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Journal of Chromatography B, 861 (2008) 218–226
JOURNAL OF CHROMATOGRAPHY B
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Renaturation, purification and characterization of streptokinase expressed as inclusion body in recombinant *E. coli*☆

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Received 16 March 2007; accepted 7 October 2007
Available online 13 October 2007

Abstract

Recombinant protein purification is facilitated using high expression systems which produce larger quantities of streptokinase protein as inclusion bodies. As the accumulation of active streptokinase is toxic to the host cells, we have optimized the conditions to achieve large amounts of streptokinase in the form of inclusion bodies. The solubility and yield of pure protein are highly dependent on various combinations of chemical additives, ionic and non-ionic detergents and salts, with solubilizing agents followed by refolding of denatured protein into active form. As the extraction of the purified streptokinase from inclusion bodies requires denaturation and a subsequent refolding step, careful balancing steps were needed to develop under different controlled conditions. Here the purified fragments of refolded proteins were screened to select the conditions that yield the active streptokinase having native conformation. The maximum specific activity of the purified streptokinase was achieved by these methods. The refolded recombinant streptokinase was analyzed by RP-HPLC showing a purity of 99%. Size exclusion chromatography profile shows that there are minimal aggregates in the active streptokinase protein and the percentage of renaturation is around 99%.

Keywords: Recombinant streptokinase (SK); *Escherichia coli* fermentation; Inclusion body isolation; Solubilization; Protein purification; Physiochemical characterization

1. Introduction

The culture filtrates of strains of beta hemolytic *streptococci* are able to cause rapid lysis of the fibrin clot. The active agent is a *streptococcal* fibrinolysin, called streptokinase, and the reaction has been termed as fibrinolysis [1]. The enzyme streptokinase has been most frequently associated with hemolytic streptococci of the Lancefield group A, human C, and G, with the C group being preferred. In particular, the strain H46A (identified by the American Type Culture Collection, Rockville, Md. As No. 12449, Rebecca C. Lancefield strain H46A, 1956) is the most frequently employed strain [2]. Native streptokinase is a single chain polypeptide with a molecular weight of 47 kDa. The protein consists of 415 amino acid residues in a single polypeptide chain. Streptokinase contains no cysteine or cystine amino acids, which shows that there is no disulphide bond formation. There are 69 carboxyl side chains, 61 basic side chains and 170 (41%) hydrophobic residues [3].

Streptokinase (SK) is a specific effective medicine for thrombolytic therapy of acute myocardial infarction. Clinical use of streptokinase worldwide includes the treatment of acute myocardial infarction, Deep Vein thrombosis, arterial thrombosis and embolism and acute retinal vein occlusion [4–7]. Streptokinase is an extracellular protein produced by haemolytic *streptococcus* group C with the ability to activate human plasminogen to form plasmin. In 1933, Tillet and Garner observed that the culture filtrates of several strains of beta haemolytic *streptococci* caused the lysis of blood clots [1]. The term streptokinase was then coined by Christensen [8] to describe the bacterial extract and the terms profibrinolysin and fibrinolysin to describe the inactive (proenzyme) and active (enzyme) forms of the plasma protein. Highly purified SK has since then been an important

Abbreviations: SK, streptokinase; HCDM, high cell density medium; VVM, volume of air/volume of medium/minute; PCV, packed cell volume; DO_2, dissolved oxygen; OD, optical density; deg. C, degree centigrade; RP-HPLC, reverse phase high pressure liquid chromatography; IB, inclusion bodies; MALDI-TOF, matrix assisted laser desorption-ionization and time of flight.

☆ This paper was presented at the Biochromatography and Nanotechnologies Conference, Vellore, Tamil Nadu, India, 12–15 February 2007.

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doi:10.1016/j.jchromb.2007.10.008

tool in fibrinolytic therapy. SK is free from cysteine, phosphorus, carbohydrates and lipids. It is now well established that the fibrinolytic activity of SK originates from its ability to activate plasma plasminogen [9].

The low yield obtained in normal SK production and the pathogenicity of its natural host were the principal reasons leading to the search for a recombinant source. Also its toxicity to the host cell is well known [10,22]. This further led to the cloning and high level expression of streptokinase in *E. coli*. In 1984, streptokinase gene from *Streptococcus equisimilis* H46A was first cloned and expressed in *E. coli* [11]. Subsequently streptokinase fragment from the genome of *S. equisimilis* H46A encoding the SK gene was sequenced [12]. The SK gene was then cloned in several Gram-negative and Gram-positive bacteria [13–17]. Several methods of recovery and purification of SK have been described previously which includes purification of wild strain as well as recombinant SK [15,16,19–22]. The recombinant target protein in general represents the major fraction of the inclusion body. Therefore refolding attempts can be undertaken directly after solubilization of the inclusion bodies [23]. Several proteins synthesized by recombinant means are yet to be used for therapeutic applications due to inability to dissolve, renature and obtain the fully functional protein. The present investigation was initiated with a view to study different aspects of refolding of an expressed recombinant streptokinase protein and characterization of the active streptokinase protein. In the present study, recombinant streptokinase inclusion bodies were solubilized and active recombinant streptokinase protein was obtained by the method of rapid dilution. The refolded protein was purified by a single step ion-exchange chromatography and characterized by different physicochemical methods.

2. Materials and methods

2.1. Chromatographic conditions

Ion-exchange chromatography: The matrix from Tosoh Co., Japan (Toyopearl DEAE 650M). The gel volume used was 50 ml. The work was carried out using pyrogen free water. The protein applied was 30–40 mg protein/ml gel proportion. The temperature used for the whole experiment is 25 deg. C. The standard buffer used was 20 mM Tris–HCl of pH 8.0.

