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Purification and Characterization of a Novel Fibrinolytic Enzyme from *Bacillus* sp. nov. SK006 Isolated from an Asian Traditional Fermented Shrimp Paste

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Bacillus sp. nov. SK006 producing four extracellular fibrinolytic enzymes was isolated from fermented shrimp paste, a traditional and popular Asian seasoning. One fibrinolytic enzyme was purified to homogeneity with a molecular mass of 43–46 kDa by SDS−PAGE and gel filtration chromatography. The specific activity was determined to be 11.2 units/mg using plasmin as a standard. The enzyme displayed optimal activity at 30 °C and pH 7.2. It was stable below 40 °C for 4 h between pH 5.0 and pH 11.0. Zinc ion stimulated the enzyme activity whereas Cu^{2+}, Ca^{2+}, Fe^{3+}, and Hg^{2+} caused its inhibition. The fibrinolytic activity was strongly inhibited by PMSF and moderately inhibited by EDTA as well as PCMB. The enzyme exhibited a higher affinity toward *N*-Succ-Ala-Ala-Pro-Phe-pNA and was able to degrade fibrin clots either by forming active plasmin from plasminogen or by direct fibrinolysis. The N-terminal amino acid sequence was found to be AQSVPYEQPHLSQ, which is different from that of other known fibrinolytic enzymes.

KEYWORDS: ***Bacillus*** **sp. nov. SK006; fibrinolytic enzyme; purification; characterization**

INTRODUCTION

Cardiovascular diseases, including acute myocardial infarction, ischemic heart disease, valvular heart disease, peripheral vascular disease, arrhythmias, high blood pressure, and stroke, are the leading causes of death throughout the world. According to data provided by the World Health Organization (WHO) in 2000, heart diseases are responsible for 29% of the total mortality rate in the world (*1*). The underlying pathophysiological process in myocardial infarction and stroke is the formation of a fibrin clot, which adheres to the unbroken wall of blood vessels. Fibrin is the main protein component of the blood clot, and it is normally formed from fibrinogen by the action of thrombin (EC 3.4.21.5). Accumulation of fibrin in the blood vessels usually increases thrombosis, leading to myocardial infarction and other cardiovascular diseases (*2, 3*).

Insoluble fibrin fiber is hydrolyzed into fibrin degradation products by plasmin, which is generated from plasminogen activator. The basis of fibrinolytic therapy is the intravenous administration of an exogenous plasminogen activator, which lyses the thrombus and restores blood flow to the area of ischemia (*4, 5*). A variety of fibrinolytic enzymes such as tissue plasminogen activator (t-PA), urokinase (u-PA, EC 3.4.21.31), and bacterial plasminogen activator streptokinase (EC 3.2.1.35) have been extensively investigated and used as thrombolytic agents (*6*). However, these enzymes are expensive, and patients may suffer from undesirable side effects such as gastrointestinal bleeding, allergic reactions, and resistance to repercussion (*7–9*). Therefore, the search for safer thrombolytic agents from other sources continues.

The fibrinolytic enzymes from *Bacillus* sp., including subtilisin NAT (formerly designated nattokinase, NK) (*10*), subtilisin J (*11*), and subtilisin E (EC 3.4.21.62) (*12–14*), as well as subtilisin amylosacchariticus (*15*), have attracted interests as thrombolytic agents because of their efficiency and safety in the fibrinolytic process (*16*).

Fermented shrimp paste, a traditional and popular seasoning in Asian countries, was shown to have a strong fibrinolytic activity (*1, 17*). In this paper, a novel fibrinolytic enzyme produced from *Bacillus* sp. nov. SK006, isolated from a traditional fermented shrimp paste, was purified and characterized. Furthermore, the physiological functions related to fibrinolytic factors were investigated.

MATERIALS AND METHODS

Materials. *Taq* DNA polymerase and STR buffer for PCR were purchased from Promega (Shanghai, China). Plasmin (3.9 units/mg), chromogenic substrates (*N*-Succ-Ala-Ala-Pro-Phe-pNA, D-Val-Leu-Lys-pNA, and D-Val-Leu-Arg-pNA), and protease inhibitors (EDC, PMSF,

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DEPC, PCMB, EDTA, and 2-mercaptoethanol) were imported from Sigma Chemical Co. (St. Louis, MO). Bovine fibrinogen and thrombin were provided by the Chinese Medicine Institute (Beijing, China). Protein standard markers [bovine serum albumin (BSA), ovalbumin, chymotrypsinogen, ribonuclease, aprotinin, and vitamin B_{12}] and chromatography columns were obtained from Pharmacia Co. (Amersham Bioscience, Sweden). Electrophoresis reagents were bought from Bio-Rad Laboratories Inc. (Nanjing, China). All other chemicals and reagents were of the highest grade commercially available.

