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preliminary communication

Purification and Characterisation of a Fibrinolytic Enzyme from *Rhizopus microsporus* var. *tuberosus*

*Shuli Zhang, Yingdong Wang, Nan Zhang, Zhe Sun, Yan Shi, Xingnan Cao and Haikuan Wang**

Key Laboratory of Industrial Fermentation Microbiology, College of Biotechnology, Tianjin University of Science and Technology, Ministry of Education, Tianjin, PR China

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Summary

Extracellular fibrinolytic enzyme from *Rhizopus microsporus* var. *tuberosus* was purified and characterised. The microorganism was isolated in a distillery from daqu, a fermentative agent used in the production of Chinese liquor and vinegar at different temperatures. The fibrinolytic enzyme was partially purified by ammonium sulphate precipitation, dialysis, DEAE Sepharose® Fast Flow ion exchange chromatography and Sephadex G-75 gel filtration chromatography. The molecular mass of the fibrinolytic enzyme was estimated to be 24.5 kDa by SDS-PAGE. The purified enzyme showed optimal activity at pH=7.0 and 37 °C by fibrin plate method. It showed stronger resistance to the inhibition by trypsin and was stable at 37 °C retaining 96.1 % residual activity after 4 h of incubation. The fibrinolytic activity of the enzyme was enhanced by Na^+, Ca^{2+}, Mg^{2+} and Mn^{2+}. Conversely, Zn^{2+} and Cu^{2+} partly inhibited enzymatic activity. Using fibrin plate method, we found that the enzyme not only degrades fibrin directly, but also activates plasminogen into plasmin to degrade fibrin. The results indicate that the pure enzyme has a potential in dissolving blood clot, and the possibility for application in the treatment of thrombosis.

Key words: Rhizopus microsporus var. *tuberosus*, fibrinolytic enzyme, purification, characterisation

Introduction

Cardiovascular diseases, such as acute myocardial infarction, ischaemic heart disease and high blood pressure, are the leading causes of death in the world (*1*). Thrombin-mediated fibrinogen conversion to fibrin and fibrin monomer cross-linking result in the formation of a clot. Thrombosis occurs when the clots are not lysed. Plasmin, which is generated from plasminogen by activators, such as tissue plasminogen activator (t-PA) and urokinase, hydrolyses insoluble fibrin fibre into fibrin degradation products. A variety of plasminogen activators have been widely studied and used as thrombolytic agents. However, these agents are very expensive and have some side effects (*2*).

Over the last decade, the search for other fibrinolytic enzymes from various sources has been under way. Several potential thrombolytic agents have been discovered from animals, *e.g.* vampire bat saliva (*3*), earthworm (*4*) and snake venoms (*5*), whose effects were confirmed in clinical trials (*6*). In addition, the fibrinolytic enzymes have also been isolated and characterised from fermented food products including Korean chungkookjang (*7*), Japanese natto (*8*) and Chinese douchi (*9*). These enzymes can convert plasminogen to plasmin.

Our study reports for the first time the purification and characterisation of the fibrinolytic enzyme from *Rhizopus microsporus* var. *tuberosus* isolated in a distillery from daqu, a fermentative agent produced at a medium temperature.

*Corresponding author: Phone: +86 022 6060 1058; Fax: +86 022 6060 2298; E-mail: hkwang@aliyun.com

Materials and Methods

Materials

Thrombin, fibrinogen from bovine plasma, urokinase and Sepharose were purchased from Sigma-Aldrich (St. Louis, MO, USA). Trypsin was purchased from Biotech (Shanghai, China). DEAE-Sepharose Fast Flow and Sephadex G-75 were purchased from GE Healthcare (Hartford, CT, USA). All other reagents were of analytical grade.