Size exclusion chromatography: The column used was Superdex 75 HR 10/300 (Pharmacia, USA), with a column volume of ~24 ml and the void volume was 8.7 ml. The standard buffer used was 20 mM Tris–HCl with 100 mM NaCl, pH 7.0. The size exclusion was carried out at a flow rate of 0.3 ml/min at 25 deg. C. All the samples were filtered through 0.22 μm filter (low binding Durapore membrane, Millipore, USA) immediately prior to analysis.

RP-HPLC: The column used was wide 10 μm pore KROMASIL C-18 of 250 mm × 4.6 mm. The column was run with a mobile phase of 0.1% trifluroacetic acid in water: acetonitrile (0–60%) using a linear gradient. The flow rate was set at 1 ml/min. The column temperature was kept at 25 deg. C. The injection volume of the sample was 20 μl, with a protein load of 200 μg. The peak was compared with the standard by monitoring at 280 nm.

2.2. Cloning

In the present work *E. coli* strain BL21(DE3)RIL was used as a host. Expression vector BBIL-SK (PUC-19, derivative) was used as an expression vector. *S. equisimilis* H46A (ATCC 12449) was used for isolation of genomic DNA. Genomic DNA from *S. equisimilis* was cloned in *E. coli*, using bacteriophase lambda pL-pR promoter. The colonies were plated on Luria Bertani (LB) medium with ampicillin. Positive clones were selected from this plate and used for making working stocks.

2.3. Cultivation conditions

Single cryovial of working culture was inoculated into 10 ml of Luria Bertini (LB) medium with 50 μg/ml ampicillin and grown for 12 h at 30 deg. C on an orbital shaker (Gallenkemp, Sanyo-UK) at 250 rpm. The culture was transferred into two 1 l flasks containing 200 ml of high cell density media (HCDM) and grown at 30 deg. C for 12 h on an orbital shaker at 250 rpm. This culture was used to inoculate 19 l fermenter (Bioengineering, Switzerland) with 10 l of HCDM (Media composition per l: 28 g glucose, 4 g $(NH_4)_2HPO_4$, 13.3 g KH_2PO_4, 1.7 g citric acid, 0.0141 g EDTA, 1.2 g $MgSO_4{\cdot}7H_2O$, 5 mg Ampicillin). Fermentation was carried out in fed-batch mode, using glucose as the carbon source. Inlet air pressure to the fermenter was set at 0.5 VVM (volume of the air/volume of the media/minute). Stirrer speed was set at 300 rpm and temperature was set at 30 deg. C. Dissolved oxygen (DO_2) level was maintained at 100% saturation. After 8 h of cultivation, when the DO_2 level fell to 50% or below, the stirrer speed and air flow were increased in steps of 100 rpm and 0.5 VVM, respectively, up to maximum rpm of 700 and 2.0 VVM. As and when the DO_2 concentration decreases to 0%, addition of 90% glucose was carried out. The cultivation was carried out for 26 h to attain maximum cell density. The culture samples were collected from the fermenter at intervals of every 4 h for monitoring optical density and packed cell volume (PCV). After 26 h of cultivation, the cells were induced by shifting the temperature to 42 deg. C. All other conditions being the same, the temperature was maintained at 42 deg. C for 4 h. At the end of the fermentation period the broth temperature was cooled to 10 deg. C and broth was harvested.

2.4. Pre-solubilization

The culture was centrifuged (Cell Sep, Sanyo, Japan) at 4000 × *g* for 30 min, maintaining the temperature at 4–6 deg. C. The cell pellet was washed with 20 mM Tris-HCl pH 8.0 containing 0.9% sodium chloride solution. The pelleted cells were resuspended in lysis buffer (20 mM Tris, 100 mM EDTA and 0.5% polysorbate-20) and the suspension was diluted with lysis buffer till the optical density (OD_{600}) of the cells reached 100 ± 10 units. The accuracy of the method is the highest when the absorbance reading in spectrophotometer is between 0.1 and

0.5 and to reach this range the cell suspension was serially diluted to give an OD between 0.1 and 0.5, for checking the OD (OD_{600}), using Hitachi (U-2000) spectrophotometer. The cells were incubated in the lysis buffer for 1 h and passed twice through Dynomill (Dynomill–KDL pilot A, Willy A, Switzerland) for cell lysis at a flow rate of 200 ml/min. Cell lysate was subjected to filtration by passing through 0.45 μm filter cassettes (Prostak, 0.45 μm tangential flow filtration unit, Millipore, USA) and clarified filtrate with inclusion bodies were collected. The cassettes were washed thoroughly to ensure that all the inclusion bodies were collected in the filtrate. Micro-filtrate was concentrated and diafiltered extensively with 20 mM Tris–HCl pH 8.0 by passing through 100 kDa cassette (Biomax cassette, Millipore, USA) to obtain a final protein concentration of 5–6 mg/ml.

2.5. Solubilization

A stock solution of protein concentration 5–6 mg/ml was taken for each experiment from five different batches of streptokinase, keeping the protein concentration constant for all the experiments. These stock solutions from five different batches were used for solubilization of inclusion bodies. Solubilization was carried out by addition of urea and guanidine hydrochloride (4 M, 6 M of urea and guanidine hydrochloride). The solubilized samples were stirred for different time intervals (4, 6, 8, 10 and 12 h). All the solubilization experiments were carried out at 4 deg. C.

2.6. Refolding by rapid dilution

The solubilized proteins have been refolded in 20 mM Tris–HCl pH 8.0 with various additives, to obtain the recombinant SK protein of highest activity. The additives used are given in Table 1. The solubilized solution containing the denatured protein was rapidly diluted (1:250 ratio) using 20 mM Tris–HCl, pH 8.0 with or without any of the additives mentioned in Table 1 using a magnetic stirrer.

