Protein Expression and Purification xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Protein Expression and Purification

journal homepage: www.elsevier.com/locate/yprep

Effect of post-solubilization conditions on the yield and efficiency of recombinant streptokinase purification at large-scale

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ARTICLE INFO

Article history:
Received 1 April 2014
and in revised form 7 June 2014
Available online xxxx

Keywords:
Recombinant streptokinase
Inclusion bodies
Downstream processing
Post-solubilization
Chromatographic purification

ABSTRACT

Streptokinase, a plasminogen activator which converts plasminogen to plasmin and consequently promotes fibrinolysis, is the leading drug for treating acute myocardial infarction in developing countries and its production is industrially demanded. In this work, the substantial influence of inclusion body (IB) post-solubilization condition on the performance of a sequential chromatography method for large-scale purification of recombinant streptokinase was demonstrated. In the preliminary experiments, various post-solubilization pH conditions were studied, and it was shown that the pH value of solubilized inclusion bodies (i.e., in 4 M urea) had a marked impact on the purity of streptokinase obtained at the end of post-solubilization process. When the pH value of the solution containing solubilized IBs was decreased from 7.5 to 6.5 and 6.0, the greatest increases (10% and 27%, respectively) in streptokinase purity occurred. The influence of different post-solubilization pH conditions on the efficiency and yield of large-scale chromatographic purification methods was next investigated. When the solubilized IBs solution with pH adjusted to 6.0 was utilized for subsequent sequential chromatography process, the complete elution peak with high overall yield (91.3%) and purity (98%) was achieved. In comparison to this, while the sequential chromatography procedure was instigated by using the solubilized IBs solution with pH 4.2, four elution fractions (EF1 to EF4) with disparate target protein purities (i.e., 57%, 77.3%, 91.4% and 86.7%, respectively) were attained, the process was incompletely effective, and the highest recovery and purity figures (81.8% and 91.4%, respectively, belonging to EF3) were much lower than those for the earlier process.

Introduction

Blood clot (thrombosis), which is the occlusion of blood cells in a matrix of the protein fibrin in the human circulation system, occurs as a result of the failure of haemostatic system and can create serious outcomes such as stroke, pulmonary embolism, deep vein thrombosis and myocardial infarctions [2]. The enzyme responsible for dissolution of the fibrin clot in mammalian circulation is plasmin which is produced from the conversion of the inactive protein plasminogen, being modulated by plasminogen activators and inhibitors [7,12,27]. Commonly-used plasminogen activators include tissue type (tPA),[1] urokinase (UK), and streptokinase (SK) [15]. Recombinant types of these agents have been utilized in clinical interventions for the clot dissolution [2,27,28]. tPA and UK are glycoproteins produced by the vascular endothelial cells and kidneys, respectively, while SK is a bacterial protein and does not exist in human circulation [3,5,30]. The high efficiency of tPA for the fibrin clot leads to the activation of the clot-bound plasminogen; therefore, tPA is a poor activator of circulatory plasminogen [2]. Unlike tPA, SK and UK are activators for both circulating and clot-bound plasminogen [2]. Amongst these plasminogen activators, streptokinase is the leading drug of choice for thrombolytic treatment prominently due to its cost-effectiveness and comparative clinical trials [16,22,25].

The fibrinolytic activity of fibrinolysin was discovered by Tillet and Garner [14,31]. The term 'streptokinase' was proposed by Christensen and MacLeod in 1945 [9] to replace the term fibrinolysin. Amongst major trials of streptokinase carried out during the early 1980s, the one conducted by GISSI (Gruppo Italiano per la Sperimentazione della Streptochinasi nell'Infarto Miocardico) was the most comprehensive appraisal for validation and establishment of a protocol for streptokinase application in acute myocardial infarction (AMI) [19,20,29,33]. Streptokinase has been widely

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[1] *Abbreviation used:* tPA, plasminogen activators include tissue type; UK, urokinase; SK, streptokinase; AMI, acute myocardial infarction; DSP, downstream processing; IB, inclusion body; rSK, recombinant streptokinase; SDS–PAGE, SDS–poly-acrylamide gel electrophoresis; GFC1, gel filtration chromatography number 1; AEC1, anion exchange chromatography number 1; DEAE, diethylaminoethanol; GFC2, gel filtration chromatography number 2; AEC2, anion exchange chromatography number 2; API, active pharmaceutical ingredients.

http://dx.doi.org/10.1016/j.pep.2014.06.007
1046-5928/

used in developing counties for treating acute myocardial infarction [10,29].

Streptokinase is a 47 kDa protein composed of 414 amino acid residues [23]. This protein has an isoelectric point of 4.7, does not contain cysteine or disulfide bonds and is naturally produced and secreted by several strains of beta-hemolytic streptococci [6,11,13,29]. Streptokinase activates plasminogen in an indirect manner by forming a 1:1 ratio complex which rapidly becomes proteolytically active. This complex subsequently takes action on plasminogen molecules in the circulation to generate plasmin which rapidly causes clot dissolution. The low yield of streptokinase production by native bacterial strains and the pathogenicity of such natural hosts are the salient incentives for exploitation of recombinant DNA technology in favor of the production of this protein [18,23,26]. Recombinant streptokinase (rSK) has been assessed in clinical trials in patients with acute myocardial infarction (AMI) and the investigations suggested that rSK was as effective as natural SK; furthermore, a pharmacovigilance program has proven the rSK safety in AMI patients in a routine practice [4,10,21,32,24].

