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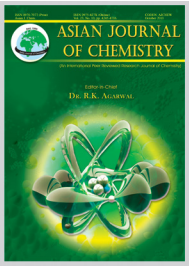

Streptokinase as Fibrinolytic Agent

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Accumulation of fibrin in the blood vessels usually results in the thrombosis, leading to myocardial infarction and other cardiovascular diseases. For thrombolytic therapy microbial fibrinolytic enzymes have now attracted much more attention than typical thrombolytic agents because of the high cost and undesirable side effect. In this study one of the most important fibrinolytic agents streptokinase was selected. The β-hemolytic *streptococci* isolated from different samples of blood and biomass from infected throat of tonsilitis patients, 50 isolates exhibited streptokinase activity. S-8, identified a new variant of *Streptococcus equisimilis* was found to be excellent streptokinase producer in liquid state fermentation biotechnology. This indigenously produced streptokinase showed 1200 U mL^{-1} activity, 415 U mg^{-1} specific activity, with 16.6 fold purification. The purified streptokinase has molecular mass 47 KDa and its apparent K_m and V_{max} values for fibrinlysis were 1.8 µmol and 5370 IU/mL, respectively. The optimum temperature and pH for catalytic activity were 37 ºC and 7.4. The activation energy requirement for the formation of ES complex was 30.2 KJ/mol. Enthalpy (ΔH*), entropy (ΔS*) and Gibb's free energy demand for streptokinase inactivation were 27.3, -91.6 and -22.8 KJ mol^{-1}. Gibb's free energy (ΔG*) demand for substrate binding and transition state stabilization were also determined.

Key Words: Streptokinase, *Streptococcus equisimilis*, Fermentation biotechnology, Plasminogen, Fibrinogen.

INTRODUCTION

According to the report of World Health Organization (WHO) 17 million people die of cardiovascular diseases every year. Intravascular thrombosis, the formation of blood clot in blood vessels is one of the main causes of variety of cardiovascular diseases[1]. 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 thrombolytic agents[2-5].

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[6,7] 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 referred 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[5].

The increasing potential of streptokinase application prompted us to screen for newer streptokinase producing organisms[8]. Also the exponential increase in the application of streptokinase in various fields in the last few decades demands extension in both qualitative improvement and quantitative enhancement. In the present work an attempt was made to isolate streptokinase producing bacteria and medium optimization for overproduction of the enzyme by using liquid state fermentation biotechnology.

The purpose of this study was 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. Determination of kinetic properties of streptokinase is the novelty of this project because no such data are available in literature.

EXPERIMENTAL

Isolation of *streptococci*

Collection of samples: A total of 6 samples collected in sterilized containers for the systematic screening of β-hemolytic *streptococci*. The samples included blood, pus and biomass from infected throat (Table-1).

TABLE-1
SOURCES OF SAMPLES (DISTRIBUTION OF STREPTOCOCCI IN DIFFERENT SAMPLES)

S. No.	Sample No.	Source of culture	Disease	No. of isolates
1	HB1	Human blood	Acute tonsillitis	6
2	HT1	Human throat	Acute tonsillitis	14
3	HB2	Human blood	Scarlet fever	12
4	HT2	Human throat	Scarlet fever	18
5	HB3	Human blood	Acute tonsillitis + scarlet fever	10
6	HT3	Human throat	Acute tonsillitis + scarlet fever	10
			Total	60

Bacterial isolation and identification: Each sample was cultured separately on sodium azide blood agar containing 5 % defibrinted sheep blood and 0.2 % sodium azide[8] and incubated at 37 ºC for 24 h under anaerobic conditions in petridishes. The discrete colonies with clear hydrolysis zones were picked up, transferred into blood agar slants and incubate at 37 ºC for 24 h. Pure growth of typical mucoid beta hemolytic colonies were analysed through Grams' staining under microscope and were confirmed for streptococcus genus through catalase test. Mucoid β-hemolytic and catalase negative colonies containing gram positive cocci were declared as pure *streptococci* and was further processed through API Strep 20 system (Bio Meriax®, France) for the conformation of species.

