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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.

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:

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

Table 1
Changes in the mean residue ellipticities (at the key wavelengths) and the content of secondary structures of rSK in dependence on IBs solubilization by various chemicals.

Chemical	$-[\theta]_{222}$	$-[\theta]_{208}$	α-Helix (%)	Antiparallel β-Sheet (%)	Parallel β-Sheet (%)	Turn + Random structures (%)
4 M U	748.8	1021	12.38 ± 0.38	35.0 ± 0.92	5.04 ± 0.56	48.26 ± 0.77
2 M U	719.2 (−4)	853.5 (−16.5)	$14.21 \pm 0.5^{*}$	$38.9 \pm 0.87^{*}$	$8.06 \pm 0.04^{*}$	$39.81 \pm 0.65^{*}$
1 M U, 0.05% SLS, 0.05% SDS	653.2 (−12.8)	1028.7 (1.0)	$14.40 \pm 0.11^{*}$	35.40 ± 0.26^{ns}	5.0 ± 0.5^{ns}	$45.84 \pm 0.39^{*}$
0.5 M U, 0.1% SDS	590.6 (−21.2)	801.2 (−21.6)	$13.80 \pm 0.12^{*}$	$39.40 \pm 0.61^{*}$	$10 \pm 0.05^{*}$	$36.87 \pm 0.59^{*}$
0.5 M U, 0.1% SLS	811.4 (8.5)	901.2 (−11.8)	$14.50 \pm 0.44^{*}$	$42.70 \pm 0.26^{*}$	$8.20 \pm 0.13^{*}$	$35.50 \pm 0.49^{*}$

Negative and positive values in parentheses indicate decreased and increased percentages of the mean residue ellipticity, respectively, compared to 4 M urea. Data for each secondary structure category was analyzed by 1-way ANOVA analysis with a Tukey Multiple Comparison Test, using Minitab17.
* The mean in the treatment is significantly different ($p < 0.05$) from the mean in 4 M urea.

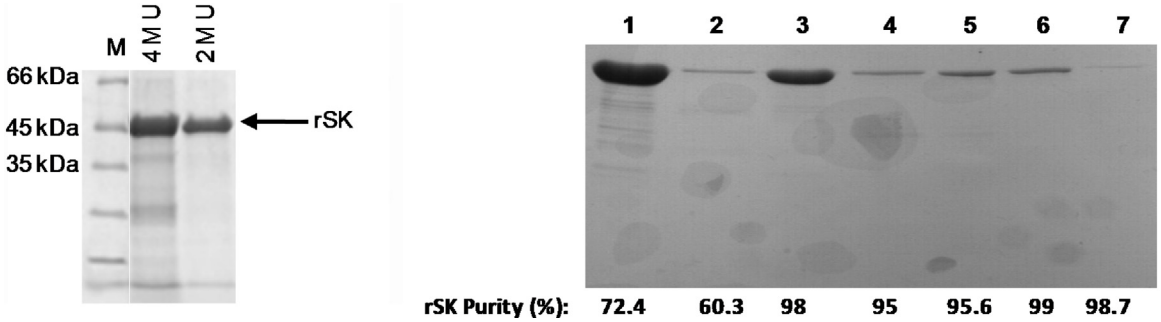

Fig. 1. SDS-PAGE analysis for the IBs' solubilization by 2 and 4 M urea (left) and the purification of rSK from solubilized IBs (in 4 M urea) on a HIC column (right). Tris-HCl buffer (20 mM) plus 4.5% $(NH_4)_2 SO_4$, pH 8.5 was employed for the equilibration and wash. Elution was achieved by 20 mM Tris-HCl buffer and 2.2% $(NH_4)_2SO_4$, pH 8.5. Lane: 1 – load; 2 – flowtrough; 3 to 7 – elution fractions.

where M is the molecular mass of the polypeptide chain (in Da), and N is the number of amino acids in the chain; the number of peptide bonds is N-1.

2.4. Statistical analyzes

In order to reveal the statistical significance in the comparison between the treatments, data shown in Table 1 was analyzed by 1-way ANOVA analysis with a Tukey Multiple Comparison Test, using Minitab17, for each secondary structure category.

