

Purification and characterization of a novel fibrinolytic protease from *Fusarium* sp. CPCC 480097

Bin Wu · Licheng Wu · Daijie Chen ·
Zhijun Yang · Minyu Luo

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Abstract A novel fibrinolytic enzyme from *Fusarium* sp. CPCC 480097, named Fu-P, was purified to electrophoretic homogeneity using ammonium sulfate precipitation and ion exchange and gel filtration chromatography. Fu-P, a single protein had a molecular weight of 28 kDa, which was determined by SDS-PAGE and gel filtration chromatography. The isoelectric point of Fu-P determined by isoelectric focusing electrophoresis (IEF) was 8.1, and the optimum temperature and pH value were 45°C and 8.5, respectively. Fu-P cleaved the α -chain of fibrin (ogen) with high efficiency, and the β -chain and γ - γ (γ -) chain with lower efficiency. Fu-P activity was inhibited by EDTA and PMSF, and the enzyme exhibited a high specificity for the chymotrypsin substrate S-2586. Fu-P was therefore identified as a chymotrypsin-like serine metalloprotease. The first 15 amino acids of the N-terminal sequence of Fu-P were Q-A-S-S-G-T-P-A-T-I-R-V-L-V-V and showed no homology with that of other known fibrinolytic enzymes. This protease may have potential applications in thrombolytic therapy and in thrombosis prevention.

Keywords *Fusarium* sp. · Chymotrypsin-like protease · Fibrinolytic activity · Fibrinogenolytic activity · Serine metalloprotease

Introduction

The incidence of thrombotic disorders including cerebral stroke, myocardial infarction, and venous thromboembolism are rapidly increasing worldwide, as is mortality due to these disorders [38]. During the past decade, thrombolytic agents have been extensively used in the therapeutic treatment of thrombosis [41]. Based on their different mechanisms of action, thrombolytic agents are divided into two types (1) plasminogen activators, such as tissue-type plasminogen activator (t-PA) [11] and urokinase [13]. This type of thrombolytic agent can activate the endogenous fibrinolytic system and generate plasmin from plasminogen. (2) Plasmin-like proteins, such as lumbrokinase [36] and various fibrolases [9], which can directly hydrolyze fibrin into fibrin degradation products, thereby dissolving the thrombi rapidly and completely. Despite their widespread use, these agents do have shortcomings such as excessive bleeding, low specificity to fibrin, and are relatively expensive [4, 18, 46]. Therefore, the search for less expensive and safer thrombolytic agents from various sources is still an urgent issue.

Effective thrombolytic agents have been identified and characterized from animals [6, 37, 49], plants [8, 35] and microorganisms [7, 16, 20]. Over the past few decades, thrombolytic agents from microorganisms have attracted more and more medical interest. These agents include NK produced by *Bacillus natto* [44], subtilisin DJ-4 [24] secreted by *B. amyloliquefacien*, subtilisin DFE [42] purified from *B. amyloliquefacines*, subtilisin CK [22] from

B. Wu · D. Chen
Department of Biological Pharmacy,
Shanghai Institute of Pharmaceutical Industry,
200040 Shanghai, China

L. Wu
Department of Life Science and Biopharmacy,
Shenyang Pharmaceutical University, 110016 Shenyang, China

Z. Yang · M. Luo (✉)
Shanghai Health Creation Center of Biopharmaceutical R&D,
201203 Shanghai, China
e-mail: hccbred@gmail.com; wbwb11@163.com

Bacillus sp. strain CK-11-4 and streptokinase produced by *Streptococcus hemolyticus* [10]. Some fungi have also been found to have high fibrinolytic activity, for example, *Aspergillus ochraceus* 513 [2], *Fusarium oxysporum* [45], *Fusarium* sp. BLB [39], *Rhizopus chinensis* 12 [31], and *P. chrysogenum* H9 [14]. In addition, Kiminori Matsubara et al. [32–34] observed fibrinolytic enzymes from the marine algae *Codium latum*, *C. divaricatum*, and *C. intricatum*.

Endophytic fungi are a special and important group of microorganisms, which produce compounds with antibacterial and anti-tumor activities [15, 43], although reports on these compounds in thrombosis prevention are relative few. In this study, we found that the endophytic fungus *Fusarium* sp. CPCC 480097 isolated from chrysanthemum stems could produce a highly active fibrinolytic enzyme. We report on the purification and characterization of this fibrinolytic enzyme.

