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ORIGINAL PAPER

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Purification and characterization of a fibrinolytic enzyme of *Bacillus subtilis* DC33, isolated from Chinese traditional *Douchi*

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Abstract *Bacillus subtilis* DC33 producing a novel fibrinolytic enzyme was isolated from Ba-bao *Douchi*, a traditional soybean-fermented food in China. The strong fibrin-specific enzyme subtilisin FS33 was purified to electrophoretic homogeneity using the combination of various chromatographic steps. The optimum temperature, pH value, and p*I* of subtilisin FS33 were 55°C, 8.0, and 8.7, respectively. The molecular weight was 30 kDa measured by SDS–PAGE under both reducing and non-reducing conditions. The enzyme showed a level of fibrinolytic activity that was about six times higher than that of subtilisin Carlsberg. The first 15 amino acid residues of N-terminal sequence of the enzyme were A-Q-S-V-P-Y-G-I-P-Q-I-K-A-P-A, which are different from that of other known fibrinolytic enzymes. The amidolytic activities of subtilisin FS33 were inhibited completely by 5 mM phenylmethanesulfonyl fluoride (PMSF) and 1 mM soybean trypsin inhibitor (SBTI), but 1,4-dithiothreitol (DTT), β-mercaptoethanol, and p-hydroxymercuribenzoate (PHMB) did not affect the enzyme activity; serine and tryptophan are thus essential in the active site of the enzyme. The highest affinity of subtilisin FS33 was towards N-Succ-Ala-Ala-Pro-Phe-pNA. Therefore, the enzyme was considered to be a subtilisin-like serine protease. The fibrinolytic enzyme had a high degrading activity for the Bβ-chains and Aα-chain of fibrin(ogen), and also acted on thrombotic and fibrinolytic factors of blood, such as plasminogen, urokinase, thrombin, and kallikrein. So subtilisin FS33 was able to degrade fibrin clots in two ways, i.e., (a) by forming active plasmin from plasminogen and (b) by direct fibrinolysis.

Keywords Amidolytic activity · Fibrin-specificity · Fibrinolytic subtilisin · Serine protease · Thrombolytic agent

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Introduction

Cardiovascular diseases such as acute myocardial infarction, ischemic heart disease, and high blood pressure are the leading causes of death in the world [1]. Among the different types of cardiovascular diseases, thrombosis is one of the most widely occurring diseases in modern life. Drugs using fibrinolytic enzymes are the most effective methods in the treatment of thrombosis. 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 studied and used as thrombolytic agents [2]. However, these agents cause excessive bleeding and recurrence at the site of the residual thrombosis [3]. They have a short half-life and are very costly to produce. Therefore, several lines of investigation are presently being pursued to enhance the efficacy and specificity of fibrinolytic therapy.

Over the last 10 years, several effective thrombolytic agents have been identified and characterized from microorganisms [4–6], earthworms [7], snake venoms [8, 9], centipede venoms [10], insects [11], and leeches [12]. The agents are of interest as useful tools for understanding fibrinolytic mechanism and as potential therapeutic drugs. Their major functions have been described as plasminogen activators or plasmin-like proteases, e.g., lumbrokinase (EC 3.4.17.13) [7], which can directly degrade fibrin or fibrinogen, thereby dissolving thrombi rapidly and completely. Nattokinase, which was purified from *natto*, a traditional Japanese fermented food, not

only directly lyses thrombi in vivo [13–15], but can also enhance fibrinolytic activity in plasma and increase the amount of t-PA by oral administration [4, 14, 16].

Douchi is a traditional and popular soybean-fermented food in China. Some fibrinolytic activities had been found in *Douchi*, and *Bacillus subtilis* DC33 producing a strong fibrinolytic enzyme has been isolated from Ba-bao *Douchi* [17]. In this study, the enzyme was purified and characterized, and its physiological function related to the thrombotic and fibrinolytic factors was investigated.

Materials and methods

Materials

DEAE-Sepharose fast flow, Phenyl Sepharose 6 fast flow, Sephadex G-50, Protein standard markers and IEF markers were purchased from Pharmacia Co. (Amersham Biosciences, Sweden). Chromogenic substrates, such as N-Succ-Ala-Ala-Pro-Phe-pNA (S7388; Succ: succinyl; pNA: p-nitroaniline), D-Val-Leu-Lys-pNA (V7127), D-Val-Leu-Arg–pNA (V6258), and D-Phe-Pip-Arg-Pna (P7027; Pip: L-pipecoly) were procured from Sigma-Aldrich (St Louis, MO, USA). Human fibrinogen (F9754), fibrin (F5386), plasminogen (P5661), urokinase (U0633), p-nitroaniline (pNA, Fluka, 72681), Nα-benzoyl-L-arginine ethyl ester hydrochloride (BAEE, B4500), subtilisin Carlsberg (EC 3.4.21.14, P5380), phenylmethanesulfonyl fluoride (PMSF, P7626), ethylene glycol-*O*,*O*′-bis-[2-amino-ethyl]-*N*, *N*, *N*′, *N*′-tetra-acetic acid (EGTA, E6263), dimethyl sulfoxide (DMSO, D5878), p-hydroxymercuribenzoate (PHMB, H0642), pepstatin A (P4265), and leupeptin (L2884) were also purchased from Sigma-Aldrich. 1,4-dithiothreitol (DTT), soybean trypsin inhibitor (SBTI), aminobenzamidine ethyl acetimidate (EAM), and PEG20000 were from Merck Co. (Beijing, China). Thrombin (EC 3.4.21.5), kallikrein (EC 3.4.4.21), and tissue-type plasminogen activator (t-PA) were obtained from the Chinese Medicine Testing Institute (Beijing). The other chemicals used were of analytical grade.

