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Journal of Chromatography B, 850 (2007) 384–391
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Hydrophobic interaction expanded bed adsorption chromatography (HI-EBAC) based facile purification of recombinant Streptokinase from *E. coli* inclusion bodies

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Received 18 January 2006; accepted 9 December 2006
Available online 26 December 2006

Abstract

The downstream processing of recombinant streptokinase (rSK), a protein used for dissolution of blood clots has been investigated employing *Escherichia coli* inclusion bodies obtained after direct chemical extraction followed by expanded bed adsorption chromatography (EBAC). Streptokinase was over-expressed using high cell density (final $OD_{600} = 40$) culture of recombinant *E. coli*, and an SK protein concentration of 1080 mg l^{-1} was achieved. The wet cell pellet after centrifugation was re-suspended in 8 M urea containing buffer resulting in direct extraction of almost 97% of cellular proteins into solution. Compared to mechanical disruption using sonication, the direct extraction helped in simultaneous cell lysis and inclusion body (IB) solubilization in a single integrated step. The post-extraction solution containing cell debris and cellular proteins was diluted and directly loaded on to an EBAC column containing Streamline phenyl, without clarification. By passing the solution four times through the column and using 1 M NaCl during loading, 82.7% rSK activity could be recovered in the 10 mM sodium phosphate buffer used for elution. A 3-fold increase in specific activity of rSK, from 0.18×10^5 in cell lysate to 0.53×10^5 IU mg^{-1} resulted after this step. rSK was further purified to near-homogeneity (specific activity = 0.96×10^5 IU mg^{-1}) by a subsequent ion-exchange step operated in packed bed mode. An overall downstream recovery of 63% rSK was achieved after EBAC and ion exchange chromatography. The paper thus describes the purification of rSK using a three-step regime involving simple, efficient and highly facile steps.

Keywords: Streptokinase; *Escherichia coli*; Downstream processing; Expanded bed adsorption chromatography; Hydrophobic interaction chromatography

1. Introduction

One of the biggest achievements of biotechnology has been the ability to virtually produce any desired commercially relevant protein in heterologus hosts like *E. coli* at levels previously unattainable in their natural hosts. However, the high level expression of foreign proteins often results in the formation of insoluble amorphous aggregates called inclusion bodies (IBs) in the *E. coli* cytoplasm. The formation of IBs poses special downstream requirements generally rendering such overall processes as fairly cumbersome and multi-step in nature, as shown in Fig. 1. One of the major challenges that the recent literature on purification involving IBs has increasingly focused on is that of process integration, i.e. combination of 2 or more steps into one simplified step. The direct chemical extraction described in literature [1,2] is one such procedure that results in the integration of cell rupture and IB solubilization. It involves the use of chaotropic concentrations of urea and EDTA containing buffers. In more recent reports, direct adsorption of the desired protein from the unclarified post-extraction solution (obtained after chemical extraction) using IMAC based EBA adsorbents have been described [3,4]. The use of IMAC based adsorbents, however, necessitate the expression of target protein with a histidine tag at the N- or C-terminal, which may not be desirable in case of injectible therapeutic proteins for human applications.

Another approach that offers advantages of direct adsorption of target protein is aqueous two-phase system (ATPS) that

Abbreviations: BME, beta mercaptoethanol; BSA, bovine serum albumin; EBAC, expanded bed adsorption chromatography; EDTA, ethylene diamine tetra acetic acid; DEAE, diethylaminoethyl; HIC, hydrophobic interaction chromatography; HI-EBAC, hydrophobic interaction expanded bed adsorption chromatography; IBs, inclusion bodies; IEX, ion exchange; rSK, recombinant streptokinase; SK, streptokinase; SP, sodium phosphate

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doi:10.1016/j.jchromb.2006.12.013