Strain and culture conditions

The conidia of *Rhizopus microsporus* var. *tuberosus*, kept at –80 °C in 20 % (by volume) glycerol, were used to inoculate PDA agar slants (in g/L of distilled water: potato 200, glucose 20 and agar 20). After 5 days of incubation at 28 °C, the spores were harvested, washed with sterilised saline and cultured in several 500-mL flasks ($3 \cdot 10^6$ spores/mL) containing 1.4 g of dextrin, 1.05 g of peptone and 70 mL of distilled water. The spores were then cultivated in shaking incubator at 150 rpm and 28 °C. After 11 h of cultivation, a 20 % (by volume) inoculum was transferred into fermentation medium (2 g of dextrin, 1 g of peptone and 1 g of soya bean in 50 mL of distilled water) and fermented at 28 °C and 150 rpm for 60 h.

The supernatant containing the enzyme was obtained by centrifugation at 4 °C and 8944×*g* for 10 min, and stored at –20 °C for purification.

Identification by molecular biology methods

The DNA of *R. microsporus* was extracted by a Fungus Genomic DNA Extraction Kit (Beijing Sunbiotech Co. Ltd, Beijing, PR China) according to the manufacturer's instructions. Polymerase chain reaction (PCR) amplifications were achieved using universal primers for ribosomal DNA regions (*10*). Primers ITS5 (5′-GGAAGTAAAAGTCGTAACAAGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) were used. The following thermocycler protocol was used: 1 cycle at 95 °C for 3 min, then 35 cycles at 95 °C for 30 s, at 55 °C for 30 s and 72 °C for 1 min, and 1 cycle at 72 °C for 7 min. The PCR reaction mixture consisted of 5 μL of 10×PCR buffer, 1 μL of dNTP (10 mmol/L), 2 μL of ITS5 (10 μmol/L), 2 μL of ITS4 (10 μmol/L), 1 μL of template DNA, 50 μL of double distilled water and 1 U of Taq polymerase. The PCR amplification products were determined by Beijing Liuhe Huada Gene Technology Co., Ltd Beijing, PR China. A homology search using the reference strains registered in DDBJ/EMBL/GenBank was performed by NCBI BLAST (*11*).

Determination of enzyme concentration and fibrinolytic activity

Protein (enzyme) concentration was determined by the method of Bradford (*12*), according to which G-250 dye reagent and bovine serum albumin were used as standards and the absorbance was measured at 595 nm.

Enzyme activity was determined using the fibrin plate method (*13,14*), with some modifications. The 9-cm Petri dishes contained fibrin plate composed of: 20 mg of blood fibrinogen in 5 mL of 0.1 M barbital buffer (pH=7.8), 20 U of thrombin solution, and 25 mg of agarose in 5 mL of medical physiological saline. For the determination of fibrinolytic activity, 10 μL of enzyme solution were carefully added onto a fibrin plate and incubated at 37 °C for 6 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. One unit of urokinase activity was defined as the amount of enzyme that dissolves 1 μg of fibrinogen clot per min per mL at 37 °C.

Enzyme purification

The enzyme was purified through the following steps: (*i*) solid ammonium sulphate was added to the supernatant containing the fibrinolytic enzyme to make 30 % saturation. This mixture was left overnight at 4 °C and then centrifuged at 7000×*g* for 30 min at 4 °C to remove particle material. Solid ammonium sulphate was added to the supernatant until it reached 65 % saturation and then left overnight at 4 °C. The precipitate was collected by centrifugation at 12 700×*g* for 30 min at 4 °C for further purification; (*ii*) the crude enzyme precipitate was dissolved in Tris-HCl buffer (20 mM, pH=7.5), desalted with dialysis bag (molecular mass of 8000 Da) at 4 °C and concentrated by lyophilisation; (*iii*) the dialysed sample was loaded onto a DEAE-Sepharose Fast Flow column (2.5 cm×20 cm) previously equilibrated with 20 mM Tris-HCl buffer (pH=7.5). After washing with two bed volumes of starting buffer, the elution was performed with a linear gradient of 0–1.0 M NaCl at a flow rate of 1 mL/min with a constant flow pump (DHL-A, Shanghai Huxi Analysis Instrument Factory, PR China). The fractions were collected by an autocollector (DBS-100, Shanghai Huxi Analysis Instrument Factory) and analysed for fibrinolytic enzyme activity and protein content. The active fractions were pooled and concentrated to 2.0 mL with PEG-20000 (BioSharp, Anhui, PR China); and (*iv*) the concentrated sample obtained using DEAE-Sepharose Fast Flow column chromatography (1.0 mL, 0.065 mg/mL) was applied on a Sephadex G-75 column (1.6 cm×80 cm, Agilent Co., Beijing, PR China), equilibrated with 0.1 M phosphate buffer (pH=7.5), and then the fibrinolytic enzyme was eluted with the same buffer (pH=7.5), at a flow rate of 1 mL per min, with a constant flow pump. The fractions of 5.0 mL were collected by an autocollector and assayed for fibrinolytic enzyme activity and protein content. The active fractions were pooled, concentrated and analysed for purity by SDS-PAGE (BioDev, Beijing, PR China) and HPLC (GE SuperdexTM Peptide 10/300 GL, 10 mm×300 mm, GE Healthcare).