Strain Isolation and Identification. Shrimp paste (1 g) from a local market was mixed with sterilized phosphate buffer (10 mM, pH 7.4) and centrifuged at 4 °C, 10000*g*, for 30 min. The supernatant (5 mL) was transferred into a 250 mL Erlenmeyer flask containing 25 mL of medium composed of the following (g/L): peptone, 10; glucose, 10; yeast extract, 5; Na_2HPO_4, 5; $MgSO_4$, 0.1. The inoculated medium was incubated at 37 °C with shaking in an orbital incubator shaker. After 36 h, a loop of the culture was spread on nutrient agar plates supplemented and incubated. After 24 h, colonies were picked and transferred to fresh nutrient agar plates. Pure colonies obtained were tested for fibrinolytic activity with a fibrin plate. Isolates with a transparent zone were considered fibrinolytic enzyme positive.

Some morphological and biochemical tests of the selected bacterium with high fibrinolytic activity were carried out by the China Center for Type Culture Collection (CCTCC) in Wuhan, China.

Genomic DNA was purified by the standard chloroform−isoamyl alcohol method (*18*). The genomic DNA from the strain was extracted, and the general primers were designed according to the conservative sequence of bacteria 16S rDNA. The gene fragment was amplified with the paired primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) according to Robb et al. (*19*). The polymerase chain reaction (PCR) mixture was composed of 2 μL of STR buffer, 0.1 μL of *Taq* DNA polymerase, 100 ng of each primer, and 5 ng of DNA template. The PCR reaction was performed in 20 μL for 20 cycles of 1 min at 94 °C, 1 min at 55 °C, and 1.5 min at 72 °C. Additional extension was carried out for 10 min at 72 °C. PCR products were purified using the Qiagen PCR purification kit and sequenced by TaKaRa Biotechnology Co., Ltd. (Dalian, China). Homology searches were performed against the sequences with the database using the BLAST program (NCBI).

Enzyme Production. The fermentation process was conducted in a Biostat B fermentor (5 L; B. Braun, Germany). The bacterium was grown aerobically at 37 °C, 180 rpm, for 36 h in a culture medium (3 L) containing the following (g/L): peptone, 15; glucose, 15; Na_2HPO_4, 6; NaH_2PO_4, 1; $MgSO_4$, 0.5; pH 7.2. The air flow rate of the process was 1.8 L/min for 0–12 h, 2.1 L/min for 12–24 h, and 2.4 L/min for 24–36 h (*20*).

The culture broth was centrifuged at 4 °C, 10000*g*, for 30 min to remove insoluble materials. The supernatant was concentrated by ultrafiltration (5 kDa cutoff) with a Labscale TM TFF system (Millipore). An equal volume of prechilled (−20 °C) ethanol was added, dropwise, with constant stirring for 1 h. The precipitated proteins were removed by centrifugation at 4 °C. Ethanol was further added to the solution up to 80% saturation with gentle stirring and left for 1 h. The precipitate formed was collected by centrifugation at 10000*g* for 20 min, dissolved in a small amount of 20 mM Tris-HCl, pH 8.0 (buffer A), and dialyzed against the same buffer for 24 h at 4 °C with two buffer changes.

Enzyme Purification. All chromatographic steps were performed at 4 °C using an Akta Prime system (Pharmacia Amersham Biotech, Sweden).

The dialyzed enzyme preparation was applied to a DEAE-Sepharose Cl 6B FF column (2.5 cm × 30 cm) equilibrated with buffer A. The column was washed with the same buffer, and bound proteins were eluted by applying a linear gradient of 0–1.0 M NaCl in the same buffer at a flow rate of 2 mL/min. Fractions exhibiting fibrinolytic activity were pooled and concentrated by freeze-drying. Samples were dissolved in a minimal volume of 10 mM phosphate buffer, pH 7.4 (buffer B), and dialyzed against the same buffer for 24 h at 4 °C with two buffer changes.

To further purify the enzyme, gel filtration was performed onto a Superdex 75 (10/300 GL) gel filtration column (1.0 cm × 30 cm), equilibrated with buffer B. Elution at a flow rate of 0.5 mL/min was performed using buffer B. The active fraction pooled and concentrated by ultrafiltration was used as the purified enzyme for further analysis. The molecular mass of the enzyme was determined using the same column calibrated previously with a range of reference proteins: BSA (66.2 kDa), ovalbumin (43.0 kDa), ribonuclease (13.7 kDa), aprotinin (6.5 kDa), and vitamin B_{12} (1.4 kDa). Blue dextran was used to determine the void volume of the column.

Protein Analysis. Protein concentration was measured by the method of Lowry (*21*) using BSA as a standard. During chromatographic purification steps, the protein concentration was estimated by observing the absorbance at 280 nm.

Sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) was carried out to determine the molecular mass and homogeneity of the enzyme, according to the procedure of Laemmli (*22*), with 10% acrylamide separating gel and 4% acrylamide stacking gel. The gel sheets were stained for proteins with 0.25% Coomassie brilliant blue (CBB) R250. The molecular mass standards for SDS−PAGE comprised rabbit myosin (200.0 kDa), calmodulin-binding protein (130.0 kDa), rabbit phosphorylase *b* (97.4 kDa), bovine serum albumin (66.2 kDa), and rabbit actin (43.0 kDa).

