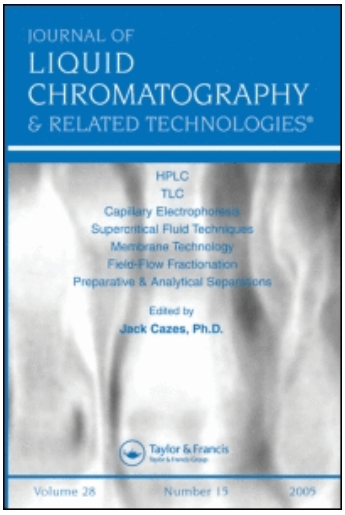


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# Renaturation of Recombinant Human Granulocyte Colony-Stimulating Factor Produced from *Escherichia coli* Using Size Exclusion Chromatography

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**Abstract:** Refolding with simultaneously partial purification of recombinant human granulocyte colony-stimulating factor (rhG-CSF) expressed in *Escherichia coli* (*E. coli*) by size exclusion chromatography (SEC) is presented in this work. The solution containing the denatured and reduced rhG-CSF in $8.0\,\mathrm{mol \cdot L^{-1}}$ urea extracted from the inclusion body was directly injected into a Superdex 75 column and the refolded rhG-CSF was obtained after elution from the column. Several factors, including the concentration of urea in the mobile phase, pH, flow rate, concentration of glutathione, and ratio of GSH to GSSG, concentration of glycerol, sample loading volume, effecting the aim protein refolding were investigated in details. With the selected optimal conditions, the denatured and reduced rhG-CSF was successfully refolded by SEC, and was partially purified during the chromatographic process. When 200 μL of denatured rhG-CSF at a concentration of $2.3\,\mathrm{mg \cdot mL^{-1}}$ was loaded on the SEC column, rhG-CSF with specific activity of $1.2 \times 10^8\,\mathrm{IU \cdot mg^{-1}}$, purity of 83%, and mass recovery of 30% was obtained.

**Keywords:** Recombinant human granulocyte colony-stimulating factor, *Escherichia coli*, Inclusion bodies, Size exclusion chromatography, Protein refolding, Purification

## INTRODUCTION

*Escherichia coli* (*E. coli*) is a common host cell organism for the production of recombinant proteins. When a heterologous protein is overexpressed, the

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production of the nascent polypeptide occurs with faster kinetics than folding of the protein, resulting in the formation of protein aggregates which are deposited as inclusion bodies in the cytoplasm of the bacterial cell.[1] In the case of proteins having disulfide bonds, the formation of protein aggregation is anticipated since the reducing environment of bacterial cytosol inhibits or causes incorrect formation of disulfide bonds. Inclusion bodies are a rich source of the recombinant protein of interest as the protein in this form can accumulate to be greater than half of the total cell protein.[2] Other advantages of producing recombinant protein as inclusion bodies include protection from proteolysis, and ease of protein purification.[3,4] However, in the form of inclusion bodies, the target protein is insoluble, misfolded, and inactive and thus, it is necessary to solubilize and refold the protein to regain its bioactivity.

A general strategy for recovery of active protein from inclusion bodies involves cell lysis, extraction and cleaning of inclusion bodies, solubilization of inclusion bodies, and refolding into the native conformation of the protein.[5,6] After dissolution of inclusion bodies in a buffer containing high concentration of denaturants, such as $8.0\,\mathrm{mol}\cdot\mathrm{L}^{-1}$ urea or $7.0\,\mathrm{mol}\cdot\mathrm{L}^{-1}$ guanidine hydrochloride (GuHCl), reducing agents, such as dithiothreitol (DTT) or $\beta$-mercaptoethanol ($\beta$-ME) are added to reduce all disulfide bonds. Then, the denatured protein is transferred into a non-denaturating environment to shift the folding equilibrium towards its native conformation. This is normally achieved by removing the denaturants through dilution, dialysis, or diafiltration in the presence of reduced glutathione (GSH) and oxidized glutathione (GSSG). However, refolding yields are typically low. Low refolding yields are attributed to mass loss of protein by aggregation, due to non-specific hydrophobic interactions. It is well established that aggregation is proportional to the initial protein concentration,[7] so protein refolding performs in dilute solutions. However, this significantly increases sample volume, making it difficult for subsequent chromatographic purification processes and increases costs. Therefore, the refolding of recombinant proteins expressed in *E. coli* is still a puzzle in the production of recombinant proteins by *E. coli*.