Bacterial strain and culture condition

Previously, 85 bacterial colonies with different fibrinolytic activities had been isolated from fermented food. Among these colonies, strain DC33 isolated from Ba-bao *Douchi* had the highest fibrinolytic activity, approximately 418 U (urokinase unit) ml^{-1} LB medium (10 g l^{-1} tryptone, 3 g l^{-1} beef powder, 5 g l^{-1} NaCl, pH 7.4). Based on its metabolic properties, the strain DC33 was identified as *B. subtilis* using API 50 CH (BioMerieux, Marcy l'Etoile, France) and the Vitek BACIL card through the criteria database of species and Bergey's Manual of Systematic Bacteriology [18] (data not shown).

Strain DC33 was incubated in a shaking water bath in an optimized medium [17] [14 g l^{-1} tryptone (Oxoid Ltd., UK), 2 g l^{-1} galactose, 1 g l^{-1} KH_2PO_4, 2 g l^{-1} Na_2HPO_4, 0.2 g l^{-1} $MgSO_4$, 3 g l^{-1} $CaCO_3$, 0.01 g l^{-1} $FeCl_3$, and 0.01 g l^{-1} $LiSO_4$, pH 7.2]. Optimum culture conditions for *B. subtilis* DC33 were 37°C, 140 rpm for 72 h.

Determination of enzyme concentration and fibrinolytic activity

Protein (enzyme) concentration was determined by the method of Bradford [19] using BSA fraction V as a standard, measuring the absorbance at 595 nm. Fibrinolytic activity of each solution was determined by both the plasminogen-free fibrin plate method and the plasminogen-rich fibrin plate method [20]. Plasminogen-free fibrin plates consisted of the fibrinogen solution [5 mg of human fibrinogen in 7 ml of 0.1 M barbital buffer (pH 7.8), 10 U of thrombin solution, and 7 ml of 10 g l^{-1} agarose] in petridishes (9 cm in diameter). Fibrin plates were heated at 80°C for 30 min to destroy other fibrinolytic factors. Plasminogen-rich fibrin plates contained 5 U plasminogen, in addition, and were not heated. To observe the fibrinolytic activity, 10 μl of enzyme solution was carefully dropped onto a fibrin plate and was incubated at 37°C for 18 h. The activity of fibrinolytic enzyme was estimated by measuring the dimension of the clear zone on the fibrin plate and plotting a calibration curve based on urokinase standard solutions.

Amidolytic activity and kinetic constants of the fibrinolytic enzyme were measured spectrophotometrically using synthetic substrates S7388, V7127, V6258, P7021. The mixture (100 μl, pH 8.0) containing different concentrations of synthetic substrate (ranging from 0.02 to 0.15 mM) and 5 μl of enzyme solution was incubated in a microplate at 37°C, respectively. At various time intervals of the reaction course, the absorbance of released pNA at 405 nm was measured (Model 550, Thermo Electron Co., Italy). The kinetic constants were determined using Hanes–Woolf plots based on the initial reaction rates [21].

Substrate specificity of the enzyme was assayed by a procedure according to Peng [22] using casein, fibrin, fibrinogen, and serum albumin as substrates, respectively. One unit of proteolytic activity (U) was defined as the amount of enzyme releasing 1 μmol of tyrosine equivalent per minute per milliliter. BAEE activity of the fibrinolytic enzyme was determined according to the method of Glazer [23].

Purification of a fibrinolytic enzyme

The fibrinolytic enzyme was purified by chromatographic procedures including hydrophobic chromatography, anion exchange, and gel filtration chromatography. All purification steps were performed at 4°C. The crude

enzyme was precipitated at 30–65% saturation of $(NH_4)_2SO_4$, and the protein was collected by centrifugation (10,000 *g*, 15 min) and dissolved in 20 mM Tris–HCl buffer (pH 7.8, Buffer A) with 1 M $(NH_4)_2SO_4$. The crude enzyme solution was applied to a Phenyl Sepharose 6 FF column (1.5×20 cm^2). Elution was carried out with a linear gradient of 0.5–0.0 M $(NH_4)_2SO_4$ in the buffer A. Next, this diluted enzyme solution was loaded on a DEAE-Sepharose FF column (1.5×20 cm^2). The column was washed with Buffer B (20 mM Tris–HCl buffer pH 9.4). Active fractions were passed through a Sephadex G-50 column (2.5×100 cm^2) using buffer A. Finally, active fractions were pooled and concentrated by lyophilization or PEG 20000.

SDS–PAGE and IEF electrophoresis

Sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS–PAGE) [24] was carried out on 120 g l^{-1} resolving and 50 g l^{-1} stacking gels in a mini-electrophoresis equipment (ATTO Co., Japan) along with the standard proteins (Pharmacia Co., Sweden). To determine fibrinolytic activity of purified enzyme, enzyme solution was heated in 50°C for 8 min and was centrifuged at 12,000 *g* for 10 min. The treated supernatants were subjected to activated SDS–PAGE. Polyacrylamide gel was washed with 20 mM Tris–HCl (pH 8.0) buffer containing 20 g l^{-1} Triton X100 to remove SDS, and the band was detected in a fibrin plate.

Isoelectric focusing electrophoresis (IEF) was performed in the Phast System using PhastGel IEF 3–9 (homogenous polyacrylamide gels containing Pharmalyte carrier ampholytes). Broad p*I* calibration kits (proteins p*I* 3.50–9.30) were used as markers in the p*I* value determinations.

Effect of metal ions and reagents

To determine the class to which the fibrinolytic enzyme belonged, the effect of different reagents and metal ions on the fibrinolytic activity was investigated in detail. Different reagents and metal ions (final concentration 0.1 mM) were incubated with chromogenic substrate S7388 (1.0×10^{-4} mg) and the enzyme (2.0×10^{-3} mg) in 100 µl of reaction solution (20 mM Tris–HCl, pH 8.0) at room temperature (21°C) for 3 min, and the absorbance of released pNA was measured at 405 nm in a microplate.