3. Results and discussion

Following solubilization of IBs with 4 M urea, purification of rSK was done by HIC. The purity of rSK in different stages of HIC has been illustrated in Fig. 1. According to this figure, the purity following solubilization with 4 M urea is 72% which is augmented by around 95% in the elution fractions of this chromatography process. It should be pointed out that following solubilization with low concentration of single or combined chemicals, the purity of rSK was normally very high (≥95%) [11, reference in press] and hence directly solubilized rSK in such cases, following refolding and dialysis processes, was utilized for CD studies. The accuracy of purity analysis by SDS-PAGE analysis was confirmed by HPLC (column used was wide 10 μm pore Vydac218TP C-18 of 250 mm × 4.6 mm).

CD is an excellent method for analyzing the secondary structure of proteins in solution, and valuable estimates of protein conformation can be acquired from data obtained only between 240 and 200 nm [12]. In the present work, CD was employed to study the structural alterations of rSK which was solubilized from IBs by means of different chemicals and then refolded/purified by chromatographic techniques. As the CD spectrum of a protein must be adequately intense for interpreting the data and the intensity of a CD spectrum is relied directly on the protein concentration; therefore, the CD spectra of rSK was collected at a protein concentration of 1 mg/mL. Also, during CD measurements, it is important to maintain the total absorbance of the sample (i.e., protein and buffer) within rational ranges to avoid excessive noise; therefore, samples must be free of optically active substances [13]. Chloride ions, as part of Tris-HCl buffer and NaCl, absorb strongly in the far-UV and hence are one of the worst interfering components while conducting CD studies [14]. In the present work chloride ions were excluded from the protein solution (subsequent to rSK refolding and purification) by dialysis against sodium phosphate buffer in order that reliable CD data can be obtained and analyzed.

The different types of regular secondary structure found in proteins give rise to characteristic CD spectra in the far-ultra violet (UV) range. The CD spectrum of alpha helical protein takes over the region between 240 and 200 nm, with two reasonably intense negative bands at about 222 and 208 nm [13,15]. The intrinsic CD spectra of rSK have been illustrated in Fig. 2 and it is evident that the spectra peak around 222 and 208 nm (with more intense band at 208 nm). It has been previously reported that α + β proteins display a more intense band at 208 nm [16], and native SK has been classified as α + β protein [17].

As it is evident from Fig. 2, the intensity of the CD signals of rSK obtained through different IBs chemical solubilization methods was changed to different extents, when compared with the signals of control (i.e., the pure protein obtained through IBs solubilization by 4 M urea). For the far-UV CD measurements, the mean residue ellipticity is often used as a unit for the presentation of CD data [14,18]; therefore, this parameter was used to make estimates for the changes in the signal of rSK at 208 and 222 nm (Table 1). Based on the values of the mean residue ellipticity at the key wavelengths, it is evident that changes in the CD signals of the protein by various chemicals are varied.

The conformational properties of native streptokinase (i.e., isolated from group C streptococci) have been assessed by various techniques [17,19,20]. For example, the content of secondary structure elements of native streptokinase has been estimated by analysis of circular dichroism, infrared and Ramnan spectra and values in the range of 14–23% α-helices, 38–46% β-sheets, 10–30% turns and 12–23% residual structures have been found [20]. In the

