p*-nitroaniline. One unit was defined as the amount of substrate hydrolyzed per minute by the enzyme.

Purification of Fibrinolytic Enzyme

To purify the bpDJ-2 with 42-kDa, *Bacillus* sp. DJ-2 was grown in TSB at 37°C for 2 days. After cultivation, the culture broth was centrifuged at 10,000 ×g for 10 min. All purification procedures were performed at 4°C with 20 mM sodium phosphate buffer, pH 6.3 (buffer A). Two liters of the crude enzyme in the culture supernatant were concentrated by ultrafiltration with PM-10 membrane (Amicon, Inc., U.S.A.). The concentrated suspension was dialyzed against 20 volumes of buffer A for one day with three buffer changes. The dialyzed suspension was loaded onto DEAE-cellulose column (2.0×10 cm) (Pharmacia Biotech, Sweden), and proteins were eluted with 300 ml of linear gradient formed from 0 to 1 M NaCl. Fractions showing the fibrinolytic activity were pooled, dialyzed against buffer A, concentrated by lyophilization, and further purified by TSK gel filtration (Toyopearl HW-55F, TOSOH, Japan) by using 0.1 M NaCl containing buffer A.

Zymography

Fibrin zymogram gel electrophoresis was carried out, as described previously [2, 11]. 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). Samples (0.1 µg) were diluted 5 times with zymogram sample buffer, which consisted of 0.5 M Tris (pH 6.8), 10% SDS, 20% glycerol, and 0.03% bromophenol blue. After 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), which contained 2.5% Triton X-100. The gel was washed with distilled water for 30 min to remove Triton X-100, and then incubated in 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 bands, corresponding to the areas where fibrin was digested, were visualized as nonstained regions of the zymogram gel.

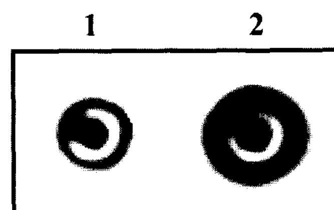

Fig. 1. Fibrinolytic activity of the purified enzyme on the fibrin plate.
Lane 1, 1.0 NIH plasmin; lane 2, the purified bpDJ-2.

SDS-PAGE and Isoelectric Focusing (IEF) Electrophoresis

SDS-PAGE was performed by the Laemmli method [12, 14]. Protein samples 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. IEF gel containing

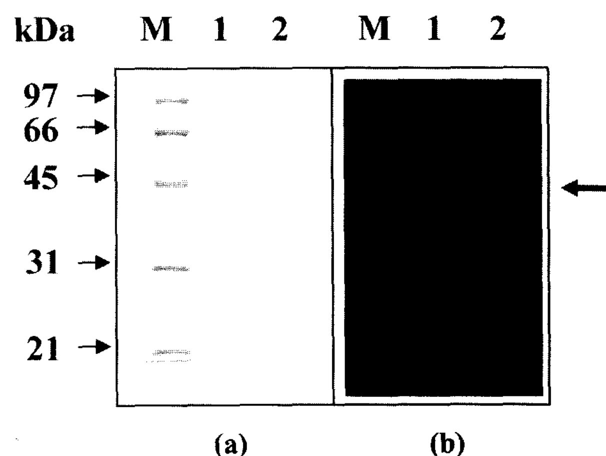

Fig. 2. SDS-PAGE and fibrin zymography of the purified fibrinolytic enzyme.
SDS electrophoresis (12%) (a) and fibrin zymography (b) were performed. Lane M, molecular mass marker proteins; lane 1, reduced purified bpDJ-2; lane 2, unreduced purified bpDJ-2.

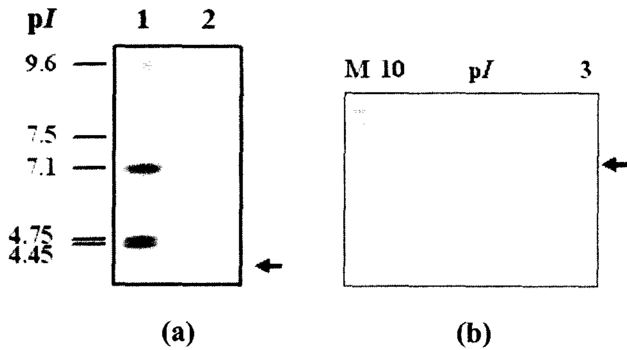

Fig. 3. IEF (a) and two-dimensional electrophoresis (b) of the purified fibrinolytic enzyme.
Lane 1, isoelectric point marker proteins; lanes 2 and 3 purified bpDJ-2; lane M, the same molecular marker in Fig. 2.