Degradation of fibrinogen and fibrin

Fibrinogenolytic activity was tested by incubating 0.5 mg of human purified fibrinogen (fibrin) in 1 ml of Tris–HCl (20 mM, pH 8.0) containing subtilisin FS33 (0.5 mg ml^{-1}) at 37°C. At various time intervals, aliquots were taken from the reaction mixture and mixed with an equal volume of sample buffer containing β-mercaptoethanol, boiled and centrifuged at 12,000 *g* for 10 min. Supernatants were electrophoresed by SDS–PAGE in 120 g l^{-1} polyacrylamide gels to determine the time sequence of fibrinogen chain cleavage.

Effect on partial thrombotic and fibrinolytic factors of blood

In order to determine whether the fibrinolytic enzyme affected body blood system, partial thrombotic, and fibrinolytic factors of blood were selected to the experiments. At different time intervals (0, 2, 5, 10, and 15 min), hydrolysis of plasminogen, thrombin, kallikrein, and urokinase by subtilisin FS33 (0.2 mg/ml dissolved in 20 mM pH 8.0 Tris–HCl buffer) were carried out on 12% gel under non-reducing conditions in SDS–PAGE, respectively.

Results

Purification and active confirmation of the Enzyme

Following the purification protocol as shown in Table 1, the fibrinolytic enzyme from *B. subtilis* DC33 was purified to electrophoretic homogeneity. After the combination of various chromatographic steps, the finally eluted proteins were subjected to SDS–PAGE and IEF. Only one band was observed in the purified sample under reducing or non-reducing conditions (SDS–PAGE; Fig. 1, lanes 1, 2). The molecular weight of the enzyme was approximately 30 kDa, and p*I* of the enzyme was 8.7. With urokinase as a standard, the final specific activity of the fibrinolytic enzyme increased more than 34.6-fold with a 13.0% recovery based on the initial culture supernatant. After activated SDS–PAGE, strong fibrinolytic activity of the purified enzyme band were observed in both plasminogen-free and plasminogen-rich fibrin plates (photographs not shown), and enhanced the activity in plasminogen-rich fibrin plates with about 6% compared to that on plasminogen-free fibrin plates. The enzyme had much higher fibrinolytic activity than the typical fibrinolytic enzyme urokinase, and six times higher than subtilisin Carlsberg.

Substrate specificity of the enzyme

Proteolytic activity of the enzyme was measured using 0.5% fibrin, fibrinogen, casein, serum albumin, and BAEE as substrates. When the activity using fibrin as substrate was taken as 100, the relative activities of the enzyme to fibrinogen, casein, serum albumin, BAEE were 132, 110, 12, 5, respectively. Specific activity of the enzyme (F/C, the ratio of fibrinolytic activity to caseinolytic activity) was 3.5 times higher than those of subtilisin Carlsberg.

Table 1 Purification stages of the fibrinolytic enzyme of *Bacillus subtilis* DC33

Steps	Total protein (mg)	Total activity[a] (U)	Specific activity (U mg^{-1})	Fold purification (*x* fold)	Recovery (%)
Cell-free supernatant	935.0	418,300	447.4	1.00	100
$(NH_4)_2SO_4$ precipitation	625.0	401,562	642.5	1.4	96.0
Phenyl Sepharose 6FF	34.85	221,125	6,345.1	14.2	52.9
DEAE-Sepharose FF	9.43	143,000	15,164.4	33.9	34.2
Sephadex G50 gel filtration	3.52	54,542	15,494.9	34.6	13.0

[a]Fibrinolytic activity was determined by the plasminogen-free fibrin plate method

Chromogenic assay of amidolytic activity using synthetic substrate

The enzyme had strong amidolytic activities on various chromogenic substrates as summarized in Table 2.

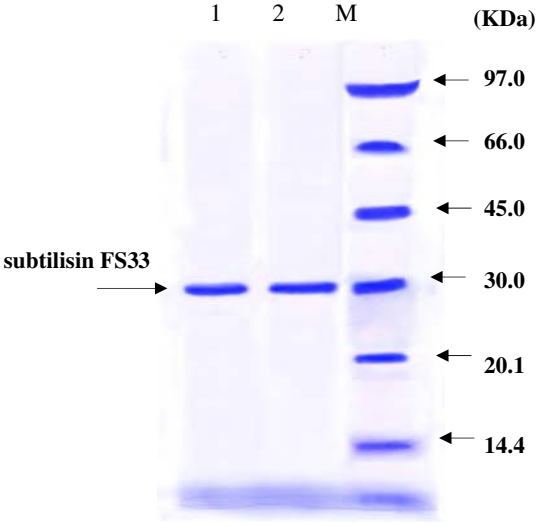

Fig. 1 SDS–PAGE patterns of the purified enzyme. SDS–PAGE was done on 12% gel under reducing (*lane 1*) and non-reducing conditions (*lane 2*), protein molecular mass markers (*lane M*): phophorylase b (97.0 kDa), albumin (67.0 kDa), ovalbumin (45.0 kDa), carbonic anhydrase (30.0 kDa), soybean trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.4 kDa)

Table 2 Amidolytic activity and kinetic parameters of the fibrinolytic enzyme on four chromogenic substrates

Chromogenic substrates	K_m (mM)	K_{cat} (S^{-1})	K_{cat}/K_m (S^{-1} M^{-1})	Amidolytic activity (μmol/min/l)
S7388	0.21	37.04	1.76×10^5	22.2
V7127	7.41	1.4	1.89×10^4	0.84
V6258	–[a]	–	–	–
P7021	47.7	0.12	2.52	0.03

[a]The activity was not detectable under the assay conditions
S7388 (N-Succ-Ala-Ala-Pro-Phe-pNA) specific substrate for subtilisin and chymotrypsin
V7127 (D-Val-Leu-Lys-pNA) specific substrate for plasmin
V6258 (D-Val-Leu-Arg–pNA) specific substrate for kallikrein
P7021 (D-Phe-Pip-Arg-pNA) specific substrate for thrombin

N-Succ-Ala-Ala-Pro-Phe-pNA, a synthetic substrate for subtilisin and chymotrypsin, was most effectively hydrolyzed with K_m of 0.21 mM, k_{cat}/K_m of 1.76×10^5 S^{-1} M^{-1}, and amidolytic activity of 22.2 μmol min^{-1} l^{-1}, respectively. The enzyme also degraded D-Val-Leu-Lys-pNA and D-Phe-Pip-Arg-pNA, but it did not degrade D-Val-Leu-Arg-pNA, a high affinity substrate for kallikrein.