2.7. Assay method for activity

The rapidly diluted protein samples were checked for streptokinase activity, in International Units (IU) against standard. The assay was performed according to the method of Robert and Thomas [24]. Chromogenic method provided was an end point method, where streptokinase in the presence of S-2251 (H-D-Val-Leu-Lys- paranitro-anilide), converts plasminogen to plasmin. In this method, a given concentration of streptokinase (1030 IU per ampoule of Third International Standard of streptokinase; National Institute of Biological Standards and Controls, UK) results in linear generation of active plasmin and accelerating hydrolysis of S-2251, hence the optical density (OD_{405} (nm)) of the solution increases exponentially. The total amount of plasmin formed (OD_{405} (nm)) is proportional to the streptokinase concentration in International Units. Sample of plasminogen (Sigma, USA) containing 0.5 IU, 50 μl of rapidly diluted protein solution was added and incubated at 37 deg. C. After 10 min of incubation, substrate containing 0.5 mM concentration of H-D-Val-Leu-Lys-paranitro-anilide (Sigma, USA) was added and the incubation was continued for another 10 min. The reaction was stopped by addition of 1% acetic acid solution. The colour development was measured at 405 nm. The activity of protein was calculated using NIBSC (National Institute of Biological Standards and Controls, UK) streptokinase standard. The buffer containing additives exhibiting maximum activity was used for all further experiments.

Table 1
Different additives used in the rapid dilution method for renaturation of SK inclusion bodies

S. No.	Buffer with/without additive
1	20 mM Tris–HCl (8.0)
2	20 mM Tris–HCl (8.0) with 1 M L-arginine
3	20 mM Tris–HCl (8.0) with 1% Triton-X-100 and 10% glycerol
4	20 mM Tris–HCl (8.0) with 10 mM Glycine and 10 mM EDTA
5	20 mM Tris–HCl (8.0) with 100 mM NaCl and 10% glycerol
6	20 mM Tris–HCl (8.0) with 10% SDS and 10% glycerol

2.8. Ion-exchange chromatography

Renatured protein was concentrated and diafiltered against 20 mM Tris–HCl buffer, pH 8.0, using 10 kDa cassette (Biomax cassette, Millipore, USA). The sample was passed through 0.22 μm filter (low binding Durapore membrane, Millipore, USA) and the protein solution was loaded onto column containing anion exchanger (Toyopearl DEAE 650M, Tosoh Co., Japan) with a bed volume of 50 ml and connected to online UV-monitor (K-prime 40, Millipore, USA). The unbound proteins were washed with the loading buffer till the optical density of the flow through was zero. The bound proteins were eluted with a linear gradient of 0–500 mM NaCl in 20 mM Tris–HCl, pH 8.0. Positive streptokinase elutes were collected according to the absorbance of the fractions monitored by the online UV-monitor. The fractions containing eluted protein were assayed for streptokinase activity and subsequently analyzed by SDS-PAGE method.

2.9. Size exclusion chromatography

Refolded recombinant streptokinase protein after ion-exchange chromatography purification was analyzed for percentage of aggregates by using Superdex 75 HR 10/300 column which separates proteins with molecular weight range from 1 to 70 kDa. The size exclusion was connected online to HPLC flow detector (multiwavelength UV–vis detector, Waters 996 Photodiode Array-PDA detector, Waters, Milford, MA). To analyze the percentage of refolded protein from the insoluble protein aggregates, following concentrations of recombinant streptokinase protein stocks were prepared.

(a) Refolded purified streptokinase protein of 1 mg/ml.
(b) Refolded purified streptokinase protein of 2 mg/ml.
(c) Refolded purified streptokinase protein of 20 mg/ml.
(d) Refolded purified streptokinase protein of 60 mg/ml.

The molecular weight markers (ribonuclease, 13.7 kDa; chymotrypsin, 25 kDa; ovalbumin, 43 kDa and bovine serum albumin, 66 kDa) were run to identify the retention time of individual markers. The size exclusion chromatography of standards were carried out in an analytical mode.

2.10. MALDI-TOF

A mass spectrum of SK protein was done by MALDI-TOF [25]. The SK protein was characterized by using 3,5-dimethyl-4-hydroxycinnamicacid (sinapinnic acid) as a matrix on Kratos seqIV TM. The molecular weight of the active SK protein was confirmed by this method.

2.11. RP-HPLC

Final bulk of SK was quantified by HPLC. The sample was analyzed by Reverse Phase HPLC (Shimadzu-VP-7.0, containing control-operating parameters through the SCL-10 AVP system controller. Shimadzu-VP-7.0, Japan). The HPLC system is equipped with a high sensitivity UV detector SPD-10AVVP, with low noise level to $\pm 0.35 \times 10^{-5}$ AU (gradient module of LC-10ATVP for solvent delivery, with a SIL-10ADVP injector, Shimadzu-VP-7.0, Japan). The column was a wide pore (10 μm) KROMASIL C-18 of 250 mm × 4.6 mm. Purity of the material was confirmed by a RP-HPLC run through a wide pore column (KROMASIL C-18). Twenty microliters sample containing 200 μg of protein was injected. Elution was done using a linear gradient (1% min^{-1}) of acetonitrile (0–60%) containing 1% trifluroacetic acid, at a flow rate of 1 ml/min. Absorbance was read at 280 nm and profile was compared with that of standard.

3. Results and discussions

3.1. Solubilization and renaturation

Harvested and resuspended cells in buffer were passed through the Dynomill for cell lysis and the lysed cells were observed spectroscopically at 600 nm. All the five batches showed ~90% cell lysis confirmed by microscopic examination, after passing twice through dynomill with a final OD (600 nm) less than 10 units. The protein profile of uninduced and induced cell lysates were analyzed on 12% gel by SDS-polyacrylamide gel electrophoresis. The expression of a prominent band of recombinant streptokinase was observed in case of induced lysate, which was absent in uninduced cell lysate (Fig. 1). Inclusion bodies were separated form cell lysate after passing through the 0.45 μm filtre cassettes (Prostak, 0.45 μm tangential flow filtration unit, Millipore, USA). The micro filtered inclusion bodies were concentrated using 100 kDa cassette, to a level where the final protein concentration of these inclusion bodies were adjusted to 5–6 mg/ml. On an average of a single batch of 10 L fermentation broth yielded 5 g of inclusion bodies. The inclusion bodies were solubilized with different concentrations of urea and guanidine hydrochloride and renaturation of the protein was attempted using 20 mM Tris–HCl, pH 8.0 containing different additives (Table 1). After renaturation, SK activity was analyzed by carrying out the assay (Section 2.7). The protein solution was incubated with plasminogen and the released plasmin was monitored by addition of S-2251 and indication of absorption at 405 nm. The results obtained are as given in Table 2.