Biochemical tests including Voges Proskauer test, Hipurate test, Esculine test, pyrolidonly test, α galactosidase test, alkaline phosphatase test, leucine amino peptidase test, arginin dihydrolase test, leucine aminopeptidase test and fermentation of sugars including D- ribose, L-arabinose, D-manitol, D- sorbitol, D-lactose, D-trehalose, inuline, D-raffinose starch and glycogen were performed to study the morphological and taxonomic characteristics of isolated strain and later Strepto Trac system were used to confirm the streptococcal species and also compared with standard H46A (ATCC 12449) of *Streptococcus equisimilis*. The selected isolates were with subjected to for extracellular streptokinase production by liquid state fermentation.

Inoculum preparation: 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 to achieve maximal yield of streptokinase under available local conditions[8,9]. The selected strain was used for growth in liquid state fermentation in order to analyze the streptokinase activity.

Medium composition: 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. Then it was filtered and the clear filtrate was used as the crude enzyme source.

Assay of streptokinase: Activity of streptokinase was determined by reported method[1] with slight modification. Plasminogen activation of streptokinase was assayed using synthetic substrate S-2251. It is chromogenic substrate for the streptokinase-activated plasminogen. The method for the determination of activity is based on the difference in the absorbance between the ρNA formed and the original substrate. The rate of ρNA formation is measured at 405 nm and the value obtained is directly proportional to plasmin activity which reflects the amount of functional plasminogen originally present in the sample. In total volume 131.5 µL containing 1.5 µL of 50 mM *Tris*-HCl (pH 7.4) and 30 µL of plasminogen (0.2 mg mL^{-1}) the reaction was initated by adding 15 µL clear extract sample, incubated for 15 min at 37 ºC and added 75 µL of S-2251 substrate (5 mg mL^{-1}), again incubated for further 10 min at 37 ºC. This reaction was stopped by adding 10 µL of acetic acid (0.4 N) and monitored at 405 nm.

Streptokinase activity: One unit was defined as the amount of enzyme activity that converts 1 µmol of substrate per minute per liter.

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[5,10-12] 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 as described by Reza *et al.*[10]. Gel filtration was also used to determine molecular mass of protein using standard markers[1,13,14]. 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 to analyse the purity and homogeneity of the enzyme[1,13,15].

Effect of inhibitors on streptokinase activity: Purified enzyme was dissolved in phosphate buffer solution (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 sulfonyl 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's plot.

Thermostability: Purified streptokinase was redissolved in 100 mM phosphate buffer (pH = 7.4) and assayed for thermostability. Optimum temperature of streptokinase was determined in 100 mM phosphate buffer (pH = 7.4) at varying temperatures (20, 25, 30, 35, 37, 40, 45, 50, 55, 60, 65 and 70 ºC) of reactive system streptokinase was also examined by incubating the enzyme in the above buffer, at certain temperature (30-50 ºC) and the enzyme samples were taken after (0, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110 and 120) min of incubation for the assay of enzyme activity.

Activation energy (Ea): Streptokinase dissolved in 100 mM phosphate buffer (pH 7.4) was incubated at varying temperatures (20-80 ºC) of reaction system to determine maximum activity at each temperature. These data were used to calculate the activation energy requirement for fibrinlysis clot[16].

Thermodynamics of enzyme: Thermal inactivation of enzyme was determined by incubating the enzyme solution in above phosphate buffer at a particular temperature. Aliquots were withdrawn at different times, cooled than assayed for enzyme activity at 37 ºC as described above. This procedure was repeated at different temperatures ranging from 35-60 ºC. The data were fitted to first order plots and analyzed. The first order rate constants (K_d) were determined by linear regression of ln (V) *versus* time of incubation (t). The thermodynamic data were calculated by rearranging the Eyring absolute rate equation to study the overall thermodynamic parameters in the range 30-60 ºC.

$$K_d = T\frac{k_B}{he^{\Delta S^*/R}e^{\Delta H^*/RT}} \quad (1)$$

$$\ln\left(\frac{K_d}{T}\right) = \ln\left(\frac{K_B}{h}\right) + \frac{\Delta S^*}{R} - \frac{\Delta H^*}{R}\frac{1}{T} \quad (2)$$