Assay of Enzyme Activity. Fibrinolytic activity was determined by both the plasminogen-free fibrin plate method and the plasminogen-rich fibrin plate method (*23*). The plasminogen-free fibrin plate was made up with the fibrinogen solution (1.5 mL, 0.75% (w/v) bovine fibrinogen in 10 mM sodium phosphate buffer, pH 7.4), 100 μL of thrombin solution (42 units/mL), and 1.0% (w/v) agarose. Fibrin plates were heated at 80 °C for 30 min to eliminate other fibrinolytic factors. Plasminogen-rich fibrin plates contained 5 units of plasminogen in addition and were not heated.

A hole (2 mm of diameter) was punched for sample application on the fibrin plate; 2 μL of the enzyme solution were carefully dropped onto the hole. After incubation of the plate at 37 °C for 18 h, the fibrinolytic activity was determined by measuring the dimension of the clear zone and expressed in plasmin units according to the plasmin standard curve.

Caseinolytic activity was assayed according to Peng et al. (*24*). One unit of caseinolytic activity (CU) was defined as the amount of enzyme releasing 1 μmol of tyrosine equivalent per minute and per milliliter.

Amidolytic activity was measured spectrophotometrically using synthetic substrates. The mixture (0.8 mL) contained 10 μL of enzyme solution, 1.5×10^{-4} mol/L chromogenic substrate, and 20 mM sodium phosphate buffer (pH 7.4). After incubation for 10 min at 37 °C, the amount of liberated *p*-nitroaniline (pNA) was calculated by spectrophotometric absorption at 405 nm. One unit of amidolytic activity (AU) was expressed as nanomoles of substrate hydrolyzed per minute and per millilter by the enzyme (*24*). The mixture containing different concentrations of synthetic substrate and 10 μL of enzyme solution was incubated at 37 °C, respectively. At various time intervals of the reaction course, the absorbance of released pNA was measured at 405 nm. The kinetic constants were determined using Hanes−Woolf plots based on the initial reaction rates.

Effects of Temperature and pH on Enzyme Activity. The optimum temperature for enzyme activity was determined by conducting the assay at various temperatures (20, 25, 30, 40, 50, 60, and 65 °C) in 10 mM phosphate buffer (pH 7.4) for 10 min. The thermostability of the enzyme was measured after preincubating the enzyme in the same buffer at the same pH but at various temperatures for different incubation times. Relative and residual activities were measured in the same conditions used to determine the fibrinolytic activity, while the control activity of the preincubated sample at 4 °C was regarded as 100%.

The optimal pH for the fibrinolytic activity of the enzyme was determined within a pH range of 3.0–11.0, using the following buffer systems: 50 mM sodium acetate buffer (pH 3.0–5.0), sodium phosphate buffer (pH 6.0–8.0), carbonate buffer (pH 9.0–10.0), and glycine−NaOH buffer (pH 11.0), respectively. Fibrin plates of different pH values were prepared using different buffers, and the pH of the enzyme was adjusted to the same value as that of the respective fibrin plate. The relative activities were expressed as a percentage of the maximum enzyme activity. All experiments were carried out at least three times.

Table 1. Purification Summary of the Fibrinolytic Enzyme Obtained from p1[a]

	total protein (mg)			total activity (units)			specific activity (units/mg)			purification (fold)			yield (%)		
procedure	p1	p2	p3	p1	p2	p3	p1	p2	p3	p1	p2	p3	p1	p2	p3
crude enzyme	225			135			0.6			1			100		
ethanol precipitation	37.7			121.4			3.22			5.4			89.9		
DEAE-Sepharose Cl 6B FF	18.7			31.1									23		
	8.0	4.4	6.3	12.0	9.3	9.8	1.5	2.11	1.56	1[b]	–	–	100[b]	–	–
Superdex 75	0.5	–	–	5.6	–	–	11.2			7.5[b]	–	–	46.7[b]	–	–

[a] A dash indicates that the enzyme activity was not determined. [b] Compared to data obtained from the DEAE-Sepharose Cl 6B FF column.

Effects of Different Metal Ions and Protein Inhibitors on Enzyme Activity. To investigate the effects of metal ions on enzyme activity, the assay was performed in a reaction mixture containing different metal ions. The activity assayed in the absence of metal ions was defined as the control. The metal ions tested comprised Cu^{2+}, Ca^{2+}, Mn^{2+}, Fe^{3+}, Mg^{2+}, Co^{2+}, Zn^{2+}, Hg^{2+}, and Fe^{2+}. To determine the influence of protease inhibitors (EDC, PMSF, DEPC, PCMB, EDTA, and 2-mercaptoethanol) on the enzyme activity, the enzyme was preincubated with the respective compound for 10 min at 37 °C, and the residual activity was determined by the standard enzyme assay. The level of inhibition was expressed as a percentage of the remaining activity (with either metal ion or inhibitor) to the control activity (without metal ion or inhibitor). The concentrations of the metal ions and the inhibitors (in the preincubation mixture) are listed in **Table 5**. The reproducibility of data was confirmed by repeating the experiments three times.