Materials and methods

Materials

Flour powder, Indian meal powder, corn powder and ground pea powder were purchased from the Beijing Comwin Pharm-Culture Co., Ltd. Human fibrinogen was purchased from the Green Cross China Biological Product Co., Ltd (Huainan, China). Human thrombin was purchased from the Shanghai Yingyue Biological Product Co., Ltd (Shanghai, China). Plasminogen, bovine serum albumin (BSA), phenylmethanesulfonyl fluoride (PMSF), tosyl-lysinechloromethylketone (TLCK), *N*- α -tosyl-phenylalanine chloromethyl ketone (TPCK), EDTA, Bz-Ile-Glu-(OR)-Gly-Arg-pNA (S-2222), H-D-Ile-Pro-Arg-pNA (S-2288), H-D-Phe-Pip-Arg-pNA (S-2238), H-D-Val-Leu-Lys-pNA (S-2251), pyroGlu-Gly-Arg-pNA (S-2444) and MeO-Suc-Arg-Pro-Tyr-pNA · HCl (S-2586) were obtained from Sigma (USA). Urokinase was obtained from the Suzhou Xinbao Pharmacia Co., Ltd (Suzhou, China). Sephadex G-25 desalting, MonoQ and Superdex 75 were products of Pharmacia Biotech (Uppsala, Sweden). Strain CPCC 480097 was held by Shanghai Health Creation Center of Biopharmaceutical R&D (Shanghai, China). Strain CPCC 480097 was deposited in CGMCC (ID. 2347) and the nucleotide sequences of internal transcriptional spacer (ITS) in the DDBJ/EMBL/GeneBank nucleotide sequence database under accession numbers: EU 363511. All other reagents were analytical grade and purchased commercially.

Culture conditions for enzyme production

The culture growth conditions of *Fusarium* sp. CPCC 480097 for enzyme production were preliminary optimized.

The fungus strain of *Fusarium* sp. CPCC 480097 was initially cultured in 50 ml of liquid medium in a flask (500 ml) containing 1.5% Indian meal powder, 1.0% flour powder, 1.0% corn powder, 1.0% ground pea powder, 0.3% CaCO₃, 0.1% MgSO₄, 0.1% NaCl and 0.1% KH₂PO₄. The culture was incubated at 28°C on a rotary shaker at 220 rpm for 144 h. After the fermentation was completed, the culture broth was centrifuged for 30 min at 8,000 g. The supernatant and the microbial cells were harvested. The microbial cells were disrupted and the cell debris was removed by centrifugation. The fibrinolytic activity in the cells was about 10% as compared with that in the culture supernatant. Therefore, the supernatant was used for subsequent purification of the enzyme.

Purification of the fibrinolytic enzyme

The culture supernatant was first treated using a two-step salting-out procedure at 4°C with ammonium sulfate, first at 40%, and then at 60%. The crude precipitate of the 60% solvent was collected by centrifugation at 8,000g for 30 min at 4°C. This precipitate was dissolved in a small volume of Tris-HCl buffer (20 mmol l⁻¹, pH 7.4, buffer A) and then loaded onto a G-25 column (16 × 25 mm) equilibrated with buffer A for desalting. The protease-containing fraction was applied to a MonoQ column (10 × 100 mm) equilibrated with buffer A, and then eluted with a linear gradient of 0–1 mol l⁻¹ NaCl in buffer A at a flow rate of 2 ml min⁻¹. The active fraction was concentrated by lyophilized after desalting. The concentrated sample was dissolved in a small volume of buffer A and loaded onto a Superdex 75 column (16 × 600 mm) equilibrated with buffer A containing 50 mmol l⁻¹ NaCl. The protein was eluted at a flow rate of 1 ml min⁻¹ with buffer A. The active fraction was pooled, lyophilized, and analyzed for purity using SDS-PAGE. For all purification steps, the eluates were monitored by spectrophotometry at 280 nm. The activity of the protease was estimated with casein (DMC) as a substrate and by fibrin plate assay as described later.