Effects of temperature and pH on the fibrinolytic enzyme activity and stability

When the enzyme activities were measured at various temperatures and pH values for the chromogenic substrate N-Succ-Ala-Ala-Pro-Phe-pNA, amidolytic activity of the enzyme was stable at below 60°C over a wide range of pH 5–12, with a maximum activity at pH 8.0. In addition, the optimum temperature of the enzyme was found to be 55°C for the hydrolysis of fibrin, which was comparable to those of CK, but higher than those of NK and KA38 [25]. The activity completely disappeared after heating at >65°C for 10 min. When the enzyme was lyophilized and stored at 4°C, its activity had not changed after 3 months. After freezing and thawing for five times, 95% of the fibrinolytic activity remained.

Effect of chemical reagents on the fibrinolytic enzyme

Table 3 shows the effects of various defined enzyme inhibitors on the amidolytic activity using N-Succ-Ala-Ala-Pro-Phe-pNA as a substrate. Amidolytic activity was completely inhibited by 5 mM PMSF and 1 mM SBTI, which are well-known inhibitors of serine proteases. The activity was also strongly inhibited by Pepstatin A. However, β-mercaptoethanol, DTT, and DMSO did not affect the activity. In fact, DMSO even had a significant enhancing effect on the protease activity. EDTA and EGTA did not affect the enzyme activity. These results indicate that the enzyme is a serine protease with a single chain (having no -S–S- bond), and is not a metalloprotease. Mercaptide-forming agents (PHMB and EAM) did not affect the enzyme activity. Consequently, we propose that hydroxy (serine) and indole (tryptophan) groups are located at, or nearby the active site of the enzyme.

Table 3 Effect of defined inhibitors on amidolytic activity of the purified fibrinolytic enzyme of *B. subtilis* DC33

Inhibitors	Concentration (mM)	Relative activity (%)	Inhibitors	Concentration (mM)	Relative activity (%)
None (control)		100	DTT	10	100
PMSF	2.0	43.0		50	98
	5.0	0	β-mercaptoethanol	10	102
SBTI	0.05	53.0		50	97
	0.25	32.9	DMSO	10	108.6
	1	0	EDTA	10	100
Pepstatin A	0.02	14.8	EGTA	10	100
	0.05	0	EAM	20	98
Leupeption	0.02	78.8	PHMB	2	106
	0.05	74.6			

The effect of metal ions such as Ca^{2+}, Co^{2+}, Cu^{2+}, Fe^{2+}, Fe^{3+}, Mg^{2+}, Zn^{2+}, Mn^{2+}, Li^{2+}, Sn^{2+}, Ag^{+}, and Ti^{2+} on the amidolytic activity was also investigated. None of these ions activated the activity; Mg^{2+}, Li^{2+}, Mn^{2+}, Ca^{2+}, and Co^{2+} ions did not decrease the enzyme activity, but Fe^{3+}, and Zn^{2+} ions inhibited the fibrinolytic enzyme by 28 and 69%, respectively, whereas Cu^{2+}, Fe^{2+}, Sn^{2+}, Ag^{+}, and Ti^{2+} fully inhibited the enzyme.

N-terminal amino acid sequencing of the fibrinolytic enzyme

N-terminal amino acid sequence of the fibrinolytic enzyme was determined by automated Edman method using a gas-phase protein sequencer (Model 476A, Applied Biosystems, USA) at Peking University (Beijing, China). As shown in Table 4, the first 15 amino acid residues of N-terminal sequence of the enzyme were A-Q-S-V-P-Y-G-I-P-Q-I-K-A-P-A. The first 15 amino acid residues of the enzyme showed high homology with that of subtilisin NAT (formerly designated Nattokinase) and subtilisin E (EC 3.4.21.62) [26]. However, no other known fibrinolytic enzyme was homologous to the 30 kDa protein with regard to N-terminal amino acid sequence. Amino acids A-Q (nrs. 1–2) and I-K-A (nrs. 11–13) are almost constant amino acid residues of N-terminal sequence of these subtilisins from *Bacillus* spp. The overall results relating to substrate specificity, effect of inhibitors and the N-terminal amino acid sequences, indicate that the fibrinolytic enzyme of *B. subtilis* DC33 is a subtilisin-like serine protease occurring as a monomer. We therefore named it subtilisin FS33.