8 M urea was prepared, according to the method of Robertson *et al.* [20].

Two-Dimensional SDS-PAGE

For further study, 2-D SDS-PAGE was performed. The IEF gel, used in the first dimension, was subjected to SDS-PAGE. After IEF electrophoresis, the gel slice was equilibrated with buffer solution [62.5 mM Tris (pH 6.8), 2.3% SDS, and 10% glycerol] for 30 min. The equilibrated gel slices were placed on the stacking gel of the SDS-PAGE gel to perform the electrophoresis in the second dimension.

Determination of N-Terminal Amino Acid Sequence of Purified Enzyme

After SDS-PAGE, the purified enzyme on the gel was transferred to a polyvinylidene difluoride membrane by

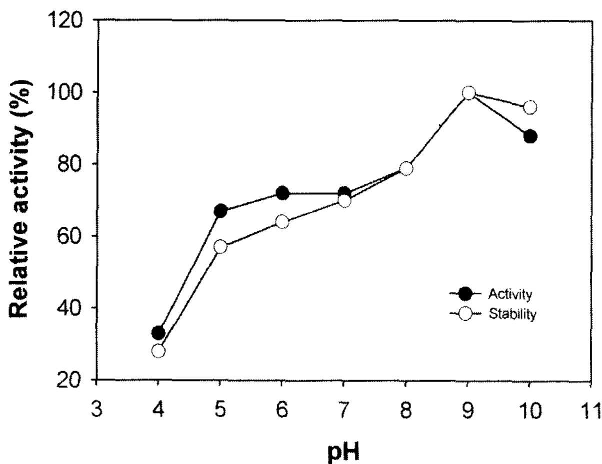

Fig. 4. Effect of pH on fibrinolytic activity and stability of the purified bpDJ-2 at 37°C.
After incubation of the enzyme over the pH range of 4.0- 10.0 for 2 h at 37°C, the residual enzyme activity was measured.

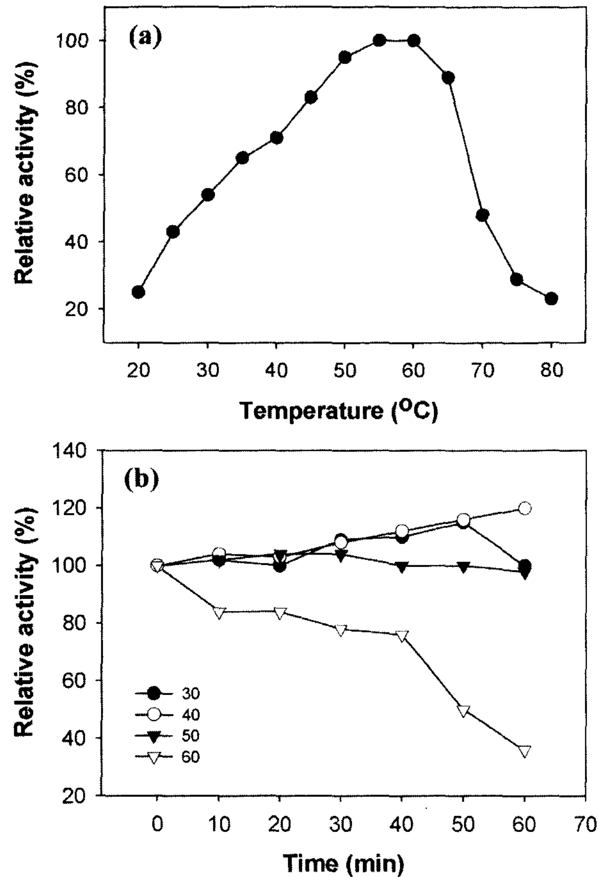

Fig. 5. Effect of temperature on fibrinolytic activity and stability of the purified bpDJ-2.
(a) The activity was assayed at various temperatures in 25 mM Tris-HCl buffer (pH 9.0). (b) To establish the thermostability of bpDJ-2, the residual enzyme activity was measured after incubation at the indicated temperatures for 1 h.

