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Investigation of Fibrinolytic Activity of Locally Produced Streptokinase

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Streptokinase is a common fibrinolytic drug and included in the World Health Organization Model list of essential medicines and widely used in the treatment of acute myocardial infraction following coronary thrombosis. Comparative clinical trials and its cost effectiveness suggest that streptokinase can be the drug of choice for thrembolytic therapy. To obtain the highest amount of the protein and production of active form of streptokinase by hemolytic bacteria need to modify and optimize methods. In the present study native streptokinase was produced indigenously from bacterial source by fermentation biotechnology. After fermentation we, obtained the activity of streptokinase, 1000 U mL^{-1}, specific activity 555.56 U mg^{-1} with 13.9 fold purification. The purified streptokinase obtained had molecular mass 47 KDa and its K_m and V_{max} values for lysis of standard fibrinogen (fibrin) clot were 1.93 µg mL^{-1} and 566.45 IU mg^{-1} protein, respectively. The activation and inactivation energy were 28.2 and 27.3 KJ mol^{-1}. The most striking findings of this study was the production and purification of native streptokinase by fermentation biotechnology. It could be the proper option to make it economically feasible for the poor heart patients in under developed countries and determine its kinetic parameters because accurate reporting of the characterization of streptokinase preparations is essential for safe and effective therapy.

Key Words: Streptokinase, Thrombolytic, Fermentation, Fibrinogen, Tissue type plasminogen activator.

INTRODUCTION

A blood clot (thrombus) developed in the circulatory system can cause vascular blockage, pulmonary embolism, deep vein thrombosis and acute myocardial infarction (AMI) including death. These circulatory disorders are increasingly becoming the leading causes of mortality in modern societies world wide. A healthy hemostatic system suppresses the development of blood clots in normal circulation, but reacts extensively in the event of vascular injury to prevent blood loss. Pathologies involving a failure of hemostasis and the development of clot require clinical intervention consisting of intravenous administration of therombolytic agents[1-4].

Thrombolytic agents have the unique ability to activate the components intrinsic to the fibrinolytic system, resulting in the degradation of blood clots, which restores blood flow through the occluded vessels[5]. Streptokinase (SK) is one such agent. Other fibrinolytic agents commonly used in the thrembolytic therapy are urokinase (UK) and tissue type plasminogen activator (tPA). These agents are commonly refereed to as plasminogen activators, since their mode of action is through the conversion of the enzymatically inert plasminogen (Pg) of the fibrinolytic system to an active protease, plasmin (PN), that dissolves the fibrin clot and solublizes degradation products, which can be removed by the phagocytes[4].

Streptokinase (E.C. 3.4.99.22) is extracellular, single chain, non-enzymatic, monomeric protein with molecular weight 45-50 KDa and is made up of 414 amino acids residues[6-10].

Streptokinase is produced by many strains of β-hemolytic streptococci[11] of lancefield group A, C and G. The group C is preferred for streptokinase production, as they do not produce erythrogenic toxin[12] Christensen[13] isolated a group C strain *Streptococcus equisimilis* H46A (ATCC 12449) from human source[3] and now it has been widely used for the production of streptokinase[4]. Streptokinase facilitates lysis of blood clots by activating the plasminogen, to the fibrinolytic enzyme plasmin. Streptokinase is as effective as recombinant type plasminogen activator in treating acute myocardial infraction[14] and it is certainly most cost effective.

The purpose of this study is to indigenously produce and purify the streptokinase *via* fermentation biotechnology and make it cost effective for the poor heart patients of Pakistan and also determine its kinetic parameters such as K_m, V_{max}, activation and inactivation energy required by the streptokinase during lysis of standard fibrin clot.

EXPERIMENTAL

Bacterical strain and culture conditions: Strain *Streptococcus equisimilis* was obtained from Avesina Research Institute, Tehran, Iran and got identified from the Department of Microbiology University of Agriculture,Faisalabad, Pakistan. It was grown on Todd-Hewit Broth and Blood Agar Media to get pure culture[15].

Inoculum prepration: The strain selected was examined accurately by cultivation in blood agar media and the pH of the medium was maintained at 7 before sterilization. The medium used for production of streptokinase was autoclaved at 121 ºC for 15 min at 15 lbs pressure. Then loop full culture of *Streptococcus equisimilis* was transferred aseptically into the flask. It was then incubated on orbital shaker (150 rpm) for 24 h at 37 ºC.