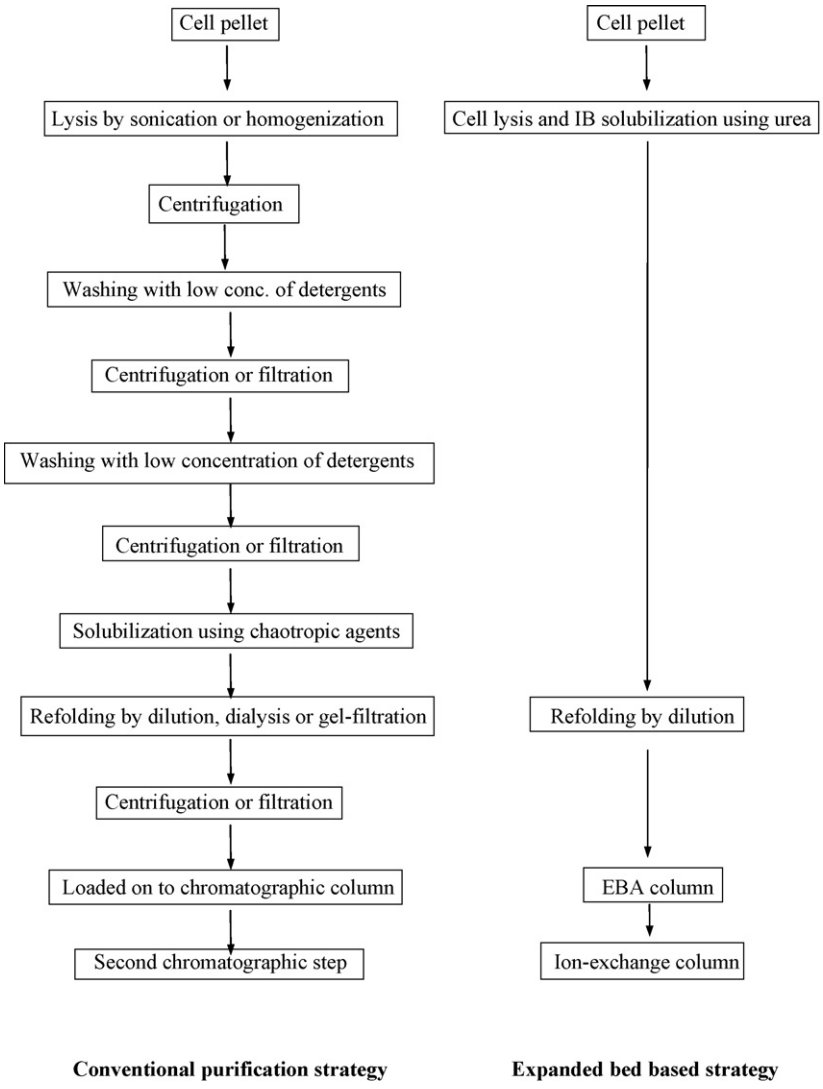

Fig. 1. Scheme of purification of a recombinant protein from IBs. A conventional IB purification protocol usually consists of large number of steps but urea extraction and EBAC based protocol comprises few steps only.

involves the use of high molecular weight hydrophobic polymers. The use of ATPS to capture recombinant protein from *E. coli* lysate, obtained after mechanical lysis had been reported [5]. However, mainly due to the high cost of the phase-forming polymers and the complexity and specificity of ATPS behavior, the complete potential of this technique is yet to be exploited [6].

In contrast, we describe here the use of direct chemical extraction along with hydrophobic interaction based expanded bed adsorption chromatography (HI-EBAC) for the purification of streptokinase, a high value therapeutic protein directly from recombinant *E. coli* IBs. Streptokinase (SK) is a clot-dissolver protein drug most widely used for the dissolution of blood clots that cause heart attack or stroke [7]. It interacts strongly with plasminogen to form a 1:1 molar complex (plasminogen activator complex), which then acts on free plasminogen to yield plasmin [8] that degrades the fibrin network of the blood clot. It has a molecular weight of approximately 47 kD and an isoelectric point of 4.7 [9] and is devoid of any disulphide bonds. SK is naturally secreted by several species of β-hemolytic streptococci, and its production by recombinant means has also been reported [10–12]. The purification of SK based on affinity chromatography using acylated plasminogen or plasmin has been described [13]. A low recovery is, however, reported due to inherent degradation of SK by plasmin, generated as a result of slow deacylation of the chemically modified plasminogen-containing matrix [14]. Large-scale purification of recombinant SK (rSK) from *E. coli* IBs has also been described using conventional multi-step procedures involving cell lysis, IB washing, solubilization, refolding and packed bed chromatography, with an overall rSK activity recovery of 51% [15].

In order to explore a facile procedure for rSK purification, we over-expressed rSK as IBs in *E. coli* and used Streamline® phenyl, a hydrophobic interaction based EBAC matrix, for its ability to directly capture rSK from unclarified feed-stock produced after direct chemical extraction. The effect of various operating parameters was first examined to achieve maximum purity and yields of the desired product by the hydrophobic

interaction-EBA chromatography (HI-EBAC) procedure. EBAC based purification has not so far been reported for SK and, if successful, can potentially bring down the number of cumbersome and costly processing steps. Further purification of rSK using DEAE-Sepharose in packed bed mode has also been described to achieve near-homogeneity level purification necessary for this therapeutic agent.