In 1992, Geng et al. first applied hydrophobic interaction chromatography (HIC) and size exclusion chromatography (SEC) to the refolding of lysozyme, ribonuclease, and bovine serum albumin,[8] though, at that time, SEC was not thought to be better than HIC in terms of refolding efficiency. Two years later, Werner et al. published a paper entitled "Refolding proteins by gel filtration chromatography", in which three denatured proteins were successfully refolded.[9] Afterwards, Batas investigated the process of protein refolding by SEC in detail.[10]

SEC, as one of the well-established chromatographic techniques, has recently been reported to have a potential for performing buffer exchange for protein refolding, while sometimes separating the intermediates of protein folding, and thus reducing aggregation.[10] The reduced diffusion of proteins in SEC media and the obstruction effect of the gel matrix have

been shown to suppress the non-specific interactions of partially folded molecules and thus reducing aggregation. Due to the buffer exchange and partial separation of the refolded protein from any aggregates, it is an important step of chromatographic separation. This technique has been applied to the denatured enzymes such as lysozyme and carbonic anhydrase, as well as inclusion body proteins such as urokinase plasminogen activator,[11] heterodimetric platelet-derived growth factor.[12] Refolding of lysozyme from a starting concentration up to $80\,mg \cdot mL^{-1}$ resulted in a 46% recovery of fully active protein.[10] This led to an increase in refolding studies using SEC.[13–15] Now, refolding by SEC represents an option to replace refolding by dilution at laboratory and industrial scale because it is relatively easy to operate.

Human granulocyte colony-stimulating factor (hG-CSF), a single chain polypeptide containing 174 amino acid residues (MW = 19,100, p$I$ = 6.1), is one of the hemopoietic growth factors, which plays an important role in stimulating proliferation, differentiation, and functional activation of blood cells.[16] The therapeutic protein hG-CSF is capable of supporting neutrophil proliferation in vitro and in vivo,[17,18] and it contains a free cysteine at position 17 and two intramolecular disulfide bonds, $Cys^{36}$-$Cys^{42}$ and $Cys^{64}$-$Cys^{74}$, both being required for its bioactivity.[19] Large quantities of recombinant hG-CSF (rhG-CSF) have been produced in genetically engineered *E. coli* and have been successfully used in human clinical studies to treat cancer patients suffering from chemotherapy-induced neutropenia.[20–22] When rhG-CSF is produced in *E. coli*, the same problems as mentioned above inevitably exist.

In the present work, SEC was successfully applied to refold denatured rhG-CSF expressed in *E. coli*, various operation factors effecting its refolding were also investigated in detail. The target protein rhG-CSF was also simultaneously partially purified during the SEC refolding process.

## EXPERIMENTAL

### Instruments

The ÄKTA explorer 100 A chromatographic system, Superdex 75 gel, and electrophoresis apparatus were obtained from Amersham Pharmacia Biotech (Uppsala, Sweden). All chromatographic data were collected and evaluated using the Unicorn 3.21 Data system. An Avanti™ J-25 centrifuge (Beckman coulter™, U.S.A.) was used for centrifugation. A 5 L fermentor (Braun, Germany) was used to express protein. A UV spectrometer (Third Analytical Instrument Co., Shanghai, China) and a CS–930 dual wavelength thin layer chromatographic scanner (Shimadzu, Japan) were used for the determination of the total amount of proteins verifying the purity of rhG-CSF, respectively.