Blood clots dissolved with subtilisin FS33 in vitro

Blood clots (male Wistar rat, clots blotted with filter paper) were divided into two groups. One was heated at 80°C for 30 min to inactivate endogenous fibrinolytic factors (such as plasmin, plasminogen, and t-PA), whereas the other group remained untreated. 0.5 g of the heated and unheated blood clots were incubated with subtilisin FS33 in micro-tubes at 37°C. It was observed that the non-heated blood clots gradually dissolved within 45 min, whereas the heated blood clots dissolved within 2 h. This indicates that the enzyme is able to

Table 4 N-terminal amino acid sequence of fibrinolytic subtilisin FS33, compared with other fibrinolytic enzymes in literature data

Fibrinolytic enzyme	N-terminal amino acid sequencing	Homology[a] (%)	Molecular weight (kDa)	Enzymes from strains	Authors
Subtilisin FS33	AQSVPYGIPQIKAPA	100	30.0	*B. subtilus* DC33	This work
Nattokinase (NK)	AQSVPYGISQ IKAPALHS	93	27.7	*B. subtilus* natto	Fujita et al. [31]
Subtilisin E	AQSVPYGĪSQIKA PA HS	93	55.8	*B. subtilis* sp.	Wong et al. [26]
Subtilisin DFE	AQSVPYGV̄SQIKAPALHS	86	28.0	*B. amyloliquefaciens*	Peng et al. [22]
SMCE	AQTVPYGIPQIKAD	86	30.0	*B. pumilus* TYO-67	Takahashi [32]
CK	AQTVPYGIPLIKADD	80	28.2	*Bacillus* sp. CK 11–4	Kim et al. [5]
Subtilisin Carlsberg	AQTVPYGIPLIKAD	80	20.0	*B. licheniformis*	Smith et al. [36]
Subtilisin IMR-NK1	AQPVPNGRTAIKA	67	31.5	*B. subtilus* IMR-NK1	Chang et al. [33]
KA38	VYPFPGPIPN	30(-P···IP-)	41.0	*B. subtilus* KA38	Kim et al. [25]
Metalloprotease	DPYEEPGPCENLQVA	No	18.0	*Bacillus* sp.	Mine et al. [1]
Katsuwokinase(KK)	IVGGYEQZAHSQPHQ	No	38.0	*B. .firmus* NA-1	Seo et al. [34]
Jerdofibrase	VIGGDECNINEHPFLVLV	No	55.0	*Trimeresurus jerdonii*	Jia et al. [8]

[a]The first 15 amino acid residues of subtilisin FS33 compared with that of other fibrinolytic enzymes (the percent of Identical amino acid residues and sequence to total numbers of amino acid residues)

degrade blood clots in the absence of endogenous fibrinolytic factors.

Mode of hydrolysis of fibrinogen and fibrin by subtilisin FS33

To elucidate the mode of reaction of subtilisin FS33, degradation products were separated with SDS–PAGE. During the degradation of fibrinogen with subtilisin FS33, Bβ-chains of fibrinogen were cleaved first, followed by slower release of the Aα- and γ-chains, producing many products with lower molecular masses within the experimental period (Fig. 2a). The enzyme decomposed Bβ-chains and also the Aα- and γ-chain to a large extent. This indicates that the proteolytic action of subtilisin FS33 differs from the typical blood clotting enzyme, thrombin, because fibrinogen was hydrolyzed, and no fibrin clot formation was observed.

Similarly, when fibrin was incubated with subtilisin FS33, the β-chain of fibrin was hydrolyzed faster than α- and γ-γ chain within the experimental period (Fig. 2b). Thus, we conclude that subtilisin FS33 has fibrinolytic activity with a relatively high substrate specificity to fibrin (ogen). The enzyme thus could be classified as a β-fibrinogenase [27].

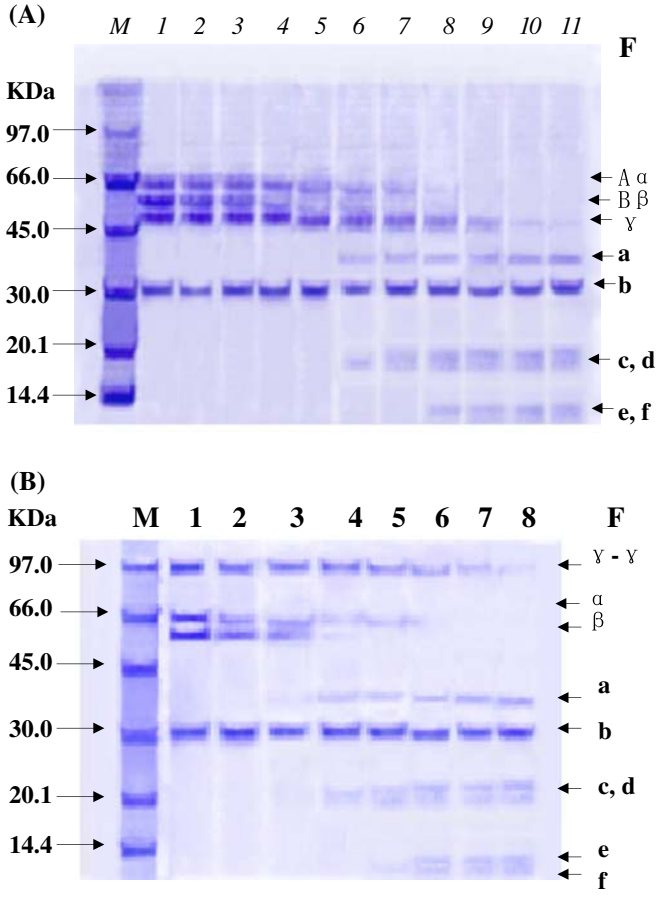

Fig. 2 **a** Hydrolysis of fibrinogen by subtilisin FS33. The hydrolytic products from fibrinogen were separated in 12% SDS–PAGE. *Lanes 1–11* represent hydrolyzed aliquots at 0, 5, 10, 30, 60 min after 1.5, 2, 3, 6, 12, 24 h reaction periods, respectively; F: *a*, *c*, *d*, *e* and *f* represent the digested fragments, respectively; *b* represents subtilisin FS33; Aα, Bβ, and γ represent the corresponding chains of fibrinogen; *lane M* represents protein molecular mass markers. **b** Hydrolysis of fibrin by subtilisin FS33. The hydrolytic products from fibrin were separated in 12% SDS–PAGE. *Lanes 1–8* represent hydrolyzed aliquots after 0, 0.5, 1, 2, 4, 8, 12 and 24 h reaction periods, respectively; F: *a*, *c*, *d*, *e* and *f* represent the digested fragments, respectively; *b* represents subtilisin FS33; α, β and γ–γ represent the corresponding chains of fibrin; *lane M* represents protein molecular mass markers