Table 1. Effect of metal ions and various inhibitors on the purified bpDJ-2 activity.

Metal ions (5 mM)/Inhibitors (1 mM)	Activity (%)
None	100
$CaCl_2$	92.3
$CdCl_2$	72.5
$CoCl_2$	105
$CuSO_4$	82.5
$NiCl_2$	84.2
$MgCl_2$	115.0
$MnCl_2$	98.4
$ZnCl_2$	70.2
PMSF	30.4
Leupeptin	91.6
EDTA	104.6
SDS	72.6

PMSF was dissolved in isopropanol and the others were dissolved in distilled water.
After incubation in 25 mM Tris-HCl buffer (pH 9.0) at 37°C for 30 min, remaining fibrinolytic activity was determined by chromogenic substrate (T-6140), as described in Materials and Methods.

Table 2. Kinetic constants of bpDJ-2, subtilisin BPN', and subtilisin Carlsberg for amidolysis of *N-p*-tosyl-Gly-Pro-Lys-*p*NA.

BpDJ-2			Subtilisin BPN'			Subtilisin Carlsberg		
k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($s^{-1}M^{-1}$)	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($s^{-1}M^{-1}$)	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($s^{-1}M^{-1}$)
68.08	0.42	1.6×10^5	82.41	0.31	2.7×10^5	102.3	0.24	4.3×10^5

The samples were monitored continuously by the liberation of p-nitroaniline at 405 nm. Kinetic constants were determined in 1.0 ml of 0.1 M Tris-HCl buffer (pH 9.0) containing 0.1 M NaCl at 37°C.

electroblotting [22] and stained with Coomassie blue. The stained material was excised and used for N-terminal sequencing directly by the automated Edman degradation method using a gas-phase protein sequencer (model Procise 491, ABI, U.S.A.).

Expression of Recombinant Full- and Mature-bpDJ-2

Chromosomal DNA from *Bacillus* sp. DJ-2 was prepared by the method described previously [3, 6, 9] and used as the template for PCR. The full- and mature-bpDJ-2 were PCR amplified by using the *Bam*HI-linked sense primers (5'-GGCCGGATCCAATGAGGAAAAAAACGAAA-3' for full and 5'-GGCCGGATCC AACTGACGGTGTGGAAT-GGAA-3' for mature) and the *Xho*I-linked antisense primer (5'-GCCCTCGAG CCACTACACTGACTTGTG-3'). PCR amplification was performed under the following conditions: 30 cycles of 95°C for 30 sec, 55°C for 30 sec, and 72°C for 1.5 min. The PCR-amplified DNA fragments were extracted from agarose gel and then ligated with the pGEM-T Easy vector (Promega, U.S.A.). After digestion with *Bam*HI and *Xho*I, the fragments were inserted into bacterial expression vector pET22b (Novagen, Germany) and then expressed in *E. coli* BL21 (DE3). The recombinant proteins obtained from inclusion bodies were dissolved in 20 mM Tris-HCl (pH 7.4) buffer containing 8 M urea, and their sizes were confirmed by SDS-PAGE.

RESULTS

Purification of Fibrinolytic Enzyme from *Bacillus* sp. DJ-2

The fibrinolytic enzyme from *Bacillus* sp. DJ-2 was purified to electrophoretical homogeneity by using commercial chromatographic techniques. The fibrinolytic activity of bpDJ-2 was measured to be 7.32 plasmin NIH units on the fibrin plate (Fig. 1). It migrated as a single band with an apparent molecular mass of 42 kDa on SDS-PAGE and fibrin zymography under both reducing and nonreducing conditions (Fig. 2), and its isoelectric point (p*I*) was determined to be 3.5-3.7 by isoelectric focusing (Fig. 3a). BpDJ-2 was further confirmed by its molecular mass and p*I* value on the 2-D gel (Fig. 3b).