ΔG* (activation Gibbs free energy of fibrinogen lysis)

$$\Delta G^* = -RT\ln\frac{(K_{cat}h)}{(K_B T)} \quad (3)$$

ΔH* (activation enthalapy of fibrinogen lysis)

$$\Delta H^* = Ea - RT \quad (4)$$

ΔS* (activation entropy of fibrinogen lysis)

$$\Delta S^* = \frac{(\Delta H^* - \Delta G^*)}{T} \quad (5)$$

$\Delta G^*_{E\text{-}T}$ (Gibbs free energy of transition state binding)

$$\Delta G^*_{E-T} = -RT\ln\frac{k_{cat}}{K_m} \quad (6)$$

$\Delta G^*_{E\text{-}S}$ (Gibbs free energy of substrate binding)

$$\Delta G^*_{E-T} = -RT\ln k_a$$

$$K_a = 1/K_m.$$

Where K_d, T, K_B, h, ΔS*, ΔH* and R are specific reaction velocity, absolute temperature, Boltzman constant, Plank's constant, entropy of activation, enthalpy of activation and ideal gas constant, respectively.

RESULTS AND DISCUSSION

About 60 *streptococci* (with hemolytic activity) samples were isolated from the above 6 samples.The distribution of *streptococci* in different samples is shown in Table-1. The isolates were pooled together and cultures, which appeared identical to the naked eye, were eliminated. All the isolates were tested or their β-hemolytic activity on PNF selective medium. About 50-β hemolytic positive isolates were obtained. Among these 30 isolates were selected for study. Among the 30 active isolates, isolate S-8 gave highest streptokinase activity (1500 U mL^{-1}). The isolate (S-8) as such, was subsequently selected study of different charactristics of the pure isolated strain and also used in the production of streptokinase by liquid state fermentation biotechnology. The characteristics of our isolate S-8 were compared with that of the reference cultures. The refrence culture used are: H46A (ATCC 12449) a group C *streptococci*.

Cultural used: Characteristics, physiological and biochemical properties, sugar fermentation, growth in different media and resistance to various inhibitors are shown in Table-2.

Api Strepto Trac was also used to confirm the strain, that is *Streptococcus equisimilis* (API # 0462415). The detailed survey of the literature indicated that our isolate S-8 belongs to *streptococci* group is close to standard *Streptococcus equisimilis*.

Production and purification of streptokinase: *Streptococcus equisimilis* was used for the production of streptokinase being non pathogenic and less fastidious in its growth requirements[4]. In present study *Streptococcus equisimilis* proved good source to obtain a better yield of streptokinase by utilizing abundantly available agricultural waste corn steep liquor because it is cost effective medium for fermentation and rich source of vitamins, minerals and carbohydrates. In crude filtrate the streptokinase activity was 1500 U mL^{-1} as presented in Table-3, which was 176.2 fold higher than the results obtained by using wild strain of *Streptococcus equisimilis*[8] and 226.4 fold higher than the activity obtained by using continuous batch culture[17]. These results showed 278.4 fold higher activity as compared[18,19] by using the same culture medium to yield the streptokinase from *Streptococcus equisimilis*.

Crude streptokinase was purified gradually by the combination of ammonium sulphate precipitation and ion exchange chromatography. The gel filtration profile of sample after ion exchange chromatography showed that enzyme was purified to homogeneity level after three separation steps with 16.6 fold increase in specific activity with 80 % recovery of protein (Table-3) and enzyme activity was obtained 120.48 fold higher than the activity obtained by using the improved strain of *Streptococcus equisimilis*, mutagenized by N-methyl-N-nitro-N-nitroso guanidine. Abdelghani *et al.*[8] and Pimienta *et al.*[20] reported the 100-150 IU mL^{-1} activity of partially purified streptokinase secreted by *Streptococcus equisimilis* strain during fermentation process[21] purified varidase streptokinase and obtained two to three fold increase in specific activity with 40-50 % ammonium sulphate precipitation technique while De Renzo *et al.*[10] purified the commercially available crude streptokinase and obtained 5-6 fold increase in purity after gel filtration chromatography. Moreover, as previous workers observed that final specific activity of streptokinase increased