Determination of enzyme purity

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to the method of Laemmli on 4 % (by mass per volume) polyacrylamide stacking gel and 12 % (by mass per volume) polyacrylamide resolving gel in an electrophoresis cell (DYCE-24DN, Beijing Wode-Life Sciences Instrument Co., Beijing, PR China) with the electrophoresis apparatus (DYY-6B, Beijing Wode-Life Sciences Instrument Co.). Protein bands were visualised by staining with Coomassie brilliant blue R250 (Amresco LLC, Solon, OH, USA) and estimated by comparing with broad range molecular mass standards from 14.4 to 66.2 kDa.

After filtration chromatography (10 μL, 10 mg/mL), the sample was subjected to HPLC (Agilent Technologies, Palo Alto, CA, USA) using a GE Superdex® Peptide 10/300 GL (10 mm×300 mm). The eluent was 50 mmol/L phosphate buffer with 0.15 mol/L NaCl at a flow rate of 0.5 mL per min at 30 °C. The purity of the sample was measured by detecting UV absorbance at 214 nm. The fractions of 5.0 mL were collected by an autocollector and assayed for fibrinolytic enzyme activity.

Characterisation of fibrinolytic enzyme activity

Effect of artificial intestinal fluid on the fibrinolytic enzyme activity

A volume of 0.5 mL of fibrinolytic enzyme solution (0.035 mg/mL) and 1 mL of artificial intestinal fluid (6.8 g of KH_2PO_4, 5 g of trypsin and 500 mL of distilled water, adjusted to pH=6.8 with 0.1 M NaOH) was mixed to determine the retaining activity after 1, 2, 3 and 4 h at 37 °C. The enzyme activity was determined as a percentage of the relative activity compared to that of the control, which was 100 %.

Effect of pH on the fibrinolytic enzyme activity and stability

The optimal pH for the fibrinolytic enzyme (0.035 mg/mL) was determined by measuring the enzyme activity within a pH range of 2.8–11.8 at 37 °C. The pH stability of the fibrinolytic enzyme was estimated by measuring the remaining fibrinolytic activity after incubating the enzyme for 24 h at 37 °C with different buffers. The residual enzyme activity was then measured at pH=7.0. The following buffer systems were used: 0.02 M disodium phosphate/citric acid buffer (pH=2.8–6.8), 0.02 M barbitone sodium/HCl buffer (pH=7.8–8.8), 0.02 M Na_2CO_3/$NaHCO_3$ buffer (pH=9.8–10.8), 0.02 M disodium phosphate/NaOH (pH=11.8) and 0.02 M KCl/NaOH buffer (pH=12.2). The enzyme activity was measured using the fibrin plate method, and expressed as a relative percentage to the maximum activity of 100 %.

Effect of temperature on the fibrinolytic enzyme activity and stability

The optimal temperature of the enzyme (0.035 mg/mL) was evaluated by measuring the enzyme activity at different temperatures (25–70 °C) for 16 h. The effect of temperature on the enzyme stability was determined by measuring the residual activity of the enzyme incubated in 50 mM sodium phosphate buffer (pH=7.0) at temperatures ranging from 25 to 70 °C for 4 h. The fibrinolytic activity was assessed by the fibrin plate method.

