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Refolding and purification of recombinant human granulocyte colony-stimulating factor using hydrophobic interaction chromatography at a large scale

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ABSTRACT

High level expression of recombinant human granulocyte colony-stimulating factor (rhG-CSF) in *Escherichia coli* (*E. coli*) usually forms insoluble and inactive aggregates, i.e. inclusion bodies. In the present work, high performance hydrophobic interaction chromatography (HPHIC) was applied to the refolding of rhG-CSF, which was solubilized by 8.0 mol L^{-1} urea from the inclusion bodies. First a laboratorial scale column (10 mm × 20 mm I.D.) was employed to study the refolding process. Several factors, including concentration of ammonium sulfate, pH of the mobile phase and flow rate, were investigated in details. The results indicated that the rhG-CSF produced by *E. coli* could be successfully refolded with simultaneous purification by using HPHIC. The refolding process was further scaled up by using a large column (50 mm × 200 mm I.D.). 200 mL of rhG-CSF solution solubilized by 8.0 mol L^{-1} urea, with a total amount of protein around 1.6 g, could be loaded onto the large column at one time. Under these conditions, the obtained rhG-CSF had a specific activity of 2.3×10^8 IU mg^{-1} and a purity of 95.4%, the mass recovery during the purification was 36.9%. This work might have great impact on practical production of rhG-CSF, and it also shed a light on protein refolding using liquid chromatography at large scales.

1. Introduction

Bacteria can produce large quantities of recombinant proteins in rapid, often inexpensive, fermentation processes. *Escherichia coli* (*E. coli*) is the most frequently used host cell in biotechnology for protein production, especially at large scales. However, when proteins are expressed in *E. coli*, they often form insoluble and inactive protein aggregates, called inclusion bodies (IBs). The typical strategy used to recover an active protein from inclusion bodies usually involves three key steps: firstly, inclusion bodies isolation and washing; secondly, solubilization of the aggregated protein; and finally, refolding of the solubilized protein. The refolding process is even more significant, it should be considered carefully to improve recovery of the bioactive protein.

Refolding is a process that leads to a transition in protein conformation from unfolded (denatured) to folded (native) state, it is generally initiated by reducing concentration of denaturant in the solubilized protein solution. The simplest method to initiate refolding is to dilute the unfolded protein in a certain refolding buffer. However, refolding yields are typically low. Recent years, liquid chromatography based protein refolding is extensively employed for various proteins [1–4]. From a scientific point of view, main branches of liquid chromatography used for protein purification, including size exclusion chromatography (SEC), hydrophobic interaction chromatography (HIC), ion exchange chromatography (IEC), and affinity chromatography (AFC), have been explored for protein refolding [5]. Stempfer et al. [6] fused a polycation tag containing hexa-arginine onto α-glucosidase, and refolded this fusion protein by IEC with a polyanionic support. Chromatographic refolding of rhodanese and lysozyme were performed under assisting of GroEL apical domain, DsbA and DsbC immobilized on cellulose, the assisted refolding yields were up to 80% for rhodanese and 100% for lysozyme, compared with 33% and 23%, respectively, obtained in the experiments without immobilized chaperones [7]. Altamirano et al. [8] immobilized molecular chaperone/disulfide isomerase/peptidylprolyl cis–trans isomerase on agrose resin and formed a tri-component stationary phase, they used this column to refold scorpion toxin Cn5, which could not be refolded at all using other methods. Its mass recovery was 87% and its bioactivity recovery was near 100%. A column packed with Triton X-100-coupled Sepharose High Performance was used to refolded green fluorescent protein-tobacco etch virus protease fusion protein using an increasing β-CD concentration gradient. The refolding yield was 46% at a protein concentration of 380 μg/mL [9]. Recombinant human granulocyte colony stimulating factor was refolded by using urea gradient size exclusion chromatography with a mass recovery of 46.1% [10]. Denatured/reduced lysozyme was refolded by an