## Chemicals

Acrylamide and bis-acrylamide, reduced glutathione (GSH), oxidized glutathione (GSSG) are of analytical grade, obtained from Sigma (U.S.A.). Tris, glycine, and SDS were obtained from Amersco (U.S.A.). Bovine serum albumin (BSA) was from Sigma Chemicals (U.S.A.). Molecular mass marker was obtained from Amersham Pharmacia Biotech (Uppsala, Sweden). All other chemicals were of analytical grade.

## Expression of rhG-CSF

*E. coli* strain DH5$\alpha$ was transformed by plasmid pBV220/G-CSF, which contains rhG-CSF cDNA. A cell culture in LB medium was seeded into modified M9 medium ($Na_2HPO_4 \cdot 7H_2O$ 12.8 $g \cdot L^{-1}$, $KH_2PO_4$ 3.0 $g \cdot L^{-1}$, $(NH_4)_2SO_4$ 1.0 $g \cdot L^{-1}$, NaCl 0.5 $g \cdot L^{-1}$, yeast extract 5 $g \cdot L^{-1}$, Trpytone 5 $g \cdot L^{-1}$, 5.0 $g \cdot L^{-1}$ glucose, and trace elements) in the presence of kanamycin (25 $mg \cdot mL^{-1}$) and ampicillin (50 $mg \cdot mL^{-1}$). The cells were allowed to grow at 30°C, maintaining the pH at 7.0. Once the OD600 value reached 1.0, the cultures were induced with 1.0 $mmol \cdot L^{-1}$ IPTG and were further grown for 4 h. The cells were then harvested by centrifugation at 7,000 rpm at 4°C.

## Recovery of G-CSF Inclusion Bodies

Recovery of rhG-CSF inclusion bodies was followed[23] by some modifications. The cells were thawed at room temperature and cleaned up with 0.020 $mol \cdot L^{-1}$ Tirs-HCl (pH 8.0), and then the suspension was centrifuged at 7,000 rpm and 4°C for 10 min after washing. The supernatant was discarded. After being frozen at −20°C for 12 h, 100 g of the frozen cells were thawed at room temperature and resuspended in 1000 mL of 0.050 $mol \cdot L^{-1}$ Tris-HCl buffer (pH 8.0) containing 1.0 m $mol \cdot L^{-1}$ EDTA. The cells were lysed by sonication in an ice water bath. The lysates were centrifuged at 14,000 rpm for 20 min to collect the insoluble protein aggregates. The pellet (protein aggregates and cell debris) was washed with 500 mL of the following solutions, 0.020 $mol \cdot L^{-1}$ Tris-HCl (pH 8.0) containing 0.010 $mol \cdot L^{-1}$ EDTA and 2.0 $mmol \cdot L^{-1}$ $\beta$-mercaptoethanol ($\beta$-ME), 0.020 $mol \cdot L^{-1}$ Tris-HCl (pH 8.0) containing 2.0 $mol \cdot L^{-1}$ urea and 2.0 $mmol \cdot L^{-1}$ EDTA, 0.020 $mol \cdot L^{-1}$ Tris-HCl (pH 8.0) containing 70% 2-propanol, respectively. Finally, the inclusion bodies were washed with 0.02 $mol \cdot L^{-1}$ Tris-HCl (pH 8.0). After each washing step, the suspension was centrifuged at 14,000 rpm and 4°C for 15 min and the supernatant was discarded. About 18.0 g of pellet fraction containing rhG-CSF inclusion bodies were obtained and stored at −20°C.

