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Production, Purification, and Biochemical Characterization of a Fibrinolytic Enzyme from Thermophilic *Streptomyces* sp. MCMB-379

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Abstract Production of the fibrinolytic enzyme was carried out using 2.5-L glass fermentor, culture of thermophilic *Streptomyces* sp., and glucose yeast extract peptone medium of pH 8.0. Five successive batches were carried out under controlled fermentation conditions viz., agitation 140 rpm, aeration 0.5 vvm, 55 °C, and 18 h. The total protein extracellularly produced in the cell-free broth was ~300–500 mg/L. The enzyme belongs to serine endopeptidase type. Studies on the fibrin degradation indicate that the enzyme degrades the fibrin into small molecular weight products as seen from HPLC profile. Phase-contrast microscopic structure of fibrin showed that enzyme cleaves the fibrin filaments. The *ex vivo* activity of the actinokinase was compared with 500 IU of urokinase and 350 IU of streptokinase. The *ex vivo* clot lysis was found to be faster as compared to the commercial available enzymes.

Keywords *Streptomyces* sp. · Thermophiles · Fibrinolytic enzyme

Introduction

Cardiovascular diseases require effective medicinal drug for their therapy. Different enzymes such as streptokinase, urokinase, staphylokinase, and nattokinase are applied to treat thrombosis, but these agents have some disadvantages such as hemorrhagic effect, immunogenicity, and high cost, due to which their uses are limited. There is still scope to search new agents which overcome these drawbacks. The cost of the enzyme production and downstream processing is of major concern for successful application of protease in the industry.

A few fibrinolytic enzymes are reported as nattokinase, purified from traditional Japanese food Natto [1]. Insoluble fibrin fiber is hydrolyzed by plasmin which is generated from plasminogen by plasminogen activator, urokinase, and streptokinase plasminogen activator [2]. These thrombolytic agents have indirect specificity to the fibrin. Some of the

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fibrinolytic enzymes which directly act on the fibrin are nattokinase, lubrokinase, and third generation of fibrinogen activator. Fibrinolytic enzymes have potential role in the therapy of thrombolytic disorders. There are several thrombolytic agents discovered till today. However, there is still a need to investigate new fibrin-specific fibrinolytic enzymes to overcome the drawbacks, side effects, etc., of the presently available drugs. The plasminogen-dependent type of enzyme has some side effects, such as bleeding complication, hemorrhage, and severe allergy to the patients. There are some enzymes still to go through clinical trials for their commercial use. Nattokinase is a fibrinolytic enzyme from the Japanese food soya sauce and is used for oral administration.

Gesheva [3] has described the production of the fibrinolytic enzyme using culture of *Streptomyces rimosus* and under nitrogen limitation in production medium. The biomass increased rapidly during 72 h and declined after 84 h. First-generation thrombolytics, streptokinase and urokinase, had no fibrin-binding capabilities and produced the systemic plasminogen activator with subsequent destruction of hemostatic proteins. *In vitro* assays highlighted advantages of fibrin binding. The clinical trials are disappointing showing small benefit of mortality with t-PA versus streptokinase [4]. One fibrinolytic metalloprotease from *Perenniporia fraxinea* mycelia was studied for its fibrinolytic properties, and the enzyme was purified and characterized [5]. The chemical modification of fibrinolytic enzyme lumbrokinase with human serum albumin fragment and characterization of the protease as a therapeutic enzyme was carried out. Selection of medium components is important for the fermentative production of fibrinolytic enzymes. As the different microbes possess diverse physiological characteristics, it is needed to optimize nutrient components and environmental conditions for microbial growth and fibrinolytic enzyme production [6, 7]

Actinokinase is a new fibrinolytic enzyme reported from thermophilic *Streptomyces megasporus*. The enzyme is resistant to broad pH range [8]. The physico-chemical properties of the enzyme are compared with reported thrombolytic agent [9]. Optimization and production of enzyme actinokinase was carried out using *S. megasporus* SD5. Optimum temperature for enzyme synthesis is 55 °C, probably because the strain was isolated from a hot spring [10]. Potent fibrinolytic enzyme from thermophilic *S. megasporus* SD5 was characterized, and it was shown that the activity is dependent on N-terminal. It was reported that this enzyme "actinokinase" could be possible clued for enzyme therapy for myocardial infarction. The cloning and expression of the actinokinase gene in *Escherichia coli* was carried out. Nitrogen sources, peptone, and yeast extract seemed to be effective for production of the actinokinase enzyme; US patent [11] and an Indian patent [12] have been granted for the process for production of the enzyme using thermophilic *S. megasporus* SD5, its mutant and variant.