TABLE-2
MORPHOLOGICAL, METABOLIC, BIOCHEMICAL AND SUGAR FERMENTATION TESTS OF THE ISOLATE S-8 AND REFERENCE CULTURES

Morphological and metabolic tests	S-8	H46A	Sugar fermentation test	S-8	H46A
Haemolysis	+	+	Inulin	–	–
PNF test	+	+	Lactose	–	–
Shape of the cells	S	S	Manitol	–	–
Endospores	–	–	Raffinose	–	–
Motility	–	–	Ribose	+	+
Gram-staining	+	+	Salicin	+	+
Heat tolerance at 60 ºC for 0.5 h	–	–	Sorbitol	–	–
Growth in media containing			Trehalose	+	+
Methylene blue (0.1 % in milk)	–	–	Cellobiose	+	+
Sodium chloride (6.5 %)	–	–	Melibiose	–	–
Bile salt (40 %)	–	–	Glycerol	+	+
Potassium tellurite	+	+	Arabinose	–	–
Growth in			Inositol	–	–
Sodium azide medium	M	M	Galactose	+	+
Crystal violet blood agar	M	M	Erythritol	–	–
Litmus milk reduction test	–	–	Dulcitol	–	–
cAMP test	–	–	Dextrin	–	–
			Adonitol	–	–
Biochemical tests:			Rhamnose	–	–
Hydrolysis of			Soluble starch	–	–
Gelatin	–	–	Potato starch	–	–
Starch	+	+	Fructose	+	+
Hippurate	–	–	Xylose	+	+
Esculin	–	±	Mannose	+	+
Arginine	+	+	Sucrose	+	+
Pyruvate fermentation	–	–	Maltose	+	+
Voges-Proskauer	–	–	Glucose	+	+
Methyl red	+	+	Final pH in glucose broth	4.8	4.9
Hydrogen sulphide	–	–			
Catalase	–	–			
Nitrate reduction	+	+			
Lancefield grouping	C	C			

M: Moderate growth, S: Spherical, +: Positive reaction, –: Negative reaction

11-fold after the purification of streptokinase by gel filtration chromatography[5,12].

Although streptokinase was discovered more than 65 years ago, yet 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 very 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[1,14] but it is very expensive, tedious, time consuming process and also much expertise and skill is needed to complete the cloning process.

In our 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 is available abundantly. 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 of 45-50 KDa[1,22,23].

Effect of inhibitors on streptokinase activity: Presences of certain substances which inhibit the enzyme activity are called enzyme inhibitor. When streptokinase was incubated with 5 mM conc. of PMSF, enzyme activity was completely inhibited similar results were reported by Kim *et al.*[24] by incubating the purified fibrinolytic enzyme produced from *Bacillus* sp. strain CK 11-4 screened from chungkook-jang, with 1 mM phenyl-methyl sulfonyl fluoride (PMSF). While EDTA, ∈ amino caproic acid (∈ ACA), 2,4 dinitrophenol (DNP) at concentration of 5 mM, also drastically decreased enzyme activity as presented in Table-4.

TABLE-3
PURIFICATION SUMMARY OF STREPTOKINASE PRODUCED BY *Streptococcus equisimilis*

Purification stage	Activity (U mL^{-1})	Protein contents (mg mL^{-1})	Specific activity (U mg^{-1})	Fold purification
Crude	1500	60	25	1.0
$(NH_4)_2SO_4$ desalted	1450	16	90.6	3.6
DEAE-Cellulose	1300	8.0	162.5	6.5
Sephadex G-150	1200	2.89	415	16.6

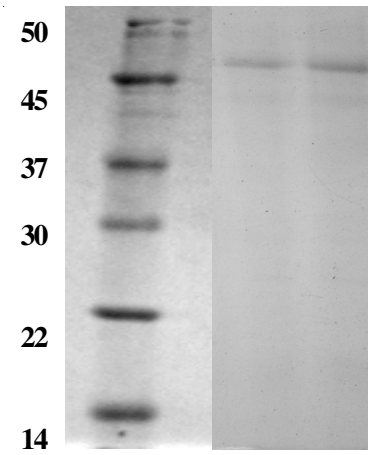

Fig. 1. SDS, PAGE after ion exchange and gel filtration chromatography

TABLE-4
EFFECT OF INHIBITORS (USED AT 5 mM)
ON ENZYME ACTIVITY AT 37 ºC

Inhibitors	Enzyme activity (IU mL^{-1})
DNP	25 ± 3
EDTA	50 ± 1
PMSF	0 ± 0
∈-ACA	40 ± 2

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

Effect of streptokinase concentration on catalytic activity: The reaction was dependent on the amount of streptokinase present in reaction mixture. The apparent K_m (1.7 µmol) for the substrate and V_{max} (5370 IU mL^{-1}) Table-5. Avilan *et al.*[25] reported the K_m of genetically engineered streptokinase about 0.70 µmol. No other literature is available about the K_m and V_{max} of the streptokinase.