2. Materials and methods

2.1. Reagents

Ampicillin was obtained from Sigma Chemical Co. St. Louis, USA, and IPTG from Promega, Australia. Plasminogen and Chromozyme-PL were from Boehringer-Mannheim, Germany while the reagents used for SDS–PAGE were from Sigma, USA. Standard molecular weight markers and purification matrices were from Amersham Biosciences, Uppasala, Sweden. All other reagents/media components were of analytical grade or the highest purity available.

2.2. Cultivation and harvesting

The *E. coli* strain BL21 (DE3) containing a pET-based expression plasmid [16] that expresses the mature 47 kD streptokinase intracellularly upon induction with IPTG was used in this study. The cells were grown in semi-synthetic media modified from [17] in 5 l Bioflo 3000 laboratory fermentor (New Brunswick Scientific, USA) at pH 6.8, 37 °C and 50% dissolved oxygen concentration. 2.5 g each of yeast extract and tryptone were added per litre of the initial cultivation media. Feed media contained glucose, yeast extract and tryptone at a concentration of 400, 100 and 100 g l^{-1}, respectively. After 4 h of batch cultivation, feeding was started at a constant rate and maintained till the end. Induction was done at a cell density (OD_{600}) of 30 using 0.5 mM IPTG (isopropyl-thiogalactopyranoside) and the cultivation temperature was increased to 42 °C. The final cell density (OD_{600}), at harvest, was 40. SK was expressed as inclusion bodies at a concentration of 1080 mg l^{-1} as determined by densitometric scanning (Software: Gene Tools from Syngene, MD, USA) of SDS–PAGE gel loaded with whole cell lysate proteins using bovine serum albumin as standard [18]. After cultivation, the cells were harvested by centrifugation of the cultivation medium at 5000 × *g* for 15 min in a Sorvall (RC 5C-Plus model) centrifuge using GS3 rotor. The cell pellets were thoroughly washed in cold sterile NaCl-TE buffer (100 mM NaCl, 10 mM Tris buffer and 1 mM EDTA) twice.

2.3. Cell lysis

Cells were lysed both by sonication and urea extraction. To 15 g wet cells obtained from 250 ml of cultivation medium, 60 ml of buffer (solubilization buffer in case of urea lysis and NaCl-TE in case of sonication) was added resulting in a dense cell suspension. Solubilization buffer was composed of 8 M urea in NaCl-TE buffer. The cell suspension in the first case was incubated for 2 h at 4 °C with constant stirring. In the second case, it was subjected to sonication at 65 W using a cell sonicator (Heat Systems Inc., NY) equipped with a titanium micro-tip probe (tip diameter 6.4 mm) with 30 s on/off cycle to prevent overheating, for a total time of 30 min. Cell suspension (50 ml) was kept in an ice bath during disruption. In case of urea, the crude protein in the lysate was analyzed directly while in case of sonication, the lysate was centrifuged to separate soluble and insoluble fractions, the latter was solubilized using 8 M urea prior to protein estimation.

Refolding of SK was done by diluting the cell lysate (in case of urea extraction) 10-fold to a final concentration of 0.5 M NaCl and 20 mM SP buffer (unless otherwise mentioned) and 0.8 M urea at 4 °C.

2.4. Purification using hydrophobic interaction based expanded bed adsorption chromatography (HI-EBAC)

All EBAC experiments were carried out using Streamline-50 column (ID = 50 mm) containing 250 ml of Streamline phenyl matrix (Amersham Biosciences, Uppasala, Sweden). The particles are based on macro porous cross-linked 6% agarose with a size distribution of 100–300 μM and average density of 1.2 g ml^{-1}. The purification experiments were carried out at 4 °C. A flow rate of 65 ml min^{-1} (200 cm h^{-1}) was maintained in the upward direction using a K-Prime 40-I purification system (Millipore) resulting in a 2.15-fold expansion of matrix, which was calculated as follows:

$$\text{Degree of expansion} = \frac{\text{Expanded bed height}}{\text{Sedimented bed height}}$$

