Potent Fibrinolytic Enzyme from a Mutant of *Bacillus subtilis* IMR-NK1

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A mutant of *Bacillus subtilis* IMR-NK1, which is used for the production of domestic "natto" in Taiwan, produced high fibrinolytic enzyme activity by solid-state fermentation using wheat bran as medium. In addition, a strong fibrinolytic enzyme was purified from the cultivation media. The purified enzyme was almost homogeneous, as examined by SDS−PAGE and capillary electrophoresis. The enzyme had an optimal pH of 7.8, an optimal temperature of 55 °C, and a K_m of 0.15% for fibrin hydrolysis. The molecular mass estimated by gel filtration was 31.5 kDa, and the isoelectric point estimated by isoelectric focusing electrophoresis was 8.3. The enzyme also showed activity for hydrolysis of fibrinogen, casein, and several synthetic substrates. Among the synthetic substrates, the most sensitive substrate was *N*-succinyl-Ala-Ala-Pro-Phe-*p*NA. PMSF and NBS almost completely inhibited the activity of the enzyme. These results indicate that the enzyme is a subtilisin-like serine protease, similar to nattokinase from *Bacillus natto*.

Keywords: *Bacillus subtilis; fibrinolytic enzyme; purification; characterization; natto*

INTRODUCTION

Bacillus species produce a variety of extracellular and intracellular proteases. Of these products, an alkaline protease (subtilisin) and a neutral metalloprotease are secreted into the medium, whereas at least two intracellular serine proteases are produced within *Bacillus* species (Hirushi and Kadota, 1976; Mantsala and Zalkim 1980; Prestidge et al., 1971; Strogin et al., 1978). Subtilisins are a family of alkaline serine endoproteases secreted by a wide variety of *Bacillus* species. For example, subtilisin Carlsberg is secreted from *B. lichentiformis*; subtilisin E is secreted from *B. subtilis*; and subtilisin Amylosaccharicticus is secreted from *B. amylosaccharicticus* (Markland and Smith, 1967, 1971; Smith et al., 1968; Stahl et al., 1984; Nedkov et al., 1985). The production of subtilisin protease has been exploited for laundry detergents, food processing, and other applications (Godfrey and Reichelt, 1983; Kame et al., 1973).

A potent fibrinolytic enzyme (nattokinase; NK) was previously isolated from a traditional fermented food in Japan, "natto", by Sumi et al. (1987). This enzyme is an extracellular serine protease produced from *Bacillus natto*, a bacteria used for the production of natto from soybeans by solid-state fermentation. Sumi et al. (1990) further demonstrated that oral administration of natto or NK capsules enhanced fibrinolysis in canine plasma in an experimental thrombosis model. Fujita et al. (1993) characterized the fibrinolytic enzyme as a subtilisin-like serine protease. Recently Kim et al. (1996) purified a strong fibrinolytic enzyme from *Bacillus* sp. strain CK-11-4 (CK), which was screened from Chungkook-Jang, a traditional Korean fermented soybean sauce. This enzyme was characterized as a thermophilic alkaline serine protease. Yasuda et al. (1999) isolated a soybean milk coagulating enzyme (SMCE) from *Bacillus pumilus* TYO-67 for the production of "tofuyo" (a traditional fermented soybean cheese in Okinawa, Japan). They characterized that enzyme as a subtilisin Carlsberg-like protease.

A mutant of *Bacillus subtilis* IMR-NK1 was isolated from Taiwan soil by Wang and Lin (1992). This microorganism produced high activities of fibrinolytic enzyme and chitosanase by solid-state fermentation using wheat bran as medium. This strain has been applied in Taiwan for the production of domestic natto from soybeans by solid-state fermentation. In this paper, we report the purification and some properties of the fibrinolytic enzyme produced by this mutant strain.

MATERIALS AND METHODS

Chemicals. Chicken ovomucoid and ovoinhibitor, trypsin inhibitors (turkey egg white, lima bean, soybean), trypsin−chymotrypsin inhibitor (soybean), leupeptin, aprotinin, *p*-aminobenzamidine, ethyl acetimidate (EAM), *p*-hydroxymercuribenzoate (PHMB), diethyl pyrocarbonate (DEPC), *N*-acetylimidazole (NAI), *N*-ethyl-5-phenylisoxazoline-3′-sulfonate (WRK), phenylmethanesulfonyl fluoride (PMSF), and the synthetic substrates were purchased from Sigma (St. Louis, MO). Polybuffer exchanger PBE 94, Polybuffer 74, Sephacryl S-100 HR, and Superdex 75 HR columns (1.0 × 30 cm) were from Pharmacia (Uppsala, Sweden). Gelatin and *N*-bromosuccinimide (NBS) were from Merck (Darmstadt, Germany). Bicinchoninic acid protein assay reagent was obtained from Pierce (Rockford, IL). Buffer salts and other chemicals used were of reagent grade.