Effect of temperature on streptokinase activity and stability: Stability of the enzyme at higher temperature up to 70 ºC was examine, the enzyme optimally active at 35-40 ºC (1000 IU/ mL). The enzyme showed thermostable properties because even at 70 ºC, 30 % activity was retained. In literature no such data are available for the comparison of indigenously produced streptokinase.

Thermostability: The protein mid point inactivation temperature activation energy conformational stability at elevated temperature, activation parameters for catalytic activity, transition state formation energy, stability of the native state ensemble are potential indices for thermostable biocatalysts. The temperature was 65 ºC (Fig. 2 and Table-5) and for streptokinase no such information could be found in literature.

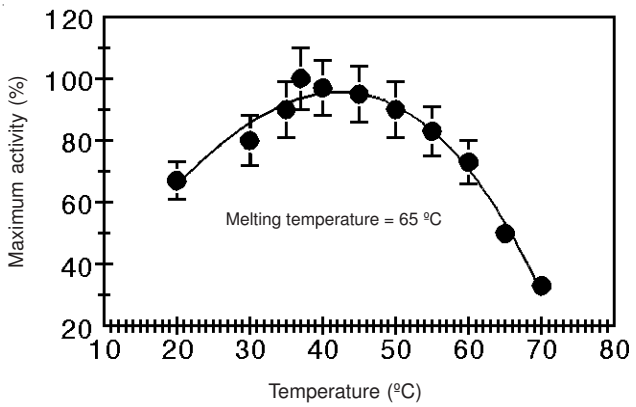

Fig. 2. Melting temperature of purified streptokinase

Enzyme displayed thermal stability at 30 ºC with half life 231 min while at 70 ºC, it was less stable and displayed a half life of 40.35 min under similar conditions (Table-6). Detailed elucidation of the mechanisms responsible for stabilization and destabilization of enzyme especially at elevated temperature is of special importance from both a scientific and commercial point of view[16].

Kinetics of fibrin lysis: The specificity constant of the enzyme was 1032.0 S^{-1} mM^{-1} shown in Table-5 and was significantly higher as compared with those of thermostable enzyme of other class[26] signifying that the high catalytic efficiency of streptokinase is due to transition state stabilization (Table-5).

This high affinity and specificity in addition to its long term stability in pH range 6-8 makes the enzyme from *Streptococcus equisimilis* a suitable biocatalyst for clinical application.

Thermodynamic of fibrin lysis: The *Streptococcus equisimilis* produced enzyme required lower Gibb's free

TABLE-5
KINETIC AND THERODYNAMIC PROPERTIES OF STREPTOKINASE FROM
Streptococcus equimillus AS COMPARED TO THOSE GIVEN BY OTHER WORKERS

Parameters	This work	Maximum value	Reference
K_m (µmol)	1.7	5.26	1
V_{max}	5370	n.a.	n.a.
V_{max}/K_m	1032.0	n.a.	n.a.
E_a (kJ mol^{-1})	28.2	53.0	n.a.
pH optimum	7.4	6.0-8.0	n.a.
Temperature optimum (ºC)	37	88	n.a.
ΔG^*(kJ mol^{-1})[a]	56.3	103	n.a.
ΔH^* (kJ mol^{-1})[b]	27.3	50.5	n.a.
ΔS^* (J mol^{-1} K^{-1})[c]	-91.6	-150	n.a.
$\Delta G^*_{E\text{-}T}$ (kJ mol^{-1})[d]	-22.8	-22.9	n.a.
$\Delta S^*_{E\text{-}S}$ (kJ mol^{-1})[e]	1.37	2.4	n.a.

