Fibrinolytic Enzyme Produced from *Bacillus subtilis* and media optimization, Purification and Characterization

*Lakshmaiah pallepati[1], Ch.Siva sai[2] ,G. Reshma Reddy[3], U.Spandana[4]
[1,2,3]Vishwabharathi college of pharmaceutical sciences perecherla, Guntur, A.P
[4]Nirmala college of pharmacy, atmakur, mangalagiri, Guntur, A.P
Corresponding Author: Dr. Lakshmaiah Pallepati

Abstract
Streptokinase is an extracellular protein, novel fibrinolytic enzymes which are isolated from *Bacillus subtilis,*it is a non-protease plasminogen activator that activates plasminogen to plasmin, the enzyme that degrades fibrin cloth through its specific lysine binding site; it is used therefore as a drug in thrombolytic therapy. The rate of bacterial growth and streptokinase production was studied in condition of excess glucose addition to culture media and its pH maintenance. The streptokinase product of the bacterial culture was preliminary extracted by salt precipitation and then purified by affinity chromatography on plasminogen substituted sepharose-4B in a condition that the plasminogen active site was protected from streptokinase-induced activation. The purity of streptokinase was confirmed by SDS-PAGE and its biological activity determined in a specific streptokinase assay. The results showed that in the fed–batch culture, the rate of streptokinase production increased over two times as compared with the batch culture while at the same time, shortening the streptokinase purification to a single step increase

Key words: Plasminogen, Purification, Streptokinase, fibrinolytic enzymes,*bacillus subtilis*

Introduction

Fibrinolytic enzyme was identified and studied among many organisms including snakes,earthworms, and bacteria:*Streptococcuspyogenes, Aeromonashydrophila,Serratia*E15, *B. natto*,*Bacillusamyloliquefacens*, Actinomycetesand fungi: *Fusariumoxysporum*; *Mucor*sp,*Armillariamellea*(JianSha et al, 2003).Fibrinolytic enzyme can be found in a variety of foods,such as Japanese Natto, Tofuyo, Korean Chungkook-Jang soy sauce and edible honey mushroom. Fibrinolyticenzymes have been purified from these foods and their physiochemical properties have been characterized.Fermented shrimp paste, a popular Asian seasoning, wasshown to have strong fibrinolytic activity. These novel fibrinolytic enzyme derived from traditional Asian foods are useful for thrombolytic therapy. They will provide an adjunct to the costly fibrinolytic enzymes that are currently used in managing heart disease, since large quantities of enzyme can be conveniently and efficiently produced. In addition, these enzymes have significant potential for food fortification and nutraceuticalapplications,such that their use could effectively prevent cardiovascular diseases (Yoshinori et al., 2005).Accounts of cardiovascular diseases have become the leading cause of death in the Western world (Viles et al.,2004). Many blood clot-dissolving agents, such as urokinase,streptokinase, and tissue plasminogen activator (t-PA), have been utilized in clinical treatments for cardiovascular diseases.Hemostasis is a complex process obtained through an optimal balance between bleeding and blood clot formation. In an unbalanced state, fibrin clots may not be lysed resulting in thrombosis. Thrombolytic agents from various sources have been extensively investigated. However, these enzymes are often expensive, thermolabile and can produce undesirable side effects (Chitte and Dey, 2000 Similar fibrinolytic enzyme-producing bacteria have also been isolated from Japanese shiokara,Koreachungkook-jang (Banerjee et al., 2004), and Chinese douchi. Nevertheless, it is still the most stable and economic way to obtain protein with fibrinolytic activity. On the basis of its food origin, relatively strong fibrinolytic activity, stability in the gastrointestinal tract, and convenient oral administration, streptokinase has advantages for commercially used medicine for preventative and

prolonged effects (Uversky et al., 2004). Streptokinase is an extra cellular protein, extracted from certain strains of beta haemolytic*Bacillus subtilis*. It is a non-protease plasminogen activator that activates plasminogen to plasmin, the enzyme that degrades fibrin