Determination of protein concentration

Protein concentration was determined by the Bradford method [5], using BSA as a standard.

Determination of molecular weight and isoelectric point

The molecular weight of the enzyme was determined by SDS-PAGE and gel filtration chromatography on Superdex 75. SDS-PAGE was performed at room temperature by the method of Laemmli [29], using 12% polyacrylamide gel, and stained with Coomassie Brilliant Blue R250. Molecular weight was analyzed by running low molecular weight

standard protein markers which comprised rabbit phosphor-ylase B (97.4 kDa), BSA (66.4 kDa), ovalbumin (44.2 kDa), carbonic anhydrase (29 kDa), trypsin inhibitor (20.1 kDa) and hen egg white lysozyme (14.3 kDa). Gel filtration chromatography was performed at room temperature, using a Superdex 75 column (AKTA FPLC) to estimate the molec-ular weight of the enzyme [30]. The elution buffer was 20 mmol l⁻¹ Tris-HCl (pH 7.4) containing 50 mmol l⁻¹ NaCl. A gel filtration protein marker comprised of glycerol-dehydes (35 kDa), carbonic anhydrase (29 kDa), trypsin-ogen-PMSF (24 kDa), and trypsin-inhibitor (20.1 kDa) was used.

Isoelectric focusing electrophoresis (IEF) was performed using a Bio-Rad Model 111 Mini IEF Cell according to the manufacturer's procedure. Broad isoelectric point (pI) cali-bration kits (proteins pI 4.45–9.60) were used as markers in the pI value determinations.

Determination of N-terminal amino acid sequence

The N-terminal amino acid sequence of the purified fibrin-olytic enzyme was determined by the automated Edman method. Sequence alignment was analyzed with those of other known fibrinolytic enzymes.

Protease activity assay

Protease activity was determined with casein as a substrate according to the modified method of Kunitz [28]. The reac-tion was initiated by adding 20 µl of enzyme solution to 1 ml of 1% (w/v) casein in 20 mmol l⁻¹ Tris-HCl buffer (pH 8.0). After 20 min at 37°C, the reaction was terminated by the addition of 3 ml of 5% (w/v) trichloroacetic acid (TCA), left for 20 min at room temperature, and centri-fuged at 10,000g for 10 min at 4°C. The absorbance of the supernatant was measured at 280 nm. One unit (U) of pro-tease activity was defined as the amount of enzyme that produced an increase in absorbance at 280 nm of 0.01 min⁻¹ in the above conditions.

Fibrin-plate activity analysis

Fibrinolytic activity was determined by both the plasmino-gen-free fibrin plate method and the plasminogen-rich fibrin plate method with minor modifications [1]. Plasmino-gen-free fibrin plate was made up of a fibrinogen solution (5 ml of 0.5% human fibrinogen in 20 mmol l⁻¹ Tris-HCl buffer, pH 7.4), 20 U of thrombin solution (same buffer), and 5 ml of 1% agarose in petri dishes (5 cm in diameter). The fibrin plate was heated at 80°C for 30 min to destroy other fibrinolytic factors. Plasminogen-rich fibrin plate con-taining 5 U plasminogen was not heated. To observe the fibrinolytic activity, 10 µl of enzyme solution was carefully

dropped onto a fibrin plate and incubated at 37°C for 18 h. The activity of the 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. Three independent experiments were carried out to obtain average measurements of the zone diameters in these experiments.

Fibrinogenolytic activity analysis

Fibrinogenolytic activity was measured by the modified method of Koh [26]. In brief, 80 µl of 2% fibrinogen (pre-pared with 20 mmol l⁻¹ Tris-HCl, pH 7.4) was incubated with 5 µg of the purified protease at 37°C. At various inter-vals a portion of the reaction solution was withdrawn and analyzed by SDS-PAGE.

Fibrinolytic activity analysis

Fibrin degradation analysis was performed as described by Datta et al. [12]. In brief, 20 µl of 2% human fibrinogen solution in 20 mmol l⁻¹ Tris-HCl buffer, pH 7.4, was mixed with 20 µl of thrombin (5 U ml⁻¹) dissolved in the same buffer. The fibrin clot was allowed to stand for 1 h at room temperature. Five microgram of the purified protease was placed on the clot surface and incubated at 37°C for various time intervals. The hydrolytic products from fibrin were analyzed by SDS-PAGE.