Effect of pH and Temperature on Fibrinolytic Activity and Stability

The effect of pH on the activity of bpDJ-2 was determined in buffers of various pH values, and results showed that bpDJ-2 was active over a wide range of pH (6.0-10.0) and was most active at pH 9.0 (Fig. 4). The enzyme was very stable in the pH range of 6.0-10.0 at 37°C for 2 h, but the enzyme activity and stability rapidly decreased at below pH 6.0 (Fig. 4). The enzyme activity increased with increasing temperature up to 55 or 60°C, thereafter sharply decreasing at 70°C (Fig. 5a). Based on the observation that about 40% of the activity still remained after incubation for 1 h at 60°C, bpDJ-2 was considered to be a relatively heat-resistant enzyme (Fig. 5b).

Effect of Inhibitors and Metal ions on Fibrinolytic Activity

The effects of inhibitors and metal ions on the fibrinolytic activity are summarized in Table 1. bpDJ-2 was inhibited by PMSF and SDS, but was not affected by leupeptin, EDTA, or EGTA. These results indicate that bpDJ-2 is a serine protease. Furthermore, the enzyme activity was inhibited by Cd^{2+}, Cu^{2+}, or Zn^{2+}, but not by Ca^{2+}, Co^{2+}, Mg^{2+}, or Mn^{2+}.

Amidolytic Activity

The fibrinolytic activity and caseinolytic activity of bpD-2 were investigated with synthetic substrate (T-6140) (Table 2). Based on K_{cat}/K_m, the amidolytic activity of bpDJ-2 was 1.7- and 2.7-fold less efficient than those of subtilisin BPN' and subtilisin Carlsberg, respectively.

Table 3. Comparison of bpDJ-2 with other proteases for enzyme activity.

Protease sources	Fibrinolytic activity (U)	Caseinolytic activity (U)	F/C (%)
BpDJ-2 from *Bacillus* sp. DJ-2	59.92	57.70	103.8 (100)
Subtilisin BPN' from *B. amyloliquefaciens*	53.36	102.62	52.0 (50)
Subtilisin Carlsberg from *B. licheniformis*	38.12	131.56	29.0 (28)

Mean values of three measurements are presented.