Clots through its specific lysine binding site; it is used therefore as a drug in thrombolytic therapy (Mohammad et al., 2009). Streptokinase is currently used in clinical medicine as a therapeutic agent in the treatment of thromboembolic blockages, including coronary thrombosis (Banerjee et al., 2004; Endrogan et al., 2006). Streptokinase (SK) a group of extracellular proteins produced by a variety of beta-hemolyticstrains, and is a plasminogen activator composed of 414 amino acids with a molecularmassof 47 kDa. Direct proteolysis, streptokinase.Forms a high affinity equimolar complex with a plasminogen (Kim et al., 2000).,

This enzyme is a strong plasminogen activator which specifically cleaves the proenzyme/ zymogen plasminogen to form the active enzyme plasmin (Kunamneni et al., 2008). It specifically catalyzes the cleavage of the Arg-Val bond in plasminogen.for the simple reasons of its being more potent as compared to tissue-plasminogen activator and non-antigenicby virtue of its human origin unlike streptokinase.Based on these observationsstreptokinase.is a strong plasminogen activator. The mechanism of action, physico-chemical properties, *in vitro* production, cloning and expression, and clinical applications of streptokinase. are shown in the present study (Adinarayana et al., 2008).

MATERIALS AND METHODS

Isolation and Identification of microorganism

Bacillus subtilis:

Bacillus subtilis producing Streptokinase (SK) was isolated from soil sample collected from various regions in Andhra Pradesh India and identified by colony morphology, Gram's staining, biochemical test and selective media. The identified microorganisms were stored at -20°C

Enzyme production

Bacillus subtillis was grown on medium containing corn steep liquor 8% and 12% serelose, 7% KH2 PO4, 0.33% K2HPO4 ,0.2% cysteine, 0.01% Glycine 0.01% tryptone, 0.01% Uracil, 0.001% adenine sulfate, 0.001% nicotinic acid, 0.001% pyridoxine-HCl, 0.0018% calcium phosphate, 0.005% thiamine-HCl, 0.002% riboflavin, 0.001% and salt mixture 2 g/lit. The pH was adjusted to 7.0 with 1 M HCl and 1M NaOH. Medium was sterilized and cooled at room temperature. One ml of culture was used as inoculum;incubated at 37°C and 170 rpm in orbital shaker. After 75 h of fermentation, cells were removed by centrifugation.

Enzyme purification

Cells were separated from culture broth by centrifugation (8,000 ×g, 15 min) and the supernatant fluid was added to 3 volume of acetone. The mixture of supernatant and acetone was allowed to stand at 4°C for 1 day. After centrifugation (10,000 × g, 15 min) of the mixture, the resultant precipitate was purified by ion exchange column chromatography (DEAE Cellulose, MERK). The active fractions were added to 660 ml of acetone and allowed to stand at 4°C for 18 h. The precipitates were collected by centrifugation and then lyophilized. For further purification, gel filtration with SephadexG200 (MERK) gel equilibrated with 10 mM glycine-NaOH buffer (pH, 9) was performed. The active fractions were precipitated with acetone and then lyophilized. Sodium dodecyl sulfate polyacrylamidegel electrophoresis (SDS-PAGE) was done by using 10 to 20% gradient polyacrylamide gel and a 4% stacking gel at 4°C.

Forward and backward extraction

Both forward and backward extraction was carried out with a volumetric phase ratio 6:6 (ml) in tightly stoppered 50 ml glass flask.In the forward extraction, 50 mM isooctane was used as the organic phase system and 1.0 mg/ml fibrinolytic enzyme in 20 mM/litre, pH 4.0 to 7.0 tris-buffer at the given salt species and concentration was used as aqueous phase system. The two phases were mixed on aorbital shaker with a speed of 240 rpm in water bath at 20°C. The mixtures were centrifuged at 4,000 rpm for 5 min. to reach a clear separation of two phases. During the investigation, fermentation broth was used as aqueous phase

Ammonium salt precipitation

The fibrinolytic enzymes were also purified by ammonium sulphate saturation. The protein fraction was precipitated with 85% ammonium sulfate. Ammonium sulfate was found to activate the fibrinolytic activity after dialysis. Fibrinolytic enzymes were partially purified by using anion exchange column chromatography (DEAE Cellulose, MERK).