Degradation of plasminogen

The digestion fragments of plasminogen produced by incubation with subtilisin FS33 were analyzed using SDS–PAGE as shown in Fig. 3. Six fragments from plasminogen were obtained after hydrolysis, with different apparent molecular weights of 43 (P1), 36 (P2), 34 (P3), 27 (P4), 15 (P5), and 12 (P6) kDa, respectively. No further fragments were found when hydrolysis was prolonged for longer than 30 min under the same conditions. Interestingly, the degradation fragment P4 of 27 kDa exhibited fibrinolytic activity in the fibrin plate. The first 8 amino acid residues of N-terminal sequence of the fragment P4 were V-V-G-G-C-V-A-H, which were identical with that of plasmin. With reference to the complete primary structure of human plasminogen [28], we deduce that the significant cleavage site was at R561–↓–V562, similar to t-PA [29] and staphylokinase [30], and that this cleavage site produced the catalytic domain (P4) of the activated plasmin. When human plasminogen was incubated with subtilisin Carlsberg, at least ten fragments from plasminogen were separated by

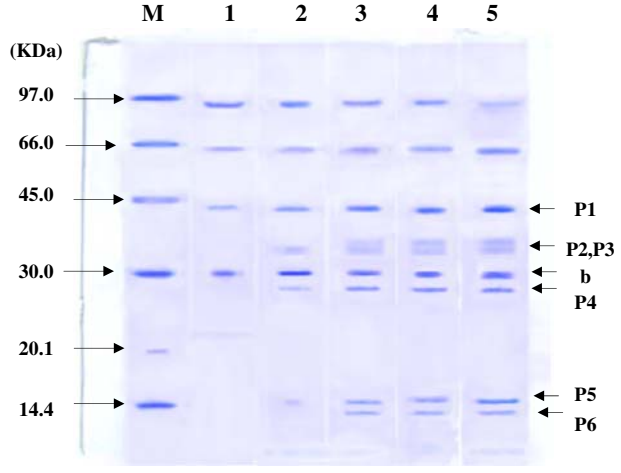

Fig. 3 Degradation of plasminogen by subtilisin FS33. The hydrolytic products from plasminogen were separated in 12% SDS–PAGE. *Lanes 1–5* represent hydrolyzed aliquots after 0, 2, 5, 10 and 15 min reaction periods, respectively; *P1*, *2*, *3*, *4*, 5 and *6* represent the digestion fragments, respectively; *b* represents the subtilisin FS33; *lane M* represents protein molecular mass markers

SDS–PAGE (photographs not shown), but these fragments had no fibrinolytic activity in the fibrin plate.

Densitometric analysis of the SDS–PAGE bands of the hydrolysis fragments indicated that approximately 5% of plasminogen was converted into the active fragment and that 80% was degraded into smaller inactive peptides by subtilisin FS33. Meanwhile, 18% of initial plasminogen activity remained according to light scattering assay. The amidolytic activity of subtilisin FS33 increased about 1.5 times at the start of the reaction when the enzyme was added to a mixture of plasminogen and substrate (H-D-Val-Leu-Lys-pNA). These results concur with our results obtained on plasminogen-free and plasminogen-rich fibrin plates. We conclude that subtilisin FS33 can transform inactive plasminogen into active plasmin. Therefore, we suggest that the fibrinolytic enzyme is able to degrade fibrin clots in two ways: (a) by forming active plasmin from plasminogen (plasminogen activator type) and (b) without plasmin (direct fibrinolysis type), the latter being a major way to the dissolution of fibrin clots as mentioned above.

Degradation of thrombin, kallikrein, and urokinase

In order to determine whether the fibrinolytic enzyme affected thrombotic, and fibrinolytic factors of blood, thrombin, kallikrein, and urokinase were incabated with subtilisin FS33 in micro-tubes (20 mM Tris–HCl buffer, pH 8.0) at 37°C for 30 min, respectively, and then their digestion products were separated by SDS–PAGE. As shown in Fig. 4, at least four fragments from thrombin were obtained after hydrolysis, with different apparent molecular weights of 34 (T1), 32 (T2), 28 (T3), and 12 (T4) kDa, respectively. These fragments of thrombin had no thrombin activity anymore. From kallikrein, lower molecular weight polypeptides were obtained, which had no activity. Two kinds of urokinase were also hydrolyzed to three fragments.

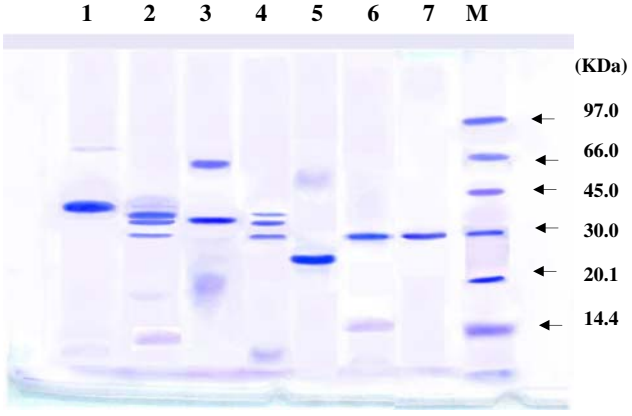

Fig. 4 Degradation of thrombin, kallikrein and urokinase by subtilisin FS33. The hydrolytic products were separated in 12% SDS–PAGE. *Lanes 1, 3, 5, 7* represent thrombin, urokinase, kallikrein and subtilisin FS33, respectively; *lanes 2, 4, 6* represent the digestion fragments of thrombin, urokinase and kallikrein by subtilisin FS33, respectively; *lane M* represents protein molecular weight markers

Discussion

Many fibrinolytic enzymes were found from Asian traditional fermented foods, such as Japanese Natto [4, 13–16], Tofuyo [34], Korean Chungkook-Jang soy sauce [5], edible honey mushroom [35], Chinese *DouChi* and *Sufu* [17, 22], as well as fermented shrimp paste [1]. *DouChi* is a typical and popular flavor-rich fermented soybean food in China and has a history dating back to more than 2,000 years. We previously discovered that fibrinolytic activity exists in *DouChi* and *Sufu* [17], and from Ba-bao *DouChi* we isolated the fibrinolytic *B. subtilis* strain DC33, which differed from other fibrinolytic enzyme producing strains previously reported.