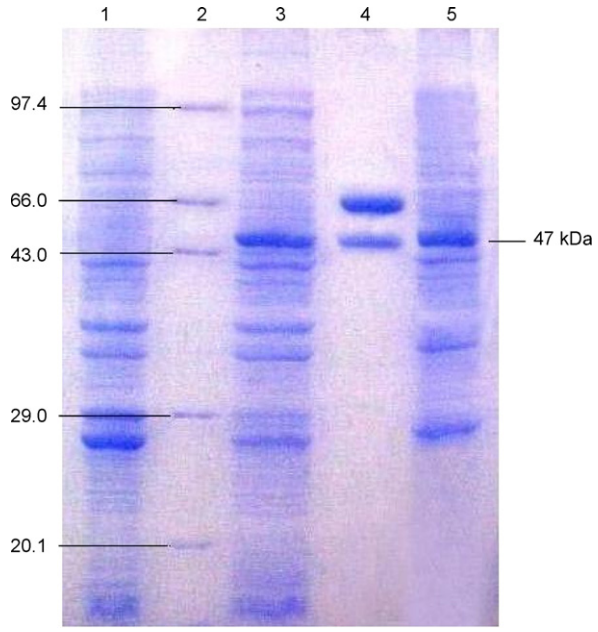

Fig. 1. SDS-PAGE analysis. SDS-PAGE analysis of uninduced cell lysate, induced cell lysate and microfiltrate samples of recombinant SK. The expression of a prominent band of SK was observed at 47 kDa in induced cell lysate, which is not seen in uninduced cell lysate. Microfiltrate sample also shows a prominent band at 47 kDa. Lane 1: uniunduced cell lysate; lane 2: molecular markers; lane 3: induced cell lysate; lane 4: NIBSC standard; lane 5: microfiltrate.

From Table 2, it can be concluded that denaturation in 6 M urea followed by renaturation in 20 mM Tris containing 1% Triton-X-100 and 10% glycerol does not deactivate streptokinase activity (Figs. 2 and 3) indicating the benefits of our protocols in solubilization inclusion bodies as well as enhancing the streptokinase activity. Initial attempts for solubilization of inclusion body were carried out using urea. Although the results obtained were satisfactory, attempts were made to improve the

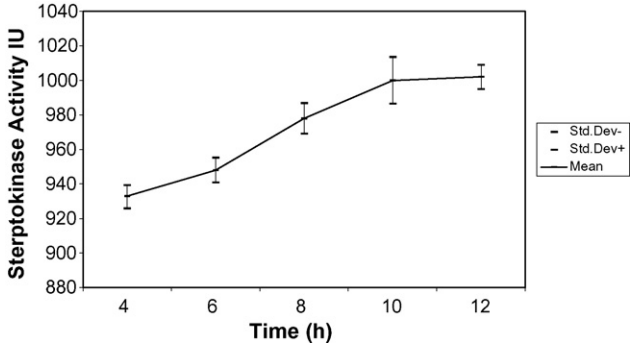

Fig. 2. Renatured streptokinase activity (IU) after 4 M urea solubilization. Mean ± standard deviation of the renatured streptokinase activity (IU) for five batches after 4 M urea solubilization at different time intervals (4–12 h) and their refolding in 20 mM Tris–HCl with 1% Triton-X-100 and 10% glycerol.

Table 2
Results of recombinant streptokinase assay in International Units (IU)

4 M Urea						6 M Urea					
Buffer with additive	Time intervals of solubilization (h) and assay mean values ± standard deviation*					Buffer with additive	Time intervals of solubilization (h) and assay mean values ± standard deviation*				
	4 h	6 h	8 h	10 h	12 h		4 h	6 h	8 h	10 h	12 h
20 mM Tris buffer (8.0)	101 ± 7.19	121 ± 9.3	154 ± 5.95	186 ± 10.36	200 ± 8.97	20 mM Tris buffer (8.0)	98 ± 9.3	100 ± 6.44	142 ± 5.61	174.4 ± 5.54	200 ± 8.33
20 mM Tris with 1 M L-arginine	554 ± 7.29	570 ± 12.38	586.6 ± 10.28	594.2 ± 10.05	610.2 ± 12.61	20 mM Tris with 1 M L-arginine	597.8 ± 10.61	601.8 ± 7.08	606.2 ± 10.68	626 ± 11.97	629.8 ± 5.40
20 mM Tris with 1% Triton-X-100 and 10% glycerol	933 ± 6.72	948 ± 7.24	978 ± 8.86	1000 ± 13.5	1002 ± 6.98	20 mM Tris with 1% Triton-X-100 and 10% glycerol	944 ± 11.02	972 ± 3.16	1004 ± 13.1	1054 ± 11.7	1058 ± 11.66
20 mM Tris with 10 mM glycine and 10 mM EDTA	476 ± 7.96	497 ± 14.28	506 ± 7.17	524.8 ± 7.29	531 ± 13.03	20 mM Tris with 10 mM glycine and 10 mM EDTA	502 ± 10.36	515 ± 4.63	521 ± 9.44	530 ± 10.17	534.8 ± 8.87
20 mM Tris with 100 mM NaCl and 10% glycerol	850.4 ± 19.32	873.8 ± 18.93	891.8 ± 15.69	902.4 ± 12.68	904.2 ± 14.82	20 mM Tris with 100 mM NaCl and 10% glycerol	875.4 ± 20.47	884.8 ± 25.63	902.4 ± 12.30	921 ± 28.5	922.2 ± 19.11
20 mM Tris with 10% SDS and 10% glycerol	876.4 ± 11.84	899 ± 9.62	910 ± 5.24	930.2 ± 6.30	932.8 ± 4.96	20 mM Tris with 10% SDS and 10% glycerol	921 ± 8.33	930 ± 7.84	950 ± 9.48	977 ± 11.59	984 ± 10.55

* Assay mean values of recombinant streptokinase shown are in International Units (IU).