Diluted sample, in 0.5 M NaCl and 20 mM SP buffer, was loaded on this EBAC column equilibrated with same buffer. After loading of the sample, washing of the bed was done with the same buffer till no more protein emerged from the column (as indicated by a near-zero absorbance at 280 nm). For maintaining a stable expanded bed, the volumetric flow rate of the buffer during equilibration, loading and washing steps was adjusted accordingly. After washing the flow was stopped and the adaptor was lowered till it touched the matrix. Elution was then performed in a conventional packed mode at a flow velocity of 100 cm h^{-1} using SP buffer (10 or 20 mM) and distilled water (DW) as mentioned in Section 3. Various EBAC experiments were conducted and the effects of parameters like sample re-circulations (one, four and twelve), NaCl concentration (molarity), molarity of the elution buffer (SP) and the amount of protein load per unit gel matrix was then studied. The flow-through, washing and elution fractions were collected separately and analyzed for protein content, SK activity, and on SDS–PAGE gel to assess purity. After each experiment, the column was regenerated by passage of 1 M NaOH (5 bed volumes) followed by DW (till the pH became neutral), and, finally equilibration buffer. The percentage recovery of SK was calculated from the total SK activity obtained in comparison to the total SK activity loaded onto the column. The purity of eluted fractions was established by SDS–PAGE and the value of specific SK activity (IU mg^{-1}).

2.5. Ion exchange (IEX) chromatography

The 10 mM SP buffer fraction from EBA chromatographic step (after optimization) was loaded on a column packed with DEAE-Sepharose® (fast flow), pre-equilibrated with same buffer. Loading (∼2 mg protein per ml of the matrix) was done at a flow rate of 2 ml min^{-1} and the column was extensively washed with the same buffer. Bound proteins were eluted by a linear gradient from 0 to 0.5 M NaCl up to five bed volumes using a flow rate of 4 ml min^{-1}. The flow-through, washing and the elution fractions were all analyzed similar to the EBAC fractions.

2.6. Analytical methods

The protein content of the lysate and purified fractions was estimated by the Bradford method [19] and was calculated from a BSA (2.5–15 μg) calibration curve. In all cases appropriate dilution were made and proper buffer controls were taken. For the estimation of urea-containing samples, a reagent blank containing identical amounts of urea, but without protein, was taken as control. The biological activity of streptokinase was quantitatively assayed using a single-step plasminogen-based chromogenic substrate assay [20]. Streptokinase activity was expressed as international units (IU) per milliliter (IU ml^{-1}). An in-house streptokinase standard calibrated against the WHO international standard was routinely used as a reference for activity. Highly pure one milligram of SK gives an activity equivalent of 1×10^5 IU. The specific activity (IU mg^{-1}) was calculated by dividing either the SK activity (IU ml^{-1}) with protein concentration (mg ml^{-1}) or the total SK activity (IU) with total protein content (mg). Discontinuous SDS–PAGE was carried out using a method described in [21] using a vertical electrophoresis system from Sigma, USA. The samples (preserved at −70 °C) were mixed in 1:1 ratio with 2X sample buffer (125 g l^{-1} Upper Tris pH 6.8, 20 g l^{-1} SDS, 20 g l^{-1} glycerol, 2 g l^{-1} Bromophenol blue, 14 g l^{-1} BME), boiled for 3 min, and 20 μl of each was loaded onto separate lanes of SDS–PAGE gels.

3. Results and discussion

3.1. Chemical extraction–integration of cell lysis and IB solubilization

Chemical extraction procedure initially described at low [2] and then at high cell concentration [22] causes not only the release of the target protein into the solution, but also other cellular proteins as well. Attempts to make this extraction procedure selective for the target protein have been described [23], these however, have not been found to be successfully applicable in a generalized manner [24]. In the present study, a complete extraction of the *E. coli* proteins at high cell concentration was carried out using 8 M urea-containing buffer. Sonication was used as a control for comparing lysis efficiency, calculated as the amount of protein released per ml of cultivation medium. Both the cell lysis procedures were performed at similar cell density that was maintained close to 160 OD_{600} units.

Lysis, using urea-containing buffer, resulted in the release of 1425 ± 50 mg of crude protein from 15 g cells (wet weight) taken for lysis, corresponding to a protein release of 5.7 mg ml^{-1} of cultivation medium. In case of sonication, 3.6 and 2.3 mg ml^{-1} protein was released from the supernatant (soluble) and pellet (insoluble) fractions, respectively, corresponding to a total of 5.9 mg ml^{-1}. Thus, assuming the total protein obtained (5.9 mg ml^{-1}) using sonication as 100%, close to 97% crude protein was obtained (5.7 mg ml^{-1}) in case of urea extraction. Since 8 M urea-containing buffer tends to crystallize at 4 °C, which could result in difficulty in large-scale processing, hence we carried out the same extraction procedure using 7 M urea, which was found to be equally efficient, whereas a further decrease to 6 M urea resulted in relatively lesser protein release.

