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ORIGINAL PAPER

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Purification and characterization of a fibrinolytic subtilisin-like protease of *Bacillus subtilis* TP-6 from an Indonesian fermented soybean, Tempeh

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Abstract We have isolated a bacterium (TP-6) from the Indonesian fermented soybean, Tempeh, which produces a strong fibrinolytic protease and was identified as *Bacillus subtilis*. The protease (TPase) was purified to homogeneity by ammonium sulfate fractionation and octyl sepharose and SP sepharose chromatography. The N-terminal amino acid sequence of the 27.5 kDa enzyme was determined, and the encoding gene was cloned and sequenced. The result demonstrates that TPase is a serine protease of the subtilisin family consisting of 275 amino acid residues in its mature form. Its apparent K_m and V_{max} for the synthetic substrate *N*-succinyl-Ala-Ala-Pro-Phe-*p*NA were 259 μM and 145 μmol mg^{-1} min^{-1}, respectively. The fibrinogen degradation pattern generated by TPase as a function of time was similar to that obtained with plasmin. In addition, N-terminal amino acid sequence analysis of the fibrinogen degradation products demonstrated that TPase cleaves Glu (or Asp) near hydrophobic acids as a P1 site in the α- and β-chains of fibrinogen to generate fragments D′, E′, and D′ similar to those generated by plasmin. On plasminogen-rich fibrin plates, TPase did not seem to activate fibrin clot lysis. Moreover, the enzyme converted the active plasminogen activator inhibitor-1 to the latent form.

Keywords *Bacillus subtilis* TP-6 · Fibrin clot lysis · Subtilisin-like protease · Tempeh · TPase

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Introduction

Since intravascular thrombosis due to fibrin aggregation in arteries is one of the main causes of cardiovascular diseases, a number of thrombolytic agents, such as streptokinase, urokinase, tissue-type plasminogen activator (t-PA), and single-chain urokinase-type plasminogen activator (scu-PA), have been developed for therapeutic use [8, 9, 22, 24]. Of these, the first-generation fibrinolytic agents such as streptokinase and urokinase can mediate unwanted side reactions. For instance, both deplete plasminogen within the thrombus, converting circulating plasminogen to plasmin [31]. Streptokinase is also immunogenic such that its use results in drug resistance, fever, and allergic reactions [20]. Second-generation agents such as t-PA and scu-PA are fibrin specific; however, they not only decrease the levels of circulating fibrinogen and plasminogen but also increase the risk of intracranial hemorrhage [19, 28]. These limitations have prompted the development of third-generation thrombolytic agents [31] that are either conjugates of t-PAs with monoclonal antibodies against fibrin, platelets, and thrombomodulin or chimeric plasminogen activators. In addition, novel molecules of animal or bacterial origin have been screened for their thrombolytic activities [18, 23].

Several microbial serine proteases with fibrinolytic activity have been considered as therapeutic agents, and traditional fermented foods in Asia have been identified as effective sources of thrombolytic agents. A strong fibrinolytic enzyme (subtilisin NAT) originating from a *Bacillus subtilis* present in Natto, a Japanese fermented soybean product, has been purified and

studied extensively [10, 25]. It was confirmed that the oral administration of subtilisin NAT produced a mild and frequent enhancement of fibrinolytic activity in plasma [26]. Recently, Urano et al. [29] reported that subtilisin NAT also cleaved plasminogen activator inhibitor type I (PAI-1) and enhanced t-PA-induced clot lysis. CKase, a strong fibrinolytic protease from *Bacillus* sp. CK 11-4, has also been isolated from Chungkook-Jang, a traditional Korean fermented soybean sauce [13]. In addition, 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 [6]. All these fibrinolytic enzymes promote t-PA-induced fibrin clot lysis while subtilisin NAT can also directly degrade fibrin clot.

In the present work, a strain of *B. subtilis* TP-6 with strong fibrinolytic activity has been isolated from Tempeh, an Indonesian indigenous fermented soy food [12]. From this strain, a novel subtilisin-like serine protease has been purified and characterized. The enzyme was highly specific for fibrin but did not seem to activate fibrin clot lysis on plasminogen-rich fibrin plates. Moreover, it did not show any degrading activity for plasminogen. Here fibrin-degradation patterns of *B. subtilis* TP-6 protease (TPase) were also compared with those of plasmin.

Materials and methods

Screening and culture conditions

Tempeh samples were collected from various regions of Java in Indonesia, enriched aerobically, and bacteria contained therein were isolated by serial dilution in nutrient broth (Difco, MD, USA) at 37°C. Culture supernatants of the isolates were obtained by centrifugation (10,000×*g*, 15 in) and assayed for fibrinolytic activity by the fibrin plate method [1]. Isolate TP6, showing the highest fibrinolytic activity, was chosen and cultured at 37°C in modified nutrient broth (pH 7.0) consisting of 5 soytone (Difco), 5 beef extract (Difco), and 20 g xylose (Sigma) per liter. For enzyme purification, growth was carried out in a 5 l fermentor (Biostat S; Braun, Germany) at 1,000 rpm with an aeration rate of 1 vvm until the end of the exponential phase of growth.