The present study is carried out for bench scale production of the fibrinolytic enzyme with validation of batches, its purification and characterization, product processing, and biochemical studies.

Materials and Methods

Chemicals

Media, glucose, yeast extract, peptone, sodium chloride, and calcium chloride were purchased from Hi-media; thrombin, fibrinogen, human fibrin, human plasma, and fibrinogen from Sigma (St. Louis, USA); and chromogenic substrate (S-2444) from Chromogenix USA. Urokinase and streptokinase were purchased from Bharat Biotech India. Other reagents are of analytical grade from Sigma Chemicals, USA.

Organism and Growth Conditions

Based on its fibrinolytic and amidolytic activities reported earlier, *S. megasporus SD5* MCMB-379 which was deposited in MACS Collection of Microorganisms (MCM, affiliated to WFCC, WDCM code no. 561), Pune India was selected for the study and grew in glucose yeast extract peptone (GYP) agar medium (pH 8). The isolate is maintained as a spore suspension [13] and used for the further study of the enzyme.

Enzyme Production

Preparation of Seed Culture

The 50-ml GYP medium, containing 0.02% $CaCl_2$, inoculated with 25 μl spore suspension (1.7×10^8 spores) was from spore stock and grown at 55 °C for 18 h. The 5% seed culture was used as an inoculum for 1-L production of the enzyme.

Production in 2.5-L Fermentor

Production of enzyme was carried using 2.5-L glass Biotron fermentor and GYP medium of pH 8.0. The fermentation was carried out at 55 °C for 18 h under controlled conditions. The agitation was maintained at 140 rpm and aeration at 0.5 vvm.

Purification

After 18 h of incubation, the cell mass was separated by centrifugation at 8,000 rpm for 15 min and then cell-free broth passed through 0.22-μm filter. The broth was loaded on the UNOsphere strong Anion-Q column (41 h × 2.4 cm diameter) of the bed height 17 cm with 78 g of resin. The column was equilibrated with two column volume of the 10 mM Tris buffer of (pH 7.8), and then the cell-free broth was passed through the column with flow rate 8 ml/min. The column was then washed with 10 mM Tris buffer of pH 7.8 and then eluted with 10 mM Tris buffer containing 0.5 to 1.0 M NaCl and of pH 8.0.The fractions were collected and then measured for protein content [14] and enzyme activity. Manual column fractions were pooled together and dialyzed in 10 mM phosphate buffer of pH 7.5. The dialyzed partial purified samples were loaded on Mono Q column which is pre-equilibrated with 10 mM phosphate buffer containing 0.5 M NaCl, pH 7.5, with flow rate of 0.5 ml/min. The fractions were checked on 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Ex Vivo Clot Lysis

Using purified fraction and crude fractions of the enzyme, *ex vivo* clot lysis was compared with that of standard commercial available enzyme.

In Vitro Fibrin Degradation Product

Human fibrin (0.1%) was dissolved in the 10 mM phosphate buffer pH 8.0 and briefly vortexed for the resuspension. The *ex vivo* clot lysis was performed according to protocol of Plough and Kjeldgaard [15]. Mixture contained 100 μl fibrin (0. 1%) suspension and 80 μl of pure

Table 1 Batchwise enzyme activity and protein content of fermented broth

Batch number	Volume of cell-free filtrate (L)	Final pH after fermentation	Enzyme activity (U) using S2424 at 405 nm	Total proteins (mg/L) for 18 h
1	1	5.9	108	490
2	1	5.7	102	510
3	1	5.5	101	450
4	1	5.8	104	430
5	1	5.6	106	490

U Amount of enzyme that catalysed the formation of 1 μmol *p*-nitroanilide min^{-1}

actinokinase enzyme (50 μg) and with 80 μl of 10 mM phosphate buffer pH 8.0. The reaction mixture was kept at 37 °C for interval time of 0, 5, 10, 15, 20, 25, 30, 35, and 40 min along with control. The samples were run on the SDS-PAGE, and the gel was stained with silver staining.

Fibrin Product after the *Ex Vivo* Clot Lysis

The clot was prepared according to Kim [16] (30 μl of 1% human fibrinogen solution in 50 mM Tris chloride buffer of pH 7.8 containing 0.1 M sodium chloride was mixed with 30 μl thrombin solution; 1.0 NIH units). In the same buffer, fibrin clot was allowed to stand for 1 h at room temperature (28±2 °C). *Ex vivo* clot was prepared using the fibrinogen and thrombin formation of clot; the enzyme (50 μg) was added and then the sample at the regular interval of time checked on the SDS-PAGE gel.