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artificial molecular chaperone-ion exchange chromatography method at extremely high protein concentrations. When the initial protein concentration was 200 mg mL^{-1}, the activity recovery was over 60% [11]. Up to date, protein refolding using liquid chromatography is usually performed in small scales, in which small columns and small volumes of protein solution are commonly used. Lanckriet and Middelberg developed a continuous chromatographic protein refolding process based on preparative continuous annular chromatography equipped with a size exclusion resin, in which an annular column with a height of 60 cm, outer diameter of 15 cm and inner diameter of 13 cm (a bed volume of 2.6 L) was employed, 105 mg denatured lysozyme was loaded with a refolding yield of 72% [12]. To the best of our knowledge, there is no refolding conducted at a quite large scale using liquid chromatography, in which hundreds of milligrams of protein could be loaded onto the column.

Granulocyte colony stimulating factor (G-CSF) is one of several glycoprotein growth factors known as colony stimulating factors (CSFs), due to they support the proliferation of hemopoietic progenitor cells. G-CSF stimulates the proliferation of specific bone marrow precursor cells and their differentiation into granulocytes. It is distinguished from other CSFs by its ability to both stimulate neutrophilic granulocyte colony formation in semi-solid agar and to induce terminal differentiation of murine myelomonocytic leukemic cells in vitro. G-CSF has been shown to improve survival rates [13–15], this has led to clinical trials of G-CSF in non-neutropenic patients at risk for or with bacterial pneumonia. The hope in these trials has been that G-CSF would stimulate host defenses and improve microbial clearance, thereby decreasing inflammatory injury and improving survival rates. Because the source of nature human G-CSF is very limited, and it has been confirmed that the recombinant G-CSF (rhG-CSF) exhibits most, if not all, of the biological properties of the native molecule [16], so it must be produced large quantity by genetic engineering to meet the clinical requirement.

In the present paper, rhG-CSF expressed in *E. coli* was refolded with simultaneous purification by using high performance hydrophobic interaction chromatography (HPHIC) at a very large scale. A large column with dimensions of 50 mm × 200 mm I.D. was employed, 200 mL of rhG-CSF solution in 8.0 mol L^{-1} urea, with a total amount of protein around 1.6 g, was subjected onto the column at one time. Quite good refolding results were obtained.

2. Materials and methods

2.1. Instruments

A LC-10Avp high pressure liquid chromatograph (Shimadzu, Japan) consists of SPD-10A detector (UV and visible wavelength), LC-10ATvp pumps, SCL-10Avp system controller, DGU-12A degas unit and workstation was used for protein refolding at small scales. An ÄKTA explorer chromatographic system was used for protein refolding at large scales. All columns used were home-made with stainless steel. An Avanti™ J-25 centrifuge (Beckman coulter™, U.S.A.) was used for centrifugation. A 5 L fermentor (Braun, Germany) was used to cultivate *E. coli* for protein expression. A UV-1700 spectrometer (Shimadzu, Japan) was used for the determination of the total amount of proteins. An electrophoresis apparatus (GE Healthcare) and a CS-930 dual-wavelength thin layer chromatographic scanner (Shimadzu, Japan) were used for the determination of the purity of rhG-CSF.

2.2. Chemicals

Bovine serum albumin (BSA) and reagents used in gel electrophoresis were obtained from Sigma Co. (St. Louis, MA, USA), low molecular mass protein markers were purchased from GE Healthcare. All other chemicals were of analytical grade obtained from local suppliers.

2.3. Expression of rhG-CSF and recovery of rhG-CSF inclusion bodies

rhG-CSF was expressed in form of inclusion bodies with *E. coli* as the host cells. The inclusion bodies were recovered by centrifugation after cell disruption, and were purified by extensive washing with different buffers. The detailed procedures can be found in the previous work [17].