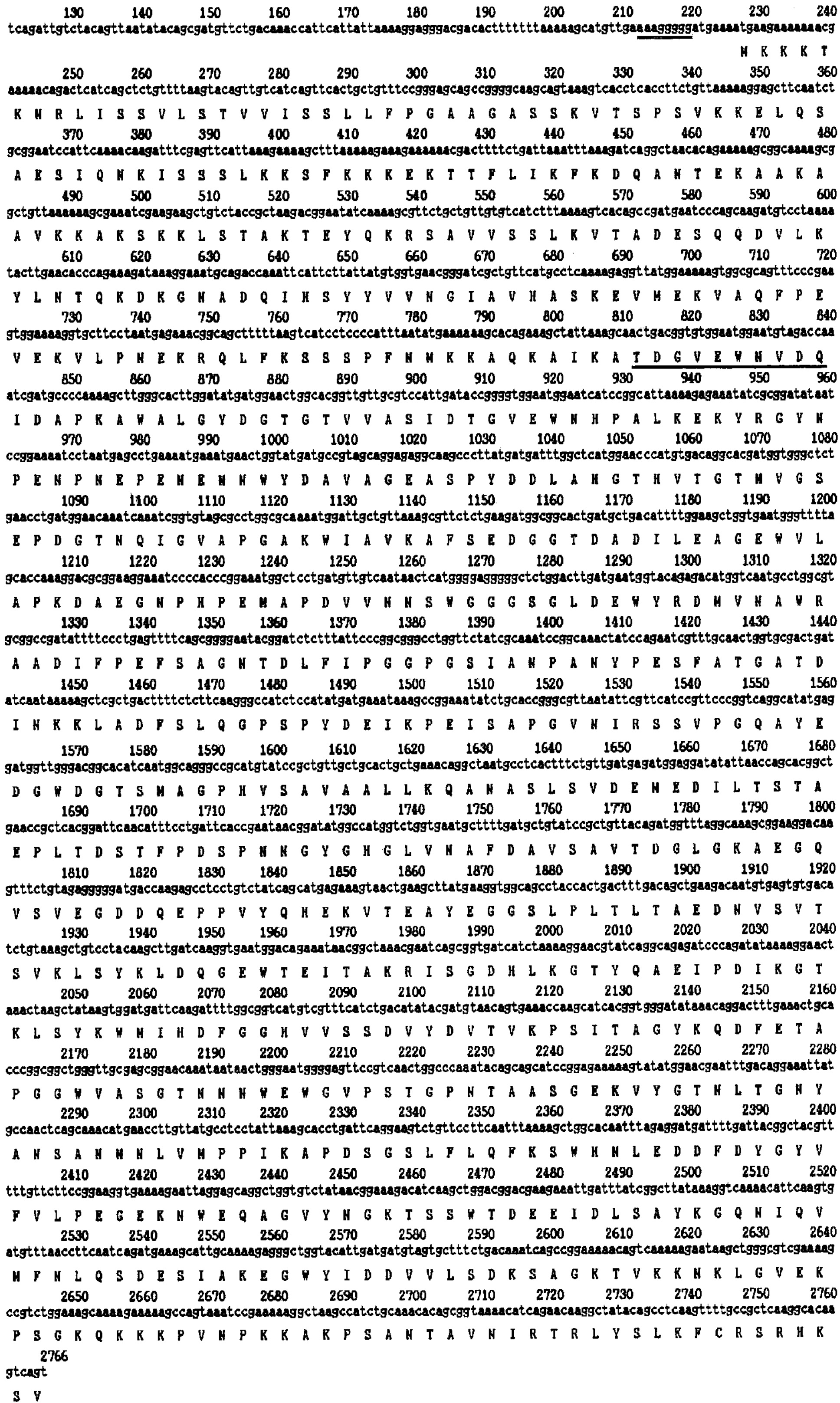

Fig. 6. Nucleotide sequence of bpDJ-2. The amino acid sequence is shown below the nucleotide sequence.
The open reading frame for bpDJ-2 extends from base 226 to base 2766. Ribosome-binding sites are double-underlined. Amino acid sequences underlined by a single straight line indicate sequence of mature-bpDJ-2.

Comparison of bpDJ-2 with Other Proteases for Fibrinolytic Activity

The fibrinolytic activity and caseinolytic activity of bpDJ-2 and other proteases were measured and the ratios of fibrinolytic to caseinolytic activity (F/C) were calculated. As shown in Table 3, the specific F/C ratio of bpDJ-2 was 2.0 and 3.6 times higher than those of subtilisin BPN' and subtilisin Carlsberg, respectively.

