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Simplified in vitro refolding and purification of recombinant human granulocyte colony stimulating factor using protein folding cation exchange chromatography

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ABSTRACT

Protein folding-strong cation exchange chromatography (PF-SCX) has been employed for efficient refolding with simultaneous purification of recombinant human granulocyte colony stimulating factor (rhG-CSF). To acquire a soluble form of renatured and purified rhG-CSF, various chromatographic conditions, including the mobile phase composition and pH was evaluated. Additionally, the effects of additives such as urea, amino acids, polyols, sugars, oxidizing agents and their amalgamations were also investigated. Under the optimal conditions, rhG-CSF was efficaciously solubilized, refolded and simultaneously purified by SCX in a single step. The experimental results using ribose (2.0 M) and arginine (0.6 M) combination were found to be satisfactory with mass yield, purity and specific activity of 71%, ≥99% and 2.6×10^8 IU/mg respectively. Through this investigation, we concluded that the SCX refolding method was more efficient than conventional methods which has immense potential for the large-scale production of purified rhG-CSF.

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1. Introduction

Recombinant human granulocyte colony-stimulating factor (rhG-CSF) is one of the most exciting new drugs developed in the past 20 years and also one of the five best-selling recombinant drugs in the world [1]. It is an effective hematopoietic growth factor which plays a vital role in proliferation, differentiation and activation of blood cells during neutropenia conditions in the treatment of cancer [2,3], bone marrow transplants [4] and in AIDS [5]. The rhG-CSF should be produced by recombinant DNA technology to satisfy therapeutic requirements. Recombinant protein expression in *Escherichia coli* (*E. coli*) has become the primary means of producing a large number of therapeutic proteins [6]. For the first time, Nagata et al. [7] and Souza et al. [38] reported the expression of rhG-CSF in *E. coli*. The protein is seen as accumulation of insoluble cytoplasmic aggregates (inclusion bodies). The process of purifying and recovering a protein from inclusion bodies involves many deliberations, including the extraction procedure. High concentrations of chaotropes like urea (8 mol/L) or guanidine-HCl (6 mol/L) are used to solubilize the target protein. Refolding is a necessary step for the solubilized inclusion bodies, and care should be taken for reduced protein loss and recovery of the bioactive protein [8]. It is generally achieved by removal of chaotropes with the exchange of refolding buffer (classical dilution) containing redox systems of reduced/oxidized thiols (GSH/GSSG). However, refolding yield is typically low with a possibility for mis-folding and subsequent loss of protein. Furthermore, handling large volume of diluted rhG-CSF protein solution for purification increases the costs of downstream processing. Therefore, development of alternative processes for efficient and simplified refolding of rhG-CSF is required.

In recent years, protein folding liquid chromatography (PFLC) has been used to refold the proteins with higher yields [9]. The PFLC technique besides preventing aggregation, it simultaneously purifies the protein during the chromatographic process owing to various kinds of biochemical and/or physicochemical interactions. It is an alternative approach to the dilution refolding method, which results in higher yield, reduces time and cost of downstream processing [10].

Ion exchange chromatography (IEC) is widely used chromatographic technique which has been used in 70% of the protein

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purification protocols [11]. The refolding and simultaneous purification of rhG-CSF by IEC is reported earlier [12] however, limited information on additives and their cooperative effect is available on protein refolding and purification. Therefore, in the present work, optimization of chromatographic conditions, the effect of external additives and their combinations on the mass yield, purity and specific activity of rhG-CSF have been investigated using protein folding-strong cation exchange (PF-SCX) chromatography method. The PF-SCX results were also compared with the classical refolding and purification methods. The purification process of rhG-CFS by SCX was supported with molecular docking [13] using Extra Precision (XP) docking protocol.

2. Experimental

2.1. Chemicals and instruments

Tris (hydroxymethyl) aminomethane, tri sodium citrate, potassium di hydrogen phosphate, sodium acetate, ammonium sulphate, arginine, glycerol, ribose, glucose, sucrose, urea, glycine and β-mercaptoethanol were purchased from Merck Millipore (Billerica, MA, USA). IPTG, bovine serum albumin (BSA), cysteinamine and PMSF were purchased from Sigma–Aldrich (Hamburg, Germany). The rhG-CSF standard was obtained from Roche (BASEL, Switzerland). Mini-PROTEAN 3 SDS-PAGE kit was obtained from Bio-Rad Laboratory Inc. (Hercules, CA, USA). The rhG-CSF was produced in a Minifors bioreactor (Infors-HT, AG, Switzerland).