16S rDNA sequencing and analysis

Genomic DNA was extracted from the cells of a 12 h culture using Genomic Tip-100 (Qiagen, Germany) according to the manufacturer's instructions. The 16S rDNA gene was amplified by PCR using primers 9F (5′-GAGTTTGATCCTGGCTCAG-3′; positions 9–27 according to *Escherichia coli* 16S rRNA numbering) and 1542R (5′-AGAAAGGAGGTGATCCAGCC-3′; positions 1542–1525) [33]. The amplified PCR products were sequenced with a Basestation DNA fragment analyzer (NJ Research, USA). The 16S rDNA sequences were aligned using the Clustal W program, version 1.81 [27].

Cloning and sequencing of TPase gene

Genomic DNA *of B. subtilis* TP6 was isolated essentially as described above. A DNA fragment containing the putative TPase gene was obtained by PCR with the genomic DNA of *B. subtilis* TP6 as template using degenerate primers [(5′-GTG AGA (A/G)GC AAA AA(A/G) (G/T)T(A/G) TGG ATC AG and 5′-A(A/T)T GTG C(A/T)G CTG CTT GTA CGT TGA T(C/T)] based on the sequence of a subtilisin (*aprT*) from *Bacillus* sp. [15]. The amplification conditions employed were one cycle of 94°C for 5 min, followed by 30 repeats of a cycle including 30 s of melting at 94°C, 30 s of annealing at 57°C, and 120 s of extension at 72°C, and then one final extension cycle at 72°C for 7 min. The PCR products amplified from the genomic DNA were directly cloned into pGEM-T Easy vector (Promega, Medigan, WI, USA), used to transform *E. coli* DH5α. Plasmids from *E. coli* DH5α were isolated using a Qiaprep Spin Miniprep kit (Qiagen) according to the manufacturer's instructions. The isolated plasmid was sequenced. The GenBank program BLAST was utilized to find similar genes or proteins and the Clustal W program, version 1.81 was then used to align such sequences.

Cloning and expression of human plasminogen activator inhibitor-1

The mature human plasminogen activator inhibitor-1 (PAI-1) was amplified from human placental cDNA (Clontech Laboratories, Inc.) using primers: 5′-**GGATCC**GTGCACCATCCCCCATCC (with the *Bam*HI site in bold) and 5′-**AAGCTT**TTAGGGTTCCATCACTTGGCCC (with the *Hin*dIII site in bold). The 1.2 kbp PCR product was digested with the above restriction enzymes as was the expression vector, pQE30 (Qiagen). The PCR product was ligated into the vector to yield pQE-PAI1, used to transform *E. coli* M15 cells.

Enzyme assay

Protease activity was measured with Hammerstein casein (Merk, Darmstadt, Germany) as substrate according to the modified method of Kunitz [14]. The reaction was initiated by adding 50 µl of enzyme solution to 450 µl of 0.5% (w/v) Hammerstein casein in 10 mM sodium phosphate buffer (pH 7.4). After 30 min at 37°C, the reaction was terminated by the addition of 500 µl of 15% (w/v) trichloroacetic acid (TCA), heat-treated for 20 min at 70°C, and centrifuged at 13,000×*g*

for 20 min at 4°C. The absorbance of the supernatant was measured at 280 nm. One unit (U) of protease activity is defined as the amount of enzyme that produces an increase in absorbance at 280 nm of 0.01 per min in the above conditions.

Fibrinolytic activity was determined using the fibrin plate assay method [1]. Human fibrinogen (0.5%, w/v, Sigma) dissolved in 10 mM sodium phosphate buffer (pH 7.4) was passed through a lysine sepharose column equilibrated with the same buffer in order to remove plasminogen [7]. Aliquots (10 ml) of the eluted plasminogen-free fibrinogen solution were mixed with 0.1 ml thrombin solution (100 NIH U ml^{-1}) and poured into the plates. After hardening for 1 h at 4°C, the fibrin plate was used to measure fibrinolytic activity: the enzyme solution (50 µl) at a suitable concentration was loaded onto a paper disk (8 mm in diameter, Millipore) that was placed on the fibrin plate and incubated for 6 h at 37°C. For quantitative analysis, the diameter of the zone of clearing was measured and the activity calculated from a reference plot of human plasmin as standard fibrinolytic enzyme.

Purification of TPase and plasminogen activator inhibitor-1

All purification steps were carried out on the ÄKTA FPLC system at 4°C. *B. subtilis* TP6 was cultured for enzyme purification in a fermentor (5 l, Biostat S) at 1,000 rpm with an aeration rate of 1 vvm to the end of the stationary phase. The culture broth was centrifuged at 10,000×*g* for 20 min and the cell-free supernatant obtained by centrifugation was fractionated with ammonium sulfate (40–70% saturation). The precipitate was resuspended in 50 mM sodium phosphate buffer (pH 6.0) and dialyzed overnight against the same buffer at 4°C. The dialysate (final concentration 2.0 M) was loaded onto an octyl sepharose FF column (30 ml) equilibrated in 50 mM sodium phosphate (pH 6.0) with 2.0 M ammonium sulfate. Proteins were eluted with a descending gradient of ammonium sulfate (2 to 0 M) and fractions containing proteolytic activity pooled. The pooled fractions were buffer-exchanged into 50 mM potassium phosphate buffer (pH 6.0) and applied to a Hiprep 16/10 SP sepharose FF column equilibrated with the same buffer. Absorbed proteins were eluted with a linear gradient of 0–0.5 M NaCl at 1.5 ml min^{-1}. Fractions with enzyme activity were pooled and concentrated approximately tenfold with a 10,000 molecular weight cutoff membrane (Amicon, Bedford, MA, USA). The protein was stored in this buffer at −20°C.