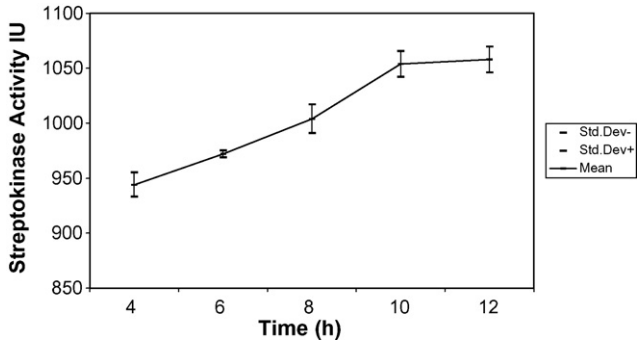

Fig. 3. Renatured streptoinase activity (IU) after 6 M urea solubilization. Mean ± standard deviation of the renatured streptoinase activity (IU) for five batches after 6 M urea solubilization at different time intervals (4–12 h) and their refolding in 20 mM Tris–HCl with 1% Triton-X-100 and 10% glycerol.

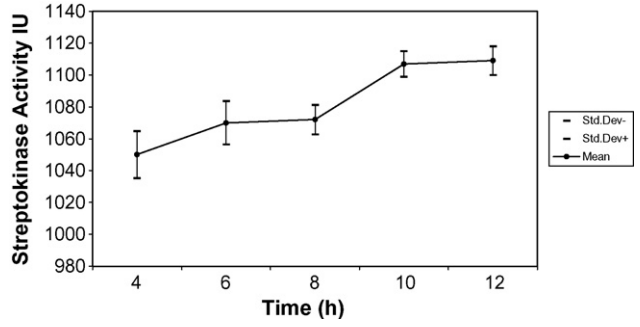

Fig. 4. Renatured streptokinase activity (IU) after 4 M guanidine hydrochloride solubilization. Mean ± standard deviation of the renatured streptokinase activity (IU) for five batches after 4 M guanidine hydrochloride solubilization at different time intervals and their refolding in 20 mM Tris–HCl with 1% Triton-X-100 and 10% glycerol.

yield of active protein. On the other hand, guanidine hydrochloride was also used as a denaturant followed by renaturation using 20 mM Tris buffer containing different additives (Table 1). The enzyme activities obtained after guanidine hydrochloride treatment followed by renaturation in Tris are shown in Table 3.

From these results we concluded that 6 M-guanidine hydrochloride treatment followed by renaturation in Tris buffer containing 1% Triton-X-100 and 10% glycerol yielded highest streptokinase activity (Figs. 4 and 5). Another notable feature is

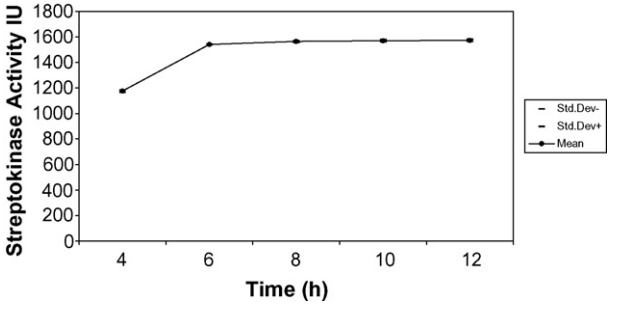

Fig. 5. Renatured streptokinase activity (IU) after 6 M guanidine hydrochloride solubilization. Mean ± standard deviation of the renatured streptokinase activity (IU) for five batches after 6 M guanidine hydrochloride solubilization at different time intervals and their refolding in 20 mM Tris–HCl with 1% Triton-X-100 and 10% glycerol.

Table 3
Results of recombinant streptokinase assay in International Units (IU)

4 M Guanidine hydrochloride						6 M Guanidine hydrochloride					
Buffer with additive	Time intervals of solubilization (h) and assay mean values ± standard deviation*					Buffer with additive	Time intervals of solubilization (h) and assay mean values ± standard deviation*				
	4 h	6 h	8 h	10 h	12 h		4 h	6 h	8 h	10 h	12 h
20 mM Tris buffer (8.0)	124 ± 8.21	150 ± 4.47	167 ± 6.04	193.6 ± 6.22	231 ± 7.07	20 mM Tris buffer (8.0)	152 ± 97.61	175 ± 8.21	200 ± 7.87	243 ± 6.32	300 ± 8.74
20 mM Tris with 1 M L-arginine	565 ± 7.48	577.8 ± 7.49	606 ± 5.83	621 ± 9.72	631 ± 15.73	20 mM Tris with 1 M L-arginine	580.2 ± 18.51	586.2 ± 19.7	600.2 ± 10.2	623 ± 12.7	636 ± 15.81
20 mM Tris with 1% Triton-X-100 and 10% glycerol	1050 ± 14.6	1070 ± 13.54	1072 ± 9.19	1107 ± 8.06	1109 ± 9.05	20 mM Tris with 1% Triton-X-100 and 10% glycerol	1176 ± 6.96	1541 ± 3.87	1564 ± 6.59	1570 ± 7.9	1573 ± 8.36
20 mM Tris with 10 mM glycine and 10 mM EDTA	498 ± 7.28	504 ± 11.46	517 ± 8.66	550 ± 9.69	569.2 ± 7.01	20 mM Tris with 10 mM glycine and 10 mM EDTA	504 ± 6.78	516 ± 6.20	523.8 ± 9.70	546 ± 9.38	565 ± 8.94
20 mM Tris with 100 mM NaCl and 10% glycerol	900.6 ± 10.57	923.2 ± 13.47	943.2 ± 14.11	974.8 ± 4.76	973 ± 7.03	20 mM Tris with 100 mM NaCl and 10% glycerol	949.2 ± 5.97	965.4 ± 9.91	973 ± 11.15	1006.8 ± 8.67	1007.4 ± 8.93
20 mM Tris with 10% SDS and 10% glycerol	921.2 ± 6.90	940 ± 12.6	949 ± 8.71	974 ± 9.61	978.2 ± 11.18	20 mM Tris with 10% SDS and 10% glycerol	975 ± 9.48	978 ± 5.78	983 ± 13.54	1010 ± 12.34	1012 ± 13.1