## Solubilization of rhG-CSF from Inclusion Bodies

Solubilization of rhG-CSF was also followed[23] by some modification. Purified inclusion bodies (4.0 g) was solubilized in 20 mL of 7.0 mol · $L^{-1}$ GuHCl, 1.0 mmol · $L^{-1}$ EDTA, 100 mmol · $L^{-1}$ $\beta$-ME, 50 mmol · $L^{-1}$ Tris (pH 8.0) with stirring at room temperature for 1 h. The suspension was centrifuged at 14,000 rpm for 15 min to remove insoluble debris and the supernatant was diluted by 20 mmol · $L^{-1}$ Tris (pH 8.0), 1.0 mmol · $L^{-1}$ EDTA to the final concentration of GuHCl to 5.0 mol · $L^{-1}$. After centrifugation, the solution was diluted again by 20 mmol · $L^{-1}$ Tris (pH 8.0), 1.0 mmol · $L^{-1}$ EDTA to the final concentration of GuHCl to 2.0 mol · $L^{-1}$. Then the precipitated protein consisting mainly of rhG-CSF was dissolved in 8.0 mol · $L^{-1}$ urea, 1.0 mmol · $L^{-1}$ EDTA, 100 mmol · $L^{-1}$ $\beta$-ME, 50 mmol · $L^{-1}$ Tris (pH 8.0). The suspension stood for 12 h at room temperature with continuous stirring. After centrifugation, the supernatant containing rhG-CSF was collected.



## Procedures for the Refolding of rhG-CSF by SEC

A chromatographic run was carried out at room temperature using a SEC column (20 × 2.6 cm I.D.) packed with Superdex 75. The SEC column was equilibrated with mobile phase containing 0.15 mol · $L^{-1}$ sodium chloride, 0.1 mol · $L^{-1}$ Tris (pH 8.0), 1.0 mmol · $L^{-1}$ EDTA, 2.5 mmol · $L^{-1}$ GSH, 0.8 mmol · $L^{-1}$ GSSG, 3.0 mol · $L^{-1}$ urea, 15% glycerol (v/v). The solubilized and denatured rhG-CSF (200 μL) in 8.0 mol · $L^{-1}$ urea solution was injected directly into the column. The renatured rhG-CSF was eluted with the same mobile phase as used for equilibration at a flow rate of 2.0 mL · $min^{-1}$. Detection was set at 280 nm. The fractions containing rhG-CSF were collected and was adjusted to pH 4.0 by hydrochloric acid and dialyzed against a storage solution containing 10.0 mmol · $L^{-1}$ sodium acetate buffer at pH 4.0. The solution containing rhG-CSF was used for determination of protein concentration and bioactivity.

## Refolding of rhG-CSF by Dilution

To run a blank without injection of a sample under the same conditions as those indicated for SEC above, collect the same eluent corresponding to that of the appearance of the rhG-CSF peak. Denatured/reduced rhG-CSF, 200 μL, in 8.0 mol · $L^{-1}$ urea was directly diluted with the collected eluent and then the solution was left for 24 h at 4°C. As in the same procedure as that indicated above, after centrifugation, the refolded rhG-CSF was adjusted to pH 4.0 by hydrochloride acid and dialyzed against 10.0 mmol · $L^{-1}$ sodium acetate buffer at pH 4.0 for determination of protein concentration and bioactivity.

## Analytical Procedures

### Electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)[24] using a Tris-SDS-glycine buffer system in the presence of a reducing agent was used to detect the purity of the purified rhG-CSF contained in the fractions after SEC. Electrophoresis was performed for 1 h at 250 V using 15% polyacrylamide gels. Protein bands were visualized by silver staining.

### Determination of Protein Concentration and Mass Recovery

The protein concentration was estimated by the Bradford quantitative protein determination assay[25] using BSA as standard. The mass recovery ($R_m$) of rhG-CSF was defined as:

$$R_m = m_{G,F}/m_{G,IB} = (C_F \cdot V_F \cdot P_F)/(C_{IB} \cdot V_{IB} \cdot P_{IB}) \quad (1)$$

where, $m_{G,F}$, the mass of rhG-CSF in the finally obtained rhG-CSF solution (mg); $C_F$, total protein concentration in the finally obtained rhG-CSF solution ($mg \cdot mL^{-1}$); $V_F$, volume of the finally obtained rhG-CSF solution (mL); $P_F$, purity of rhG-CSF in the finally obtained rhG-CSF solution; $m_{G,IB}$, the mass of rhG-CSF in the injected solution of inclusion bodies (mg); $C_{IB}$, total protein concentration in the injected solution of inclusion bodies ($mg \cdot mL^{-1}$); $V_{IB}$, volume of the injected solution of inclusion bodies (mL); $P_{IB}$, purity of rhG-CSF in the injected solution of inclusion bodies.