Expression of PAI-1 and purification of the active, non-glycosylated, recombinant PAI-1 were performed as described previously [17]. *E. coli* M15 cells containing pQE-PAI1 were grown in 2 l of Luria-Bertani (LB) medium at 37°C containing 100 µg of ampicillin and 50 µg of kanamycin per ml, induced in mid-exponential phase ($OD_{600} = 0.6$) with 0.1 mM of isopropyl-β-D-thiogalactopyranoside, grown at 30°C for an additional 10 h, and harvested by centrifugation (4,500×*g*, 10 min, 4°C). To purify the active PAI-1, cell pellets were resuspended in 20 mM sodium acetate buffer (pH 5.6) with 0.5 M sodium chloride and 0.05% Tween 80, disrupted by sonication and the lysates centrifuged at 10,000×*g* for 20 min to remove cell debris. The supernatants were filtered through a 0.2 µm filter and loaded onto His-Bind resin column (5 ml, Novagen) equilibrated with 20 mM sodium acetate buffer (pH 5.6) with 0.5 M sodium chloride, 0.01% Tween 80, and 5 mM imidazole. The absorbed proteins were eluted with buffer containing 300 mM imidazole. The pooled fractions were dialyzed overnight with 50 mM sodium phosphate buffer (pH 7.4). The purified active PAI-1 was filtered and stored at −20°C before use. Protein concentration was determined with the bicinchoninic acid protein assay reagent kit (Sigma) with bovine serum albumin as standard.

Electrophoresis and N-terminal amino acid sequencing

SDS-PAGE was carried out with a Modular Mini-Protein II Electrophoresis System (Bio-Rad, Hercules, CA, USA) according to standard protocols [16]. Broad range molecular weight markers (Bio-Rad) were used to determine the molecular mass of proteins. After electrophoresis, proteins were visualized using Coomassie brilliant blue R-250 (Sigma) or silver nitrate (Bio-Rad). For blotting, proteins on the gel (12% SDS-PAGE) were transferred to a Sequi-Blot PVDF membrane (Bio-Rad) in 10 mM CAPS buffer (pH 11.0), 10% methanol, using a Mini Trans-Blot electrophoretic transfer apparatus (Bio-Rad). N-terminal amino acid sequencing of protein bands of interest was performed at the Tufts University, Analytical Core Facility, Boston, MA, USA.

Results and discussion

Isolation and identification of a fibrin-degrading bacterium

From five Tempeh samples, 200 microorganisms showing fibrinolytic activity were screened. Amongst them, isolate TP6 was chosen for further investigation in light of it exhibiting relatively strong activity for fibrin degradation than for casein as revealed by comparing the diameters of halos formed in the solid-plate assay with fibrin versus casein. The fibrin-degrading isolate TP6 is Gram positive, rod shaped, endospore forming, and actively motile. The 16S rDNA sequence of isolate TP6 (1,489 bp) showed a high level of sequence similarity (99%) to that of *B. subtilis*. Thus, the isolate was identified as a strain of *B. subtilis* and designated as *B. subtilis* TP6.

Purification of an extracellular protease (TPase) from *B. subtilis* TP6

The fibrinolytic protease was purified from a culture supernatant (3 l) of *B. subtilis* TP6. As summarized in Table 1, purification of TPase was performed in three steps, i.e., ammonium sulfate fractionation followed by octyl sepharose hydrophobic and SP sepharose cation-exchange chromatography. Proteolytic and fibrinolytic activities were evaluated at each step of the purification as described in Materials and methods. TPase was thus purified 200-fold, with a yield of 26%, as compared with the crude enzyme. The purity of the protease was examined by 12% SDS-PAGE. A single protein was visualized by silver nitrate staining and its apparent M_r was estimated as 29 kDa (Fig. 1). The N-terminal sequence of the first 24 residues of the purified TPase was determined to be Ala-Gln-Ser-Val-Pro-Tyr-Gly-Val-Ser-Gln-Ile-Lys-Ala-Pro-Ala-Leu-His-Ser-Gln-Gly-Phe-Thr-Gly-Ser (Fig. 2). The result demonstrated that TPase exhibits a high level of sequence similarity to other subtilisin-like serine proteases such as subtilisin BPN from *B. amyloliquefaciences* [30] and subtilisin E from *B. subtilis* [21].

Nucleotide sequence of TPase

The gene encoding TPase was amplified by PCR from the genomic DNA of *B. subtilis* TP6 using the degenerate primers as described above. Based on the amino acid sequence deduced from the DNA sequence of TPase, the enzyme is apparently composed of 275 amino acids with a molecular mass of 29 kDa in the mature form (Fig. 2). The DNA sequence of TPase exhibits high levels of sequence identity to subtilisin K54 from *B. subtilis* K-54 (97%), subtilisin BPN′ from *B. amyloliquefaciences* (95%) [30], subtilisin E (82%), and NAT (82%). In addition, the deduced amino acid sequence of TPase has three conserved amino acid residues (Ser 221, His 64, and Asp 32) essential for the catalytic activity of serine proteases [21, 34]. Therefore, the above results suggest that the purified fibrinolytic protease, TPase, is a subtilisin-like serine protease.

Biochemical characterization of TPase

The temperature dependence of the purified enzyme was determined after 30 min of incubation at various temperatures (25–70°C). TPase exhibited maximal activity at 50°C and pH 7.0 under the assay conditions used. For pH stability study, the enzyme activity was measured after a 24 h pre-incubation at 37°C in different pH buffers (pH 4–7). The enzyme was active and stable in the acidic range pH of 6.0–6.5.