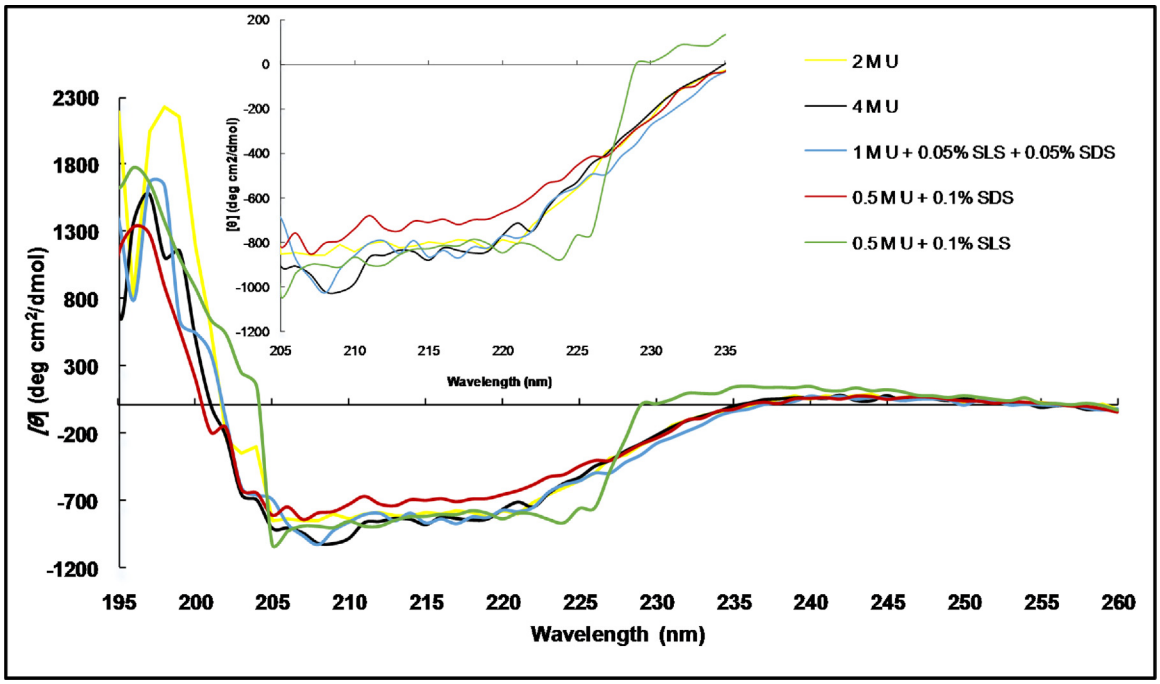

Fig. 2. Far-uv intrinsic CD spectra of pure rSK (with purity ≥95%) following solubilization of proteins from IBs by various chemicals. Urea at the concentrations of 2 M and 4 M was used singly and also this chemical at low concentrations (0.5 M and 1 M) was used in combination with 0.05% or 0.1% of the anionic detergents for solubilization of IB proteins.

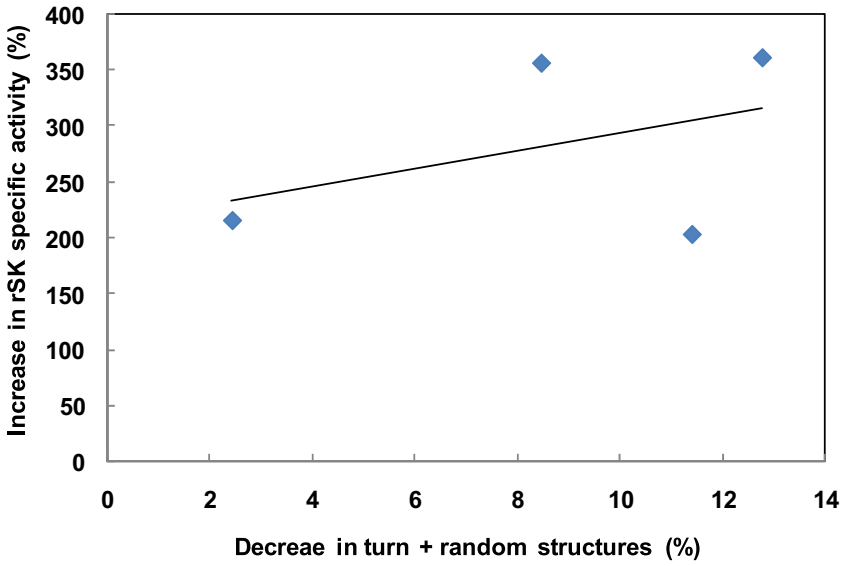

Fig. 3. Correlation between rSK biological activity and the percentage of random structures obtained from CD analysis.

current study, the secondary structures of rSK were determined by CDNN (secondary structures content analysis of the CD spectra) following various chemical solubilization methods (Table 1). According to the rSK secondary structural distribution in Table 1, the target protein obtained following IB solubilization by different chemicals contained different amount of secondary structures, being 12.4–14.5% α-helices, 40–51% β-sheets and 35.5–48.3% turns + residual structures. It is evident from the results presented in Table 1 that with reducing the concentration of chemicals in such treatments, the percentage of turn plus unstructured is decreased (by 13%), while the percentage of other structures has been increased. As the hydrophobic regions in random structures are more exposed, the propensity of aggregation in such structures is increased [21]. Our previous studies [11, reference in press] have shown that use of low concentration of chemicals for solubilization of rSK from IBs can increase the biological activity (potency) of the target protein by 360% (3.6 times), compared to IB solubilization by high concentration of urea. The correlation between rSK biological activity and the percentage of random structures obtained from CD analysis has been shown in Fig. 3. According to the results presented in Fig. 3, reducing the percentage of random structures in the protein following solubilization by low concentration of chemicals could be the reason for the increase observed in specific activity. In native streptokinase, the regions of highest probability