Escherichia coli (*E. coli*) is the most commonly used host in industry for high expression of valuable heterologous recombinant proteins such as streptokinase [8,18]. In downstream processing (DSP), efficient purification of streptokinase from the feedstock obtained from the bacterial host cultivation is a key part in order to get a great yield of highly-pure pharmaceutical-grade target protein with an appropriate biological activity. The conventional route for purifying proteins produced in inclusion body (IB) form involves cell disruption, separation of the insoluble IBs, washing, solubilization using a highly concentrated chaotrope such as urea or guanidine hydrochloride, and eventually several chromatographic purification stages followed by refolding of the target protein [17]. The overall objective of this work was to investigate the effect of different post-solubilization pH conditions on the efficacy of chromatographic processes for purification of recombinant streptokinase. For this purpose, the post-solubilization process was first studied in various pH conditions and the optimized pH condition to reach the highest achievable purity of the target protein was determined. Secondly, the effect of different solubilization pH conditions on the efficiency and yield of large-scale chromatographic purification processes was investigated.

Materials and methods

Recombinant streptokinase was produced as inclusion bodies through batch fermentation process using *E. coli* W3110 (ATCC 27325) transformed with a plasmid containing the gene for streptokinase under the control of the tryptophan expression system. Following harvesting by centrifugation at 10,000*g* for 10 min, the bacterial cells (11 kg wet weight) were resuspended in 40 L of TE buffer (10 mM Tris–HCl and 1 mM EDTA, pH 7.2), in a 22% (w/v) ratio, and disrupted using a continuous flow high pressure homogenizer (Avestin Co., Canada) operated at 600 Bar and 5 °C. The supernatant was separated from the cell debris by centrifugation at 10,000*g* for 40 min, and the pellet (4 kg wet weight) containing inclusion bodies was washed (at a 10% w/v ratio) three times consecutively. The first and second washes were done using TE buffer (10 mM Tris–HCl and 1 mM EDTA, pH 7.2) containing 1% Triton X-100 and 1 M urea, respectively, and the third wash was made using TE buffer. In order for inclusion body solubilization, the washed pellet (1100 g wet weight) was resuspended in 5.5 L of 4 M urea prepared in TE buffer, pH 7.5, in a 20% (w/v) ratio. Subsequent centrifugation (10,000*g*, 20 min), the supernatant containing solubilized proteins was used for further experiments.

Protein precipitation studies

For this purpose, the pH of the samples (20 ml) containing solubilized proteins was reduced to different values, ranged between 4.0 and 7.0, by gradual addition of 3 M HCl. The samples were centrifuged at 10,000*g* for 15 min (Eppendorf AG, Biopaths, Germany) and all supernatants were analyzed by SDS–poly-acrylamide gel electrophoresis (SDS–PAGE) and Lowry total protein quantification method (BSA was used as the standard protein).

Purification of recombinant streptokinase by chromatographic techniques

Following inclusion body solubilization in 4 M urea prepared in TE buffer (pH 7.5) in a 20% (w/v) ratio (i.e., 1100 g washed pellet in 5.5 l urea), the pH of the solubilized inclusion body was reduced to 6.0 or 4.2 to investigate the effect of different pH values at this stage on the performance of subsequent chromatographic methods. After 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.

Gel filtration chromatography number 1 (GFC1)

In order to remove the chaotropic agent (i.e., 4 M urea) from the samples containing solubilized proteins and to achieve the renaturation of recombinant streptokinase, 7 L of the solution obtained from the pH reduction process was loaded onto a column which was packed with 27 L of Sephadex G-25 (GE Healthcare, Life Sciences, Uppsala, Sweden) and equilibrated with 20 mM Tris buffer and 200 mM NaCl, pH 8.5. After loading, elution was made with 10 L of the equilibration buffer and the eluate was utilized for further purification steps.

Anion exchange chromatography number 1 (AEC1) for separation of the host's DNA

For this purpose, the eluate (10 L) collected from GFC-1 process (pH 8.5 and conductivity 20 mS/cm) was loaded onto a column which was packed with 1.6 L of diethylaminoethanol (DEAE) Sepharose Fast Flow (GE Healthcare, Life Sciences, Uppsala, Sweden) and equilibrated with 20 mM Tris buffer and 200 mM NaCl, pH 8.5. The host's DNA was adsorbed to the chromatographic matrix during the loading process; the column flow through contained recombinant streptokinase was collected and utilized in the next chromatographic purification step.

Hydrophobic interaction chromatography

For this purpose, $(NH_4)_2SO_4$ at a final concentration of 4.5% (w/v) was added to the flow through sample collected from AEC-1 column. The sample was loaded onto a column which was packed with 4.8 L of Fractogel TSK-Butyl 650 S (Tosoh Bioscience, Japan) and equilibrated with 20 mM Tris buffer and 4.5% $(NH_4)_2SO_4$, pH 8.5. The washing of the column was performed using with 20 mM Tris buffer and 2.2% $(NH_4)_2SO_4$, pH 8.5. Elution was made using 3.5 L of 20 mM Tris buffer containing 1.1% $(NH_4)_2SO_4$, pH 8.5. The eluate was analyzed using SDS–PAGE and Lowry total protein quantification method.

Gel filtration chromatography number 2 (GFC2)

The eluate collected from the HIC process was desalted by GFC-2 to remove $(NH_4)_2SO_4$ present in the sample. For this purpose, the

Please cite this article in press as: A. Beiroti et al., Effect of post-solubilization conditions on the yield and efficiency of recombinant streptokinase purification at large-scale, Protein Expr. Purif. (2014), http://dx.doi.org/10.1016/j.pep.2014.06.007