Degradation of Fibrinogen. Fibrinogenolytic activity was tested by incubating 0.5 mg of bovine fibrinogen in 1 mL of Tris-HCl (20 mM, pH 7.8) containing the enzyme (0.5 mg/mL) at 37 °C. At various time intervals (0, 15, 30, 60, 120, and 180 min), aliquots were taken from the reaction mixture and mixed with an equal volume of sample buffer containing 2-mercaptoethanol, boiled, cooled, and centrifuged at 10000*g* for 10 min. Supernatants were treated by SDS−PAGE with 12% (w/v) polyacrylamide gel to determine the time sequence of fibrinogen chain cleavage. The molecular mass standards used were rabbit phosphorylase *b* (97.4 kDa), BSA (66.2 kDa), rabbit actin (43.0 kDa), bovine carbonic anhydrase (31.0 kDa), and trypsin inhibitor (20.1 kDa).

N-Terminal Amino Acid Sequence. The enzyme was separated from any minor impurities by SDS−PAGE and transferred onto a polyvinylidene difluoride membrane (PVDF; Bio-Rad, Richmond, CA) by electroblotting (*25*) at a constant current of 250 mA for 1 h. The membrane was stained with 0.25% CBB R-250 solution, and the stained portion was excised for N-terminal sequencing by stepwise Edman degradation with a 492 cLC protein sequencer (Applied Biosystems).

Activity Staining of the Fibrinolytic Enzyme. Polyacrylamide gel electrophoresis (native PAGE) was carried out with the crude enzyme solution on 12% polyacrylamide gel under constant electric current (20 mA) for approximately 50 min at 4 °C, and no fixing solution nor staining solution was used in order to preserve activity. The gel was put on the fibrin plate carefully and incubated at 30 °C for about 10 h.

RESULTS

Isolation and Identification of the Strain. During the screening program, several strains of moderate fibrinolytic activity were isolated from fermented shrimp paste. Based on the level of production and crude enzyme properties, one strain was selected for further optimization of the extracellular enzyme production. The strain was identified as *Bacillus* sp. nov. SK006 (CCTCC No. M 205071) by CCTCC.

Enzyme Purification and Confirmation of Enzyme Activity. The crude enzyme solution was subjected to a DEAE-Sepharose Cl 6B FF column. Among the five different fractions which were eluted from the gel matrix, three peaks exhibited fibrinolytic activity (i.e., p1, p2, and p3). The first peak (p1) contained the majority of the enzyme activity. The partially purified enzyme in p1 was loaded onto a Superdex 75 column. The elution profile of Superdex 75 gel filtration chromatography showed a sharper peak having fibrinolytic activity from 10 to 11 mL. This fraction was collected and concentrated by ultrafiltration. The purification results are summarized in **Table 1**. The enzyme was purified 7.5-fold with a 46.7% recovery and 11.2 units/mg of specific activity. The molecular mass of the enzyme was estimated to 43 kDa by size exclusion chromatography on the Superdex 75 column. The homogeneity of the purified enzyme was ascertained using SDS−PAGE as shown in **Figure 1**. A single band of protein was obtained at approximately 46 kDa. A strong fibrinolytic activity of the enzyme was observed in both plasminogen-rich (specific activity of about 11.2 units/mg) and plasminogen-free fibrin plates (data not shown), the activity being slightly enhanced in plasminogen-rich fibrin plates compared to that observed in plasminogen-free fibrin plates.

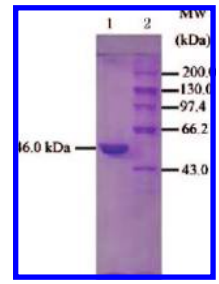

Figure 1. SDS−PAGE of the fibrinolytic enzyme. SDS−PAGE analysis of the enzyme was performed in a 10% (w/v) polyacrylamide gel using the method described by Laemmli (*22*) with slight modification. Lane 1: the purified enzyme from *Bacillus* sp. nov. SK006. Lane 2: molecular weight markers with indicated molecular masses in kDa on the right. Molecular mass markers from the top to the bottom are rabbit myosin (200.0 kDa), calmodulin-binding protein (130.0 kDa), rabbit phosphorylase *b* (97.4 kDa), BSA (66.2 kDa), and rabbit actin (43.0 kDa), respectively.

The fibrinolytic and caseinolytic activities of various proteases were measured by the method described previously. After the activity of each enzyme was converted into caseinolytic activity for unification of the enzyme unit, the ratio of fibrinolytic activity to caseinolytic activity was calculated. As shown in **Table 2**, fibrinolytic activity of the enzyme from *Bacillus* sp. nov. SK006 was 1.5-, 4.8-, and 19.2-fold higher than those of protease from *Bacillus* sp. strain CK 11-4 (*24*), subtilisin BPN′, and subtilisin Carlsberg (*33*), respectively. Hence, the enzyme has a relatively high specificity for fibrin as substrate.