Crude Enzyme. Crude fibrinolytic enzyme from a mutant of *B. subtilis* IMR-NK1 was kindly supplied by Dr. Ching-Fwu Lin of the Institute for Microbial Resources, Taiwan. The mutant of *B. subtilis* was isolated from Taiwan soil and cultured at 30 °C in solid state using wheat bran as a cultivation medium for 60 h. The enzyme produced was extracted with 1% NaCl, concentrated by ultrafiltration, and dried by lyophilization.

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Table 1. Purification of the Fibrinolytic Enzyme from *B. subtilis* IMR-NK1

procedure	total activity (units)	total protein (mg)	specific activity (units/mg)	purifn (fold)	yield (%)
crude enzyme[a]	1180000	2460	479	1	100
ammonium sulfate fractionation	1080000	580	1860	3.9	92
Sephacryl S-100 HR gel filtration and lyophilization	532000	234	2270	4.7	45
PBE 94 chromatofocusing	84500	25	3380	7.1	7.2
Superdex 75 HR gel filtration	77000	17.5	4400	9.2	6.5

[a] Twenty grams of crude fibrinolytic enzyme was dissolved in 200 mL of 25 mM imidazole−HCl (20 mM $CaCl_2$, pH 7.4).

Purification of Fibrinolytic Enzyme. All purification steps were carried out at 4 °C. Twenty grams of crude enzyme was dissolved in 200 mL of 25 mM imidazole−HCl buffer (20 mM $CaCl_2$, pH 7.4) and stirred for 30 min. The precipitate was removed by centrifugation at 8600*g* for 10 min. The supernatant was fractionated by the addition of $(NH_4)_2SO_4$. The precipitate formed between 40 and 80% saturation of $(NH_4)_2SO_4$ was collected by centrifugation and dissolved in 4 mL of extraction buffer. After centrifugation, the supernatant was applied to a Sephacryl S-100 HR column (2.6 × 75 cm). The column was eluted with 25 mM imidazole−HCl buffer (pH 7.4) at a flow rate of 20 mL/h. Fractions (10 mL/fraction) containing the enzyme activity were pooled, lyophilized, and dissolved in 4 mL of water. After dialysis with 25 mM imidazole−HCl buffer (pH 7.4) and centrifugation, the supernatant was applied to a PBE 94 Polybuffer exchanger column (0.9 × 30 cm). The column was washed with 50 mL of 25 mM imidazole−HCl (pH 7.4) and eluted with 250 mL of diluted (1:8) Polybuffer (pH 4.0) at a flow rate of 10 mL/h (5 mL/fraction). Fractions containing fibrinolytic activity were collected and precipitated with an 80% saturation of $(NH_4)_2SO_4$. The precipitate was dissolved in 2 mL of 0.05 M phosphate buffter (0.15 M NaCl, pH 7). Of this, 0.3 mL was applied to a Superdex 75 HR column (1.0 × 30 cm). The column was eluted with the above buffer using an FPLC system (Pharmacia) at a flow rate of 30 mL/h. Fractions containing the enzyme activity were collected.

Determination of Fibrinolytic Activity. Fibrinolytic activity was measured by the hydrolysis of fibrin according to the procedure described by Anson (1939) with some modifications. The incubation mixture contained 2.5 mL of 1.2% fibrin (1.2 g of fibrin was dissolved in 80 mL of 0.2 N NaOH and adjusted to pH 7.8 with 6 N HCl; the final volume was adjusted to 100 mL with water), 2.5 mL of 0.1 M Tris-HCl (10 mM $CaCl_2$, pH 7.8), and a suitable amount of enzyme in a total volume of 6 mL. The incubation was carried out at 30 °C for 10 min. The reaction was stopped by adding 5 mL of 0.11 M trichloroacetic acid containing 0.22 M sodium acetate and 0.33 M acetic acid. The absorbance at 275 nm of the trichloroacetic acid (0.05 M) soluble product was determined. A fibrinolytic unit was defined as the amount of enzyme that gave an increase in absorbance at 275 nm equivalent to 1 μg of tyrosine per minute at 30 °C.

Determination of Protein. Protein was measured according to the bicinchoninic acid (Smith et al., 1985) using bovine serum albumin as the standard.

PAGE. PAGE was performed on 12% acrylamide gel plates in a mini-gel system (Hoefer SE 250) at pH 4.3 using β-alanine−acetate buffer (pH 4.5) as the electrode buffer according to the method of Reisfeld et al. (1962). The distribution of protein on the gel plate was revealed by staining with Coomassie blue. For activity staining, PAGE was done on 12% acrylamide gel plates containing 0.14% fibrin. After electrophoresis, the gel was washed with 0.05 M Tris-HCl (20 mM $CaCl_2$, pH 7.8) and then immersed in the same buffter at 37 °C overnight. The enzyme activity was revealed as a colorless band with blue background after washing with 0.11 M trichloroacetic acid and staining with Coomassie blue. SDS−PAGE was carried out with PhastGel gradient 8−25 according to the manufacturer's instruction (PhastSystem user manual) or with 12% polyacrylamide gel according to the method of Laemmli (1970). After electrophoresis, the gels were stained with PhastGel Blue R and destained with destaining solution (methanol/acetic acid/water = 3:1:6).