Effects of temperature and pH on enzyme fibrinolytic activity

The optimal temperature for the purified enzyme was deter-mined by measuring residual activity in Tris-HCl buffer (pH 7.4) for 10 min at different temperatures (20–60°C). To measure its thermal stability, the purified protease was incubated in the same buffer for 4 h at 4–80°C. The remain-ing activity was determined using the casein method.

The optimal pH for the purified enzyme was determined by incubating the enzyme at room temperature in the fol-lowing buffers, glycine-HCl (pH 2.0–6.0), Tris-HCl (pH 6.0–9.0), and glycine-NaOH (pH 9.0–11.5). All buffers were 20 mmol l⁻¹. The effect of pH on enzyme stability was determined by incubating the enzyme for 4 h at 4°C in the above buffer. The remaining activity was determined using casein as a substrate.

Effects of metal ions and protease inhibitors on enzyme activity

The effects of metal ions on enzyme activity were investi-gated using MgCl₂, ZnCl₂, CoCl₂, CaCl₂, and CuSO₄. The purified enzyme was pre-incubated in both the absence and

presence of metal ions at various concentrations for 30 min at 37°C, and the protease activity was determined using casein as a substrate.

The effects of the protease inhibitors (EDTA, PMSF, TPCK, and TLCK) were also examined. The enzyme was pre-incubated with these protease inhibitors for 30 min at 37°C. After incubation, the effects were assessed with casein as a substrate.

Amidolytic activity of the enzyme

Amidolytic activities were measured spectrophotometrically, using chromogenic protease substrates such as S-2222 (for factor Xa), S-2288 (for t-PA), S-2238 (for thrombin), S-2251 (for plasmin and streptokinase-activated plasminogen), S-2444 (for urokinase), and S-2586 (for chymotrypsin). Activities were evaluated by mixing the purified enzyme [1 µg/200 µl of 20 mmol l⁻¹ Tris-HCl (pH 7.4)] with 300 µl of each substrate (0.5 mmol l⁻¹). After continuous measurement for 5 min at 37°C, the amount of *p*-nitroaniline released was determined by measuring the change in absorbance at 405 nm.

Results

Purification of the fibrinolytic enzyme

The fibrinolytic enzyme was purified using the steps listed in Table 1. Protease activity and fibrinolytic activity were used as indices of purification. After ammonium sulfate precipitation, a Mono-Q column was used to purify the enzyme, which yielded one major peak showing protease activity and fibrinolytic activity in the washed-out fraction. This result indicated that the enzyme did not bind to the Mono-Q and had a pI higher than 7.4. The washed-out fraction was further purified using a Superdex 75 column and eluted as four peaks. Only the third peak was noted to have protease and fibrinolytic activity, and showed a single band on SDS-PAGE (Fig. 1a). This active protein was named Fu-P. The enzyme was purified 158-fold, with a final yield of 6.8% after three purification steps as summarized in Table 1.

Table 1 Purification steps of Fu-P from *Fusarium* sp. CPCC 480097

Purification step	Protein (mg)	Total activity (U)	Specific activity (U mg ⁻¹)	Purification (fold)	Yield (%)
Culture supernatant	4,200	2 × 10 ⁶	480.2	1	100
40–60% (NH ₄) ₂ SO ₄	219.3	8.1 × 10 ⁵	3,697.5	7.7	40.2
Mono-Q	13.8	2.1 × 10 ⁵	15,462.2	32.2	10.7
Superdex 75	1.8	137,000	76,111	158.5	6.8

Molecular weight and isoelectric point (pI)

The molecular weight of Fu-P was estimated to be 28 kDa by SDS-PAGE (Fig. 1a). This value was consistent with the molecular weight estimated by a Superdex 75 column, using AKTA FPLC (date not shown). Therefore, we concluded that the enzyme was a monomeric protein. The molecular weights of fibrinolytic enzymes from microbes have been reported to range from 18 to 52 kDa. The molecular weight of Fu-P was similar to those of the fibrinolytic enzyme from *Fusarium* sp. BLB [39], Nattokinase [16], subtilisin DFE [42], CK [22], subtilisin QK-2 [27], subtilisin DJ-4 [24], SW-1 [47] and subtilisin FS33 [48]. The pI of Fu-P was 8.1, as determined by IEF, which was comparable to that of subtilisin DEF [42], but lower than those of NK [16], subtilisin DJ-4 [24], SW-1 [47] and subtilisin FS33 [48] (Table 2).