Amidolytic activity of the enzyme was compared with those of other fibrinolytic enzymes (**Table 3**). A synthetic substrate for subtilisin and chymotrypsin (*N*-Succ-Ala-Ala-Pro-Phe-pNA) was most effectively hydrolyzed with amidolytic activity of 39.30 nmol min^{-1} mL^{-1}, K_m of 0.45 mM, and k_{cat}/K_m of 6.4 × 10^4 s^{-1} M^{-1} (**Table 4**), respectively.

Effects of Temperature and pH on Enzyme Activity. The enzyme activity, measured as a function of temperature from 20 to 65 °C, showed that the activity was the highest at 30 °C

Table 2. Comparison of the Purified Enzyme with Other Proteases for Fibrinolytic Activity[a]

protease	caseinolytic activity (units)	fibrinolytic activity (units)	F/C (%)
protease from *Bacillus* sp. nov. SK006	59 ± 4.32	110 ± 5.73	1.86 (100)
protease from *Bacillus* sp. strain CK 11-4[b]	352	257	0.73 (39.2)
subtilisin BPN′ [b]	438	142	0.32 (17.2)
subtilisin Carlsberg[b]	325	30	0.092 (4.9)

[a] Data are expressed as mean values of three replications with their corresponding standard deviations. [b] Reference *24*.

(**Figure 2a**). These results revealed that the enzyme was inactivated rapidly at temperature higher than 50 °C and was inactivated totally at 65 °C within 10 min. As shown in **Figure 2b**, the enzyme still kept 60% of its activity following treatment at 40 °C for 4 h, indicating that the enzyme was highly stable below 40 °C. However, the fibrinolytic enzyme produced by *Bacillus* sp. strain CK11-4 (*24*) and that from *Bacillus* sp. KA38 (*26*) showed higher optimal temperatures (70 and 40 °C, respectively) while being more sensitive to thermal inactivation. However, the enzyme contained in fermented shrimp paste (*17*) exhibited a similar optimum temperature value.

As shown in **Figure 3**, the enzyme remained active at pH values ranging from 5.0 to 11.0. The fibrinolytic activity of the enzyme was optimal at pH 6.0–8.0. However, when the pH value was lower than 5.0, the enzyme activity decreased remarkably. These results suggest that the enzyme is active in a very wide range of pH.

Effect of Metallic Ions and Protease Inhibitors on Enzyme Activity. As shown in **Table 5**, only Zn^{2+} stimulated the enzyme activity, whereas Cu^{2+}, Ca^{2+}, Fe^{3+}, and Hg^{2+} caused its inhibition. Mg^{2+} and Co^{2+} had a negligible influence on the enzyme activity. Mn^{2+} and Fe^{2+} inhibited the enzyme up to approximately 30%. In addition, the enzyme was inhibited by PMSF and PCMB. However, chemicals selective for glutamic acid or aspartic acid (EDC), histidine (DEPC) residues, or disulfide bonds (2-mercaptoethanol) had little effect on the enzyme activity.

Mode of Hydrolysis of Fibrinogen. To elucidate the mode of reaction of the enzyme, degradation products were separated with SDS−PAGE. During the degradation of fibrinogen, B β-chains of fibrinogen were cleaved first, followed by slower release of the γ-chains, producing many products with lower molecular masses within the experimental period. The A α-subunit was resistant to the enzyme digestion (**Figure 4**). This may be due to the fact that the three chains have different physical and/or chemical compositions.

N-Terminal Amino Acid Sequence. The N-terminal amino acid sequence of the purified enzyme was analyzed via the automated Edman method, after SDS−PAGE and electroblotting. The N-terminal amino acid sequence of the first 13 residues was determined as AQSVPYEQPHLSQ. The amino acid sequence of the enzyme was analyzed using a BLAST search against GenBank (http://www.ncbi.nlm.nih.gov/BLAST). A comparison with other existing fibrinolytic enzymes (**Table 6**) proved the observed N-terminal amino acid sequence to be different.

Activity Staining of the Fibrinolytic Enzyme. When polyacrylamide gel was put on the fibrin plate and incubated at 30 °C for about 10 h, four transparent bands were observed on the plate (**Figure 5**). Among the active fractions eluted from the DEAE-Sepharose Cl 6B FF column, the first peak (p1) contained the highest enzyme activity. Therefore, the band corresponding to the biggest transparent zone was supposed to be formed by the enzyme (contained in p1) which was intensively investigated in this study.