* Assay mean values of recombinant streptokinase shown are in International Units (IU).

that optimum streptokinase activity could be achieved when the inclusion bodies were solubilized in 6 M guanidine hydrochloride with incubation period of 6 h followed by renaturation (Fig. 5).

3.2. Ion-exchange chromatography

Although high activity was obtained after renaturation in the 20 mM Tris 8.0, with 1% Triton-X-100 and 10% Glycerol, electrophoretic analysis indicated impurities in the range of 20–40 kDa. Therefore in order to separate the impurities, further purification was done using ion-exchange chromatography. Ion-exchange chromatography was carried out using Toyopearl DEAE matrix (Tosoh Co., Japan) and 20 mM Tris 8.0 as the loading buffer. The bound proteins were eluted with linear gradient of 0–500 mM NaCl. The contents of the preload pro-

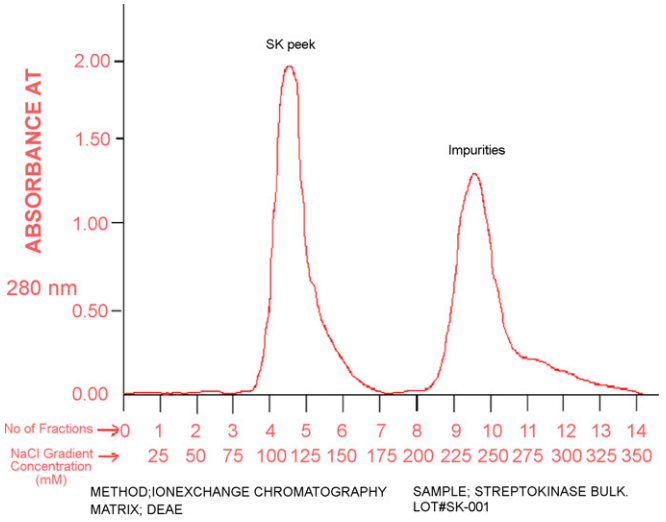

Fig. 6. Ion-exchange chromatography analysis. Bound proteins were eluted with a linear gradient of 0–500 mM NaCl. Pure streptokinase protein was eluted at 100 mM concentration of NaCl.

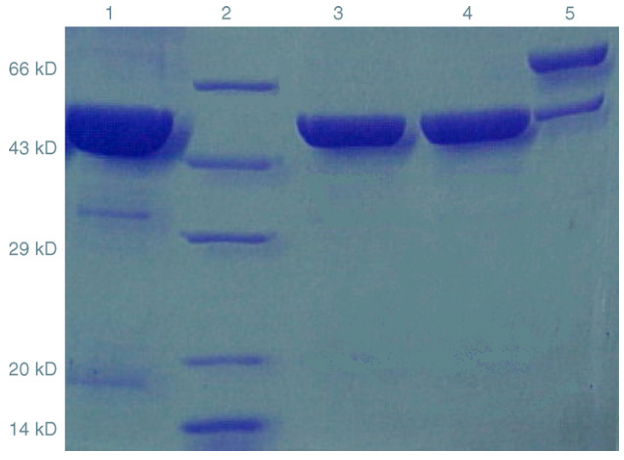

Fig. 7. Ion-exchange chromatography analysis by SDS-PAGE. SK eluted is indicated by the presence of single band corresponding to the molecular weight of 47 kDa. Lane 1: refolded SK feed sample of the column; lane 2: molecular markers; lane 3 and 4: purified SK elute fractions; lane 5: NIBSC standard.

tein sample in the eluted fractions were shown as two peaks at 280 nm absorbance (Fig. 6) and the eluted samples were analyzed by SDS-gel electrophoresis and also assayed for streptokinase activity (Fig. 7). Streptokinase was eluted from DEAE column at a salt concentration of 100 mM and the SDS-PAGE analysis of eluted streptokinase showed a single band corresponding to the molecular weight of 47 kDa, indicating that the eluted fractions of refolded streptokinase was very pure (Fig. 7). The final yield of pure streptokinase protein was estimated by Lowry method [18] which was 2 g/101 batch, with a specific activity of 1.5×10^5 IU/mg.