Production of streptokinase: The growth on blood agar stab (BAS) was scraped and transferred to the production medium[15] with slight modification to achieve maximal yield of streptokinase under available local conditions. The selected strain was used for growth in liquid state fermentation (LSF) in order to analyze the streptokinase, activity.

Medium composition: The abundantly available agriculture waste, corn steep liquor (CSL) 5 % (w/v) was used as an economical substrate along with glucose 5 %, yeast extract 5 %, KH_2PO_4 0.1 %, K_2HPO_4 0.1 %, $MgSO_4 \cdot 7H_2O$ 0.1 %, $NaHCO_3$ 0.1 %, $CH_3COONa \cdot 3H_2O$ 1.5 %, $FeSO_4 \cdot 7H_2O$ 0.05 %, $MnCl_2 \cdot 7H_2O$ 1 %, to achieve higher streptokinase yield by (LSF). These were autoclaved at 121 ºC for 15 min. Then 5 % inoculum was added aseptically in each flask (triplicate were used) for incubation in shaker at 37 ºC and 120 rpm for 24 h.

Sample harvesting: After growth for 24 h, streptokinase was harvested from liquid state fermentation. It was filtered and the clear filtrate was used as the crude enzyme source.

Streptokinase assay: The activity of streptokinase was measured on the basis of standard (fibrin) fibrinogen blood clot lysis[16] with slight modifications.

Streptokinase: The commercially available lypholyzed streptokinase vial (15,00000, 750000, 25,0000 and 5000 I.U) each in 5 mL phosphate buffered saline (PBS) was added and mixed properly. These suspension were used as stock from which appropriate dilutions were made to observe the thrombolytic activity and used as a standard and compared the activity of indigenously produced streptokinase.

Procedure: Venous blood drawn from healthy volunteers (n = 38) was transferred in different sterile microcentrifuge tube (500 µL) and incubated at 37 ºC for 1 h. After clot formation,serum was completely removed and each tube having clot was again weighed to determine the clot weighed (clot weight = weight of clot containing = tube-weight of tube alone). Each tube properly labeled and 200 µL of streptokinase along with various dilutions was added to the tubes. Water was also added to one of the tubes containing clot as a control. All the tubes were then incubated at 37 ºC for 90 min and observed for clot lysis. After incubation fluid obtained was removed and tubes were again weighed to observe difference in weight after clot disruption. Difference obtained in weight taken before and after clot lysis was expressed as percentage of clot lysis. Test was repeated three times with all the dilutions of the streptokinase in blood samples of different healthy volunteers.

Streptokinase activity: One unit of streptokinase was defined as the amount of the enzyme that would liquefy a standard clot of fibrinogen at pH 7.4 at 37 ºC in 90 min.

Estimations of protein contents: For determination of protein concentration the absorbance of the solution was measured at 280 nm by spectrophotometer through a 1 cm light path[10] then multiplied the obtained value by extinction coefficient of streptokinase[4,9,10,11] because the extinction coefficient gives a fairly accurate measure of protein concentration.

Purification of streptokinase: Enzyme extract was purified by a combination of ammonium sulphate precipitation, ion exchange and gel filtration column chromatography. Gel filtration[9] was also used to determine molecular mass of protein using standard markers[12,17]. After each step of purification activity of enzyme was determined.

Electrophoresis: Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE; 10 %) of different streptokinase preparations was done as described by Reza *et al.*[12] and Hermentin *et al.*[17] to analyze the purity and homogeneity of the enzyme[18].

Effect of inhibitors on streptokinase activity: Purified enzyme was dissolved in phosphate buffer soloution (pH 7.4) and mixed with each salt solution to give a final inhibitor concentration of 5 mM. Enzyme samples were separately incubated at 37 ºC for 10 min with each of the following inhibitors; 2,4-dinitrophenol (DNP), EDTA, phenyl-methyl sulphonyl fluoride (PMSF), ∈-amino caproic acid (∈-ACA).

Effect of substrate concentration: Streptokinase was assayed in phosphate buffer of pH 7.4, with variable amounts (0.48-1.19 µg mL^{-1}) of fibrinogen solution. The data were plotted according to Lineweaver-Burk[19].