for interacting with plasminogen are hydrophilic areas occupying the surface of the molecule [17]. It is conjectured that such hydrophilic regions are masked when the hydrophobicity of the molecule is increased. Thus, structural changes which lead to a decrease in rSK hydrophobicity would further expose the hydrophilic regions of the protein; as a result, an increased biological potency would be achieved by facilitating rSK interaction with plasminogen. However according to the results demonstrated in Fig. 3, the correlation between the reduction in random structures of rSK and the rise in protein specific activity was not necessarily linear. Although the reduction in random structure throughout the process using 0.5 M U, 0.1% SDS was more than most of the treatments, the increase in the protein specific activity was the lowest. Streptokinase contains three human plasminogen-binding domains, namely α (residues 1–150), β (residues 151–287) and γ (residues 288–414), amongst which α domain plays an integral role in plasminogen activation [22]. Alterations in the secondary structures can occur inside and/or outside of the protein's plasminogen-activation site (i.e. α domain); hence, the proteins with significantly reduced random structure may or may not show significant increase in their biological activity depending on the site in which alteration is occurred. Other studies have also demonstrated that proteins with significantly modified secondary structures may not have altered biological activity [23].

Overall, this study shows that the use of low concentration of single or combined chemicals can decrease the random structures, which subsequently lowers the hydrophobicity along with the chance of protein aggregation which could be linked to final improvements in renaturation and biological potency of the target protein.

4. Conclusions

Extreme care must be taken when using IB expression and refold pathways so that the refolded recombinant protein reaches the biological potency close to the natural (native) protein. Structural characterization of recombinant streptokinase following its recovery from inclusion bodies using "harsh" or "mild" chemical solubilization has not been conducted yet. We have previously used low and very low concentrations of chemicals (i.e., urea and detergents) for solubilization of IBs isolated from *E. coli*, and substantial improvement in biological potency of final refolded rSK has been observed [11, reference in press], compared to the protein obtained by IBs solubilization in high concentration of urea. In the present work, we demonstrated that the use of low concentrations of chemicals (singly or in combination) for solubilization of IBs could lead to dissimilar structural conformations in the final recovered target protein. The use of low concentrations of chemicals distinctively reduced the percentage of turn plus random structures in the protein molecules which could result in decrease in hydrophobicity and increase in streptokinase-plasminogen interaction, phenomena which could be linked to final improvement in the renaturation and biological potency of the target protein under solubilization of inclusion body proteins by low concentrations of chemicals.

Declarations of interest

None.