DISCUSSION

Food is commonly considered to be one of the most abundant sources of fibrinolytic enzymes (*17–21*). In recent years, fibrinolytic enzymes have been discovered in a variety of foods, such as Japanese natto (*10*), Korean Chungkook-Jang soy sauce (*24*), edible honey mushroom (*27*), tofuyo (*28*), and Chinese douchi and sufu (*29, 30*), as well as fermented shrimp paste (*17*). Among the food sources, fermented food products have been the focus of research. In this study, it was discovered that fibrinolytic activity exists in fermented shrimp paste. Furthermore, a fibrinolytic strain was isolated and was found to be different from other fibrinolytic enzyme-producing strains previously reported. The strain was motile, rod-shaped, catalase-positive, spore-forming, and aerobic. Amplification of the 16S rDNA gene resulted in a 1456 bp DNA fragment. The BLAST search of the rDNA gene sequence against sequences in public databases showed 100% homology with AY601723 and AB195282 (accession number) 16S rDNA gene sequence. On the basis of 16S rDNA gene sequencing, the strain was closely related to the species of the genus *Bacillus*, though, to some extent, some characteristics were different. According to the morphological and physiological characteristics and the comparative sequence analysis of the 16S rDNA gene of strain with other 16S rDNA available in the GenBank database, the strain isolated from traditional fermented shrimp paste was identified as *Bacillus* sp. nov. SK006 (CCTCC No. M 205071).

An extracellular fibrinolytic enzyme produced by *Bacillus* sp. nov. SK006 was purified to electrophoresis homogeneity by a combination of chromatographic stages. The molecular mass of the enzyme, which was estimated to be 43–46 kDa by SDS−PAGE and gel filtration chromatography, was similar to that of subtilisin KA38 (41 kDa) (*26*) but lower than that of subtilisin E (55.8 kDa) (*14*) and higher than those of subtilisin NAT (27.7 kDa) (*10*), subtilisin CK (28.2 kDa) (*24*), subtilisin DFE (28.0 kDa) (*30*), subtilisin IMR-NK1 (31.5 kDa) (*31*), and subtilisin KK (38 kDa) (*28*).

As shown in **Table 3** the enzyme had the highest affinity for *N*-Succ-Ala-Ala-Pro-Phe-pNA, which is a well-known substrate for subtilisin or chymotrypsin. Fibrinolytic enzymes from *Bacillus subtilis* DC33 (*29*) and *Bacillus subtilis* IMR-NK1 (*31*) also showed relatively high activities for the substrate. Some kinetic constants for substrate hydrolysis by some fibrinolytic enzymes, which are shown in **Table 4**, indicated that the enzyme from *Bacillus* sp. nov. SK006 is the most efficient for the hydrolysis of *N*-Succ-Ala-Ala-Pro-Phe-pNA. The enzyme also degraded D-Val-Leu-Lys-pNA (for plasmin). However, it showed a lower activity for D-Val-Leu-Arg-pNA (for kallikrein) compared with the other investigated fibrinolytic enzymes.

Additional evidence of the uniqueness of the enzyme was found in the dependence of the fibrinolytic activity on metal ions and/or protease inhibitors. It was hypothesized that Mn^{2+} and Fe^{2+} intrude on the enzyme-catalyzed system by binding to carboxyl groups of the enzyme. A stimulation of enzyme activity by Zn^{2+} predicted that the Zn^{2+} ion might be present in the active center of the enzyme. This suggests strongly that the inhibition of enzyme activity caused by EDTA results from the chelation of a metal relevant for its activity, possibly Zn^{2+}. A similar observation was made with the fibrinolytic enzyme from *Bacillus* sp. KA38 (*26*).

Table 3. Comparison of Enzyme Specificity with Other Fibrinolytic Enzymes for Some Chromogenic Substrates[a]

	amidolytic activity					
chromogenic substrates	this study (nmol min^{-1} mL^{-1})	BK-17[e] (nmol min^{-1} mL^{-1})	CK[f] (μmol min^{-1} mL^{-1})	DC33[g] (nmol min^{-1} mL^{-1})	IMR-NK1[h] (nmol min^{-1} mL^{-1})	NK[i] (nmol min^{-1} mL^{-1})
N-Succ-Ala-Ala-Pro-Phe-pNA[b]	39.30 ± 0.50	ND	ND	22.2	16.8	ND
D-Val-Leu-Lys-pNA[c]	5.47 ± 0.23	90	424.3	0.84	1.7	68.5
D-Val-Leu-Arg-pNA[d]	0.38 ± 0.04	42	16.9	—	ND	13.5

[a] Data are expressed as mean values of three replications with their corresponding standard deviations. ND indicates not determined. A dash indicates that the enzyme activity was not detectable under the assay conditions. [b] Synthetic substrate for subtilisin and chymotrypsin. [c] Synthetic substrate for plasmin. [d] Synthetic substrate for kallikrein. [e] Reference *2*. [f] Reference *24*. [g] Reference *29*. [h] Reference *31*. [i] Reference *32*.

Table 4. Comparison of Kinetic Constants of the Enzyme with Other Fibrinolytic Enzymes[a]

	FE from *B. subtilis*		
	this study	IMR-NK1[b]	NK from *B. natto*[b]
k_{cat} (s^{-1})	28.75 ± 0.23	21.08	17.85
K_m (mM)	0.45 ± 0.03	0.34	0.52
k_{cat}/K_m (s^{-1} M^{-1})	6.4×10^4	6.2×10^4	3.4×10^4

[a] Data are expressed as mean values of three replications with their corresponding standard deviations. Kinetic constants were determined using *N*-Succ-Ala-Ala-Pro-Phe-pNA as substrate in 20 mM sodium phosphate buffer (pH 7.4) at 37 °C. [b] Reference *31*.