Enzyme assay and characterization

The relative activity and quantitative estimation of fibrinolytic enzymes were estimated by Lowry's method spectrophotometricallyat 560 nm; L-arginine, casein, BSA, mixture of amino acids and phenylthiohydantoins (PTHs) was used as standard.

Effect of pH

Eight hundred (800) µl of serum was added to 100 µl of casein (3%) solution. The mixture was incubated at 37°C for 1 h then centrifuge. The precipitate was washed twice with 1 ml of phosphate buffer and vortexes. The purified enzymes were dissolved in sodium phosphate buffer (pH, 7.5). The enzyme solution (100 µl) was added to the serum casein solution and absorbance was taken by UV-spectrophotometer at 560 nm.

Effect of inhibitor/ activator

Purified enzyme was dissolved in 10 mM Glycine-NaOH buffer (Ph, 9.0) and mixed with each salt solution to give a final inhibitor and activator concentration of 0.5 mM. Enzyme samples were separately incubated at 37°C for 10 min with each of the following inhibitors: PMSF, EDTA, AgNO3, HgCl2 and SDS; residual activity was then determined. MgSO4, FeSO4, MnSO4, MnCl2 and CaCl2were used as activator.

Effect of temperature

Effect of temperature on the fibrinolytic activity was exa-mined at pH 8.5. Casein and serum was used as a substrate.

Enzyme assay

Fibrinolytic activity was determined by serum, casein and fibrin plate method. The Casein solution [2.5 ml of 2% (w/v) human fibrinogen (Merck) in 0.1 M Sodium Phosphate buffer, pH 7.4] was missed with 2 ml of human serum after sterilization of agarosesolution in Petri dish (100 by 15 mm). After the dishes were allowedto stand for 30 min at room temperature, three holes were made on a fibrin plate by suction by using steel gel puncture (0.5 cm). 50 and 100 µl enzyme solution was dropped into each hole and incubated at 37°C for 18 h. After measuring the dimension of the clear zone, the number of units was determined. One unit of the enzyme activity was defined as the amount of enzyme in 25 µl of enzyme solution that produced a clear zone of 1mm2 at pH 7.7 and 35°C for 18 h.

Caseinolytic activity

Caseinolytic activity was assayed using the following procedure: A mixture (1 ml) containing 0.7 ml of 0.1 M sodium phosphate buffer (pH 7.5), 0.1 ml of 2% casein, and 0.1 ml of enzyme solution was incubated for 5 min at each temperature, mixed with 0.1 ml of 1.5 M trichloroacetic acid, allowed to stand at 4°C for 30 min and then centrifuged at RT. The absorbance at 560 nm for the

Step	Total activity (u)	Total protein(mg)	Specific activity(u/mg)	Purification fold	Recovery(%)
Crude	2506988	290	8645	1.00	100.0
Ammonium sulfatetreatmen((060%)	2287299	31.07	73618	8.52	91.2
UnoQSepharoseStrongAnionExchanger	872296.2	3.87	225400	26.07	34.8
Butyl Sepharose FF	187500	0.669	280280	32.42	7.5

table : Summary of the purification fibrinolytic enzyme from *Bacillus subtilis*

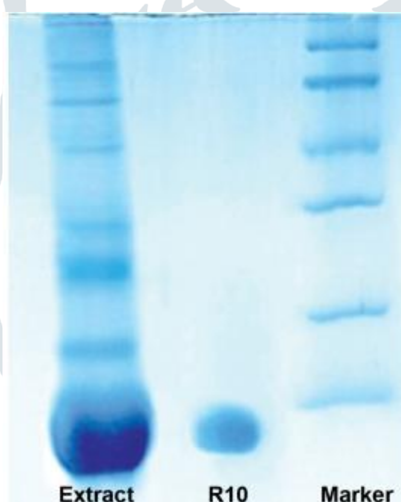

Fig: SDS-PAGEofthepurifiedfibrinolyticenzymefrom *Bacillussubtilis*

Results

Streptokinase enzyme *bacillus subtilis* organism used for production of streptokinase enzyme was gram positive, cocci, aerobic bacteria. The biochemical test for streptococcus showed positive result in TSI, Gelatin, Nitrate, Citrate and Starch hydrolysis test while negative result was observed in MRVP, urease and indole production test.