The N-terminal amino acid sequence of the fibrinolytic enzyme

The N-terminal amino acid sequence of Fu-P was observed to be QASSGTPATIRVLVV. Multiple sequence alignment showed that Fu-P appeared to differ strongly from other reported fibrinolytic proteases (identity below 20%, Table 3). Thus, we strongly suggest that Fu-P is a novel protein.

Fibrinogenolytic and fibrinolytic activity

Fu-P formed a similar-sized clear lysis zone on both plasminogen-rich and plasminogen-free fibrin plates (Fig. 1b). The fibrinolytic activity measured by these two methods showed a difference of less than 2% difference, which indicated that Fu-P was not a plasminogen activator.

The fibrinogenolytic activity of Fu-P analyzed by SDS-PAGE showed that the α band disappeared first, followed by the β band, and then the γ chain (Fig. 1c). Similarly, when fibrin was incubated with Fu-P, the α band was rapidly hydrolyzed, and the β chain and γ-γ chain were slowly hydrolyzed (date not shown). This hydrolytic pattern was similar to that of other proteases purified from *C. divaricatum* and snake venom [3, 50], but was different from the

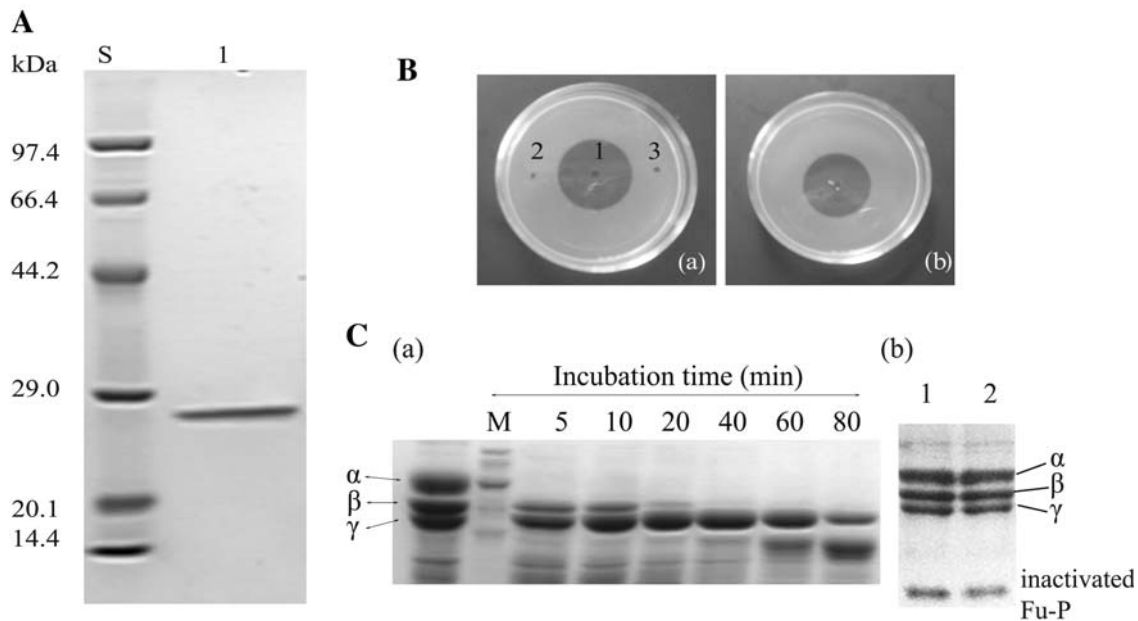


Fig. 1 SDS-PAGE of purified Fu-P (**a**) and the mode of fibrinolytic action (**b**) and hydrolysis of fibrinogen by Fu-P (**c**). **a** Lane S a standard low molecular mass marker. Lane 1 recycled protein. **b** Analysis of fibrinolysis by Fu-P on plasminogen-rich fibrin plate (**a**) and plasminogen-free fibrin plate (**b**). 1 purified Fu-P (5 μg), 2 fermentation medium

fibrinolytic enzymes of the medicinal mushroom *Cordyceps militaris* [25] and *B. subtilis* FS33 [48].