Capillary Zone Electrophoresis (CZE). A P/ACE system 5010 (Beckman) was used for CZE. The Superdex 75 column purified fibrinolytic enzyme was examined in 50 mM phosphate buffer (pH 2.5) using an uncoated fused silica capillary (75 μm × 57 cm, 7 cm to the detector). Runs were made at 20 kV at 20 °C, and detection was performed at 214 nm.

Fibrin Plate Assay. Fibrin plates were prepared by adding agarose to a level of 1% in 1.5% fibrin. After the added agarose was dissolved with heating, the medium (10 mL) was poured into a Petri dish. A hole (2 mm in diameter) on a solidified plate was punched for sample application. After incubation at 37 °C overnight, the colorless zone was observed either directly or after washing with 0.11 M trichloroacetic acid.

Amidolytic Activity Determination. The reaction mixture containing 0.5 mL of 1.7 mM synthetic substrate, 0.5 mL of 25 mM Tris-HCl buffter (10 mM $CaCl_2$, pH 7.8), and 0.2 mL of enzyme was incubated at 37 °C for 10 min, and then 0.5 mL of 0.2 M acetic acid was added to stop the reaction. The absorbance was determined at 405 nm of the *p*-nitrophenol formed.

N-Terminal Amino Acid Sequence Analysis. After dialysis and lyophilization, the purified enzyme was used for automated Edman degradation with an Applied Biosystems 477 A protein sequencer.

Isoelectric Point (p*I*) Determination. The p*I* of the purified fibrinolytic enzyme (NBS blocked) was measured by isoelectric focusing (IEF) on PhastGel IEF 3-9 and compared with standards from an IEF calibration kit (Pharmacia LKB) according to the manufacturer's instructions (PhastSystem user's manual). Standard IEF conditions included prefocusing of carrier ampholytes at 75 Vh and focusing of the sample at 410 Vh at 2.5 mA and 15 °C. Samples were applied to the gel using the 8 × 1 μL comb.

RESULTS

Purification of Fibrinolytic Enzyme. The purification accomplished by the processes employed is summarized in Table 1. At the final step the enzyme was purified 9.2-fold over the first crude enzyme with a yield of >6.5%. Chromatofocusing was performed on a Polybuffer exchanger column equilibrated with 25 mM imidazole−HCl buffer (pH 7.4). As shown in Figure 1, the enzyme was not adsorbed by the exchanger and emerged immediately from the column. This implies that the enzyme has an isolectric point >7.4. At the final stage of purification, the recovery of the enzyme activity was rather low, especially after the chromatofocusing step. Because the activity did not stick to the chromatofocusing column, it is unlikely to have lost activity in this step. Perhaps much of the activity loss occurred during lyophilization and dialysis before the enzyme was applied to the chromatofocusing column.

Polyacrylamide Gel and Capillary Electrophoresis. The acidic electrophoretic patterns at individual steps of gel filtration and chromatofocusing are shown in Figure 2A, where a major protein band can be seen in both steps. The zymogram of the proteolytic enzyme is shown in Figure 2B, where the enzyme activity bands (lanes 1 and 3) match the major protein band. If the enzyme was treated with PMSF before electrophoresis,

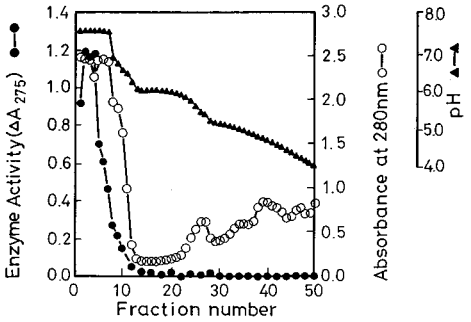

Figure 1. Chromatofocusing of the fibrinolytic enzyme of *B. subtilis* IMR-NK1 on Polybuffer exchanger (PBE 94) column. The column was equilibrated with 25 mM imidazole−HCl buffer (pH 7.4). Twenty five milligrams of Sephacryl S-100 HR gel filtration purified fibrinolytic enzyme in 25 mL of equilibrium buffer was applied. The column was washed with 50 mL of 25 mM imidazole−HCl (pH 7.4) and eluted with 250 mL of diluted (1:8) Polybuffer (pH 4.0) at a flow rate of 10 mL/h. Fractions of 5 mL were collected.