This establishes that urea extraction is as effective as sonication in releasing intracellular proteins and may prove to be an efficient and scaleable alternative to mechanical disruption. The time taken for lysis will very likely be scale independent as long as an optimum cell concentration is maintained. The removal of the intermittent washing and centrifugation cycles conventionally used in IB processing will definitely add to the cost benefits of urea extraction. Moreover, the same vessel that is used for IB solubilization can be utilized for this combined cell lysis and IB solubilization procedure. Hence, it shows promise as an economical and facile alternative to mechanical disruption because of its ease of operation, rapidity, efficiency and its apparent independence of requirements for specialized equipment.

Further, the total amount of active SK was estimated after the refolding of SK into its biologically active form which was achieved by 10-fold dilution of cell lysate as mentioned in Section 2. In our preliminary studies using washed SK-IBs, obtained after following the traditional scheme of IB purification, a minimal 10-fold dilution was observed to be consistent with high recoveries of biologically active refolded protein. In case of urea extraction process, remarkably, the same conditions proved to be satisfactory. In case of other proteins, however, refolding conditions may have to be standardized. One may also note that the results obtained with SK clearly indicate that even though the IBs obtained directly were of lesser purity, they were in no way inhibitory to the refolding efficiency.

3.2. Purification of SK using expanded bed adsorption chromatography (EBAC)

For exploiting the ease of operation and rapidity of urea extraction fully, it is generally followed by EBAC that allows efficient capture of protein of interest directly from the unclarified crude feedstock. In the recent literature some reports on the use of affinity based EBAC in conjunction with chemical extraction [3,4,25,26] are present. In this study, however, a hydrophobic interaction based EBAC was attempted using phenyl-based Streamline matrix. The various operating parameters during loading and elution were optimized for maximizing SK recovery.

Since EBAC is usually carried out at flow velocities roughly ten times higher than the conventional packed beds [27], hence it was likely that re-circulation of the sample through the column

might be necessary for optimal binding. In order to investigate this aspect, the loaded sample was re-circulated once, four and twelve times through the column using 0.5 M NaCl and 20 mM SP buffer. The re-circulation was achieved by collecting the flow-through after first pass and then circulating it continuously through the column in expanded bed mode. Elution was carried out in packed bed mode first by passing 20 mM SP buffer followed by distilled water (DW), and SK recovery was evaluated (Table 1). When the sample was passed once through the column negligible activity could be obtained in both buffer and DW fractions. In contrast, the total activity of SK after elution improved significantly from 0.9 to 31.5% when the sample was re-circulated four times through the column. A further increase in sample re-circulations to twelve times however, did not bring any further improvement (29.2%) in SK recovery.

From the results it is evident that the kinetics of adsorption and time taken for reaching equilibrium in EBAC may be different from that observed in packed bed mode. Hence, either the time of contact or repetitive recycling, or both, can be exploited to achieve equilibration/saturation in binding. In our case, four re-circulations of the sample were found to be efficient in allowing sufficient interaction time between protein and the matrix particles at the employed linear flow rate of 200 cm h^{-1}.

One may also note that in case of four re-circulations, SK activity was present in both buffer and DW fraction. Also, the specific activity of the buffer fraction (0.69×10^5 IU mg^{-1}) was higher than that of water fraction (0.2×10^5 IU mg^{-1}) indicating that a much purer protein elutes in buffer. DW elution in HIC is likely to result in impure protein, since it causes rapid elution of most bound proteins due to decreased hydrophobic interactions. From this we reasoned that for obtaining purer SK, enriched elution of this protein could be achieved in buffer of a low but optimized salt concentration. Hence, the molarity of SP buffer (used for elution) was decreased and its effect on SK recovery was studied. Indeed, by decreasing the elution buffer molarity from 20 to 10 mM, most of the SK (around 32%) now eluted in buffer fraction (i.e. 83×10^5 IU) while only 0.02% (i.e. 4×10^5 IU) was found in DW fraction (Table 1). A further decrease in elution buffer molarity to 2 mM also yielded the same results as that of 10 mM and caused no further improvement in yield or purity (data not shown). Analysis of the protein profile obtained in both cases (Fig. 2) also indicated that SK was completely absent in DW fraction (lane 6) when 10 mM buffer was used for elution. Since it was desirable from the point of view of stability that SK should be present in buffer rather than pure water, hence the elution buffer molarity was maintained close to 10 mM in subsequent experiments. Thus, the use of low molarity buffer resulted in selective elution of SK ahead of DW. One may also note that in the case of 10mM, the specific activity of SK in the buffer fraction decreased to 0.54 compared to 0.69×10^5 IU mg^{-1} obtained in 20 mM buffer. This was considered acceptable since at the EBAC stage, the emphasis was on maximum SK capture and elution in a single fraction. This fraction could then be taken to the next, ion-exchange chromatographic step that would deliver SK with desired purity.