Activation energy (Ea): Streptokinase dissolved in phosphate buffer (pH 7.4) was incubated at varying temperatures (20-70 ºC) of reaction system[20] to determine the maximum activity at each temperature. These data were used to calculate the activation energy requirements for lysis of fibrinogen clot.

RESULTS AND DISCUSSION

Production and purification of streptokinase: *Streptococcus equisimilis* was used for the production of streptokinase being non pathogenic and less fastidious in its growth requirements[3]. In present study *Streptococcus equisimilis* proved good source to obtain a better yield of streptokinase by utilizing abundantly available agriculture waste corn steep liquor (CSL) because it is cost effective medium for fermentation and rich source of vitamins, minerals and carbohydrates. In crude filtrate the streptokinase activity was 1200 U mL^{-1} as presented in Table-1, which was 176.2 fold higher than the results obtained[16] by using wild strain of *Streptococcus equisimilis*. These results were also 226.4 and 278.4 fold greater than the results reported by using continuous batch culture[21,22] and with same culture medium by using *Streptococcus equisimilis* strain[23] obtained increase in yield of streptokinase with *Streptococcus equisimilis* by using corn steep liquor.

Crude streptokinase was purified gradually by the combination of ammonium sulphate precipitation and ion exchange

TABLE-1
PURIFICATION SUMMARY OF STREPTOKINASE

Purification stage	Activity (U/mL)	Protein (mg/mL)	Sp. act (U/mg)	Purification (fold)
Crude	1200	30.0	40.00	1.0
$(NH_4)_2SO_4$ (desalted)	1100	6.4	171.15	4.2
Ion exchange Chromatography	1050	4.5	233.30	5.8
Gel filtration chromatography	1000	1.8	555.56	13.9

chromatography. The gel filtration profile of sample after ion exchange chromatography showed that enzyme was purified to homogeneity level after three separation steps with 13.9 fold increase in specific activity with 7.2 % recovery of protein (Table-1) and enzyme activity was obtained 120.48 fold higher than the activity[16] by using the improved strain of *Streptococcus equisimilis*, mutagenized by N-methyl-N-nitro-N-nitroso guanidine (NTG)[24] reported the 100-150 IU mL^{-1} activity of partially purified streptokinase secreted by *Streptococcus equisimilis* strain during fermentation process[25] from purified varidase streptokinase and obtained two to three fold increase in specific activity with 40-50 % ammonium sulphate precipitation technique while purified the commercially available crude streptokinase obtained 5-6 fold increase in purity after gel filtration chromatography[10] observed that final specific activity increased 11-fold after the purification of streptokinase by gel filtration chromatography.

Although streptokinase was discovered more than 65 years ago relatively very little has been published on the preparation of highly purified material.

Native streptokinase is useful for cost effective thrombolytic therapy and large quantities of streptokinase could be produced inexpensively *via* bacterial fermentation, but unfortunately limited literature is available about the production of streptokinase by fermentation biotechnology.

Cloning of streptokinase now-a-days extensively used for the production of recombinant streptokinase[12,19,26] but it is very expensive, tedious, time consuming process and also much expertise and skill is needed to complete the cloning process.

In present studies, it has been proved that fermentation biotechnology is the best way to produce clinically important fibrinolytic enzyme streptokinase in large quantity in under developed countries like Pakistan where large agro industrial waste are available. In order to increase the activity of streptokinase different procedures might be used for improving the strain potential, like mutagenesis and further purification of bacterial strain from different sources.

Molecular mass of streptokinase: SDS-PAGE of streptokinase (Fig. 1) showed one protein band with molecular mass of 47 KDa. The molecular mass value of enzyme was within the range[4,12,17].

Effect of inhibitors on streptokinase activity: Presence of certain substances which inhibit the enzyme activity are called enzyme inhibitor. When streptokinase was incubated with 5 mM conc. of phenyl-methyl sulphonyl fluoride, enzyme activity was completely inhibited, similar results were reported by Kim *et al.*[27] with the purified fibrinolytic enzyme produced from *Bacillus* sp. strain CK 11-4 screened from chungkookjang, with 1 mM phenyl-methyl sulphonyl fluoride (PMSF), activity of while EDTA, ∈ amino caproic acid, 2,4-dinitro phenol at conc. of 5 mM, also drastically decreased enzyme activity as presented in Table-2.