### Bioactivity Assay of rhG-CSF

The bioassay for the bioactivity of the renatured rhG-CSF was determined by a cell proliferation assay using mouse myeloblastic NFS-60 cell as described previously.[26]

## RESULTS AND DISCUSSION

### Refolding of rhG-CSF by SEC

The hydrophobic amino acids of rhG-CSF molecules in the unfolded state are very easy to interact with each other and thus aggregate during refolding. SEC was applied to refold rhG-CSF in the present work, because it can accomplish the buffer exchange process between $8.0\ mol \cdot L^{-1}$ urea and low concentration of urea in the mobile phase, which is necessary to rhG-CSF refolding, and partially diminished aggregation in a single step. The mechanism for protein refolding by SEC has been demonstrated by Batas et al.[10] The

denatured rhG-CSF in 8.0 mol · $L^{-1}$ urea was directly injected into the column equilibrated with the mobile phase, and then the renatured rhG-CSF were also eluted with the same mobile phase. As shown in Figure 1, three peaks were obtained. The results obtained from bioactivity and reduced SDS-PAGE in each peak indicate peak 2 mainly to be the refolded rhG-CSF with relatively high bioactivity containing some impure proteins, while peak 1 corresponds to the aggregated rhG-CSF with very low bioactivity and contaminating more impure proteins (data not shown). Peak 3 is only smaller impure proteins, and some small molecules, such as $\beta$-ME. Therefore, the SEC refolding method can perform buffer exchange from solubilizing to refolding solutions for rhG-CSF refolding, with simultaneously removing the formed aggregates by one chromatographic run, resulting in partial purification of rhG-CSF.

As is well known, both the correct refolding and formation of native disulfide bonds are strongly dependent on the renaturing conditions used. Thus, several factors effecting the refolding of rhG-CSF were investigated in detail during the following experiments.



## Urea Concentration

A key to protein refolding is to have an environmental condition where either the denaturant concentration is completely removed, or its concentration is

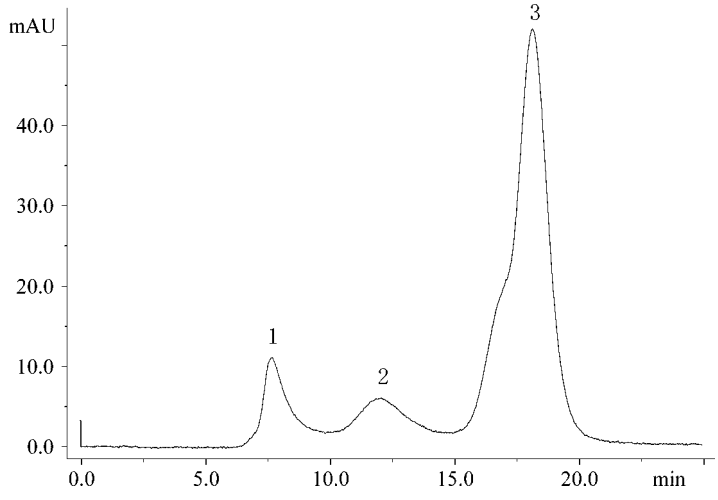


***Figure 1.*** Refolding of rhG-CSF by SEC. Conditions: mobile phase: 0.10 mol · $L^{-1}$ Tris, pH 8.0, 1.0 mol · $L^{-1}$ urea, 1.0 mmol · $L^{-1}$ EDTA, 1.0 mmol · $L^{-1}$ GSH, 0.1 mmol · $L^{-1}$ GSSG; flow rate: 2.0 mL · $min^{-1}$; detection: 280 nm; the solid line presents elution profile of rhG-CSF; 1, aggregates of rhG-CSF and contaminants; 2, refolded rhG-CSF; 3, smaller contaminants and $\beta$-ME.