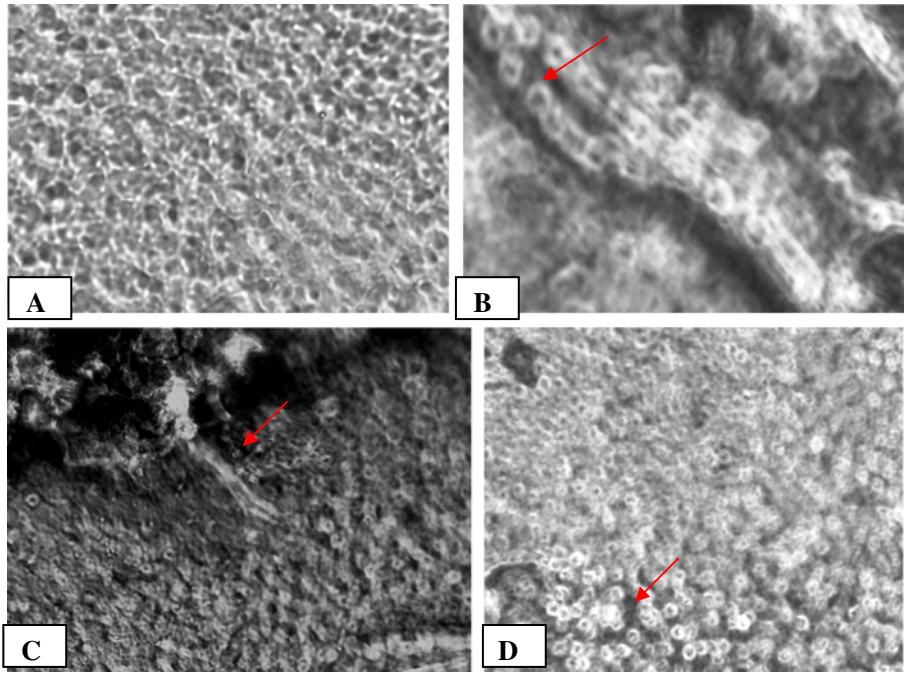

Fig. 1 Phase-contrast microscopy photographs of fibrin clot lysis using actinokinase enzyme: **a**) control fibrin and **b**) lysis at 10 min. *Arrow* shows the lysis point **c**) lysis at 20 min and **d**) lysis at 30 min

Table 2 Comparative *in vitro* clot lysis of actinokinase with known enzymes

Time in minutes	5	10	15	20	25	30	40	60	80
Actinokinase, % clot lysis	20	55	85	100					
Urokinase, % clot lysis	5	50	60	80–90	90	95	100		
Streptokinase, % clot lysis	5	10	10	15	20	40	40	90	100

Concentration in 500 IU

Percentage of clot lysis calculated as described by Prasad et al. [23]

HPLC Profile of Fibrin Degradation Products

Previously described fibrin degradation products of *ex vivo* clot lysis were detected on HPLC using C18 nucleosil, reverse-phase column with flow rate of 0.5 ml/min at 280 nm and solvent system 1:1 acetonitrile and water.

Results

The five fermentation batches of production of actinokinase enzyme were successfully carried out using 2.5-L glass fermentor (Table 1). It is seen that pH of fermentation process dropped towards acidic side (5–5.5) after 18 h of incubation. The change in pH is the indicator for enzyme production. Gas chromatographic analysis showed production of acetic, propionic, and *n*-butyric acids during the fermentation process. There is no report on the development of acidic pH during the production of other available fibrinolytic enzymes. The actinokinase enzyme resists to broad pH range. The protein content of cell-free broth was about 500 mg/L and pure enzyme recovered was 30–40 mg/L.

The novel fibrinolytic enzyme from *Rhizopus chinesis* 12 requires 5 days of incubation at 30 °C [17]. *Bacillus natto* strain produced the enzyme Nattokinase within 22–120 h of incubation time at 37 °C under different culture conditions [18].

The enzyme is not plasminogen activator and directly acts on the fibrin monomer. The action of actinokinase enzyme is different than that of streptokinase and urokinase which are plasminogen activator. Plasmin is generated during the activation process and cleaves the fibrin network.

