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Structural characterization of recombinant streptokinase following recovery from inclusion bodies using different chemical solubilization treatments

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ABSTRACT

Circular dichroism (CD) in far-UV region was employed to study the extent of changes occurred in the secondary structures of recombinant streptokinase (rSK), solubilized from inclusion bodies (IBs) by different chemicals and refolded/purified by chromatographic techniques. The secondary structure distribution of rSK, obtained following different chemical solubilization systems, was varied and values in the range of 12.4–14.5% α-helices, 40–51% β-sheets and 35.5–48.3% turns plus residual structures were found. With reducing the concentration of chemicals during IB solubilization, the content of turns plus random coils was diminished and simultaneously the amounts of α- and β-sheets were increased. These changes in the secondary structures would lower the hydrophobicity along with the chance of protein aggregation and expose the hydrophilic regions of the protein. Therefore, these alterations in the secondary structures, occurred following efficient IBs solubilization by low concentration of chemicals, could be related to enhancement in rSK biological potency previously observed.
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1. Introduction

Escherichia coli (*E. coli*) has been used as an integral host for production of recombinant proteins in biopharmaceutical industry; however, production of recombinant proteins in *E. coli* has been associated with serious challenges. One major part of challenges is related to the fact that the overexpression of recombinant proteins in *E. coli* often leads to the formation of misfolded protein aggregates, called inclusion bodies (IBs), mainly because of differences in folding pathways and physicochemical conditions between expression and natural hosts [1]. Although recombinant protein production via inclusion body formation system looks attractive due to attributes such as relatively straightforward IB generation to high yields, good supply of soluble protein after applying a solubilization treatment and potential clear-cut purification of target protein to high purities, IB proteins mostly contain non-functional target proteins which need to be solubilized by denaturants (e.g., high concentrations of chaotropic agents including urea and guanidinium chloride) and then refolded to functional (bioactive) counterparts [2,3]. The solubilization of IBs protein by denaturation is a simple process; however, the successful recovery of correctly-folded bioactive proteins from completely-denatured IB proteins is often a difficult step. For instance, large-scale refolding of recombinant streptokinase (rSK) from IBs separated from *E. coli* cells and solubilized in 4 M urea has led to a final product with biological activity much lower than the natural protein (i.e., the protein obtained from beta-hemolytic streptococci) [3].

Due to the inefficiency of the aforementioned route of production for most proteins prone to forming IBs, directing such proteins into the periplasmic space (the region between the cytoplasmic and outer membrane) of *E. coli* has been considered as a potent alternative route of production [4,5]. Nevertheless, recombinant protein expression in the periplasm has been also come across with serious problems, relevant to the stress on the bacterial cells during cultivation process in bioreactor. For example, in such system, the accumulation of the recombinant protein in various locations within and outside the bacteria host cells and unplanned cell lysis cut the benefits of periplasmic expression and compromise process robustness [6,7]. Hence, periplasmic production of recombinant proteins, in actual fact, is complicated and a number of stress minimization strategies

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should be established in order to optimize issues such as cell viability, process robustness (e.g., definite product accumulation in the periplasm which assures simple purification), productivity, etc.

In parallel with endeavors for the periplasmic expression of the aggregation-prone recombinant proteins as soluble form, further investigations (e.g., attempts for finding new solubilization and refolding methods) have been carried out on the IBs production route to increase the recovery of bioactive recombinant proteins. In the past it was actually believed that IBs were formed by inactive protein; consequently, the procedures used for protein solubilization were based on a denaturalization and refolding process. However, currently, it has been broadly proven that IBs are formed by active proteins (at least partially) and, therefore, the protocols used for IBs solubilization have been modified. For example, alkyl alcohols (such as 6 M n-propanol), which have helix-stabilizing properties and unfold the proteins due to their hydrophobic interactions as well as ability for lowering the dielectric constant of the medium, have been used for solubilization of IB proteins and successful recovery of bioactive target protein [8]. Also, simultaneous use of relatively low concentration (2 M) of urea and high pH (pH 12) has been effective for recovery of bioactive protein in high yield from *E. coli* [9]. These kinds of solubilization strategies have been utilized based on the understanding of the fact that protein molecules in inclusion body aggregates contain native-like structure. Therefore, native-like secondary structure protection by 'mild solubilization' of proteins from IBs, unlike conventional harsh solubilization methods (e.g. 4–8 M urea and guanidinium chloride) which completely unfold the solubilized proteins, could improve the recovery of bioactive target protein [10]. Hence, if high yield recovery of bioactive protein from IBs becomes successful by employing such strategies, the production of recombinant proteins via IB route in *E. coli* would be more desirable than before for biotechnology industry.

In the current study, low and very low concentrations of chemicals (including urea and anionic detergents) were used as 'mild solubilization' materials in the process of rSK solubilization from IBs isolated from *E. coli* cells. The obtained protein following solubilization and refolding process (with purity higher than 95%) was used for circular dichroism (CD) studies. In order to compare the secondary structures of final target protein subsequent to solubilization with different chemicals, rSK obtained through conventional solubilization (i.e., 4 M urea) and purification methods was used as a reference for structural studies.