low enough to make protein molecules stay in solution and be flexible in order to reorganize their three-dimensional structure. Sometimes, a suitable concentration of denaturant in solution is favorable to protein refolding, due to prevention from, or diminishing of, the aggregation of the denatured protein molecules. For protein refolding by SEC, the denaturant concentration in the mobile phase is also very important. Batas et al. used the mobile phase containing a low concentration of urea to refold high concentrations of denatured enzymes by SEC successfully.[10] As can be seen from Figure 2, mass recovery of rhG-CSF increases with increasing the concentration of urea in the mobile phase lower than $3.0\,\mathrm{mol}\cdot\mathrm{L}^{-1}$ due to reducing aggregation; further increases in the urea concentration have not had a remarkable effect on the mass recovery. It can also be seen from this figure, that the specific bioactivity of rhG-CSF increases with the urea concentration first, until its maximum value at $3.0\,\mathrm{mol}\cdot\mathrm{L}^{-1}$ urea, but decreases after this point. The explanation for this is because a suitable urea concentration suppresses aggregation of the rhG-CSF refolding intermediates, and makes the denatured rhG-CSF molecules be flexible to reorganize their three-dimensional structure. Too low a concentration of urea results in the partial aggregation of the denatured or partially folded rhG-CSF molecules with each other. However, too high a concentration of urea makes native proteins unfold and, thus, the denatured rhG-CSF could not refold efficiently to its native state in this circumstance.



## pH

The effect of pH on the yield and rate of protein refolding, especially on the formation of disulfide bonds, is very important. The basic thiol exchange reaction involves the ionized form of thiol and is, therefore,

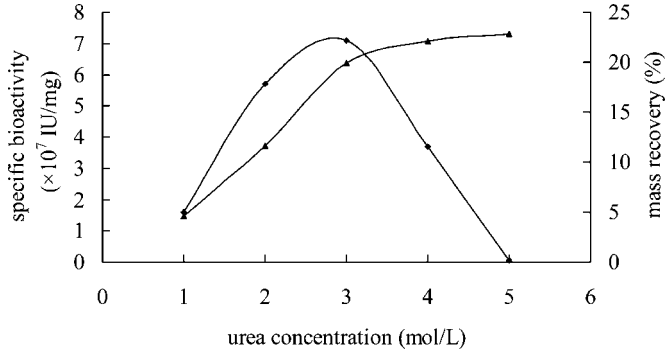


***Figure 2.*** Effect of urea concentration on the refolding of rhG-CSF by SEC. Conditions are as the same as those indicated in Figure 1 except for urea concentration. ◆, specific activity; ▲, mass recovery.

pH-dependent.[27] In general, a basic circumstance helps the formation of disulfide bonds, whereas protein refolding at acidic environment would hamper the reoxidation. The effect of pH on the specific bioactivity and mass recovery of rhG-CSF was tested using Tris-HCl buffer in the range from 7.0 to 10.0 and the obtained results are shown in Figure 3. It can be seen that mass recovery of rhG-CSF did not change significantly with the pH range investigated in the mobile phase. There is an increase in the specific bioactivity of rhG-CSF from pH 7.0 to 8.0, with a maximum at pH 8.0, but a decrease is found with a further increase of pH. This is probably because lower pH does not make the thiol groups on the cysteine residues in rhG-CSF sufficiently ionize to form disulfide bonds, while under the condition of a higher pH, the chance of the formation of wrong paired disulfide bonds would increase and also have little opportunity to rearrange by the redox system of GSH/GSSG.[28] Moreover, high pH would also cause the degradation of peptide chains,[29] which therefore decreases the refolding yield. A weakly basic environment (pH 8.0) enhanced the ionization of thiol in cysteines of rhG-CSF and GSH.



## Flow Rate

Flow rate is an important parameter during SEC purification process. However, we do not know whether it affects protein refolding, or not, by SEC. If it does, it would affect the rate of the buffer exchange, the contact time between denatured protein molecules and stationary phase, and also the redox reaction. With increasing the flow rate from 1.0 to 4.0 $mL \cdot min^{-1}$, as can be seen from Figure 4, the mass recovery increased somewhat. This coincides with the results obtained by Liu et al.[30] and Fahey et al.[31] due to the total amount of aggregation decrease as flow rate increases. However, the specific bioactivity of rhG-CSF decreases when the flow rate increases.

