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Refolding with Simultaneous Purification of Recombinant Human Granulocyte Colony-stimulating Factor from *Escherichia coli* Using Strong Anion Exchange Chromatography

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Abstract: The urea denatured recombinant human granulocyte colony-stimulating factor (rhG-CSF) which was expressed in *Escheriachia coli* (*E. coli*) was refolded with simultaneous purification by strong anion exchange chromatography (SAX) in the presence of low concentration of urea. The effect of urea concentration on this refolding process was investigated. The obtained refolded rhG-CSF has a high specific activity of 2.3×10^8 U/mg, demonstrating that the proteins were completely refolded during the chromatographic process. With only one step by SAX in 40 min, purity and mass recovery of the refolded and purified rhG-CSF were 97% and 43%, respectively.

Keywords: Recombinant human granulocyte colony-stimulating factor, inclusion bodies, protein refolding, purification, strong anion exchange chromatography.

Human granulocyte colony-stimulating factor (hG-CSF), a single chain polypeptide containing 174 amino acid residues (MW=18,800, p*I*=6.1), is one of the hemopoietic growth factors which plays an important role in stimulating proliferation, differentiation, and functional activation of bloodcells[1]. It contains a free cysteine at position 17 and two intramolecular disulfide bonds, Cys^{36}-Cys^{42} and Cys^{64}-Cys^{74}, and the two disulfide bonds in G-CSF molecule are both required for its bioactivity[2].

When hG-CSF is produced by *E. coli*, it is called rhG-CSF, the formation of disulfide bonds is either incorrect or inhibited because the reducing environment of bacterial cytosol, and it is accumulated in the form of inclusion bodies. So it must be dissolved by a solution containing high concentration of denaturants firstly and then refolding companying with oxidization. The denatured recombinant G-CSF (rhG-CSF) is often refolded to its native state by removing the denaturants by dilution, dialysis or diafiltration in the presence of reduced glutathione (GSH) and oxidized glutathione (GSSG). However, refolding yields of rhG-CSF are typically low. In addition, a large volume of solution after dilution brings difficult operation to the subsequently chromatographic purification steps. rhG-CSF is a very hydrophobic protein, easy to aggregate and form precipitates during refolding. Therefore its refolding is still a

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puzzle in the production of rhG-CSF expressed in *E. coli*.

Liquid chromatography for buffer exchange to induce protein refolding is an alternative to the dilution refolding and has been paid much attention in recent years[3,4]. Protein refolding by ion exchange chromatography (IEC) was reported in the literatures[5,6,7], and indicates to be an efficient method. Proteins should be refolded with simultaneous purification in one IEC run, if an optimization of chromatographic condition is employed.

In the present work, rhG-CSF expressed in *E. coli* was solubilized in 8.0 mol/L urea, and the denatured, the reduced rhG-CSF was refolded and oxidized in the presence of low concentration of urea using strong anion exchange chromatography (SAX). In addition, the purification of rhG-CSF was also completed during the chromatographic process.

Experimental

The ÄKTA explorer 100A chromatographic system, Q-Sepharose Fast Flow gel, and electrophoresis apparatus were obtained from Amersham Pharmacia Biotech (Uppsala, Sweden). The chromatographic data were collected and evaluated using the Unicorn 3.21 Data system. An Avanti^{TM} J-25 centrifuge (Beckman coulter$_{TM}$, U.S.A.) was used for centrifugation.

The target protein, rhG-CSF was expressed in *E. coli* in the form of inclusion bodies. Cells were disrupted by ultrasonic. After washing several times, the inclusion bodies were solubilized in 8.0 mol/L urea, 1.0 mmol/L EDTA, 100 mmol/L β-mercapto-ethanol, 50 mmol/L Tris (pH 8.0).

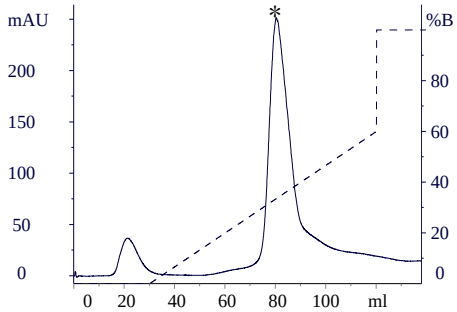
Chromatographic run was carried out at room temperature using a SAX column (20×1.2 Cm I.D.) packed with Q-Sepharose Fast Flow with a bed volume of about 20 mL and connected to an ÄKTA explorer 100A chromatographic system. After the SAX column had been equilibrated with 100% solution A, 400 μL of denatured rhG-CSF solution was injected directly into the SAX column. The column was washed with 30 mL of 100% solution A, and then rhG-CSF was eluted with 90 mL of a linear gradient from 0%B to 60% B at a flow rate of 4.0 mL/min. Finally, the column was regenerated by 20 mL of 100% solution B. Detection was at 280 nm. The fraction containing rhG-CSF was collected and stood for 24 h at 4 °C for fully oxidized, and then dialyzed against a storage solution containing 10.0 mmol/L sodium acetate at pH 4.0. The solution containing rhG-CSF was used for the determination of protein concentration and bioactivity.