The fibrin clot dissolution was observed using the phase-contrast microscopy and thinness of the fibrin filaments observed during fibrin clot dissolution by the actinokinase enzyme (1–3 μg/

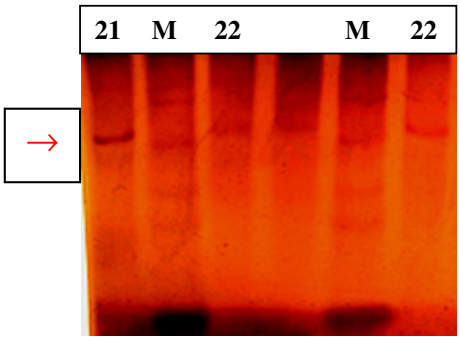

Fig. 2 SDS-PAGE gel of pure actinokinase enzyme

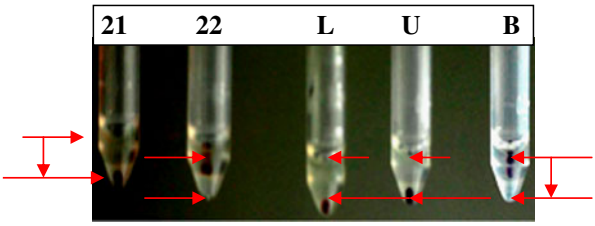

Fig. 3 *Ex vivo* clot lysis, using dropping beads methods. *21* and *22* were FPLC fractions. *L* and *U* were Centriplus fractions. *B* was blank

clot volume of 125 μl (*w*/*v*) during incubation at 10, 20, and 30 min of time (Fig. 1). The fibrin chains are cleavage by the actinokinase when exposed to the fibrin clot. The actinokinase is fibrin specific in nature and cleaves the fibrin filament. The fibrin specificity of actinokinase on the structural basis is demonstrated and pointed, using phase-contrast microscopy. Plasmin and urokinase fibrinolysis on structural basis was illustrated by electron microscopy [19]. Novelty of actinokinase lies with respect to the mode of action and the source from thermophilic *Streptomyces* sp. which is isolated from untouched ecosystem.

Purified enzyme was compared with international units of urokinase and streptokinase. Actinokinase of 500 IU dissolved the clot within 20 min while urokinase and streptokinase took 40 and 80 min, respectively (Table 2). *Ex vivo* clot lysis was carried out with fast protein liquid chromatography (FPLC)-purified (Fig. 2) fractions of pure actinokinase enzyme (20–200 μg/ml) which dissolved the clot quickly within 10 min (Fig. 3) while partially purified enzyme took 20 min for complete lysis (Fig. 4). The *ex vivo* lysis is using clot volume (125 μl *w*/*v*) with comparable units of actinokinase with that of the known enzymes. The thermophilic *Streptomyces* sp. secretes enzyme extracellularly with 12.5% pure enzyme yield. Fibrin degradation products using actinokinase enzyme showed that the enzyme binds strongly to the fibrin monomer and subsequently lyses the clot. Fibrin degradation products using fibrin as a substrate (Fig. 5) and *ex vivo* clot lysis are seen, and fibrin degradation shows the degradation products. It is also seen that the enzyme acts on the α- and β-chain with subsequent lysis of the γ-chain. *Ex vivo* clot lysis degradation products obtained using actinokinase enzyme were checked on HPLC at a 5-min interval of time. The HPLC profile of degradation products is illustrated in (Fig. 6).

It was observed that actinokinase enzyme hydrolyzed the fibrin α-chain, followed by β-chain, and slowly hydrolyzed the γ-γ-chain, and the chains get hydrolyzed which are shown in 12% SDS-PAGE. The fibrinolytic pattern indicated that the enzyme hydrolyzed the fibrin α-chain, followed by β-chain. It also hydrolyzed γ-γ-chain, and all chains get hydrolyzed slowly.