N-Terminal Amino Acid Sequence of bpDJ-2

The N-terminal amino acid sequence of the purified bpDJ-2 was analyzed by the automated Edman degradation method

```
                 10        20        30        40        50
Bpf     MRKKTKNRLISSVLSTVVISSLLFPGAAGASSKVTSPSVKKELQSAESIQ
BpDJ-2  MKKKTKNRLISSVLSTVVISSLLFPGAAGASSKVTSPSVKKELQSAESIQ
                 60        70        80        90       100
        NKISSSLKKSFKKKEKTTFLIKFKDLANPEKAAKAAVKKAKSKKLSAAKT
        NKISSSLKKSFKKKEKTTFLIKFKDQANTEKAAKAAVKKAKSKKLSTAKT
                110       120       130       140       150
        EYQKRSAVVSSLKVTADESQQDVLKYLNTQKDKGNADQIHSYYVVNGIAV
        EYQKRSAVVSSLKVTADESQQDVLKYLNTQKDKGNADQIHSYYVVNGIAV
                160       170       180       190       200
        HASKEVMEKVVQFPEVEKVLPNEKRQLFKSSSPFNMKKAQKAIKATDGVE
        HASKEVMEKVAQFPEVEKVLPNEKRQLFKSSSPFNMKKAQKAIKATDGVE
                210       220       230       240       250
        WNVDQIDAPKAWALGYDGTGTVVASIDTGVEWNHPALKEKYRGYNPENPN
        WNVDQIDAPKAWALGYDGTGTVVASIDTGVEWNHPALKEKYRGYNPENPN
                260       270       280       290       300
        EPENEMNWYDAVAGEASPYDDLAHGTHVTGTMVGSEPDGTNQIGVAPGAK
        EPENEMNWYDAVAGEASPYDDLAHGTHVTGTMVGSEPDGTNQIGVAPGAK
                310       320       330       340       350
        WIAVKAFSEDGGTDADILEAGEWVLAPKDAEGNPHPEMAPDVVNNSWGGG
        WIAVKAFSEDGGTDADILEAGEWVLAPKDAEGNPHPEMAPDVVNNSWGGG
                360       370       380       390       400
        SGLDEWYRDMVNAWRAADIFPEFSAGNTDLFIPGGPGSIANPVNYPESFA
        SGLDEWYRDMVNAWRAADIFPEFSAGNTDLFIPGGPGSIANPANYPESFA
                410       420       430       440       450
        TGATDINKKLADFSLQGPSPYDEIKPEISAPGVNIRSSVPGQTYEDGWDG
        TGATDINKKLADFSLQGPSPYDEIKPEISAPGVNIRSSVPGQAYEDGWDG
                460       470       480       490       500
        TSMAGPHVSAVAALLKQANASLSVDEMEDILTSTAEPLTDSTFPDSPNNG
        TSMAGPHVSAVAALLKQANASLSVDEMEDILTSTAEPLTDSTFPDSPNNG
                510       520       530       540       550
        YGHGLVNAFDAVSAVTDGLGKAEGQVSVEGDDQEPPVYQHEKVTEAYEGG
        YGHGLVNAFDAVSAVTDGLGKAEGQVSVEGDDQEPPVYQHEKVTEAYEGG
                560       570       580       590       600
        SLPLTLTAEDNVSVTSVKLSYKLDQGEWTEITAKRISGDHLKGTYQAEIP
        SLPLTLTAEDNVSVTSVKLSYKLDQGEWTEITAKRISGDHLKGTYQAEIP
                610       620       630       640       650
        DIKGTKLSYKWMIHDFGGHVVSSDVYDVTVKPSITAGYKQDFETAPGGWV
        DIKGTKLSYKWMIQDFGGHVVSSDIYDVTVKPSITVGYKQDFETAPGGWV
                660       670       680       690       700
        ASGTNNNWEWGVPSTGPNTAASGEKVYGTNLTGNYANSANMNLVMPPIKA
        ASGTNNNWEWGVPSTGPNTAASGEKVYGTNLTGNYANSANMNLVMPPIKA
                710       720       730       740       750
        PDSGSLFLQFKSWHNLEDDFDYGYVFVLPEGEKNWEQAGVYNGKTSSWTD
        PDSGMLFLQFKSWHNLEDDFDYGYVFVLPEGEKNWEQAGVYNGKTSSWTD
                760       770       780       790       800
        EEIDLSAYKGQNIQVMFNLQSDESIAKEGWYIDDVVLSDKSAGKTVKKNK
        EEIDLSAYKGQNIQVMFNLQSDESIAKEGWYIDDVVLSDKSAGKTVKKNK
                810       820       830       840     848
        LGVEKPSGKQKKKPVNPKKAKPSANTAVNIRTRLYSLKFCRSRHKSV
        LGVEKPSGKQKKKPVNPKKAKPSANTAVNIRTRLYSLKFCRSRHKSV
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Fig. 7. Comparison of the amino acid sequence of bpDJ-2 with that of bpf.
The different amino acid sequences between bpDJ-2 and bpf are underlined.

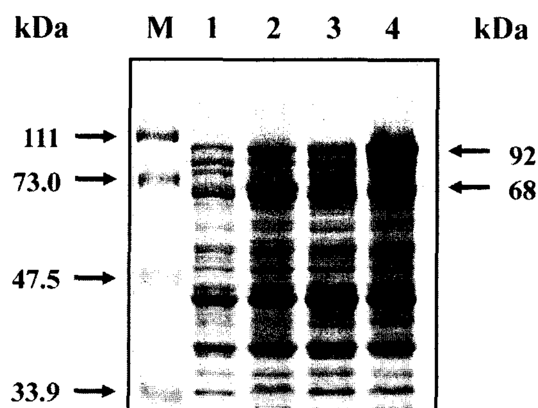

Fig. 8. SDS-PAGE (12%) analysis of expressed full- and mature-bpDJ-2.
Lanes 1 and 3, *E. coli* BL21 protein as a control; lane 2, expressed mature-bpDJ-2; lane 4, expressed full-bpDJ-2.

after SDS-PAGE and electroblotting. The sequence of the first 18 residues was found to be TDGVEWNVDQIDAPKAW, which is identical to that of bpf of *B. subtilis*, but different from that of bpf and extracellular 90 kDa serine proteinase [7, 21].

Cloning and Sequencing of bpDJ-2 Gene