Effects of pH and temperature on enzyme activity

The optimum pH of the purified protease was 8.5 (date not shown). Fu-P was stable over a pH range of 6–9 for 4 h at 4°C (date not shown). The optimum temperature of the enzyme was 45°C and the enzyme activity was stable below 37°C (date not shown).

Effects of metal ions and protease inhibitors on enzyme activity

The effects of protease inhibitors on the fibrinolytic activity of Fu-P were also examined and are shown in Table 3. Fu-P activity was strongly inhibited by the serine protease inhibitor PMSF and the metalloprotease inhibitor EDTA. However, TLCK had a weak inhibitory effect. These results indicated that Fu-P was a serine metalloprotease. Most known fibrinolytic enzymes from microbes are serine proteases and are not inhibited by EDTA. KA38 [23], SW-1 [47], an enzyme from *R. chinensis* 12 [31] and AMMP [30] are metalloproteases. However, the characteristics and the N-terminal amino acid sequence of Fu-P were different from those of these four enzymes.

The effects of metal ions on enzyme activity were also investigated (Table 4). The enzyme activity was found to

as a control, 3 20 mmol l⁻¹ Tris-HCl buffer as a control. **c a** The hydrolytic products from fibrinogen were separated in 12% SDS-PAGE; **b** Lane 1, SDS-PAGE of fibrinogen incubated with inactivated Fu-P for 0 min; Lane 2 SDS-PAGE of fibrinogen incubated with inactivated Fu-P for 80 min. which showed that the fibrinogen alone was stable

be enhanced by Ca²⁺ (1 mmol l⁻¹), Zn²⁺ (1 mmol l⁻¹), but inhibited by Co²⁺ (3 mmol l⁻¹), Cu²⁺, and Zn²⁺ (3 mmol l⁻¹). Based on metal ion interactions with other fibrinolytic enzymes, all reported metallo fibrinolytic enzymes are inhibited by Cu²⁺ and Co²⁺. Some metallo fibrinolytic enzyme activities were enhanced by Ca²⁺ at 1 mM, such as the enzyme from the thermophilic fungus *Oidiodendron flavum* and AMMP. The fibrinolytic activity of KA38 was also enhanced by Zn²⁺ at 1 mM, but was decreased with increasing amounts of Zn²⁺. The mechanism of metal ions on fibrinolytic activity is not clear and requires further study.

Amidolytic activity of the enzyme

The amidolytic activity of Fu-P was measured using several chromogenic substrates (date not shown). The enzyme exhibited the highest degree of specificity for S-2586 (for chymotrypsin). According to the effects of inhibitors on Fu-P and its activities with chromogenic substrates, Fu-P can be classified as a chymotrypsin-like serine metalloprotease.

Discussion

Within the last few years, studies on fibrinolytic enzymes from microorganisms have attracted significant attention because of their potential use in thrombosis therapy. This

Table 2 Comparison of the characteristics of Fu-P with other fibrinolytic enzymes

Enzyme	M.W. (kDa)	pI	Optimal pH	Optimal temperature (°C)	pH stability	Temperature stability (°C)	Comments	Reference
Fu-P	28	8.1	8.5	45	6–9	<37	Chymotrypsin-like serine metalloprotease	This work
NK	27.7	8.6	ND	ND	7–12	<50	Subtilisin-family serine protease	[16]
Subtilisin DJ-4	29	10.0	ND	40	4–11	Room temperature for 48 h	Plasmin-like serine protease	[24]
Subtilisin DFE	28	8.0	9.0	48	6–10	<50	Subtilisin-family serine protease	[42]
CK	28.2	ND	10	70	7–10.5	<50	Thermophilic alkaline serine protease	[22]
From <i>Fusarium</i> sp. BLB	27	ND	9.5	50	2.5–11.5	<50	Serine protease belong to neither chymotrypsin, trypsin, or subtilisin	[39]
From <i>chinesis</i> 12	18	8.5	10.5	45	6.8–8.8	37 for 24 h	Metalloprotease	[31]
AMMP	21	ND	6.0	33	ND	ND	Chymotrypsin-like metalloprotease	[30]
KA38	41	ND	7.0	40	ND	ND	Metalloprotease	[23]
SW-1	30	8.5	ND	ND	4–9	4–37	Serine metalloprotease	[47]
Subtilisin QK-2	28	ND	8.5	55	3–12	40 for 30 min	Subtilisin-family serine protease	[27]
Enzyme from <i>Oidiodendron flavum</i>	22	ND	8.0	ND	6–11	<65	ND	[40]
Subtilisin FS33	30	8.7	8.0	55	ND	ND	Subtilisin-like serine protease	[48]