We purified the extracellular fibrinolytic enzyme of *B. subtilus* DC-33 to electrophoretic homogeneity by a combination of chromatographic stages. As shown in Fig. 1, the molecular weight of subtilisin FS33 was estimated to be 30 kDa by SDS–PAGE. This is similar as reported by Takahashi [32] for SMCE (30.0 kDa) of *B. pumilus* TYO-67, but lower than published for subtilisin E (55.8 kDa), subtilisin IMR-NK1 (31.5 kDa) [33], KK (38 kDa) [34], and KA38 (41 kDa) [25] and higher than for subtilisin NAT (27.7 kDa, formerly designated nattokinase, NK), CK (28.2 kDa), and subtilisin DFE (28.0 kDa). In spite of the strong homology of N-terminal sequences of subtilisin FS33, NK, and subtilisin E, they exhibit different molecular weights and amidolytic activities. Amino acids A-Q (nrs. 1–2) and I-K-A (nrs. 11–13) are almost constant amino acid residues in the N-terminal sequence of the known subtilisins from *Bacillus* spp.

Inhibition studies can provide a first insight into the nature of the enzyme, its cofactor requirements, and the nature of the active center [31]. The amidolytic activity of subtilisin FS33 was inhibited completely by PMSF and SBTI, but the enzyme activity was not affected by DTT, β-mercaptoethanol, ions, and metalloprotease inhibitors (EDTA and EGTA). These results indicated that the enzyme is a monomeric serine protease (having no -S–S-), and is not a metalloprotease. Mercaptide-forming agents (PHMB and EAM) had no effect on the activity of the enzyme. Serine and tryptophan therefore are essential in the active site of the enzyme.

As shown in Table 2, subtilisin FS33 had the highest affinity for N-Succ-Ala-Ala-Pro-Phe-pNA; the studies achieved almost the same level of NK and subtilisin IMR-NK1 [33], their amidolytic activities for the substrate were 14.2 and 16.8 μmol min^{-1} l^{-1}, respectively. However, subtilisin FS33, NK, and CK react very differently with D-Val-Leu-Lys-pNA, with amidolytic activities of 0.84, 285.6, and 424 μmol min^{-1} l^{-1}, respectively. Subtilisin FS33 degraded D-Phe-Pip-Arg-pNA, but not D-Val-Leu-Arg-pNA, which distinguishes it also from other known subtilisins.

Subtilisin FS33 had a very strong activity for decomposing the Bβ-chains and Aα-chain of fibrin (ogen), and the enzyme thus can be termed a β-fibrinogenase [27]. Subtilisin FS33 can form a clear lysis zone on plasminogen-rich and plasminogen-free fibrin plates. After incubation with subtilisin FS33, plasminogen was decomposed into the active fragment P4. These results indicate that the fibrinolytic enzyme is able to degrade fibrin clots in two ways, namely by forming active plasmin from plasminogen (plasminogen activator type), and by direct fibrinolysis. Subtilisin FS33 is a proteolytic enzyme and differs from typical blood clotting enzymes such as thrombin, because fibrinogen was hydrolyzed, but no fibrin clot formed.

The overall results relating to substrate specificity, effect of inhibitors, the molecular weight, the N-terminal amino acid sequences, action on thrombotic, and fibrinolytic factors of blood, indicate that subtilisin FS33 differs from other known fibrinolytic enzymes. This novel protease had much higher fibrinolytic activity than urokinase and subtilisin Carlsberg. The analysis of the cleavage site of Bβ-chain, which showed a preference for hydrophilic amino acids, is presently under investigation. The safety of the purified fibrinolytic enzyme, as an orally administered thrombolytic agent, its induction of in vivo lysis of the thrombi, and potential side effects need further research.