Effect of metal ions on the fibrinolytic enzyme activity

The effect of different metal ions was investigated by using $MgSO_4$, $CuSO_4$, KCl, $CaCl_2$, $ZnSO_4$, $MnSO_4$, $FeSO_4$ and NaCl solutions. The fibrinolytic enzyme (0.035 mg/mL) was incubated with different metal ion saline solutions (1 and 10 mM) for 18 h at 37 °C. The residual activity was measured by the fibrin plate method. The activity of the fibrinolytic enzyme assayed in the absence of metal ions was taken as 100 %.

Activation of plasminogen by the fibrinolytic enzyme

The commercially available fibrinogen contains plasminogen which can be activated into plasmin. The plasminogen on the fibrin plate can be deactivated by heating, when it loses its fibrinolytic activity. After the prepared plasminogen-positive fibrin plate was incubated at 85 °C for 30 min and cooled to room temperature, plasminogen-free fibrin plate was made. If the fibrinolytic enzyme (0.035 mg/mL) dissolved much more fibrin on plasminogen-positive fibrin plate than on plasminogen-free fibrin plate, it meant that the activated fibrinolytic enzyme dissolved protein fibre in an indirect way. However, the results on plasminogen-free fibrin plate show that the enzyme degrades fibrin directly. The purified enzyme from *R. microsporus* and urokinase solution (10 μL) was added onto the plasminogen-free and plasminogen-positive fibrin plates in order to determine the nature of fibrin degradation.

Results and Discussion

Identification of the Rhizopus microsporus

The strain was identified as *Rhizopus microsporus* based on an analysis of ITS-5.8S rDNA sequences, which revealed 99 % homology. The strain was deposited in China General Microbiological Culture Collection Center, CGMCC, Beijing, PR China (CGMCC No. 4368).

Purification and confirmation of activity of the enzyme

The fibrinolytic enzyme from *R. microsporus* var. *tuberosus* was partially purified as shown in Table 1. After the ammonium sulphate precipitation, a DEAE-Sepharose Fast Flow column and a Sephadex G-75 column chromatography were used to partially purify the enzyme. Two protein peaks were detected. The major fraction with fibrinolytic activity was collected and applied onto the Sephadex G-75 column. Only a minor peak showed fibrinolytic activity (figure not shown).

The purity of the enzyme preparation was measured by SDS-PAGE, which showed one dominant protein band with the molecular mass of about 24.5 kDa (Fig. 1). This is

Table 1. Purification of the extracellular fibrinolytic enzyme from *Rhizopus microsporus* var. *tuberosus*

Purification step	γ(protein) / mg/mL	Activity / U/mL	Specific activity / U/mg	Yield / %	Purification fold
Cell-free supernatant	7.5±0.8	324.7±16.2	43.2±2.2	100	1
$(NH_4)_2SO_4$ precipitation	0.67±0.07	182.6±9.1	272.0±13.6	56.2	6.3
DEAE-Sepharose Fast Flow	0.065±0.007	68.7±3.4	1062.2±23.1	21.2	24.6
Sephadex G-75	0.035±0.004	57.6±2.9	1645.2±32.9	17.8	38.1

lower than that of the enzyme that coagulates the protein in soya bean milk (30.0 kDa) from *Bacillus pumilus* TYO-67 (*15*) and higher than that from *Rhizopus chinensis* 12 (16.6 kDa) (*16*). In addition, according to the results of HPLC, the fibrinolytic enzyme was partially purified, showing only one major peak (Fig. S1).

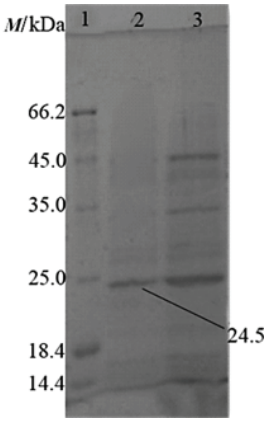

Fig. 1. SDS-PAGE profile of enzyme purification. 1=marker, 2=sample after gel filtration chromatography, and 3=sample after ion exchange chromatography