2.4. Solubilization of rhG-CSF from inclusion bodies

2.0 g of wet inclusion bodies were solubilized in 20 mL of 0.05 mol L^{-1} Tris buffer at pH 8.0 containing 2% Sarkosyl, 10 mmol L^{-1} GSH, 1 mmol L^{-1} GSSG. After centrifugation, the solubilized rhG-CSF was precipitated by 60 mL acetone and centrifuged again. The precipitate was re-dissolved in 20 mL of 8.0 mol L^{-1} urea, 0.05 mol L^{-1} Tris (pH 8.0), 1.0 mmol L^{-1} EDTA, then centrifuged again. The supernatant was collected and kept at 4 °C for subsequent use.

2.5. Refolding and purification of rhG-CSF using HPHIC at small scales

All chromatographic runs were carried out at ambient temperature. Solution A consisted of 3.0 mol L^{-1} $(NH_4)_2SO_4$, 0.05 mol L^{-1} Tris, pH 8.0, solution B consisted of 0.05 mol L^{-1} Tris, pH 8.0. The small scale HPHIC column (10 mm × 20 mm I.D.) was equilibrated with an expected percentage of solution B to achieve a certain ammonium sulfate concentration. 400 μL of the solubilized and denatured rhG-CSF in 8.0 mol L^{-1} urea was directly loaded onto the column via an injection valve. After washing the column with the equilibration buffer to UV absorbance achieved baseline, the elution of rhG-CSF was started by a linear gradient with a decreasing slope of ammonium sulfate concentration of 0.12 mol L^{-1} per minute, then the column was further eluted with the solution B for 15 min. UV detection was set at 280 nm. The fractions containing the rhG-CSF were pooled and adjusted to pH 4 by diluted hydrochloride acid, after that the solution was dialyzed against a storage solution containing 10.0 mmol L^{-1} sodium acetate at pH 4.0. And then it was used for the determination of protein concentration and bioactivity.

2.6. Refolding and purification of rhG-CSF using HPHIC at a large scale

The chromatographic run was carried out at ambient temperature. The large HPHIC column (50 mm × 200 mm I.D.) was equilibrated with solution A consisting of 1.5 mol L^{-1} $(NH_4)_2SO_4$, 0.05 mol L^{-1} Tris, pH 8.0 by using pump A. Then the pump A continuously subjected the solution A to the chromatographic system, while pump B was used to feed rhG-CSF solution in 8.0 mol L^{-1} urea, the flow rate ratio of pumps A to B was 9: 1, and the total flow rate was set at 10.0 mL min^{-1}. After sample loading, the column was washed with solution A to UV absorbance achieved baseline. The elution of rhG-CSF was completed by a linear gradient from 100% solution A to 100% solution B (0.05 mol L^{-1} Tris, pH 8.0) in 50 min, with a delay of 100 min. UV detection was set at 280 nm. The fractions containing the rhG-CSF were collected and treated as above for the determination of protein concentration and bioactivity.

2.7. Analytical procedures

The analytical methods followed Ref. [17]. The purity of the rhG-CSF was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The protein concentration was estimated by the Bradford method. The bioassay for measuring the bioactivity of the renatured rhG-CSF was based on the cell proliferation assay.

3. Results and discussion

Though protein refolding using liquid chromatography has been extensively reported, there is no protein refolding conducted at large scales using this method. In this work, high performance hydrophobic interaction chromatography (HPHIC) was applied to the refolding of rhG-CSF at a quite large scale. In order to investigate effect of experimental variables, such as concentration of ammonium sulfate, pH of the mobile phase and flow rate, a laboratorial scale column (10 mm × 20 mm I.D.) was employed first to investigate the refolding process.

3.1. Concentration of ammonium sulfate

During HPHIC, the solvent-accessible hydrophobic regions on the surface of proteins interact with the hydrophobic ligands on the adsorbent. According to the retention mechanism of proteins in HPHIC, this adsorption is salt-promoted so that the binding of proteins onto the stationary phase is usually carried out at high salt concentrations. Elution and separation of proteins, according to differences in the surface hydrophobicity of proteins, are in general brought about by decreasing the salt