Table 1
Effect of sample re-circulations and elution buffer molarity on rSK recovery in EBAC

Sample re-circulations	SP buffer molarity (M)	SP buffer fraction				Distilled water fraction				Total SK activity recovered (%)
		SK activity ($\times 10^5$ IU)	Total protein (mg)	Specific activity of SK ($\times 10^5$ IU mg^{-1})	Percent activity recovered (%)	SK activity ($\times 10^5$ IU)	Total protein (mg)	Specific activity of SK ($\times 10^5$ IU mg^{-1})	Percent activity recovered	
Once	20	ND	ND	–	–	2.25	75	0.06	0.9	0.9
Four	20	59 ± 3	85 ± 2.5	0.69 ± 0.06	22.7 ± 2.1	23 ± 2	115 ± 3.5	0.2 ± 0.02	8.8 ± 1.2	31.5 ± 3.3
Twelve	20	58 ± 3.8	99 ± 3	0.58 ± 0.06	22.3 ± 2.4	18±4	135 ± 4.1	0.13 ± 0.04	6.9 ± 1.9	29.2 ± 3.1
Four	10	83 ± 4	153 ± 4.6	0.54 ± 0.05	31.9 ± 3.1	4	147 ± 4.4	0.03	0.02	32.1 ± 3.9

ND: Not Detectable. Diluted cell lysate containing $260 \pm 10 \times 10^5$ IU of SK was loaded on to the EBAC column using 0.5 M NaCl. The effect of number sample re-circulations and the decrease in molarity of elution buffer from 20 to 10 mM was checked. The percentage recovery was calculated by dividing the recovered SK activity with the total loaded onto the column. The deviation in values obtained in different set of experiments are represented.

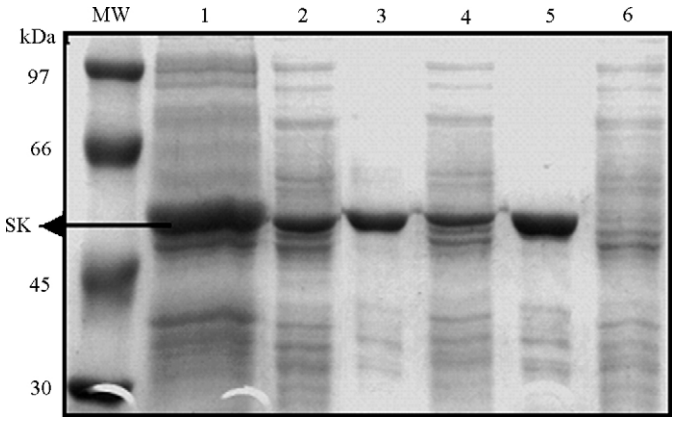

Fig. 2. Effect of molarity of elution buffer on the amount of streptokinase recovery. Lanes represent: (MW) standard molecular weight markers (1) cell lysate; (2) flow-through; (3) 20 mM SP buffer fraction; (4) distilled water; (5) 10 mM SP buffer; and (6) distilled water.

Till now, by using four sample re-circulations and 10 mM buffer for elution, close to 30% of loaded SK could be recovered, the rest around 65% remaining largely unbound as suggested by the activity analysis. In order to specifically improve the capture of SK, the NaCl concentration during loading and washing steps was increased to either 1.0 or 2.0 M. This is because the molarity of the salt used for loading not only affects the hydrophobicity of a given protein in general, but also the binding environment of protein–matrix interactions [28]. Elution was performed with 10 mM buffer and DW was passed afterwards as before.

By the use of 1.0 M NaCl during loading, more than 80% of total loaded SK activity could be recovered in the 10 mM buffer eluate, compared to only 31.9% in case of 0.5 M (Table 2). At the same time, a further increase to 2 M caused only a marginal improvement in SK recovery compared to 1 M. The specific activity, however, decreased to 0.45×10^5 IU mg^{-1} in case of 2.0 M, while the specific activities in case of 0.5 and 1.0 M salt were closely similar ($\sim 0.54 \times 10^5$ IU mg^{-1}). The DW fraction contained negligible amount of activity in all cases. Fig. 3A–C also show absence of SK band in the flow-through fractions in case of both 1 and 2 M salt. Clearly, NaCl concentration was the most significant factor that influenced the amount of SK recovered during HI-EBAC. It may be mentioned that the Hoefmeister series suggests that in addition to NaCl other more stabilizing salts like $(NH_4)_2SO_4$ could also be employed for optimal results. However, in every case an optimal concentration range needs to be determined that would allow efficient and selective binding of desirable protein over the impurities. Based on the SK recovery and purity obtained, 1 M NaCl was found to be optimum in the present study.