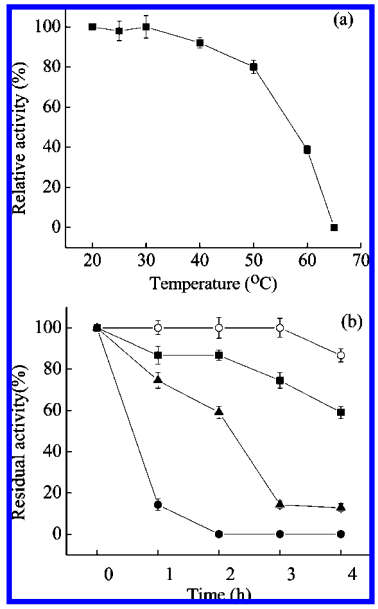

Figure 2. Effects of temperature on the activity (a) and stability (b) of the fibrinolytic enzyme. The optimum temperature for the enzyme activity was assayed at various temperatures for 10 min. The thermostability of the enzyme was determined after incubating the enzyme in 10 mM phosphate buffer (pH 7.4) at various temperatures for different incubation times. Residual activity was measured, and the relative activity of the preincubated sample at 4 °C was regarded as 100%. Key: (○) 30 °C, (■) 40 °C, (▲) 50 °C, and (●) 60 °C. Each point represents the mean ($n = 3$) ± standard deviation.

Results from metal ions and protease inhibitors showed that some characteristics of the enzyme were different from those of other fibrinolytic enzymes previously reported (*17, 24, 26, 30*). The enzyme was strongly inhibited by PMSF, indicating that it is a serine protease. Similar results have been obtained from some fibrinolytic enzymes (*17, 24, 30*). The enzyme was also inhibited by Cu^{2+}, Hg^{2+}, and PCMB, which were supposed to react with the free SH group of the critical cysteine, causing a strong reduction of the enzyme activity. However, reagents selective for glutamic acid or aspartic acid (EDC), histidine

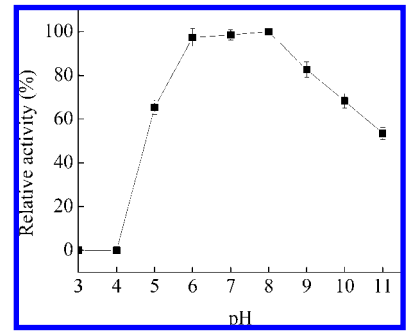

Figure 3. Effect of pH on the activity of the fibrinolytic enzyme. The enzyme was mixed with various buffers: 50 mM sodium acetate buffer (pH 3.0–5.0), sodium phosphate buffer (pH 6.0–8.0), carbonate buffer (pH 9.0–10.0), and glycine−NaOH buffer (pH 11.0), respectively. Relative activity was expressed as a percentage of the maximum enzyme activity under the assay conditions. Each point represents the mean ($n = 3$) ± standard deviation.

Table 5. Effect of Metal Ions and Protease Inhibitors on the Activity of Purified Enzyme[a]

metal ion (2 mM)	relative activity (%)	chemical reagents	concn (mM)	relative activity (%)
Cu^{2+}	49.93 ± 2.19	EDC	2	95.83 ± 1.82
Ca^{2+}	78.53 ± 3.44		1	99.03 ± 0.84
Mn^{2+}	70.2 ± 2.3	PMSF	2	15.03 ± 1.70
Fe^{3+}	81.17 ± 3.65		1	13.83 ± 1.36
Mg^{2+}	92.3 ± 4.55	DEPC	2	95.3 ± 1.31
Co^{2+}	93.0 ± 4.3		1	96.03 ± 1.29
Zn^{2+}	116.43 ± 3.71	PCMB	2	47.8 ± 1.25
Hg^{2+}	49 ± 3.24		1	81.63 ± 1.01
Fe^{2+}	69.93 ± 2.41	EDTA	10	53.3 ± 3.42
control	100.0		5	65.3 ± 2.26
		2-mercaptoethanol	2	95.4 ± 1.45
			1	98.6 ± 1.23

[a] Data are expressed as mean values of three replications with their corresponding standard deviations. The enzyme was mixed with the metal salts or inhibitors in 10 mM phosphate buffer (pH 7.4). The mixture was kept at 37 °C for 10 min. The residual activity was calculated on the basis of the activity of enzyme without any metal ion or inhibitor under the same reaction condition.

(DEPC) residues, or disulfide bonds (2-mercaptoethanol) had little effect on enzyme activity.