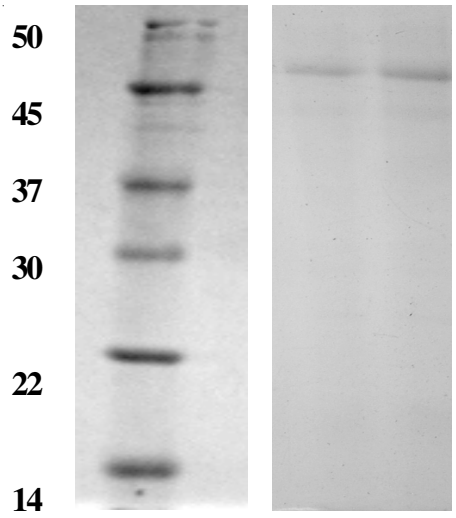

Fig. 1. SDS, PAGE after ion exchange and gel filtration chromatography

TABLE-2
EFFECT OF INHIBITORS (USED AT 5 mM) ON ENZYME ACTIVITY AT 37 ºC

Inhibitors	5 mM
DNP	20 ± 3
EDTA	60 ± 1
PMSF	0 ± 0
∈-ACA	60 ± 2

Each value is a mean three readings ± stands for standard deviation among replicates.

K_m and V_{max} of streptokinase: The lineweaver Burk plot (Fig. 2) of the data revealed a K_m (1.93 µg mL^{-1}) and (V_{max} 566.45 IU mL^{-1}). In literature no such data are available about streptokinase for comparison.

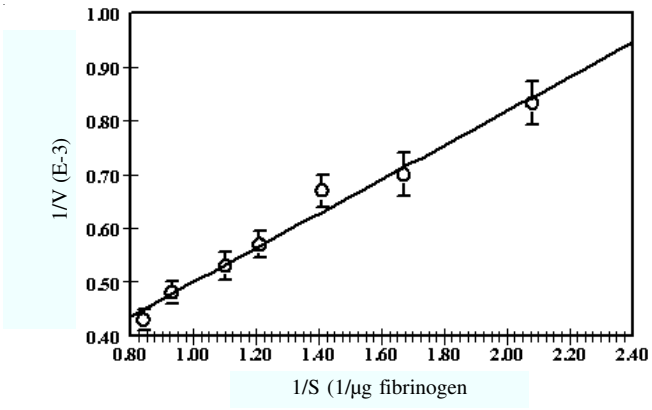

Fig. 2. Lineweaver burk plot for calculation of K_m and V_{max} of streptokinase. Each point is the mean of three independent readings

Activation and deactivation energy: The activation energy required for catalysis of fibrin was 28.2 KJ mol^{-1} while for deactivation phase enzyme showed low activation energy demand (27.3 KJ mol^{-1}) for unfolding, as presented in Fig. 3.

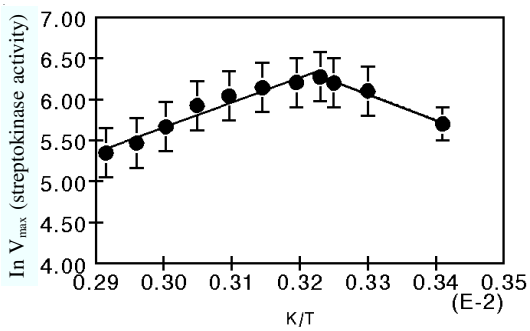

Fig. 3. Streptokinase, dissolved in phosphate buffer (pH 7.4), was incubated at varying temperature (20-70 ºC) of reaction system to determine enzyme activity at each temperature. These data were used to calculate the activation energy requirement for lysis of fibrinogen clot using Arrhenius plots. Each value is the mean of three readings. Standard deviation among replicates was too small to visible

Conclusion

The most striking findings of this study was the production and purification of native streptokinase by fermentation biotechnology. It could be the proper option to make it economically feasible for the poor heart patients in under developed countries like Pakistan and determine its kinetic parameters because accurate reporting of the characterization of streptokinase preparations is essential for safe and effective therapy.

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