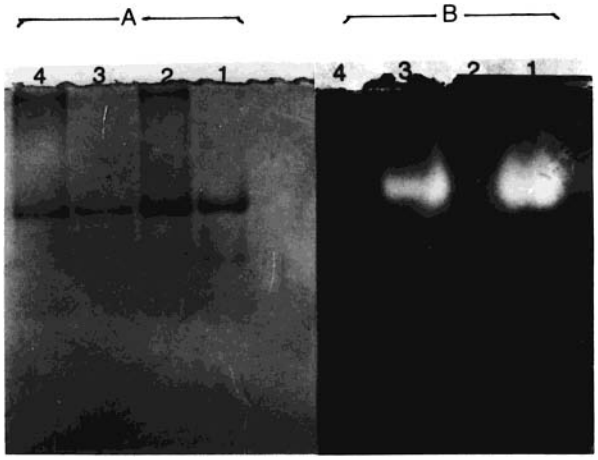

Figure 2. PAGE of the fibrinolytic enzyme of *B. subtilis* IMR-NK1: (A) Electrophoresis was carried out in 12% polyacrylamide gel in 0.35 M β-alanine−acetate buffer (pH 4.5). The protein in the gel plate was revealed by staining with Coomassie brilliant blue G-250. (B) Electrophoresis was carried out in 12% polyacrylamide gel containing 0.14% fibrin. After immersion of the gel plate in 0.05 M Tris-HCl (pH 7.8) to degrade the fibrin, the fibrinolytic activity in the gel plate was revealed as a colorless band with blue background by washing with 0.11 M trichloroacetic acid and staining with Coomassie brilliant blue G-250. Lanes 1 and 2 are PBE-94 purified enzyme with (lane 2) or without (lane 1) PMSF treatment; lanes 3 and 4 are Sephacryl S-100 HR purified enzyme with (lane 4) or without (lane 3) PMSF treatment.

the activity band disappeared (lanes 2 and 4). SDS−PAGE of the chromatofocused purified fibrinolytic enzyme revealed two major protein bands with molecular masses of approximately 43 and 30 kDa (Figure 3). However, only one protein band with a molecular mass of approximately 30 kDa was observed as the enzyme was further purified by FPLC on Superdex 75 HR (Figure 4). The purified enzyme was almost homogeneous, as analyzed by CZE (Figure 5).

Molecular Mass. According to Andrews (1965), the molecular mass of the fibrinolytic enzyme was 31.5 kDa, as estimated by gel filtration on Superdex 75 HR using the FPLC System. This value is close to the value estimated by SDS−PAGE (approximately 30 kDa) as described above. Therefore, the enzyme is a monomeric protein.

Optimal pH and pH Stability. The optimal pH of the fibrinolytic enzyme for hydrolysis of fibrin was

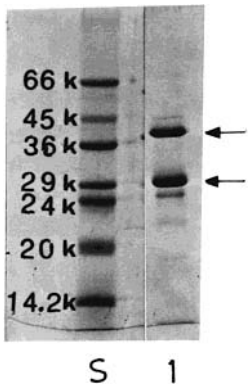

Figure 3. SDS−PAGE of partially purified fibrinolytic enzyme of *B. subtilis* IMR-NK1: (lane 1) chromatofocused purified enzyme; (lane S) low molecular weight standard proteins [bovine serum albumin (67 kDa); egg albumin (45 kDa); glyceraldehyde-3-phosphate dehydrogenase (45 kDa); carbonic anhydrase (29 kDa); trypsinogen (24 kDa); soybean trypsin inhibitor (20 kDa); α-lactalbumin (14.2 kDa)]. One hundred micrograms of chromatofocused purified fibrinolytic enzyme (2 mM PMSF treated) was heated in boiling water for 5 min with 50 μL of an aqueous solution containing 1% SDS and 2% 2-mercaptoethanol. Electrophoresis was carried out with 12% acrylamide gel according to the method of Laemmli (1970).

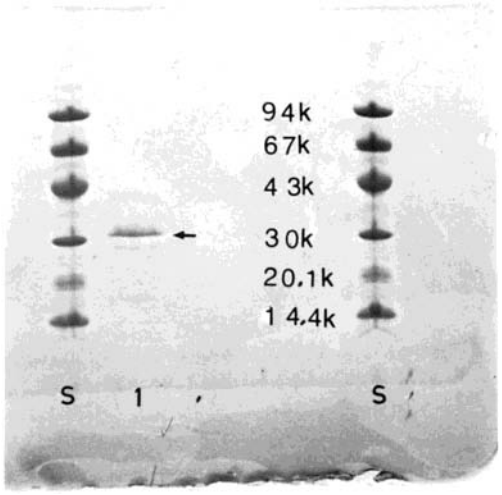

Figure 4. SDS−PAGE of purified fibrinolytic enzyme of *B. subtilis* IMR-NK1: (lane 1) purified fibrinolytic enzyme; (lane S) low molecular mass standard proteins [phosphorylase *b* (94 kDa); bovine serum albumin (67 kDa); ovalbumin (43 kDa); carbonic anhydrase (30 kDa); soybean trypsin inhibitor (20.1 kDa); α-lactalbumin (14.4 kDa)]. Fifty micrograms of purified fibrinolytic enzyme (trichloroacetic acid denatured) was heated in boiling water for 5 min with 50 μL of an aqueous solution containing 1% SDS and 1 M 2-mercaptoethanol. Electrophoresis was carried out with PhastGel gradient 8−25 according to the manufacturers' instructions (PhastSystem user's manual).