To investigate the effects of protease inhibitors and organic solvents on TPase activity, the enzyme was pre-incubated in the presence of various reagents for 30 min at 37°C, and its residual activity was measured (Table 2). TPase was inhibited by 1 mM PMSF (53%), 1 mM EDTA (36%), and 10% β-mercaptoethanol (46%). Other inhibitors and organic solvents such as pepstatin A, leupeptin, DTT, methanol, ethanol, and isopropanol caused no inhibitory effect on the enzyme.

The effect of metal ions on the enzyme activity was also studied. Metal ions were removed from the purified TPase by treatment with 5 mM EDTA at room temperature for 1 h, followed by overnight dialysis against 10 mM sodium phosphate buffer (pH 7.4) at 4°C with several changes of buffer. After that, various metal ions, such as Ca^{2+}, Ni^{2+}, Mn^{2+}, Mg^{2+}, Cu^{2+}, Zn^{2+}, Fe^{2+}, Co^{2+}, Na^{+}, and K^{+}, were added to the dialyzed enzyme. The proteolytic activity of an EDTA-treated TPase (100%) was slightly restored by the addition of 1 mM Ca^{2+} (123%) and Fe^{2+} (127%) (data not shown). However, the EDTA-treated enzyme in the presence of Ca^{2+} had only 34% of as-isolated enzyme activity. It has been reported that subtilisins from *Bacillus* species were inhibited by the deletion of metal ions and did not sufficiently recover upon addition of metal ions [5]. In addition, those are classified as serine endopeptidases and inhibited by PMSF. The enzymes are most active at neural or alkaline pH with a molecular mass of 15–30 kDa. The universal feature of subtilisins is the presence of one or more calcium-binding sites. Because calcium binding acts as a major contributor to conformational stability, the removal of Ca^{2+} ions results in a loss of conformational stability of these enzymes. TPase also seems to contain the calcium-binding site, termed site A, based on the high degree of sequence homology to other subtilisins. Therefore, together with the above sequence data, the biochemical results suggest that TPase is a subtilisin-like serine protease.

Substrate specificity and kinetic parameters of TPase

The substrate specificity of purified TPase was analyzed using synthetic substrates (Table 3). Amidolytic activity

Table 1 Purification of TPase from *B. subtilis* TP-6

Purification step	Total protein (mg)	Total activity[a] (AU)	Specific activity (AU mg^{-1})	Fold purification	Yield (%)
Culture supernatant	12,320	73,447	6	1	100
Ammonium sulfate precipitation	970	67,994	70	12	93
Octyl sepharose	44	41,614	945	158	57
SP sepharose	16	19,165	1,197	200	26

[a]Protease activity was measured with Hammerstein casein as a substrate (see Materials and methods)

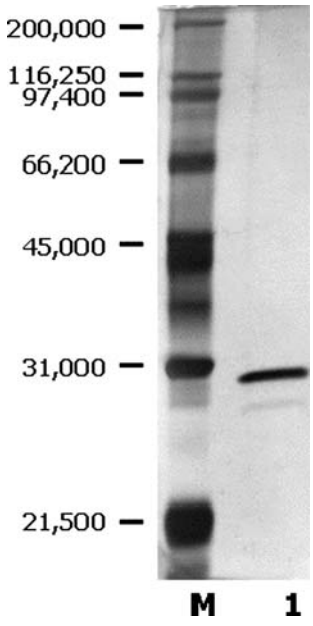

Fig. 1 SDS-PAGE (12%) of the purified TPase from *B. subtilis* TP-6. *M* molecular weight markers (Bio-Rad), *1* purified TPase from SP sepharose

was highest (20,000 nmol min^{-1} ml^{-1}) with *N*-succinyl-Ala-Ala-Pro-Phe-*p*NA. In contrast, it had very low activity with other substrates such as D-Val-Leu-Lys-*p*NA or *N*-succinyl-Ala-Ala-Ala-*p*NA. To compare its activity with fibrinolytic proteases such as nattokinase (NK) from *B. natto* and a fibrinolytic protease from *B. subtilis* IMR-NK1, the kinetic parameters of TPase were determined for *N*-succinyl-Ala-Ala-Pro-Phe-*p*NA as a representative substrate for the subtilisin-like protease under the same conditions as described previously (Table 4) [6]. The K_m and k_{cat} values of TPase were 0.26 mM and 25.71 s^{-1}, respectively. Although the k_{cat} value of TPase was similar to that of NK and MK1, the K_m value of TPase is higher than that of NK, resulting in the increased catalytic efficiency ($k_{cat}/K_m = 9.9 \times 10^4$ s^{-1} M^{-1}), i.e., approximately three times higher than that of NK. From this result, it is evident that TPase has significantly higher substrate specificity toward the synthetic substrate than does NK, one of the best fibrinolytic protease from the genus of *Bacillus* identified so far. As TPase is a fibrinolytic serine protease and a member of the subtilisin family like NK, the activity of TPase on a synthetic substrate may have direct relevance to its strong fibrinolytic activity rather than the others. These results strongly suggest that TPase is the most promising candidate for fibrin clot lysis.