The AKTA Prime Plus chromatographic system, preparative grade resins and cross flow filtration system (Quix Stand) were obtained from GE Health care (Uppsala, Sweden). The chromatography data was collected and evaluated using PrimeView Evaluation^{TM} v5.31 software. Hollow fiber cartridge (3 kDa) was obtained from GE Healthcare Bioscience Corp. (Westborough, MA, USA). RP-HPLC with SPD 20A Prominence UV/Vis detector was from Shimadzu (Kyoto, Japan). The Jupiter 5 μ C4 column (250 mm × 4.6 mm) obtained from Phenomenex (Torrance, CA, USA).

2.2. Production of rhG-CSF

The large scale production of rhG-CSF in *E. coli* was carried out in a fed batch mode with a working volume of 3-L. The production was accomplished at the following conditions: temperature 37 °C, pH 6.9, 40% dissolved oxygen (DO). The culture was induced with 1.0 mM IPTG at an OD_{600} of 0.9 (=dry cell weight 5.2 g/L) of the culture.

2.3. Solubilization and refolding of the inclusion bodies

The cells were collected from the fermented broth and lysed by repeated steps of homogenization and sonication using lysis buffer with 1 mM PMSF. The inclusion bodies were dissolved in 8.0 mol/L urea containing 10 mM Tris–HCl pH 8.8, 1 mM EDTA and 0.1 M DTT [14]. Before subjecting to the refolding, the protein solution was divided into two aliquots: one part has been used for refolding dilution process, which is a classical method and the other for in vitro refolding by SCX method. The refolding protein solution pH was adjusted to 12.5 by the addition of 2 N NaOH. The refolding buffer comprises 100 mM Tris pH 8.8, 0.1 M L-cysteine hydro chloride, 0.05 M L-cysteine 2 hydro chloride 0.1% Tween 20, 2 mM EDTA, 5% sucrose and 0.2 M urea. This refolding process was carried out at 18 °C with continuous stirring.

2.4. Cross flow filtration

The refolded rhG-CSF solution was simultaneously concentrated and clarified by using cross flow filtration (CFF) system with a constant volume of 500 mL. The protein solution was concentrated using 3 kDa hollow fiber molecular weight cut-off (MWCO) cartridge with flow path length of 30 cm with 650 cm^2 of the cross section area. The maximal flow rate from cartridge was maintained at 10 mL/min. The clarification was achieved by using constant pump speed at 350 rpm. The degree of concentration of the rhG-CSF is characterized by concentration factor (f) and retention factors (R) which are calculated using the following equations.

$$f = \frac{V_F}{V_C} \tag{1}$$

where V_F = feed volume and V_c = final retentate volume.

The retention factor is also important for the characterization of a target product, which is defined as, the distribution of components between permeate and retentate.

$$R = C_F - \frac{C_p}{C_F} \tag{2}$$

where C_F = concentration of feed and C_p = concentration of permeate.

2.5. Preparation of chromatography system and columns

The ÄKTA Prime plus chromatography system was used for the purification. The Bioprocess glass (BPG) column XK 16/20 (20 cm length × 1.6 cm ID) with thermostatic jacket was connected to the system. Sephacryl S-200, Sephadex G-25, SP-Sepharose FF and Capto SP ImpRes resins were used for the fractionation of rhG-CSF. 30 mL of each resin was packed with 0.3 Mpa pressure in 20% aqueous ethanol solution. The column was initially washed with 50 mL of 1.0 M NaCl solution at a constant flow rate of 2.0 mL/min. For each run, 2 mL of sample was directly loaded onto the column, and the pressure was maintained throughout the run between 0.1 and 0.2 MPa. 50 mL of the linear gradient program was run for all the purifications from 10% to 100% salt solution at a flow rate of 1.0 mL/min with the detection at 280 nm. All the purifications have been carried out at 20 °C temperature.

2.6. Purification strategies of rhG-CSF

2.6.1. Classical refolding dilution and purification

The experiments were carried out with various kinds of chromatography resins such as the Sephacryl S-200, Sephadex G-25 and Sepharose FF. The BPG column was loaded with respective resins with a bed height equivalent to 75% of the column length. The pH of the refolded sample was adjusted to 4.5 by the addition of 2 M phosphoric acid buffer. The refolded protein solution (2 mL) was loaded onto the column and equilibrated with 25 mM citrate buffer (pH 4.6) until the absorbance value returned to baseline. The rhG-CSF was eluted from the column using a linear gradient of 1.0 M NaCl solution.

2.6.2. On-column (in vitro) refolding with simultaneous purification of rhG-CSF

Preparative grade Capto SP ImpRes was used for on-column refolding with simultaneous purification of rhG-CSF. The IEC column was packed with a strong cation exchanger: Capto SP ImpRes with a 30 mL of bed volume. Two types of solutions were used for the purification of rhG-CSF. Solution A (equilibration buffer): 25 mM citrate buffer (pH 8.5); solution B (elution buffer): 1.0 M NaCl, 1 mmol/L EDTA, 3.0 mM GSH, 0.5 mM GSSG in solution A. The denatured protein sample was directly loaded onto the column and

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