assayed in a universal buffer (Britton and Robinson type) from pH 3 to 11, with the enzyme having an optimal pH at 7.8. The enzyme was then incubated at various pH values for 1 h at room temperature, and then the remaining activity was assayed, indicating that the enzyme was stable at pH 5−10. It also showed activities for hydrolysis of casein with an optimal pH of 7−9.5 and a synthetic substrate, *N*-succinyl-Ala-Ala-Pro-Phe-*p*NA, which had an optimal pH of 7−11.

Optimal Temperature and Thermostability. The optimal temperature of the fibrinolytic enzyme was measured at various temperatures from 30 to 70 °C, and the enzyme was found to have an optimal temperature

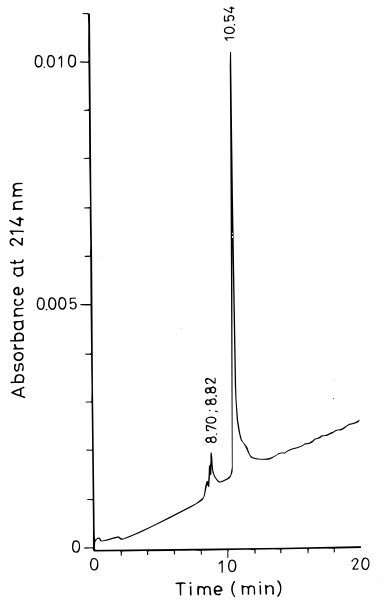

Figure 5. CZE of the Superdex 75 HR purified fibrinolytic enzyme of *B. subtilis* IMR-NK1. CZE was performed in 50 mM phosphate buffer (pH 2.5) using an uncoated fused silica capillary (75 μm × 57 cm, 7 cm to the detector).

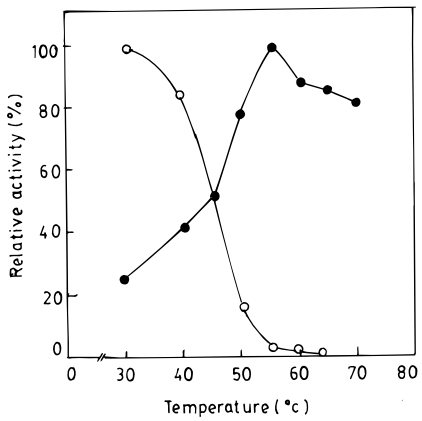

Figure 6. Effect of temperature on the fibrinolytic activity and stability of the purified fibrinolytic enzyme of *B. subtilis* IMR-NK1: (●) profile of temperature-dependent activity; (○) profile of thermal stability.

of 55 °C for the hydrolysis of fibrin. The effect of temperature on the stability of the enzyme was studied by maintaining the enzyme at various temperatures (30−65 °C) for 40 min at pH 7.0, after which time the remaining activities were then measured. The enzyme was relatively labile between 30 and 40 °C and significantly inactivated above 40 °C (Figure 6).

Michaelis Constant. A Michaelis−Menten type curve was observed from the plot of the fibrinolytic enzyme activity with fibrin concentration. The K_m value obtained from a Lineweaver−Burk double-reciprocal plot was 0.15%.

Substrate Specificity. The proteolytic activity of the fibrinolytic enzyme was measured using 0.5% of fibrin, fibrinogen, casein, hemoglobin, elastin, mucin, gelatin, and serum albumin as substrate. The enzyme had activity toward fibrin, fibrinogen, casein, hemoglobin, and elastin. When the activity using fibrin as a substrate was taken as 100, those of fibrinogen, casein, hemoglobin, and elastin were 124, 118, 24, and 6, respectively. The ratio of fibrinolytic/caseinolytic activity was 80.6%. Mucin and gelatin were slightly active as substrates for the enzyme, although serum albumin was inactive (Table 2). Fibrinolytic activity was also found when the purified enzyme was assayed by using the fibrin plate method (Figure 7).