Enzyme assay

The activity of Streptokinase enzyme was achieved about 467.73U when compared to the casein. The fibrinolytic activity of Streptokinase was also measured by casein, serum and fibrin plate technique. One unit of enzyme activity was defined as the amount of enzyme in 25 µl of enzyme solution that produced a clear zone of 1 mm2 at pH 7.7 and 35°C for 18 h. The 2.5 U for 50 µl and 5 U of activity for 100 µl were achieved by Streptokinase.

Effect of temperature

The effect of temperature on the fibrinolytic activity of Streptokinase was examined at pH 7. The temperature showing maximal enzyme activity was 27 to 37°C and showed 38.4, 34.5, 32.2 and 11.2% residual activity at 8, 55 and -20°C and 100°C, respectively. It was concluded that the enzyme was active in the range of 27 to 45°C (Figure 6).

Effect of pH

The optimum pH for fibrinolytic activity of Streptokinase was around 4 to 7 and the enzyme activity decreased rapidly at level below pH 3. The enzyme was very stable in the range of 5 to 7 at 30°C for 25 h. Above pH 11, enzyme activity abruptly decreased

SDS-PAGE

The protein bands found on SDS PAGE for Streptokinase were approximately 58, 47 and 40 kDa (Fig) It was concluded that the molecular weight of purified protein band 47 kDa was approximately to Streptokinase 47 kDa.

Recovery of Streptokinase dissolved blood clotting

The recovery of enzyme was checked by dissolving human blood clotted. The experiment was done in laboratory in clean slide; about 100 mg of coagulated blood was dissolved by 200 µl enzyme within 2 h at 37°C temperature.

DISCUSSION

The article describes the purification and characterization of Streptokinase and from *bacillus Subtilis,*assessment for its application as a thrombosis agent. As mention above, intravenous administration Streptokinase has been widely used for thrombosis therapy. Fibrinolytic therapy by oral drug administration has been recently investigated in animal models in which enteric-coated capsule were given to normal and experimental dogs with saphenous vein thrombosis. (Sumi *et al.,* 1990) reported that intravenous administration did not show any clear thrombolytic effect but oral administration enhanced fibrinolytic activity.In another study (Endrogan *et al* 2006) reported that streptokinase was given to human subject by oral administration, fibrinolytic activity On *Bacillus subtilis* producing fibrinolytic enzyme were isolated from soil obtained from various regions of Andhra pradesh. *Bacillus subtilis* showed strongest fibrinolytic, thermophilic and hydrophilic activity. According to (Wonkeuk *et a.,l* 1996) the fibrinolytic activity of SK, fibrinolytic enzyme obtained from *Bacillus strain* .These several studies that have reported on the intestinal absorption of serum albumin. (Bernik and Oller 1973) observed activation of these reports. Structurally, with greater than 85% homology at the amino acid level. In contrast, the streptokinase secreted by a *bacillus subtils* isolated from human host exhibited at the amino acid only 29.4% identity as described by (Wang *et.al*., 1998) In view of this report, it can be suggested that the fibrinolytic enzymes isolated from bacteria can be given orally for use as a thrombolytic agent. *Bacillus subtilis* have been recognized as being safe for humans. Further studies will test for other species

Conclusion.

Fibrinolytic enzymes such as Streptokinase and used as thrombolytic agent but too costly and also used through intravenous instillation, needs large scale production by some alternative methods and high purity. So, isolation, production, purification, assay and characterization of fibrinolytic enzymes from bacterial sources are very effective and useful. In the future, the research will progress into the production of highly purified fibrinolytic enzymes from bacterial sources.

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