References

1. Mine Y, Wong AHK, Jiang B (2005) Fibrinolytic enzymes in Asian traditional fermented foods. Food Rev In 38:243–250
2. Moukhametova LI, Aisina RB, Lomakina GY, Varfolomeev SD (2002) Properties of the urokinase-type plasminogen activator modified with phenylglyoxal. Russ J Bioorg Chem 28(4):278–283
3. Bode C, Runge M, Smalling RW (1996) The future of thrombolysis in the treatment of acute myocardial infarction. Eur Heart J 17:55–60
4. Fujita M, Hong K, Ito Y, Fujii R, Kariya K, Nishimuro S (1995) Thrombolytic effect of nattokinase on a chemically induced thrombosis model in rat. Biol Pharm Bull 18:1387–1391
5. Kim W, Choi K, Kim Y (1996) Purification and characterization of a fibrinolytic enzyme produced from *Bacillus sp*. strain CK11-4 screened from Chungkook-Jang. Appl Environ Microbiol 62:2482–2488
6. Zheng ZL, Zuo ZY, Liu ZG (2005) Construction of a 3D model of nattokinase, a novel fibrinolytic enzyme from *Bacillus natto*: a novel nucleophilic catalytic mechanism for nattokinase. J Mol Graph 23:373–380
7. Wang F, Wang C, Li M (2005) Crystal structure of earthworm fibrinolytic enzyme component B: a novel, glycosylated two-chained trypsin. J Mol Biol 348:671–685
8. Jia YH, Jin Y, Lu QM, Li DS (2003) Jerdonase, a novel serine protease with kinin-releasing and fibrinogenolytic activity from *Trimeresurus jerdonii* venom. Acta Biochem S 35(8):689–694
9. De-Simone SG, Correa-Netto C, Antunes OAC (2005) Biochemical and molecular modeling analysis of the ability of two *p*-aminobenzamidine-based sorbents to selectively purify serine proteases (fibrinogenases) from snake venoms. J Chromatogr B Analyt Technol Biomed Life Sci 822:1–9
10. You WK, Sohn YD, Kim KY, Park DH (2004) Purification and molecular cloning of a novel serine protease from the centipede, *Scolopendra subspinipes mutilans*. Insect Biochem Mol Biol 34:239–250
11. Ahn MY, Hahn BS, Ryu KS (2003) Purification and characterization of a serine protease with fibrinolytic activity from the dung beetles, Catharsius molossus. Thromb Res 112:339–347
12. Chudzinski-Tavassi AM, Kelen EM, De Paula Rosa AP (1998) Fibrino(geno)lytic properties of purified hementerin, a metalloprotease from the leech Haementeria depressa. Thromb Haemost 80:155–160
13. Sumi H, Hamada H, Tsushima H (1987) A novel fibrinolytic enzyme (nattokinase) in the vegetable cheese Natto, a typical and popular soybean food in the Japanese diet. Experientia 43:1110–1111
14. Sumi H, Hamada H, Nakanishi K, Hiratani H (1990) Enhancement of the fibrinolytic activity in plasma by oral administration of nattokinase. Acta Haematol 84:139–143
15. Sumi H, Yanagisawa Y, Yatagai C (2004) Natto bacillus as oral fobrinolytic agent: Nattokinase activity and the ingestion effect of *Bacillus natto*. Food Sci Tec 10(1):17–20
16. Fujita M, Hong K, Ito Y (1995) Transport of NK across of the rat intestinal tract. Biol Pharm Bull 18(9):1194–1196
17. Wang CT, Ji BP, Li B, Ji H (2003) Isolation of *Douchi* fibrinolytic enzyme producing strain DC33 from Chinese *Douchi* and primary analysis of the enzyme property. Food Sci (China) 6:34–38
18. Krieg Nr, Holt JG (1984) Bergey's manual of systematic bacteriology, Williams and Wilkins, Baltimore 1:1104–1139
19. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254
20. Astrup T, MuEllertz S (1952) The fibrin plate method for estimating fibrinolytic activity. Arch Biochem Biophys 40:346–351
21. Price Nc, Stevens L (1989) Fundamentals of enzimology 2nd edn. Oxford university press, Oxford
22. Peng Y, Huang Q, Zhang RH, Zhang YZ (2003) Purification and characterization of a fibrinolytic enzyme produced by *Bacillus amyloliquefaciens* DC-4. Comp Biochem Physiol B Biochem Mol Biol 134:45–52
23. Glazer AN (1967) Esteratic reactions catalyzed by subtilisins. J Biol Chem 242(3):433–436
24. Laemmli UK (1970) Cleavage of structural proteins during assembly of the head of bacteriophage T4. Nature 227:680–685
25. Kim HK, Kim GT, Kim DK, Choi WA (1997) Purification and characterization of a novel fibrinolytic enzyme from Bacillus sp. KA38 originated from fermented fish. J Ferment Bioen 84:307–312
26. Wong SL, Price CW, Goldfarb DS, Doi RH (1984) The subtilisin E gene of Bacillus subtilis is transcribed from a sigma-37 promoter in vivo. Proc Natl Acad Sci USA 81:1184–1188
27. Peng Y, Yang XJ, Zhang YZ (2005) Microbial fibrinolytic enzymes: an overview of source, production, properties and thrombolytic activity in vivo. Appl Microbiol Biotechnol 0175–7598, 1432–0614 (Online). DOI: 10.1007/s00253-005-0159-7
28. Matsuo O, Rijken DC, Collen D (1981) Thrombolysis by human tissue plasminogen activator and urokinase in rabbits with experimental pulmonary embolus. Nature 291(5816): 590–591
29. Wiman B, Wallen P (1975) On the primary structure of human plasminogen and plasmin. Purification and characterization of cyanogen-bromide fragments. Eur J Biochem 57:387–394
30. Lijnen HR, Cock FD, Hoef BV, Schlott B, Collen D (1994) Characterization of the interaction between plasminogen and staphylokinase. Eur J Biochem 224:143–149
31. Fujita M, Nomura K, Hong K, Ito Y, Asada A (1993) Purification and characterization of a strong fibrinolytic enzyme (nattokinase) in the vegetable cheese natto, a popular soybean fermented food in Japan. Biochem Biophys Res Commun 197:1340–1347

32. Takahashi M, Sekine T, Kuba N, Nakamori S, Yasuda M (2004) The production of recombinant APRP, an alkaline protease derived from *Bacillus pumilus* TYO-67, by in vitro refolding of pro-enzyme fixed on a solid surface. J Biochem 136(4):549–556
33. Chang CT, Fan MH, Kuo FC, Sung HY (2000) Potent fibrinolytic enzyme from a mutant of *Bacillus subtilis* IMR-NK1. J Agric Food Chem 48:3210–3216
34. Seo JH, Lee SP (2004) Production of fibrinolytic enzyme(KK) from soybean grits fermented by *Bacillus firmus* NA-1. J Med Food 7(4):442–449
35. Kim JH, Kim YS (1999) A fibrinolytic metalloprotease from the fruiting bodies of an edible mushroom, Armillariella mellea. Biosci Biotechnol Biochem 63:2130–2136
36. Smith EL, Delange RJ, Evans WH, Landon M (1968) Subtilisin Carlsberg V. The complete sequence, comparison with subtilisin BPN', evolutionary relationships. J Biol Chem 243:2184–2191