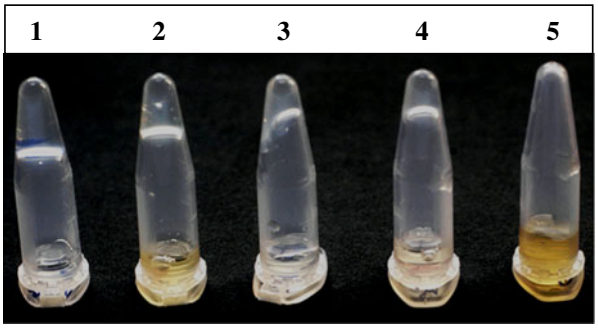

Fig. 4 *1*) control, *2*) 5 min, *3*) 10 min, *4*) 15 min, *5*) 20 min

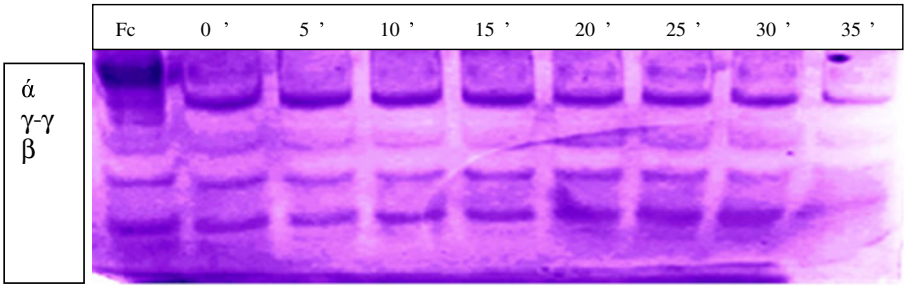

Fig. 5 SDS-PAGE showing the degradation of fibrin by actinokinase, Fc-control fibrin, time of interval of incubation in minutes

Discussion

The currently available thrombolytic agents are non-specific in nature for cleavage on other blood physiological factors that lead to the hemorrhage. These side effects are reported in the literature [20].

The enzyme such as urokinase is obtained from human urine, but its yield is very low, while streptokinase is obtained from the pathogenic culture like *Streptococcus*. The actinokinase is reported from non-pathogenic culture of *Streptomyces* sp. The fibrinolytic enzymes reported from different sources and their characterization, production, and fermentation conditions are reported [21].

The fibrinolytic enzyme reported here is plasminogen independent type that acts directly on the fibrin. The enzyme is fibrinolytic in nature and releases the degradation products. The enzyme is extracellular in nature so its purification and yield are economically feasible. The urokinase extraction and purification steps are difficult, and yield is also low that ultimately

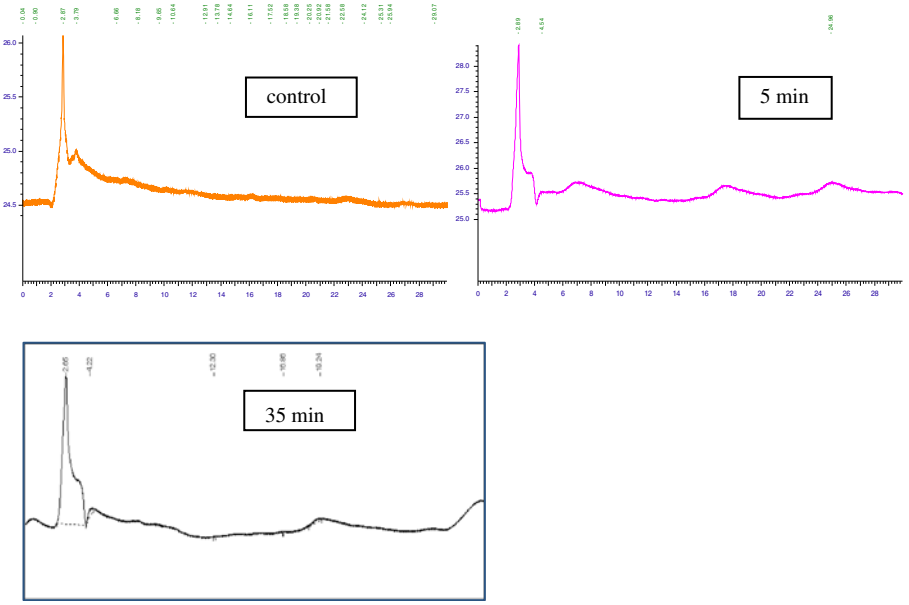

Fig. 6 Fibrin clot degradation profile after 5 and 35 min on HPLC system along with control

leads to an increase in the price of the product. Streptokinase is obtained from the pathogenic strain so the enzyme is antigenic in nature, which leads to immunological reaction in the patient. The secondary immune response is reported among the patients who had streptokinase therapy. Thus, there is a need to check the effect of actinokinase in the animal model.

The fibrinolytic metalloprotease from *P. fraxinea* hydrolyzed the fibrin chain [5], and degradation of fibrin was studied. The fibrinolytic protease from *Flammulina velutipes* hydrolyzed fibrin digesting α-chain over β- and γ-γ-chain. This hydrolysis pattern is similar to that of plasmin [22]. This paper emphasizes the production of the enzyme with good yield, extracellular in nature for simple recovery and enzyme characterization, and potent in nature with respect to *ex vivo* clot lysis. The studies indicate that the enzyme actinokinase will be an effective thrombolytic agent against cardiovascular diseases.

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