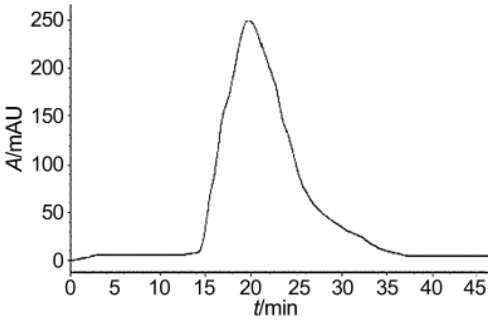

Fig. S1. The purity of the fibrinolytic enzyme determined by HPLC gel-filtration chromatography

After all purification steps, the enzyme was purified 38.1-fold, with a final yield of 17.75 % and the specific activity of (1645.1±32.9) U/mg (Table 1).

Characterisation of the fibrinolytic enzyme

The enzyme was stable in the artificial intestinal fluid and retained more than 80 % of its original activity after 4 h of preincubation (Table 2). It is indicated that the enzyme could be resistant to trypsin *in vivo*, and could have a potential to be developed as oral drug. Oral administration of the fibrinolytic enzyme nattokinase has been reported to enhance fibrinolytic activity in plasma and the production of endogenous plasminogen activators, such as t-PA (*17*).

Table 2. Effect of artificial intestinal fluid on the fibrinolytic enzyme

t(preincubation)/h	1	2	3	4
Residual activity/%	95.2±8.6	94.5±9.7	90.4±8.4	83.3±8.7

The pH=7.0 was found to be optimum for the fibrinolytic activity of the enzyme (Fig. 2a). Since the enzyme had a wide range of pH adaptability, it remained active even when the pH of the fibrin plate was adjusted to pH< 3.4 and >11.2. The pH stability of the enzyme was tested in the pH range of 3.0 to 12.0 by measuring the residual activity after incubation for 24 h at each pH (Fig. 2b). The enzyme was stable in the pH range from 6.0 to 8.0 for 24 h at 37 °C, which was the optimum temperature for the enzyme activity (Fig. 2c) and the enzyme showed approx.

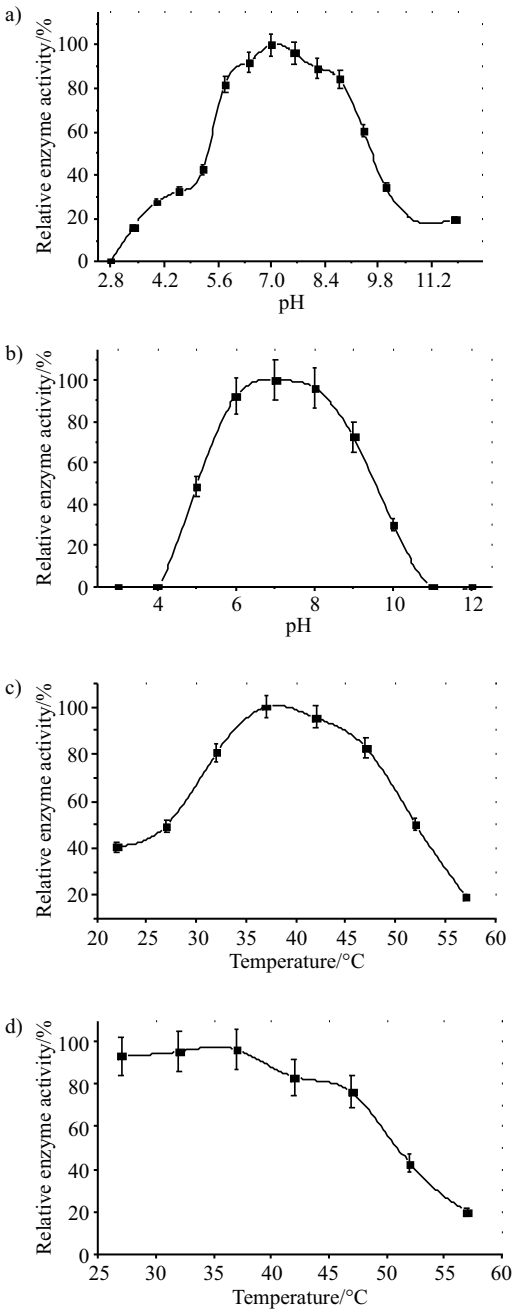

Fig. 2. Characterisation of the influence of pH and temperature on the enzyme activity and stability: effect of pH on a) the activity and b) stability (24 h at 37 °C); effect of temperature on c) the activity and d) stability (4 h at 27–57 °C). The values of the activities that correspond to 100 % were: a) 292.23, b) 286.34, c) 314.26 and d) 302.58 U/mL