ND indicates the missing information

Table 3 Comparison of the N-terminal amino acid sequence of Fu-P with other fibrinolytic enzymes

Fibrinolytic enzyme	N-terminal amino acid sequence	Identity (%)	Reference
Fu-P	QASSGTPATIRVLVV	100	This work
<i>Rhizopus chinensis</i> 12	SVSGIGLMHALG	20	[31]
<i>Streptomyces griseus</i>	IVGGTRAAQGEFFMMVRLSM	19	[21]
Metalloprotease	DPYEPPGPCENLQVA	18	[38]
<i>Fusarium</i> sp. BLB	IVGTTAASGGDFPIIVSIYYQGRAR	16	[39]
<i>Fusarium oxysporum</i>	IVGGTSASAGDFPIVVISRNGGPWC	15	[45]
Bacillokinase II	ARAGEALDIYD	13	[19]
CK	AQTVPYGIPLIKAD	12	[22]
KA38	VYPFPGPOP	12	[23]
AMMP	MFSLSSRFFLYTLCLSAVAVSAAP	12	[30]
NK	AQSVPYGISQIKAPALHSQGYTGS	8	[16]
Subtilisin DFE	AQSVPYGVSQIAPALHSQGFYGS	8	[42]
Subtilisin DJ-4	AQSVPYGVSQIKAP	6	[24]
SW-1	R/N/FP/DGMTMTAIANQNTQIN	5	[47]

Table 4 Effect of protease inhibitors and metal ions on activity of Fu-P

Inhibitor or metal ions	Concentration (mmol l ⁻¹)	Relative activity (%)	Concentration (mmol l ⁻¹)	Relative activity (%)	Concentration (mmol l ⁻¹)	Relative activity (%)
Control		100		100		100
PMSF	0.5	50.2 ± 2.2	1.5	30.2 ± 2.6	4.5	6.4 ± 2.2
TLCK	0.5	97.3 ± 2.0	1	97.2 ± 2.2	2	95.5 ± 2.1
TPCK	0.5	101.4 ± 3.2	1	96.3 ± 2.3	2	88.6 ± 3.3
EDTA	0.5	24.2 ± 2.4	1.5	16.6 ± 2.1	4.5	4.7 ± 2.4
K ⁺	1	98.7 ± 2.4	3	101.8 ± 3.2	5	96.4 ± 3.6
Zn ²⁺	1	120.5 ± 2.1	3	55.5 ± 2.0	5	0
Ca ²⁺	1	116.1 ± 3.5	3	98.3 ± 3.2	5	100.4 ± 2.5
Cu ²⁺	1	20.2 ± 2.4	3	12.3 ± 2.3	5	0
Mg ²⁺	1	97.3 ± 2.8	3	96.2 ± 2.5	5	103.5 ± 2.6
Mn ²⁺	1	99.5 ± 3.7	3	100.2 ± 2.2	5	102.2 ± 2.7
Co ²⁺	1	97.1 ± 3.1	3	73.2 ± 2.7	5	42.1 ± 2.9

The results shown represent averages from three independent experiments. Error bars represent the standard deviation

article described the purification and characterization of a fibrinolytic enzyme (Fu-P) from *Fusarium* sp. CICC 480097 isolated from chrysanthemum stems.

The overall findings of Fu-P in relation to the molecular weight, the isoelectric point, the N-terminal amino acid sequences, effect of inhibitors and metal ions, substrate specificity, and fibrino(gen)olytic activity indicate that Fu-P differs from other known fibrinolytic enzymes and is a novel fibrinolytic enzyme (Tables 2, 3).