The first six amino acid residues of the enzyme showed high homology with those of subtilisin NAT (formerly designated nattokinase) (*10*), subtilisin E (*14*), subtilisin DFE (*30*), and the enzyme from *B. subtilis* BK-17 (*2*). However, no other known fibrinolytic enzyme was homologous to the enzyme with regard to N-terminal amino acid sequence. Amino acids A-Q (numbers 1 and 2) and I-K-A (numbers 11–13) are almost constant amino acid residues of the N-terminal sequence of these subtilisins from *Bacillus* spp. Results obtained from the effect of inhibitors and the N-terminal amino acid sequence indicated that the

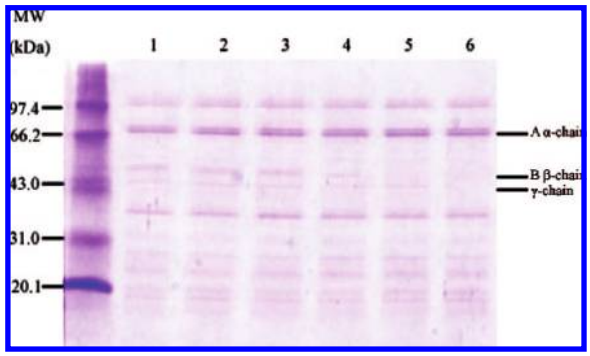

Figure 4. Fibrinogen hydrolysis by the fibrinolytic enzyme. Lanes 1–6 represent hydrolyzed aliquots after 0, 15, 30, 60, 120, and 180 min of incubation at 37 °C, respectively. Molecular mass markers from the top to the bottom were rabbit phosphorylase *b* (97.4 kDa), BSA (66.2 kDa), rabbit actin (43.0 kDa), bovine carbonic anhydrase (31.0 kDa), and trypsin inhibitor (20.1 kDa), respectively.

Table 6. Comparison of the N-Terminal Amino Acid Sequence of the Enzyme with Other Existing Fibrinolytic Enzymes

	N-terminal amino acid sequence	ref
Bacillus sp. nov. SK006	AQSVPYEQPHLSQ	this study
nattokinase (NK)	AQSVPYGISQ IKAPALHS	*32*
subtilisin E	AQSVPYGISQIKA PA HS	*14*
subtilisin DFE	AQSVPYGVSQIKAPALHS	*30*
Bacillus subtilis BK-17	AQSVPYGVSQIKAPA	*2*
Shungkook-Jang (CK)	AQTVPYGIPLIKAD	*24*
subtilisin Carlsberg	AQTVPYGIPLIKAD	*33*
subtilisin IMR-NK1	AQPVPNGRTAIKA	*31*
Katsuwokinase	IVGGYEQZAHSQPHQVSLNSG	*34*
Armillariella mellea	XXYNGXTXSPQTTLV	*27*
fermented shrimp paste	DPYEEPGPCENLQVA	*17*
Bacillus sp. KA38	VYPFPGPIPN	*26*

fibrinolytic enzyme of *Bacillus* sp. nov. SK006 is different from subtilisin-like serine proteases.

A strong fibrinolytic activity of the enzyme was observed in both plasminogen-rich and plasminogen-free fibrin plates, with a slightly enhanced activity in plasminogen-rich fibrin plates compared to that in plasminogen-free fibrin plates. This revealed that the enzyme is able to degrade fibrin clots in two ways, i.e., (a) by forming active plasmin from plasminogen and (b) by direct fibrinolysis.

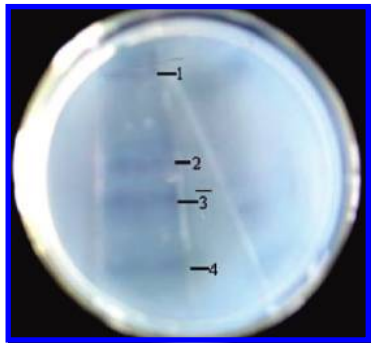

Figure 5. Activity staining of the fibrinolytic enzyme. Native PAGE was carried out on a 12% polyacrylamide gel under constant electric current at 20 mA for approximately 50 min at 4 °C, and no fixing solution nor staining solution was used in order to preserve activity. The gel was put on the fibrin plate carefully and incubated at 30 °C for about 10 h. The transparent band indicated with a bar was supposed to be formed by the enzyme eluted from p1.

Characterization of the fibrinolytic enzyme confirmed its novelty with respect to molecular mass, thermostability, amino acid sequence, and quantitative yields of the end products.

In addition, when the gel obtained from native PAGE was pasted on the fibrin plate, four transparent bands could be observed, suggesting the presence of four isoenzymes. Further research on these enzymes is being carried out in our laboratory. In addition, the enzyme test *in vivo* as well as the cloning, sequencing, and expression of its gene from chromosomal DNA of *Bacillus* sp. nov. SK006 is envisaged in the future.

ABBREVIATIONS USED

BSA, bovine serum albumin; CBB, Coomassie brilliant blue; pNA, *p*-nitroaniline; EDC, *N*-ethyl-*N*′-(3-diethylaminopropyl)-carbodiimide; PMSF, phenylmethanesulfonyl fluoride; DEPC, diethyl pyrocarbonate; PCMB, *p*-chloromercuribenzoic acid; EDTA, ethylenediaminetetraacetate; MW, molecular weight; PVDF, polyvinylidene difluoride; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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