Analysis of fibrinogen degradation

The target protein of the coagulation cascade is fibrinogen, a 340 kDa soluble plasma protein consisting of disulfide-bonded α-, β-, and γ-chains [4, 32]. Following vascular injury, the thrombin-mediated polymerization of fibrinogen (i.e., fibrin) comprises the major protein component of the hemostatic plug. In contrast, the

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GTGAGAAGCAAAAAGTTGTGGATCAGTTTGCTGTTTGCTTTAGCGTTAATCTTTACGATGGCGTTCGGCAGCACGACTTCTGCCCAGGCT      90
MetArgSerLysLysLeuTrpIleSerLeuLeuPheAlaLeuAlaLeuIlePheThrMetAlaPheGlySerThrThrSerAlaGlnAla

GCAGGGAAATCAAACGGGGAAAAGAAATATATTGTCGGATTTAAGCAGACAATGAGCACGATGAGCGCCGCCAAGAAAAAAGATGTCATT     180
AlaGlyLysSerAsnGlyGluLysLysTyrIleValGlyPheLysGlnThrMetSerThrMetSerAlaAlaLysLysLysAspValIle

TCTGAAAAAGGCGGGAAAGTGCAAAAGCAATTCAAATATGTAGACGCAGCTTCAGCTACATTAAATGAAAAAGCTGTAAAAGAGCTGAAA     270
SerGluLysGlyGlyLysValGlnLysGlnPheLysTyrValAspAlaAlaSerAlaThrLeuAsnGluLysAlaValLysGluLeuLys

AAAGACCCTAGCGTCGCTTACGTTGAAGAAGATCACGTTGCACAGGCGTACGCGCAGTCCGTGCCTTACGGCGTATCACAGATTAAAGCC     360
LysAspProSerValAlaTyrValGluGluAspHisValAlaGlnAlaTyr[AlaGlnSerValProTyrGlyValSerGlnIleLysAla]

CCTGCTCTGCACTCTCAAGGCTTCACCGGATCAAATGTTAAAGTAGCGGTTATCGACAGCGGTATCGATTCTTCTCATCCTGATTTAAAG     450
[ProAlaLeuHisSerGlnGlyPheThrGlySer]AsnValLysValAlaValIleAspSerGlyIleAspSerSerHisProAspLeuLys

GTAGCAGGCGGAGCCAGCATGGTTCCTTCTGAAACAAATCCTTTCCAAGACAGAAACTCTCACGGAACTCACGTTGCCGGTACAGTTGCG     540
ValAlaGlyGlyAlaSerMetValProSerGluThrAsnProPheGlnAspArgAsnSerHisGlyThrHisValAlaGlyThrValAla

GCTCTTAATAACTCAGTCGGTGTATTAGGCGTTGCGCCAAGCGCATCTCTTTACGCGGTAAAAGTTCTCGGCACTGACGGTTCCGGCCAG     630
AlaLeuAsnAsnSerValGlyValLeuGlyValAlaProSerAlaSerLeuTyrAlaValLysValLeuGlyThrAspGlySerGlyGln

TACAGCTGGATCATTAACGGAATTGAGTGGGCGATCGCAAACAATATGGACGTTATTAACATGAGCCTCGGCGGACCTTCTGGTTCTGCA     720
TyrSerTrpIleIleAsnGlyIleGluTrpAlaIleAlaAsnAsnMetAspValIleAsnMetSerLeuGlyGlyProSerGlySerAla

GCGTTAAAAGCGGCAGTTGACAAAGCCGTTGCTTCCGGCGTCGTAGTGGTTGCGGCAGCCGGTAACGAAGGCACTTCCGGCGGCTCTAGC     810
AlaLeuLysAlaAlaValAspLysAlaValAlaSerGlyValValValValAlaAlaAlaGlyAsnGluGlyThrSerGlyGlySerSer

ACAGTGGGCTACCCTGGTAAATACCCTTCTGTCATTGCAGTAGGCGCTGTTAACAGCAGCAACCAAAGAGCATCTTTCTCAAGCGTAGGT     900
ThrValGlyTyrProGlyLysTyrProSerValIleAlaValGlyAlaValAsnSerSerAsnGlnArgAlaSerPheSerSerValGly

TCTGAGCTTGATGTCATGGCACCAGGCGTCTCTATCCAAAGCACGCTTCCTGGAAACAAATACGGCGCGTACAATGGTACGTCAATGGCA     990
SerGluLeuAspValMetAlaProGlyValSerIleGlnSerThrLeuProGlyAsnLysTyrGlyAlaTyrAsnGlyThrSerMetAla

TCTCCGCACGTTGCCGGAGCGGCTGCTTTGATTCTTTCTAAGCACCCGAACTGGACAAACACTCAAGTCCGCAGCAGTTTAGAAAACACC    1080
SerProHisValAlaGltAlaAlaAlaLeuIleLeuSerLysHisProAsnTrpThrAsnThrGlnValArgSerSerLeuAluAsnThr

ACTACAAAACTTGGTGATGCTTTCTACTACGGAAAAGGGCTAATCAACGTACAAGCAGCTGCACATTAA                          1149
ThrThrLysLeuGlyAspAlaPheTyrTyrGlyLysGlyLeuIleAsnValGlnAlaAlaAlaHis***
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Fig. 2 Nucleotide and deduced amino acid sequences of the gene encoding TPase. The N-terminal amino acid residues of TPase purified from *B. subtilis* TP-6 are indicated in *bold*