96.1 % residual activity after 4 h of incubation at 37 °C (Fig. 2d). The optimum pH (7.0) and temperature (37 °C), which are very close to physiological conditions, suggest the possibility of clinical applications of the enzyme.

The effect of metal ions showed that Zn^{2+} and Cu^{2+} hinder the enzyme activity, whereas Na^{+}, Ca^{2+}, Mn^{2+} and Mg^{2+} showed obvious stimulation of the enzyme (Fig. 3).

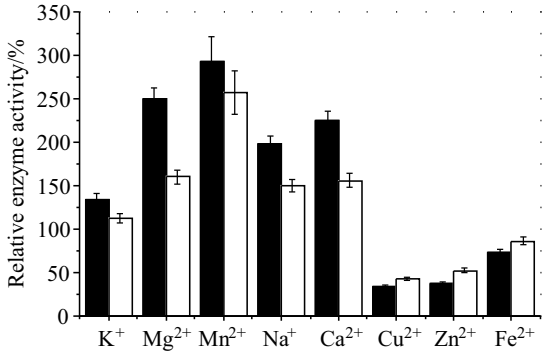

Fig. 3. Effect of metal ions on the activity of the fibrinolytic enzyme. The activity value that corresponds to 100 % was 292.64 U/mL. Metal salt concentration: —□—1 mM, —■—10 mM

On the plasminogen-positive fibrin plate, the fibrinolytic enzyme formed a clear zone (18.32 mm×18.28 mm) which was larger than that (16.08 mm×15.28 mm) on the plasminogen-free fibrin plate. This showed that the fibrinolytic enzyme produced by *R. microsporus* var. *tuberosus* could activate plasminogen into plasmin to degrade fibrin (Fig. 4). The clear zone on the plasminogen-free fibrin plate indicates that the fibrinolytic enzyme from *R. microsporus* var. *tuberosus* could degrade fibrin directly, in contrast to urokinase.

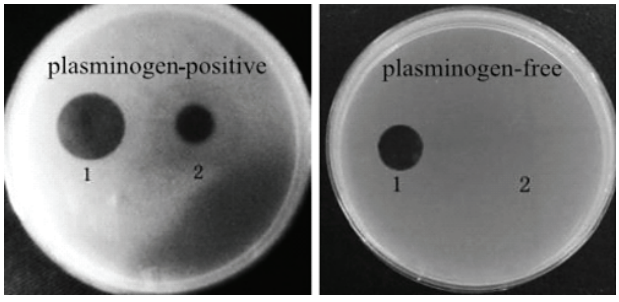

Fig. 4. Activation of plasminogen by the fibrinolytic enzyme. 1=fibrinolytic enzyme, and 2=urokinase

Conclusions

The fibrinolytic enzyme from *Rhizopus microsporus* var. *tuberosus* was partially purified, and thus purified enzyme had maximum activity at 37 °C and pH=7.0, and showed stronger resistance to the inhibition by trypsin. It was stable at 37 °C and retained 96.1 % of its original activity after being incubated for 4 h. The activity of the enzyme is promoted in the presence of Na^{+}, Ca^{2+}, Mg^{2+} and Mn^{2+}, whereas the presence of Zn^{2+} and Cu^{2+} partially inhibits its activity. The enzyme not only degrades fibrin directly, but also activates plasminogen into plasmin to degrade fibrin. The results indicate the potential of the pure enzyme in dissolving blood clot, and the possibility for application in the therapy of thrombosis.

Further research and more clinical trials are needed to verify the safety of the application of fibrinolytic enzyme as an oral thrombolytic agent, detect its potential side effects and study its induction of *in vivo* lysis of thrombi or its industrialised production.

Acknowledgements

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