Table 2. Relative Activity of the Fibrinolytic Enzyme from *B. subtilis* IMR-NK1 for Hydrolysis of Several Proteins

protein	rel activity (%)	protein	rel activity (%)
fibrin	100	elastin	6
fibrinogen	124	mucin	2
casein	118	gelatin	2
hemoglobin	24	albumin	0

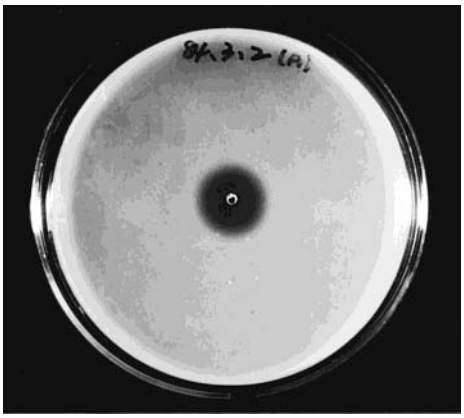

Figure 7. Fibrinolytic activity of the purified fibrinolytic enzyme of *B. subtilis* IMR-NK1. Here, 0.2 μg (in 2 μL) of the Superdex 75 HR purified enzyme was applied to the fibrin plate.

Table 3. Comparative Amidolytic Activity of the Fibrinolytic Enzyme from *B. subtilis* IMR-NK1 for the Hydrolysis of Several Synthetic Substrates

synthetic substrate (0.71 mM)	substrate hydrolysis (nmol/min/mL)
N-succinyl-Ala-Ala-Pro-Phe-*p*NA[a] (for subtilisin or chymotrypsin)	16.8
N-benzoyl-Val-Gly-Arg-*p*NA	7.1
D-Val-Leu-Lys-*p*NA (for plasmin)	1.7
N-succinyl-Ala-Ala-Ala-*p*NA	0.5
Ala-Ala-Ala-*p*NA	0
N-succinyl-Ala-Ala-Val-*p*NA	0
Gly-Phe-*p*NA	0
Gly-Pro-*p*NA	0
D-Phe-Val-*p*NA	0

[a] *p*NA, *p*-nitroanilide.

Amidolytic Activity. The amidolytic activity of the fibrinolytic enzyme was investigated with several synthetic substrates. The most sensitive substrate for the enzyme was *N*-succinyl-Ala-Ala-Pro-Phe-*p*NA (for subtilisin or chymotrypsin), with lesser effects for *N*-benzoyl-Val-Gly-Arg-*p*NA and D-Val-Leu-Lys-*p*NA (for plasmin). There was almost or completely no activity on other synthetic substrates (Table 3). NK from *B. natto* (Fujita et al., 1993) also showed the highest activity for subtilisin substrate *N*-succinyl-Ala-Ala-Pro-Phe-*p*NA. Some kinetic constants for hydrolysis of the subtilisin substrate for this enzyme and NK are shown in Table 4, indicating that the enzyme is more efficient than NK for the hydrolysis of *N*-succinyl-Ala-Ala-Pro-Phe-*p*NA.

Effectors. After the fibrinolytic enzyme had been incubated with amino acid side chain modification reagents for 30 min, the enzyme activity was almost completely inhibited by NBS (2 mM) and PMSF (2 mM). However, other reagents, CHD (2 mM), DNFB (2 mM),

Table 4. Kinetic Constants of the Fibrinolytic Enzyme from *B. subtilis* IMR-NK1 and NK from *B. natto*[a]

fibrinolytic enzyme from *B. subtilis* IMR-NK1			NK from *B. natto*		
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})
21.08	0.34	6.2×10^4	17.85	0.52	3.4×10^4

[a] Kinetic constants were determined using *N*-succinyl-Ala-Ala-Pro-phe-*p*NA as substrate in 1.2 mL of 10.4 mM Tris-HCl buffer (4.2 mM $CaCl_2$, pH 7.8) at 37 °C for fibrinolytic enzyme from *B. subtilis* IMR-NK1 and in 1.0 mL of 100 mM phosphate buffer (0.1 M NaCl, pH 7.4) at 37 °C for NK (Fujita et al., 1993).

Table 5. Effect of Various Reagents on the Activity of the Fibrinolytic Enzyme from *B. subtilis* IMR-NK1 for Fibrin Hydrolysis

reagent	concn (mM)	rel activity (%)	reagent	concn (mM)	rel activity (%)
none		100	NAI	2	99
CHD	2	98	PHMB	1.6	104
DNFB	2	93	WRK	20	99
DEPC	24	80	PMSF	2	23
EAM	20	98	NBS	2	0

Table 6. Effect of Various Inhibitors on the Activity of the Fribrinolytic Enzyme from *B. subtilis* IMR-NK1 for Fibrin Hydrolysis

inhibitor	concn	rel activity (%)
none		100
chicken ovoinhibitor	2 mg/mL	28
trypsin inhibitor (turkey egg white)	2 mg/mL	41
leupeptin	0.5 μg/mL	59
p-aminobenzamidine	2 mM	62
chicken ovomucoid	2 mg/mL	79
trypsin inhibitor (lima bean)	2 mg/mL	93
trypsin inhibitor (soybean)	2 mg/mL	93
trypsin−chymotrypsin inhibitor(soybean)	2 mg/mL	95
aprotinin	2 mg/mL	104