Results and Discussion

The chromatogram of the refolding with simultaneous purification of rhG-CSF is shown in **Figure 1**. When sample in 8.0 mol/L urea solution was injected into the SAX column, the denatured rhG-CSF was firstly adsorbed on the stationary phase of SAX. With decreasing in the concentration of the denaturant in the SAX column, rhG-CSF began to refold to its native state from its denatured form in the presence of GSH/GSSG.

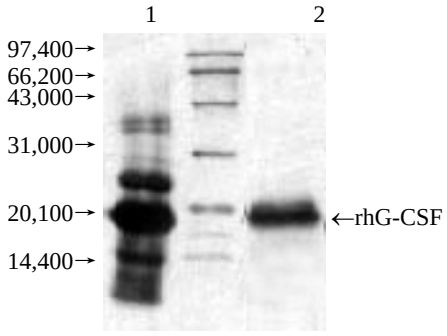
The electrostatic interaction between the denatured rhG-CSF molecules and the stationary phase suppressed the non-specific interactions of the unfolded, or partially folded molecules to prevent rhG-CSF molecules from aggregating with each other. With increasing the concentration of sodium chloride, the denatured rhG-CSF refolded gradually and then desorbed at high concentration of sodium chloride to refold into its native state. At the same time, impure proteins and rhG-CSF were separated during the chromatographic process. So rhG-CSF can be refolded and purified simultaneously.

Figure 1 Chromatogram of rhG-CSF refolding with simultaneous purification by SAX.



Conditions: solution A: 0.050 mol/L Tris, pH 8.0, 3.0 mol/L urea, 1.0 mmol/L EDTA, 1.0 mmol/L GSH, 0.10 mmol/L GSSG; solution B: solution A containing 1.0 mol/L NaCl; gradient: a linear mode from 0% solution B to 60% solution B, then regenetated with 20 mL of 100% B; flow rate: 4.0 mL/min; detection: 280 nm; the solid line presents elution profile of rhG-CSF, the dot line denotes gradient profile of solution B. * indicates rhG-CSF.

Figure 2 SDS-PAGE analysis of rhG-CSF.



Lane 1, rhG-CSF inclusion body extract; 2, molecular weight marker; 3, rhG-CSF refolded with simultaneously purified by SAX.

The effect of urea on the refolding process was tested in the SAX refolding process. The results show that there is an increase in the specific activity of rhG-CSF as urea concentration changing from 0 to 3.0 mol/L. When the urea concentration in mobile phase is 3.0 mol/L, the specific activity of the refolded with simultaneously purified rhG-CSF is 2.3×10^8 U/mg. If the urea concentration is greater than 3.0 mol/L, a great decrease in the bioactivity recovery of rhG-CSF was found. This is because that a suitable concentration of urea can improve refolding and make denatured rhG-CSF

flexible to reorganize its structure. However, too low concentration of urea cause denatured or partially folded rhG-CSF molecules to aggregate with each other, while too high concentration of urea denatures proteins, of course, including rhG-CSF.

As mentioned above, the specific activity of rhG-CSF after refolded with simultaneously purified by SAX was 2.3×10^8 U/mg, indicating that the two disulfide bonds, Cys^{36}-Cys^{42} and Cys^{64}-Cys^{74} were bonded correctly, and rhG-CSF is completely refolded during the SAX chromatographic process. The sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (in **Figure 2**) shows that the purity of the purified rhG-CSF by SAX is about 97%. The mass recovery was measured to be 43%. The molecular weight of refolded rhG-CSF was measured with MALDI-TOF-MS to be 19,182 Dalton. It should be pointed out that the back pressure of the column increased from 0.27 MPa to 0.32 MPa after running 30 times due to the formation of a little precipitates of proteins. Thus the column must be cleaned according to the instructions of Q Sepharose Fast Flow.

The results obtained in this work show that SAX is an efficient tool for the refolding with simultaneous purification of rhG-CSF produced by *E. coli*. The advantage of the SAX method is that the refolding and purification of rhG-CSF is completed by only one chromatographic run in 40 min. Compared to the usual method, in which denatured rhG-CSF was firstly diluted into refolding buffer, then removed precipitate by centrifugation, and loaded the large volume sample solution on several chromatography columns for purification, the whole time for the downstream processing of rhG-CSF production by using the presented method was largely shortened. This is especially important for large scale production of rhG-CSF. In addition, the cost for rhG-CSF production could be significantly decreased also.

Acknowledgments

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