Table 2 Effect of various inhibitors on the fibrinolytic activity of TPase

Inhibitors	Concentration	Relative activity (%)
None	1 mM	100
E-64	0.1 mM	100
Pepstatin A	1 mM	100
Leupeptin	1 mM	97
PMSF	1 mM	53
DTT	1 mM	98
EDTA	1 mM	36
	5 mM	30
	10 mM	27
2-Mercaptoethanol	10%	46
	1%	78
Ethanol	10%	94
	1%	100
Methanol	10%	97
	1%	100
Isopropanol	10%	94
	1%	100

solubilization of fibrin is the result of selected bond cleavages catalyzed by plasmin. Such cleavage of fibrinogen by plasmin yields a characteristic degradation pattern [11]. Although the cleavage sites of fibrinogen and fibrin have been identified and the products generated by the digestion of fibrin by plasmin well characterized, no bacterial enzyme able to mimic this protease-mediated fibrin degradation has been proposed. Thus, the fibrinolytic pattern of TPase was analyzed and compared with that offered by human plasmin using human fibrinogen as substrate. Purified TPase was incubated for 6 h at 37°C with 0.25% (w/v) human fibrinogen, and aliquots of the reaction mixture were withdrawn periodically and analyzed on SDS-PAGE. As shown in Fig. 3a, TPase completely degraded the α-chain of fibrinogen in 5 min and the β-chain was no longer intact after 10 min. In contrast to the human plasmin treatment, the γ-chain seemed to be degraded by TPase, the γ-chain was not completely digested even after 6 h of treatment. As shown in Table 3, TPase showed relatively low activity for D-Val-Leu-Lys-*p*NA as a target substrate as compared with human plasmin, further reflecting the different substrate specificity of TPase from human plasmin (Fig. 3b). Thus, this result indicates that a specific TPase substrate recognition site might be present in the α- and β-chains of fibrinogen rather than the γ-chain different from that of plasmin even though the time course of fibrinolysis had a similar pattern to that of human plasmin. As shown in Fig. 3, however, both enzymes yield similar cleavage patterns with the target substrate, fibrinogen, although substrate recognition by TPase may be less selective than plasmin. Thus, TPase seems to be a remarkable fibrin-specific and plasmin-like protease.

Amino acid sequence of fibrinogen degradation products

The N-terminal amino acid sequences of the first ten residues of the fibrinogen degradation products (FDPs) obtained upon TPase treatment were determined to analyze the catalytic site of the enzyme. Twenty microliters of fibrinogen solution (10 mg ml^{-1}) in 10 mM sodium phosphate buffer (pH 7.9) was incubated with 20 μl purified TPase for 6 h at 37°C. The reaction mixture was subjected to 12% SDS-PAGE and transferred to PVDF membrane for amino acid sequence analysis. The N-terminal amino acid sequences of the major protein bands cleaved by TPase were determined as follows: GGGVRGPRVV (26 kDa), GGGVRGPRVV (31 kDa), YSSESEKHQ (36.9 kDa), and AATLK-SRKML (37.4 kDa). Each amino acid sequence was aligned with that of human fibrinogen. The resultant fragments corresponded to the internal sequences of the α1, α2, β1, and γ1 fragments, respectively. The relative molecular weights of these fragments determined by

Table 3 Comparative amidolytic activity of TPase

Synthetic substrate (0.5 mM)	Substrate hydrolysis (nmol min^{-1} ml^{-1})	Relative activity (%)	Target enzymes	References
N-succinyl-Ala-Ala-Pro-Phe-*p*NA	20,488	100	Subtilisin or chymotrypsin	[6, 10]
N-succinyl-Ala-Ala-Pro-Leu-*p*NA	5,626	27.4	Subtilisin or chymotrypsin	[6, 10]
D-Val-Leu-Lys-*p*NA	13.5	0.1	Plasmin	[2, 13]
N-succinyl-Ala-Ala-Ala-*p*NA	1.7	0	Gelatinase	[6]

*p*NA, *p*-nitroanilide

Table 4 Kinetic constants of the fibrinolytic enzymes from *B. subtilis* TP6, NK from *B. natto*, and MK1 from *B. subtilis* IMR-NK1

TPase from *B. subtilis* TP6			NK from *B. natto*			MK1 from *B. subtilis* IMR-NK1		
k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (s^{-1} M^{-1})	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (s^{-1} M^{-1})	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (s^{-1} M^{-1})
25.71	0.26	9.9×10^4	17.85	0.52	3.4×10^4	21.08	0.34	6.21×10^4

Kinetic constants were determined using *N*-succinyl-Ala-Ala-Pro-Phe-*p*NA as a substrate in 1.0 ml of 100 mM sodium phosphate buffer (0.1 M NaCl, pH 7.4) at 37°C for TPase and NK. MK1 was incubated in 1.2 ml of 10.4 mM Tris–HCl buffer (4.2 mM $CaCl_2$, pH 7.8) [6]

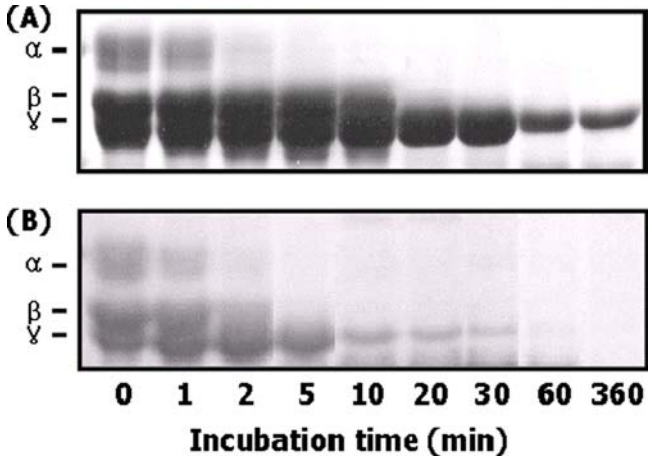

Fig. 3 SDS-PAGE of human fibrinogen degraded by TPase (**a**) and plasmin (**b**). Fibrinogen (0.25%, w/v) was incubated in 20 mM sodium phosphate buffer (pH 7.4) at 37°C with purified TPase (0.2 μg) and plasmin (1 μg). Aliquots of the reaction mixtures were withdrawn after various periods of incubation and analyzed by SDS-PAGE (12%)