n.a. = Not available for streptokinase information on a, b, c, d, e on sterptokinase are not available in literature and have been compared with those for thermostable or themostabilized enzymes in lierature. 1. Avilan et al., 1997, [c]ΔG^* (activation free energy of fibrin hydrolysis) = -RT. In $(V_{max}/K_m\, h)/(K_B\, T)]$, where h = planck constant (6.63×10^{-34} Js), K_B = Boltzman constant (1.38×10^{-23} J K^{-1}) and R= Gas constant (8.314 JK^{-1} mol^{-1}). [f]ΔH^* (activation enthalpy of clot hydrolysis) = E_a-RT, [g]ΔS^* (activation entropy of clot hydrolysis) = $(\Delta H^* - \Delta G^*)/T$, [h]$\Delta G^*_{E\text{-}T}$ (free energy of transition state binding = -RT ln V_{max}/K_m, [i]$\Delta G^*_{E\text{-}S}$ (free energy of substrate binding) = -RT In K_a, where $K_a = 1/K_m$.

TABLE-6
KINETIC AND THERMODYNAMIC PARAMETERS FOR IRREVERSIBLE THERMAL INACTIVATION OF STREPTOKINASE

Temperature (K)	K_d (min^{-1}.)	$t_{1/2}$ (min)	ΔH^* (kJ mol^{-1})	ΔG^* (kJ mol^{-1})	ΔS^* (J mol^{-1} K^{-1})
303	3.0	231	40.68	99.20	-193.14
333	16.0	43	40.43	104.64	-192.82
343	22.0	32	40.35	106.96	-194.20

energy ($\Delta G^*_{E\text{-}T}$) to form transition state than that required by thermostabilized enzyme by microorganisms[27] no literature is available according these data. Similarly, enzyme released the higher amount of transition state binding energy ($\Delta G^*_{E\text{-}S}$), as compared with others[16] signifying that the high catalytic efficiency of streptokinase is due to transition state stabilization. This enzyme showed highest enzyme substrate stabilization ($\Delta G^*_{E\text{-}S}$) whereas other clinically important enzymes belong to different class showed least enzyme substrate stabilization energy, respectively Table-5[27].

Thermodynamics of irreversible thermal inactivation: Enthalapy of denaturation (ΔH^*) of the *Streptococcus equisimilis* derived streptokinase was obtained to be 40.68 KJ/mol at 30 ºC. It was showed that free energy of thermal denaturation observed to be in the range of 99.20-106 KJ mol^{-1} at 20-70 ºC (Fig. 3). When entropy of inactivation (ΔS^*) was calculated at each temperature, it gave negative values, indicating that enzyme is thermodynamically stable. streptokinase showed ΔS^* values of 193.14 J mol^{-1} K^{-1} and -194.20 J/mol/K, respectively at 20 and 70 ºC. The results are arranged in Table-6.

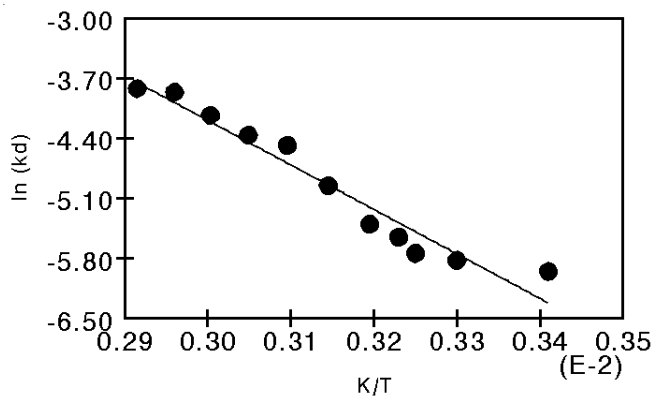

Fig. 3. Arrhenius plot for activation energy of thermal denaturation of streptokinase

Streptokinase isolated from *Streptococcus equisimilis* strain is thermally stable and could be used for clinical application. The negative entropy of deactivation observed for streptokinase suggested that there was negligible disorderness. A high value for free energy of thermal denaturation at 70 ºC indicated that the streptokinase exhibited the resistance against thermal unfolding at higher temperature.

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