In order to establish the maximum amount of cultivation medium that can be processed using 250 ml of Streamline matrix, without affecting recovery and purity, cell lysate obtained from 500 and 1000 ml of cultivation medium were loaded on to the same column in separate experiments. Results show (Table 2) that on increasing the protein load from 250 to 500 ml of cultivation medium, SK yield (79%) and purity levels (Specific activity 0.54×10^5 IU mg^{-1}) were not adversely

Table 2
Effect of increase in molarity of loading buffer and the amount of loaded protein on streptokinase recovery in EBAC

S. No.	Molarity of NaCl used (M)	Fermentation broth (ml)	Total activity loaded ($\times 10^5$ IU)	SK activity recovered ($\times 10^5$ IU)	Total protein recovered (mg)	Specific activity ($\times 10^5$ IU mg^{-1})	Percent SK activity recovered in 10 mM SP(%)
1.	0.5	250	260 ± 10	83 ± 4	153 ± 4.6	0.54 ± 0.05	31.9 ± 3.1
2.	1.0	250	260 ± 10	215 ± 6	405 ± 8	0.53 ± 0.03	82.7 ± 5.3
3.	2.0	250	260 ± 10	222 ± 7	491 ± 8	0.45 ± 0.02	85.4 ± 6.6
4.	1.0	500	520 ± 20	410 ± 10	759 ± 10	0.54 ± 0.02	78.8 ± 5.2
5.	1.0	1000	1040 ± 22	440 ± 15	880 ± 12	0.50 ± 0.02	42.3 ± 2.7

The effect of NaCl concentration of 0.5, 1 and 2 M during loading was checked. Also, the effect of loading total cell proteins from 250, 500 and 1000 ml fermentation broth on to 250 ml Streamline-phenyl was also examined.

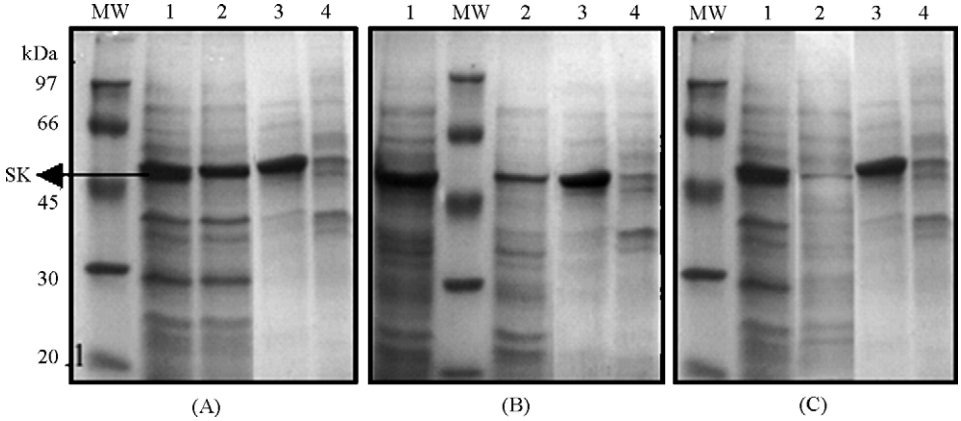

Fig. 3. Effect of NaCl concentration in the loading buffer on streptokinase recovery in EBAC. The effect of varying the NaCl concentration to 0.5 (A), 1.0 (B) and 2.0 (C) M was studied in three purification runs. In each panel lanes represent: (MW) standard molecular weight markers; (1) loaded sample; (2) unbound protein released on washing with NaCl; (3) SP buffer (10 mM) elution fraction; and (4) water elution fraction.

Table 3
Purification of streptokinase using HI-EBAC and ion-exchange chromatography

Sample	Total protein (mg)	Total SK activity ($\times 10^5$ IU)	Specific SK activity ($\times 10$ IU mg^{-1})	SK recovery w.r.t. activity	
				Step yield (%)	Overall (%)
Cell lysate	1425 ± 50	260 ± 10	0.18 ± 0.02	100	100
HIC-EBAC elution fraction	405 ± 8	215 ± 6	0.53 ± 0.03	82.7 ± 5.3	82.7 ± 5.3
IEX elution	171 ± 3	164 ± 6	0.96 ± 0.05	76 ± 5	63.1 ± 4.9

Recombinant cells from 250 ml broth were lysed using urea containing buffer and loaded on to EBAC column. Elution fraction (10 mM SP buffer) from this step was loaded on a packed-bed column with ion exchange matrix (DEAE-Sepharose fast flow) for further purification.

affected. In contrast, with a further increase to 1000 ml, the amount of SK activity recovered decreased to 42.3%. Thus, a matrix volume to cultivation medium ratio of 1:2 was found to result in maximum yield and purity of the protein under the employed conditions. This ratio could serve as a basis during further optimization of the operations at a higher scale.