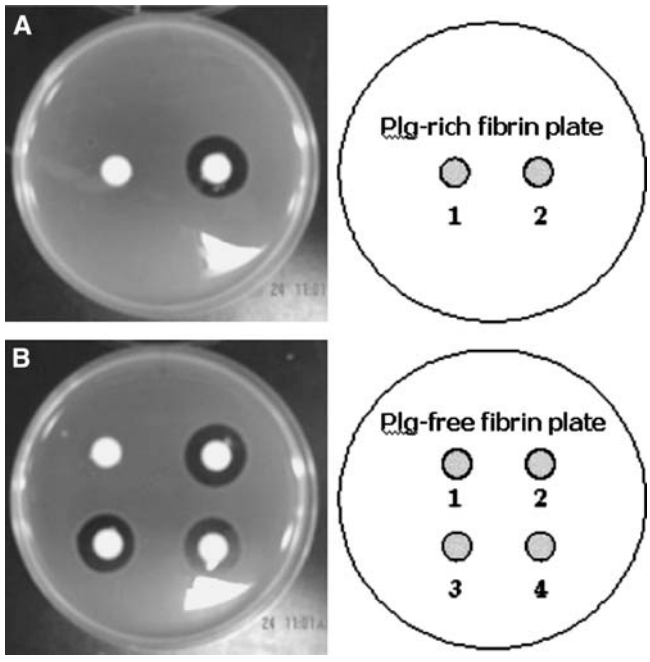

Fig. 5 Analysis of fibrinolysis by TPase on plasminogen-rich fibrin plate (**a**) and plasminogen-free fibrin plate (**b**). *1* 100 mM sodium phosphate buffer (pH 7.4) as a control, *2* TPase (2.25 μg) in 100 mM sodium phosphate buffer (pH 7.4), *3* TPase (2.25 μg) in 100 mM sodium phosphate buffer (10 μl) as a control, *4* TPase (2.25 μg) and plasminogen (0.05 U) in 100 mM sodium phosphate buffer after 6 h of pre-incubation. One unit of plasminogen activity was defined as an increase in absorbance at 275 nm of 1.0 from α-casein in 20 min at 37°C

SDS-PAGE analysis were identical to those calculated from theoretical analysis based on the amino acid sequence of fibrinogen. These results indicate that the C-terminal regions of the *β*- and *γ*-chains of fibrinogen were not cleaved, as shown in Fig. 3a. In contrast with the non-reduced fragments from the fibrin clot cleaved by TPase, non-reduced fragments from fibrinogen showed higher molecular weight than was expected (Fig. 4a). For a more detailed analysis of fibrinogen degradation patterns, 10 mg fibrinogen was digested with 100 U TPase for 3 h and loaded onto a Superdex 200 gel column. The partially digested fibrinogen was eluted as three major peaks, which were analyzed by SDS-PAGE under non-reducing and reducing conditions. The approximate molecular weight of each peak

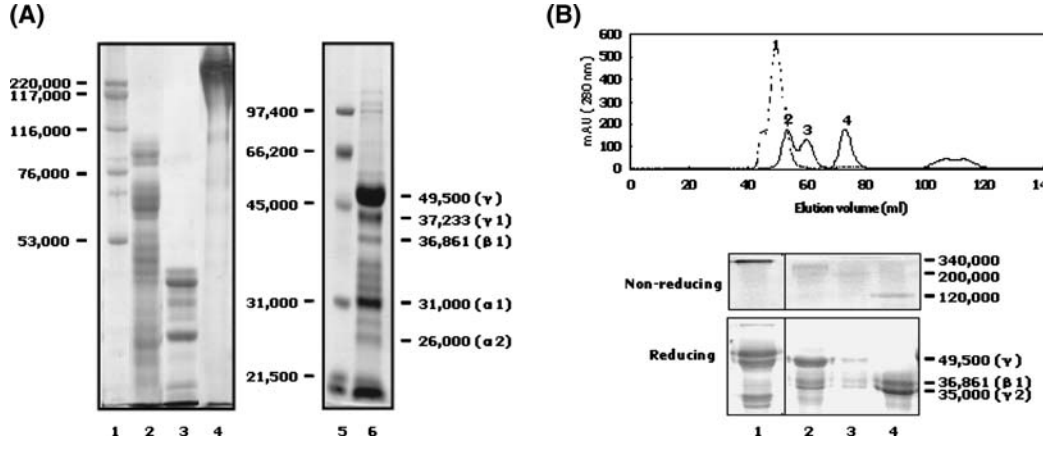

Fig. 4 **a** SDS-PAGE analysis of the cleavage of fibrin clots and fibrinogen by TPase. Fibrinogen (0.25%, w/v) was incubated with purified TPase (0.2 μg) in 20 mM sodium phosphate buffer (pH 7.4) at 37°C for 6 h. For fibrin clots, thrombin (10 mU) was added to the fibrinogen solution (0.5%, w/v). *Lane 1* high molecular weight standard proteins, *lane 2* non-reduced fibrin clot fragments, *lane 3* reduced fibrin clot fragments, *lane 4* non-reduced fibrinogen fragments, *lane 5* low molecular weight standard proteins, *lane 6* reduced fibrinogen fragments. **b** Size-exclusion chromatography analysis of the TPase-treated fibrinogen. Each eluted protein peak was analyzed with SDS-PAGE as non-reducing and reducing conditions. *1* Fibrinogen as a control, *2–4* the major fibrinogen degradation products by TPase