Several techniques have been described for recovery and purification of streptokinase from wild type *streptococcus* and recombinant streptokinase expressing in host *E. coli*. De Renzo et al. [19] reported highly purified preparations of streptokinase with the use of commercially available streptokinase preparations as the starting material. They reported a specific activity of 90,000–100,000 IU/mg of SK protein, after chromatography of streptokinase preparation twice on DEAE-cellulose [19]. Perez et al. [20] expressed recombinant streptokinase as inclusion bodies and produced by fermentation in *E. coli*. Using two methods both hydrophobic and ion-exchange chromatography, Perez et al. purified recombinant streptokinase protein at a purity of 99%, with a specific activity of 87,854 IU/mg [20]. Zhang et al. [21], purified recombinant streptokinase expressed in *E. coli* in the form of inclusion bodies. Inclusion bodies were dissolved in 6 M guanidinium hydrochloride solution and renatured by dialysis. This renatured protein solution was passed

Table 4
Streptokinase protein purification table

Purification step	Protein (ml^{-1})	Sample volume (ml)	Total protein (mg)	Specific activity (IU/mg)
Solubilization	5 mg	40	200	–
Rapid dilution	20 μg	10,000	200	77,000
Feed sample (sample before column)	192 μg	1,000	192	80,208
SK elute samples from DEAE column	800 μg	50	40	150,000

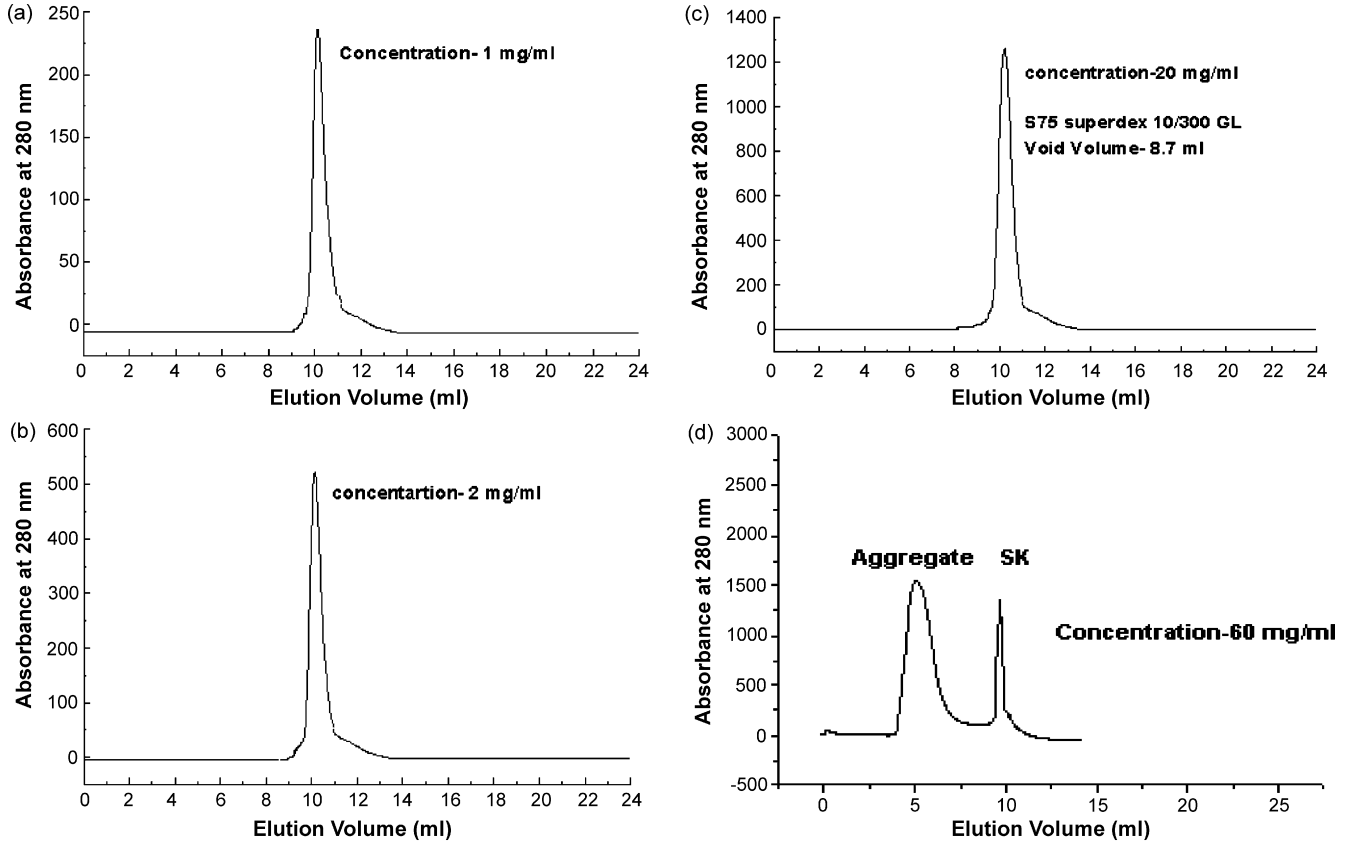

Fig. 8. Size exclusion chromatography profiles of renatured and purified streptokinase protein. (a) Purified streptokinase protein concentration of 1 mg/ml absorbance peak has reached up to 240 units at 280 nm. According to marker runs on the column the streptokinase has a molecular mass of about 47 kDa with a single peak. (b) Purified streptokinase protein concentration of 2 mg/ml absorbance peak has reached up to 500 A units at 280 nm. The streptokinase has a molecular mass of about 47 kDa with a single peak in comparison with molecular markers. (c) Purified streptokinase protein concentration of 20 mg/ml absorbance peak has reached up to 1200 A units at 280 nm. According to marker runs on the column the streptokinase has a molecular mass of about 47 kDa, with a single peak. (d) Purified streptokinase protein concentration of 60 mg/ml was loaded to the column. The profile shows two peaks, one aggregated protein and a small peak of streptokinase. The aggregated peak has eluted in the void volume.

through Q-sepharose column and subsequently passed through a sepharose G-10 column for final purification and obtained a purity of 95.7%, with a specific activity of 1×10^5 IU/mg [21]. Briefly, all the above-cited publications have carried out purification of recombinant streptokinase comprising of two chromatography methods. In the present study, recombinant streptokinase has been partially purified by cell lysis and passing through 0.45 μm filtration. The inclusion bodies were concentrated, solubilized and active recombinant streptokinase protein was obtained by the method of rapid dilution. Final purification of recombinant streptokinase protein was done by "single step" ion-exchange chromatography, with a purity of 99% and a specific activity of 1.5×10^5 IU/mg protein (Table 4), which is higher than the values so far reported in the literature.