To summarize, SK recovery improved from negligible to 80% after optimization of loading and elution conditions in HI-EBAC. The specific activity of SK also increased to 3-fold from 0.18 to 0.53 $\times$ 10^5 IU mg^{-1} (Table 3). The important factors were found to be NaCl concentration and sample re-circulations.

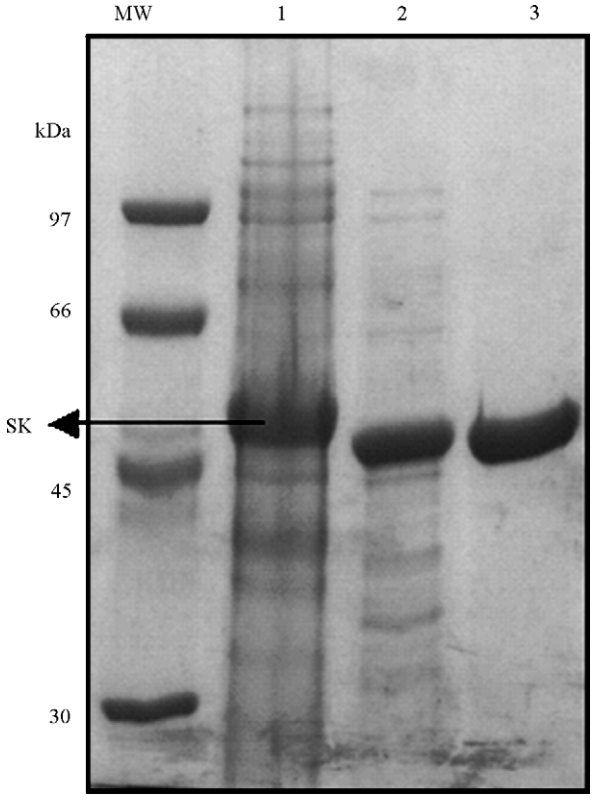

Fig. 4. Purification of streptokinase using the optimized three-step process. Cell pellet was lysed using 8 M urea containing buffer; the lysate was loaded on to HIC column in expanded bed mode under optimized conditions. The HI-EBAC fraction after elution was loaded on to ion exchange (IEX) column for final purification. Lanes represent: (MW) standard molecular weight markers; (1) cell lysate; (2) HIC purified fraction; (3) IEX purified fraction.

3.3. Ion exchange (IEX) chromatography

In order to purify the partially pure SK obtained after EBAC further, an ion-exchange chromatography step was carried out in packed bed mode. Elution was done with a NaCl gradient and SK was found to elute at 0.25–0.35 M NaCl. Out of the total loaded SK activity, 76% was recovered after elution in this step.

An overall yield of 63 ± 5% streptokinase activity was obtained after EBAC and IEX (Table 3). The specific activity after IEX increased to 0.96 ± 0.05 $\times$ 10^5 IU mg^{-1}, which was the same that was expected from highly pure SK. Fig. 4 establishes the complete purity of the SK protein and shows the final protein to be homogenous by SDS–PAGE.

4. Conclusions

In this paper, we explore, and successfully establish, the feasibility of using EBAC in conjunction with chemical extraction for a minimal-step downstream processing scheme (Fig. 1) for SK from *E. coli* inclusion bodies. This approach has so far been tried only with affinity-based matrices [3,4]. Here, for the first time we have established the utility of hydrophobic interaction based EBAC (HI-EBAC) procedure for achieving the same end, and could thereby achieve a purification yield of around 80% and a 3-fold increase in purity. The protein could then be further purified to homogeneity using an ion exchange packed bed based chromatographic step. This approach can be adapted for many different recombinant systems and proteins, and after optimization of the operating parameters, can potentially lead to yields and purity levels comparable to those achieved by traditional multi-step routes.

Acknowledgements

The authors would like to thank the Council of Scientific and Industrial Research, India for overall support and awarding Junior and Senior research fellowships to one of the authors (D.G.). Authors are grateful to Mr. Dinesh Krishnan for the excellent technical guidance and support.

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