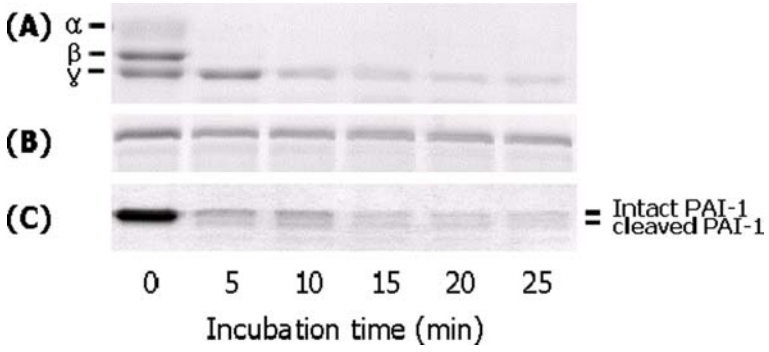

Fig. 6 SDS-PAGE of degradation patterns of fibrinogen (**a**), plasminogen (**b**), and PAI-1 (**c**) by TPase. Ten microliters of each substrate solution (500 μg ml^{-1}) in 10 mM sodium phosphate buffer (pH 7.4) was incubated with the purified enzyme (1.5 μg) at 37°C

was about 250, 150, and 100 kDa, respectively. Moreover, the C-terminal regions of β- and γ-chains (36.9 and 35 kDa) almost remained (Fig. 4b).

Effect of plasminogen addition on the fibrinolytic activity of TPase

The solubilization of fibrin clots is performed directly or indirectly by plasmin, plasminogen activator (PA), and PAI-1. Plasmin is the central enzyme in fibrin lysis. It is formed from its inactive precursor plasminogen by the action of PA. Most reported fibrinolytic drugs are PA-like. In blood plasma, plasmin is regulated by inhibitors from the serpin (serine protease inhibitor) family. Serpins contain a reactive center that mimics the protease's natural susbstrate so that the protease forms an inactive stoichiometric complex with the inhibitor. The principal inhibitors of PAs are PAI-1. Recently, subtilisin NAT has been reported to enhance fibrinolysis through cleavage and inactivation of PAI-1 [29]. Because PAI-1 is the primary inhibitor of fibrinolysis and regulates total fibrinolytic activity by its relative ratio to PA, its inactivation is directly related to the enhancement of fibrinolysis.

To investigate the mechanism by which TPase induces fibrinolysis, the relative fibrinolytic activity of TPase on plasminogen-free and plasminogen-rich fibrin plates was examined (Fig. 5). On plasminogen-rich fibrin plates (10 ml) containing 1 ml plasminogen solution (5 U ml^{-1}), the fibrinolytic activity of TPase did not show any difference from the activity seen with plasminogen-free and plasminogen-rich fibrin plates with respect to the halo size, indicating that TPase is not a PA. In addition, the proteolytic patterns obtained using fibrinogen and plasminogen as substrates were compared (Fig. 6a, b). As shown in Fig. 6b, TPase showed little proteolytic activity towards plasminogen even after a 6 h incubation. These results indicate that TPase acts directly on the fibrin clot rather than on PA. Interestingly, TPase cleaved PAI-1, resulting in the concomitant inactivation of the inhibitor (Fig. 6c). This property could be potentially applicable for the use of TPase as thrombolytic agent that can degrade PAI-1 but not plasmin.

Conclusion

Recent pharmaceutical and clinical studies have focused on improving the effectiveness, tolerability, and convenience of thrombolytic agents. A common feature of newer thrombolytic agents is their prolonged half-life in plasma, making them suitable for single or repeated bolus injections. Although the ideal thrombolytic agent has not yet been developed, its desired clinical properties include rapid opening of the thrombosed vessel without re-occlusion, without activation of systematic fibrinogenolysis, and without risk of hemorrhage [3]. Collen and collaborators reported that plasmin is comparable or superior to t-PA and is associated with less bleeding. The direct fibrinolytic enzyme, plasmin, has been compared with t-PA in rabbit models of local thrombolysis and fibrinolytic hemorrhage [10]. In the model of restricted blood flow into the thrombosed aorta, which limits local plasminogen supply, plasmin was superior to t-PA in clot lysis and vascular reperfusion. These results suggest that direct-acting fibrinolytic enzymes are more effective and are unique thrombolytic drugs distinct from the PAs. Since TPase, a bacterial protease originating from a fermented food, shows a high degree of specificity toward fibrin without degrading plasminogen, it could be useful in thrombolytic therapy as it is a directly acting thrombolytic agent. TPase was also found to cleave and inactivate human PAI-1. Though the clinical results are necessary for the thrombolytic application, the newly isolated TPase will be the potential source of fibrinolytic enzyme.

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