Fu-P had strong fibrinolytic activity. The specific fibrinolytic activity was 76,111 U/mg. The units for the activity of fibrinolytic enzymes have been defined differently in many studies, making it difficult to compare absolute values. However, we noticed that the activity of Fu-P was much higher than that of subtilisin DJ-4 (4,664 U/mg),

subtilisin QK-2 (41,000 U/mg), subtilisin FS33 (15,494.4 U/mg) and the enzyme from *R. chinensis* (2,143.4 U/mg), which were assayed by the same method. Fu-P also had a higher fibrinolytic activity than the typical fibrinolytic enzyme urokinase (Fig 2). The specific protease (caseinolytic) activity of the purified Fu-P was 40,920 U/mg. The ratio of fibrinolytic activity to caseinolytic activity was 1.86. This relative value was higher than that of other fibrinolytic enzymes, such as subtilisin DJ-4 (0.98) [24], CK (0.73) [22] and subtilisin Carlsberg (0.092) [22]. Therefore, Fu-P has relatively high substrate specificity for fibrin.

To investigate the mechanism of Fu-P induced fibrinolysis, the relative fibrinolytic activity of Fu-P on plasminogen-free and plasminogen-rich fibrin plates was examined. The results indicated that Fu-P acted via direct cleavage of

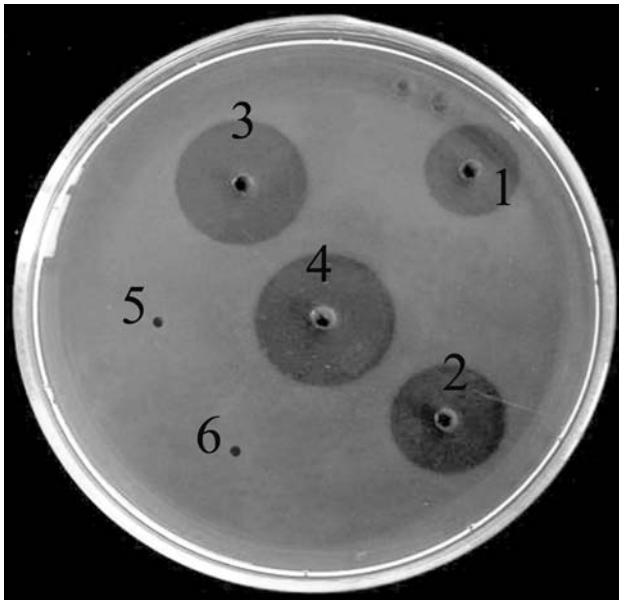


Fig. 2 Comparison of the fibrinolytic activity of Fu-P and UK. The fibrinolytic activity was examined by fibrin plate assay. 1 UK (0.5 µg) 2 UK (1 µg) 3 UK (2 µg) 4 Fu-P (2 µg) 5 buffer (20 mmol l⁻¹ Tris-HCl, pH 7.4) 6 distilled water. The calculated fibrinolytic activity of Fu-P was approximately 12% higher than that of UK according to the fibrin plate data

fibrin and not by plasminogen activation like UK, SK and tPA. Therefore, secondary effects such as platelet activation related to plasmin formation could be avoided. This is a specific advantage of Fu-P over clinically used plasminogen activators. Fu-P also exhibited fibrinogenolytic activity by rapidly hydrolyzing the fibrinogen the α band, and then the β band and the γ band. Thus Fu-P could be termed a fibrinogenase-like enzyme. Fibrinogen, the final molecule in the coagulant cascade before the deposition of fibrin, is involved in primary haemostasis, platelet aggregation and is a major determinant of plasma viscosity [17]. A reduction in fibrinogen level decreases the incidence of thrombosis. From preliminary experiments with mice, we also found that Fu-P showed a significant in vivo inhibition of thrombus formation and in vitro degradation of blood clots (data not shown). Therefore, Fu-P may be used in thrombolytic therapy, and may also be used to prevent the formation of venous blood clots.

Taking account of these results, the fibrinolytic enzyme from *Fusarium* sp. CPCC 480097 may be a potential candidate for thrombolytic therapy or thrombosis prevention. Further investigation of this enzyme is underway.

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