3.3. Characterization

The size exclusion chromatography standards were carried out in an analytical mode using Superdex 75 HR 10/300 column at a flow rate of 0.3 ml/min. Fig. 8 shows that there is a single peak corresponding to 47 kDa in three concentrations of streptokinase protein, i.e. 1, 2 and 20 mg/ml. In case of 1 mg/ml streptokinase protein concentration the absorbance of the peak has reached up to 240 A units at 280 nm (Fig. 8a). In case of 2 mg/ml streptokinase protein concentration the absorbance of the peak has reached up to 500 A units at 280 nm (Fig. 8b) and in case of 20 mg/ml streptokinase protein concentration, the absorbance of the peak has reached up to 1200 A units at 280 nm (Fig. 8c). While in case of 60 mg/ml, the streptokinase eluted at void volume, which indicates the presence of aggregates at this concentration (Fig. 8d). These results show that the refolded and chromatographically purified SK protein (1, 2 and 20 mg/ml) is free from contaminants, resulting in 99% active recombinant streptokinase protein without aggregates. MALDI-TOF analysis of refolded and purified streptokinase protein shows 47 kDa which is similar to theoretically calculated mass (Fig. 9). The RP-HPLC of active streptokinase protein shows a single peak, with 99% of pure streptokinase protein indicating the presence of homogenous conformers (Fig. 10).

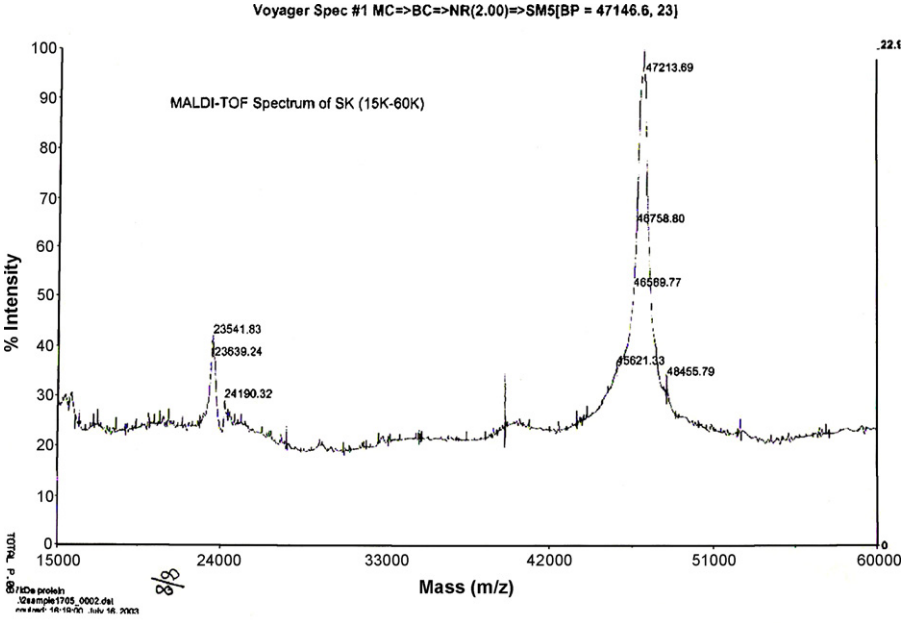

Fig. 9. MALDI-TOF. Graph submitted shows the mass range from 12 to 60 kDa. The sample showed a single streptokinase protein at 47 kDa till *m/z* (mass-to-charge ratio) range of 100 kDa.

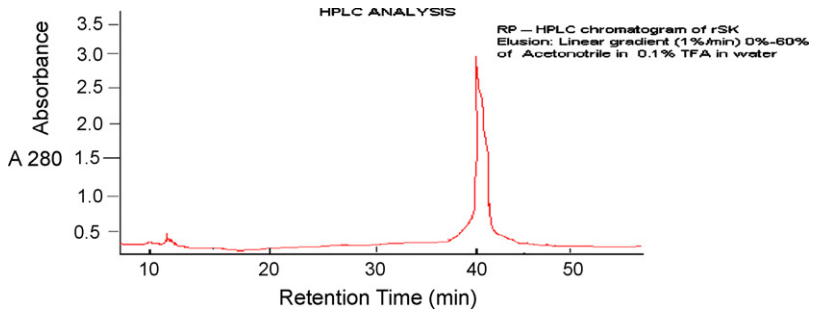

Fig. 10. Bulk of streptokinase quantification by HPLC. The active pure protein analyzed by HPLC, using kromasil C-18 column (250 mm × 4.6 mm), with a mobile phase of 0.1% trifluroacetic acid in water: and acetonitrile (0–60%) which shows a single peak with a retention time of 40 min.

4. Conclusions

This paper shows different solubilization conditions of streptokinase inclusion bodies, renaturation methods by different buffering conditions with additives and finally purification by ion-exchange chromatography. Here, we have discussed in depth the method to solubilize the inclusion body of streptokinase. Most of the solubilization methods followed so far do not give an enhancement in streptokinase activity [20–22].

We, therefore, conclude that 6 M-guanidine hydrochloride treatment followed by renaturation in Tris buffer containing 1% Triton-X-100 and 10% glycerol yields highest enzyme activity. Most of the streptokinase activity is optimum when the solubilization mixture was recovered after 6 h of incubation. The purified recombinant streptokinase protein has shown yield of 2 g, with a specific activity of 1.5×10^5 IU/mg protein. Characterization of pure streptokinase using size exclusion chromatography, MALDI-TOF and RP-HPLC shows the size similarity to the standard, similar mass with theoretically calculated mass and 99% purity, respectively.

Acknowledgement

One of the authors, Dr. Cherish Babu is thankful to Bharat Biotech International Limited for being sponsored for the PhD programme.

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