DEPC (24 mM), EAM (20 mM), NAI (2 mM), PHMB (1.6 mM), and WRK (20 mM), showed no significant effect on the activity of the enzyme (Table 5). These results indicate that hydroxyl (serine) and indole (tryptophan) groups are located at or near the active site of the enzyme. As shown in Table 6, some naturally occurring proteinaceous proteinase inhibitors, such as chicken ovoinhibitor (hen egg white), trypsin inhibitor (turkey egg white) and ovomucoid (hen egg white), inhibited the activity of the enzyme. A *Streptococcus*-secreted peptide proteinase inhibitor, leupeptin (acetyl-L-Leu-L-Leu-L-arginal), and a synthetic trypsin inhibitor, *p*-aminobenzamidine, also inhibited the activity of the enzyme. However, other naturally occurring proteinaceous proteinase inhibitors showed no effect on the activity of the enzyme. Matsushima et al. (1958) reported that chicken ovoinhibitor inhibited microbial proteolytic enzymes prepared from *B. subtilis* var. *biotecus* and from an unidentified *Aspergillus*. Rhodes et al. (1960) and Tomimatsu et al. (1966) further purified the ovoinhibitor and found that the ovoinhibitor inhibited both subtilisin Carlsberg and subtilisin BPN′.

Isoelectric Point. The isoelectric point (p*I*) of the fibrinolytic enzyme was 8.3 as determind by IEF electrophoresis (Figure 8). The subtilisin-like enzymes of NK (Sumi et al., 1987), SMCE (Yasuda et al., 1999), and subtilisin Carlsberg (Ottesen and Svendsen, 1970) showed isoelectric points of 8.3, 9.75, and 7.8, respectively. The p*I* of the fibrinolytic enzyme from this strain is identical to that of NK from *B. natto*.

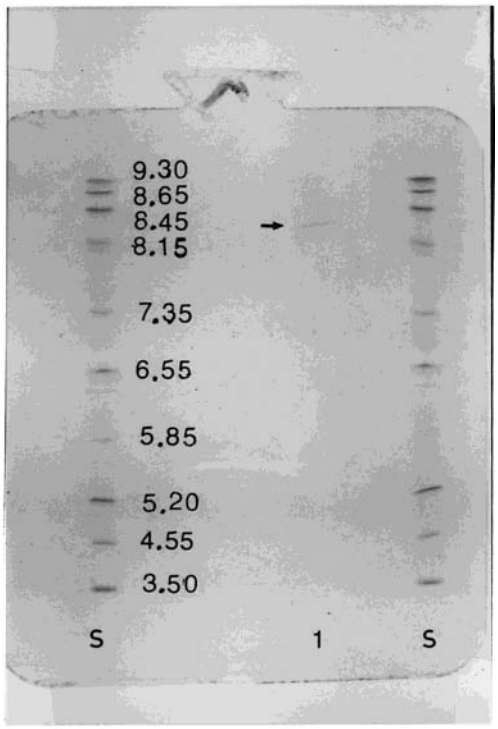

Figure 8. IEF electrophoresis of the purified fibrinolytic enzmye of *B. subtilis* IMR-NK1: (lane 1) purified fibrinolytic enzyme; (lane S) standard proteins [lentil lectin, basic band (p*I* = 8.65); lentil lectin, middle band (p*I* = 8.45); myoglobin, basic band (p*I* = 7.35); human carbonic anhydrase β (p*I* = 6.55); bovine carbonic anhydrase β (p*I* = 5.85); β-lactoglobulin A (p*I* = 5.2); soybean trypsin inhibitor (p*I* = 4.55); amyloglucosidase (p*I* = 3.5)]. IEF was performed on PhastGel IEF 3-9 according to the PhastSystem user's manual. A sample (0.5 μg/μL) was applied to the gel using the 8 × 1 μL comb.

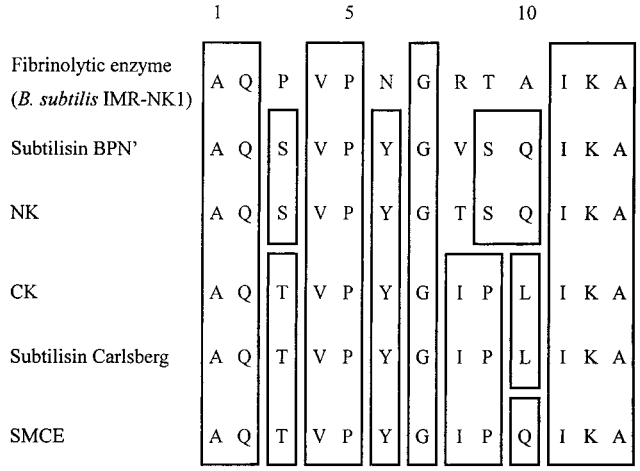

Figure 9. Comparison of N-terminal amino acid sequence of the fibrinolytic enzyme of *B. subtilis* IMR-NK1, NK, CK, subtilisin BPN, subtilisin Carlsberg, and SMCE: NK, fibrinolytic enzyme from *B. natto* (Fujita et al., 1993); CK, fibrinolytic enzyme from *Bacillus* sp. strain CK-11-4; subtilisin BPN′, extracellular protease from *B. amyloliquefacies*; subtilisin Carlsberg, extracellular protease from *B. licheniformis;* SMCE, soybean milk coagulating enzyme from *B. pumilus* TYO-67.