The gene for bpDJ-2 from *Bacillus* sp. DJ-2 was cloned and its nucleotide sequence was determined. The nucleotide sequence analysis revealed only one large open reading frame, composed of 2,541 base pairs and 847 amino acid residues (Fig. 6). bpDJ-2 is identical to that of the bacillopeptidase F (bpf), except for eleven amino acid substitutions, which are five in the propeptide region and six in the mature protein (Fig. 7) [30].

Expression of Recombinant Full- and Mature-bpDJ-2

The full- and mature-bpDJ-2 genes were cloned and expressed. The recombinant proteins obtained from inclusion bodies were dissolved in 20 mM Tris-HCl (pH 7.4) buffer containing 8 M urea. The expressed full- and mature-bpDJ-2 proteins, as shown in Fig. 8, were detected on SDS-PAGE at expected sizes of 92 and 68 kDa, respectively.

DISCUSSION

This article describes the purification and characterization of bpDJ-2 produced by *Bacillus* sp. DJ-2 which was isolated from *Doen-Jang*, a traditional Korean soybean fermented food. The molecular mass of bpDJ-2, 42 kDa calculated by both SDS-PAGE and fibrin zymography (Fig. 1), was similar to that of Katsuwokinase (38 kDa) [18, 26] and KA38 (41 kDa) [8], but there was no sequence homology among them. In addition, the enzyme activity of bpDJ-2 was the same, whether in the presence or absence of a reducing agent, 2-mercaptoethanol (Fig. 1). bpDJ-2 was active over a broad range of pH, with higher activity in alkaline pH, therefore, it was classified as an alkaline protease in spite of its acidic p*I* value of 3.5-3.7. The optimum temperature of bpDJ-2 was 55 to 60°C, which was comparable to that of IMR-NK1 [1], but lower than that of CK [13].

The kinetic constant of bpDJ-2 for the plasmin substrate (T-6140) indicated that it was 1.7- and 2.7-fold less efficient than those of subtilisin BPN' and subtilisin Carlsberg, respectively (Table 2). On the other hand, the specific F/C ratio of bpDJ-2 was 2.0 and 3.6 times higher than those of subtilisin BPN' and subtilisin Carlsberg, respectively (Table 3). Furthermore, the specific F/C ratio of bpDJ-2 was 103.8, which is higher than that of CK (73.0) [11] and IMR-NK1 (80.6) [1], but lower than that of subtilisin DFE (192) [18] (Table 3).

The N-terminal 18 amino acids sequence of bpDJ-2 is identical to that of bpf from *Bacillus subtilis* [21, 23, 30]. Compared with previously reported bpf types, bpDJ-2 is a new form of bpf. Roitsch and Hageman [21] isolated two forms of bpf with molecular masses of 33 and 50 kDa, which have p*I* of 4.5 to 5.4. In order to generate the 80 and 50 kDa proteins, proteolytic cleavage must have occurred at Lys-Ala in positions 194 to 195 (Ala-195 bpf) and 197 to 198 (Ala-198 bpf), respectively [30]. Furthermore, a new extracellular 90 kDa serine proteinase of p*I* 3.9, which is identical to that of Ala-195 bpf, was also purified [7]. However, the bpDJ-2 protein purified by us contains Ala-Thr at positions 198-199 (Thr-199 bpf), and the molecular mass and p*I* are 42 kDa and 3.5-3.7, respectively. These variations are possibly due to an additional proteolytic processing of this version of bpDJ-2, since the cells were cultivated. However, it remains to be elucidated whether this processing step is mediated by bpDJ-2 or by other proteases produced by *Bacillus* sp. DJ-2. Based on the above results, it is strongly suggested that the fibrinolytic enzyme of *Bacillus* sp. DJ-2 is a new type of enzyme.

The recombinant full- and mature-bpDJ-2 proteins were detected at expected sizes of 92 and 68 kDa on SDS-PAGE, respectively (Fig. 8). Based on the data of purified and expressed bpDJ-2, it is highly likely that proteolytic processing occurred also at the COOH terminus of bpf. At present, the relationship between characteristics and the N- and C-terminals of the peptidase has yet to be confirmed.

Acknowledgments

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