Acknowledgments

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References

[1] C. Wyre, T.W. Overton, Use of a stress-minimisation paradigm in high cell density fed-batch *Escherichia coli* fermentations to optimise recombinant protein production, J. Ind. Microbiol. Biotechnol. 41 (2014) 1391–1404.
[2] T.W. Overton, Recombinant protein production in bacterial hosts, Drug Discov. Today 18 (5) (2014) 590–601.
[3] A. Beiroti, A. Kavianpour, M. Sepahi, F. Arsalani, P. Tavakoli Zaniani, R. Jalalirad, Effect of post-solubilization conditions on the yield and efficiency of rSK purification at large-scale, Protein Expr. Purif. 101 (2014) 127–132.
[4] D.P. Humphreys, M. Sehdev, A.P. Chapman, R.G.B.J. Smith, L.M. King, D.J. Glover, D.G. Reeks, P.E. Stephens, High-level periplasmic expression in *Escherichia coli* using a eukaryotic signal peptide: importance of codon usage at the 5′ end of the coding sequence, Protein Expr. Purif. 20 (2000) 252–264.
[5] P. Natale, T. Brüser, A.J.M. Driessen, Sec- and Tat-mediated protein secretion across the bacterial cytoplasmic membrane—distinct translocases and mechanisms, Biochim. Biophys. Acta 1778 (2008) 1735–1756.
[6] R. Jalalirad, Production of antibody fragment (Fab) throughout *Escherichia coli* fed- batch fermentation process: changes in titre, location and form of product, Electron. J. Biotechnol. 16 (3) (2013), doi:http://dx.doi.org/10.2225/vol16-issue-fulltext-15.
[7] C.C. Hsu, O.R.T. Thomas, T.W. Overton, Periplasmic expression in and release of Fab fragments from *Escherichia coli* using stress minimization, J. Chem. Technol. Biotechnol. 91 (2016) 815–822.
[8] S.M. Singh, A. Sharma, A.K. Upadhyay, A. Singh, L.C. Garg, A.K. Panda, Solubilization of inclusion body proteins using n-propanol and its refolding into bioactive form, Protein Expr. Purif. 81 (2012) 75–82.
[9] R.H. Khan, K.B.C. Appa Rao, A.N.S. Eshwari, S.M. Totey, A.K. Panda, Solubilization of recombinant ovine growth hormone with retention of native-like secondary structure and its refolding from the IBs of *Escherichia coli*, Biotechnol. Prog. 14 (1998) 722–728.
[10] A. Singh, V. Upadhyay, A.K. Upadhyay, S.M. Singh, A.K. Panda, Protein recovery from IBs of *Escherichia coli* using mild solubilization process, Microb. Cell Fact. 14 (2015) 41–51.
[11] A. Mohammadian, H. Kaghazian, A. Kavianpour, R. Jalalirad, Solubilization of inclusion body proteins using low and very low concentrations of chemicals: implication of novel combined chemical treatment designs in enhancement of post-solubilization target protein purity and biological activity, *J. Chem. Technol. Biotechnol.* (in press). doi:https://doi.org/10.1002/jctb.5525.
[12] N.J. Greenfield, Methods to estimate the conformation of proteins and polypeptides from circular dichroism data, Anal. Biochem. 235 (1996) 1–10.
[13] A.J. Miles, B.A. Wallace, Synchrotron radiation circular dichroism spectroscopy of proteins and applications in structural and functional genomics, Chem. Soc. Rev. 35 (2006) 39–51.
[14] S.M. Kelly, T.J. Jess, N.C. Price, How to study proteins by circular dichroism, Biochim. Biophys. Acta 1751 (2005) 119–139.
[15] W.C. Johnson, Secondary structure of proteins through circular dichroism spectroscopy, Ann. Rev. Biophys. Biophys. Chem. 17 (1988) 145–166.
[16] W.C. Johnson, Extending circular dichroism into the vacuum UV and its application to proteins, Photochem. Photobiol. 44 (3) (1986) 307–313.
[17] J.T. Radek, F.J. Castellino, Conformational properties of streptokinase, J. Biol. Chem. 264 (17) (1989) 9915–9922.
[18] G. De Sanctis, F. Ascoli, M. Brunori, Folding of apominimyoglobin, Proc. Natl. Acad. Sci. U. S. A. 91 (1994) 11507–11511.
[19] H. Fabian, D. Naumann, R. Misselwitz, O. Ristau, D. Gerlach, H. Welfle, Secondary structure of streptokinase in aqueous solution: a Fourier transform infrared spectroscopic study, Biochemistry 31 (28) (1992) 6532–6538.
[20] H. Welfle, R. Misselwitz, H. Fabian, W. Damerau, Conformational properties of streptokinase—secondary structure and localization of aromatic amino acids, Int. J. Biol. Macromol. 14 (1992) 9–18.
[21] S.M. Singh, A.K. Panda, Solubilization and refolding of bacterial inclusion body proteins, J. Biosci. Bioeng. 99 (4) (2005) 303–310.
[22] L.V. Mundada, M. Prorok, M.E. DeFord, M. Figuera, F.J. Castellino, W.P. Fay, Structure-function analysis of the streptokinase amino terminus (residues 1–59), J. Biol. Chem. 278 (27) (2003) 24421–24427.
[23] R. Jalalirad, Selective and efficient extraction of recombinant proteins from the periplasm of *Escherichia coli* using low concentrations of chemicals, J. Ind. Microbiol. Biotechnol. 40 (10) (2013) 1117–1129.