N-Terminal Amino Acid Sequence. The N-terminal amino acid sequence of the first 13 residues of the fibrinolytic enzyme is shown in Figure 9. Comparison of this result with those of subtilisin BPN′, subtilisin Carlsberg, CK, NK, and SMCE shows that all of the

enzymes had overlapping identical residues. The amino acid sequences of 5 enzymes are identical in 8 of the 13 residues.

DISCUSSION

This paper describes the purification and characterization of a fibrinolytic enzyme from a mutant of *B. subtilis*, which was used for the production of domestic natto in Taiwan. The molecular mass of the enzyme was calculated to be ~30 kDa by both SDS−PAGE and gel filtration, indicating that the enzyme is a monomer. Sumi et al. (1987) reported that NK from *B. natto* consisted of a total of 275 amino acid residues (MW = 27728) and exhibited a high homology with the subtilisins. Kim et al. (1996) reported that CK from *Bacillus* sp. strain CK-4 had a molecular mass of 28.2 kDa, as determined by SDS−PAGE. Yasuda et al. (1999) purified a subtilisin Carlsberg-like protease, SMCE, from *B. pumilus* TYO-67. The purified SMCE showed a molecular mass of ~30 kDa. Most subtilisins from *Bacillus* species have a molecular mass of ~30 kDa (Hagihara, 1960; Ottesen and Svendsen, 1970). The fibrinolytic enzyme from this strain showed activities for hydrolysis of fibrin and casein, and the optimal pH for the hydrolysis of fibrin was 7.8. However, the optimal pH for the hydrolysis of casein was 7−9.5. The ratio of fibrinolytic/caseinolytic activity was 80.6%, which is slightly higher than that of CK from *Bacillus* sp. strain CK 11-4 (73.0%) but much higher than that of subtilisin BPN′ (32.4%) and subtilisin Carlsberg (9.2%) (Kim et al., 1996). The optimal pH for digestion of casein is lower than those of subtilisin BPN′ (optimal pH 10−10.5) and subtilisin Carlsberg (optimal pH 10−11.5) (Hagihara, 1960). The enzyme also showed amidolytic activity for the hydrolysis of several synthetic substrates. The most sensitive substrate was *N*-succinyl-Ala-Ala-Pro-Phe-*p*NA, which is a well-known substrate for subtilisin or chymotrypsin.

The chemical modification agents PMSF (a serine specific modification reagent) and NBS (a protein-oxidizing agent) significantly or completely inhibited the fibrinolytic activity of the enzyme. The oxidizing reaction is usually specific for tryptophan in the absence of sulfhydryl groups, which are even more reactive (Means and Feeney, 1971; Patchornik et al., 1958). However, a mercaptide-forming agent (PHMB) had no effect on the activity of the enzyme. Therefore, serine and tryptophan are essential for the active site of the enzyme. The N-terminal sequences of the first 13 amino acids of the enzyme exhibited considerable homology with the sequences of 5 subtilisin or subtilisin-like serine proteases. All of the data suggest that the fibrinolytic enzyme from this strain is a subtilisin-like serine protease, similar to that of NK from *B. natto.* However, this enzyme had a much higher proteolytic coefficient (k_{cat}/K_m) than that of NK from *B. natto* for *N*-succinyl-Ala-Ala-Pro-Phe-*p*NA. Furthermore, the enzyme showed a much lower optimal pH for the hydrolysis of casein and a much higher ratio of fibrinolytic/caseinolytic activity than those of alkaline serine proteases (subtilisin) from *Bacillus* species.

There are several studies that have reported on the intestinal absorption of serum albumin (Warshaw et al., 1974), lipase (Papp et al., 1977), elastase (Katayama and Fujita, 1972), and *Serratia* protease (Miyata et al., 1980). Recently, Sumi et al. (1990) reported that natto and NK may be potent natural agents for oral fibrinolytic therapy. They demonstrated that the enhancement of the fibrinolytic activity in the plasma by the fibrinolytic enzyme orally administered was related to the production of endogeneous plasminogen activators. Bernik and Oller (1977) observed activation of a plasminogen proactivator of the human kidney by trypsin treatment. In view of these reports, the fibrinolytic enzyme and the fermented natto from this strain probably can be also used as potent natural agents for oral fibrinolytic therapy or thrombosis prevention. However, further investigation in vivo is required to confirm their physiological function.

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