2. Materials and methods

2.1. Preparation and solubilization of IBs

rSK was produced as IBs through batch fermentation process using transformed *Escherichia coli* W3110 (ATCC 27325), extracted and washed as described previously [3]. Subsequent to separation from the supernatant and washings, IBs (4 g wet weight) were resuspended in 20 mL of TE buffer (10 mM Tris–HCl and 1 mM EDTA, pH 7.2) and the suspension was distributed, in 0.5 mL fractions, into Eppendorf microtubes. The microtubes were centrifuged at 15,800 × *g* for 10 min and the pellets were frozen at −20 °C until required for further experiments.

For protein solubilization from the washed IBs, urea at the concentrations of 2 M and 4 M was used singly and also this chemical at low concentrations (0.5 M and 1 M) was used in combination with low and very low concentrations (0.1% or 0.05%) of the anionic detergents sodium dodecyl sulfate (SDS) and sodium lauroyl sarcosinate (SLS). All chemicals were freshly prepared in 20 mM Tris-HCl, pH 7.5, and the IBs were suspended in the buffered chemicals in a 20% (w/v) ratio, for 2.5 h at room temperature with brief intermittent vortexing every 10 min. Following centrifugation (10,000 × *g*, 20 min), the supernatant derived from each chemical solubilization was used for subsequent refolding and purification processes.

2.2. Refolding and purification of the solubilized rSK by chromatographic methods

Refolding of solubilized rSK and its purification (if required) were carried out according to the procedure described in Beiroti et al. [3]. Briefly, the pH of the solubilized inclusion body was reduced to 6.0 in order for reducing proteins accompanying streptokinase. Following centrifugation (10,000 × *g*, 20 min) and filtration through 0.45 filters, the pH of the solubilized inclusion body solution was increased to 7.5 and this solution was used in further chromatographic methods. In order to refold target protein via removing the chaotropic agent (urea) and other chemicals from the samples containing solubilized proteins, 7 mL of the solution obtained from the pH reduction process was loaded onto a column which was packed with 27 mL of Sephadex G-25 (GE Healthcare, Life Sciences, Uppsala, Sweden) and equilibrated with 20 mM Tris-HCl buffer and 200 mM NaCl, pH 8.5. After loading onto the gel filtration column, elution was made with 10 mL of the equilibration buffer and the eluates were analyzed by SDS-poly-acrylamide gel electrophoresis (SDS-PAGE) for estimation of target protein purity. In the case that the purity of the refolded target protein was lower than 95%, additional purification was done by hydrophobic interaction chromatography (HIC). For this purpose, $(NH_4)_2SO_4$ at a final concentration of 4.5% (w/v) was added to the elution sample collected from the gel filtration column and the sample was loaded onto a HIC column, which was packed with 4.8 mL of Fractogel TSK-Butyl 650 S (Tosoh Bioscience, Japan) and equilibrated with 20 mM Tris-HCl buffer and 4.5% $(NH_4)_2$ SO_4), pH 8.5. The washing of the column was performed with 20 mM Tris-HCL buffer and 2.2% $(NH_4)_2SO_4$, pH 8.5. Elution was made using 3.5 mL of 20 mM Tris-HCl buffer containing 1.1% $(NH_4)_2SO_4$, pH 8.5. The eluate was analyzed using SDS-PAGE and Lowry total protein quantification method (BSA was used as the standard protein). Streptokinase purity in elution fractions was evaluated by densitometry analysis of the SDS-PAGE gel using AlphaEaseFC™ software.

2.3. Circular dichroism (CD) studies

After inclusion body solubilization with various chemicals and target protein refolding/purification), the elution fractions with >95% purity were used for CD studies. The protein content in such elution fractions was adjusted at 1 mg/mL, by dilution or concentration, and the solutions were dialyzed overnight against 25 mM sodium phosphate buffer, pH 7.0 to eliminate the components (e.g., chloride ions as part of Tris-HCl buffer) which interfered with CD measurements. The CD spectra of 25 mM sodium phosphate buffer alone and rSK plus buffer were measured using a spectropolarimeter (AVIV-215). The baseline CD spectrum of the buffer was taken away from the spectrum containing the protein to yield the true protein CD spectrum (i.e., intrinsic spectrum). The mean residue ellipticity $[\theta]$mrw at wavelength λ is quoted in units of deg cm^2/dmol and is calculated as follows:

$$[\theta]\text{mrw},\ \lambda = \text{MRW} \times \theta\lambda / 10 \times \text{d} \times \text{c}$$

where θ is the observed ellipticity (degrees) at wavelength λ, d is the path length (cm), and c is the concentration (g/mL).

MRW is the mean residual weight for the peptide bond and is given by:

$$\text{MRW} = M/(N\text{-}1)$$

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