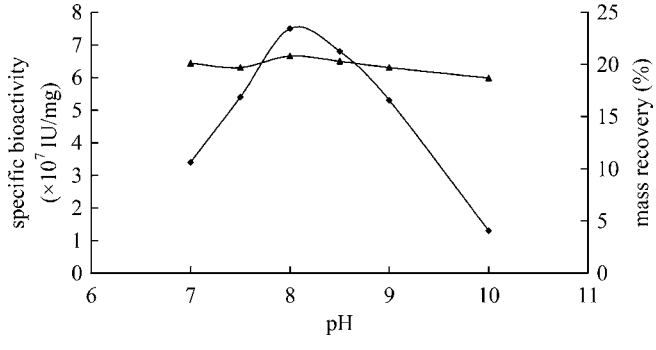


***Figure 3.*** Effect of pH on refolding of rhG-CSF by SEC. Chromatographic conditions: 3.0 $mol \cdot L^{-1}$ urea, other conditions are the same as those shown in Figure 1 except for pH value. ◆, specific activity; ▲, mass recovery.

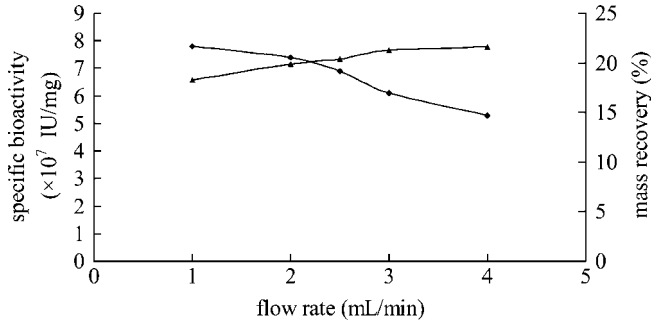


***Figure 4.*** Effect of elution flow rate on refolding of rhG-CSF by SEC. Chromatographic conditions: 3.0 mol · $L^{-1}$ urea, other conditions are the same as those in Figure 1 except for flow rate. ◆, specific activity; ▲, mass recovery.



This might be attributed to the decrease in the resolution between aggregated and refolded forms of rhG-CSF because total bioactivity only comes from the refolded rhG-CSF, but the total mass comes from the sum of the refolded and aggregated forms of rhG-CSF. Though the specific bioactivity of rhG-CSF obtained at a flow rate of 1.0 mL · $min^{-1}$ is the maximum in the investigated range, its corresponding mass recovery is the minimum, and it took a longer time to elute rhG-CSF from SEC column than that at higher flow rate. Accounting for this point, the following refolding experiments were all carried out at a flow rate of 2.0 mL · $min^{-1}$.

## Glutathione Concentration and Ratio of GSH to GSSG

The aim protein rhG-CSF in its native state contains a free cysteine at position 17 and two intramolecular disulfide bonds, $Cys^{36}$-$Cys^{42}$ and $Cys^{64}$-$Cys^{74}$,[32] and the two disulfide bonds are both required for its three-dimensional structure, and consequently its bioactivity.[33] The cysteines in solubilized rhG-CSF produced by *E. coli* are all in the reduced forms, which must be oxidized to form two native disulfide bonds. Previously, it was found that the reoxidation of protein disulfide bonds could be effectively catalyzed in the presence of GSH/GSSG.[33] The effect of total concentration and ratio of GSH to GSSG on rhG-CSF refolding by SEC was investigated in the present work. Figures 5 and 6 separately show the results. As can be seen from Figure 5, the total glutathione concentration has little effect on the specific activity of rhG-CSF. When its concentration is 3.3 mmol · $L^{-1}$, as the ratio of GSH to GSSG is 10/1, the specific bioactivity of the renatured rhG-CSF has a maximum value, while lower or higher concentration results in decreased specific bioactivity. The mass recovery did not change significantly with increasing the total concentration of glutathione in the range from 1.1 to 9.9 mmol · $L^{-1}$.

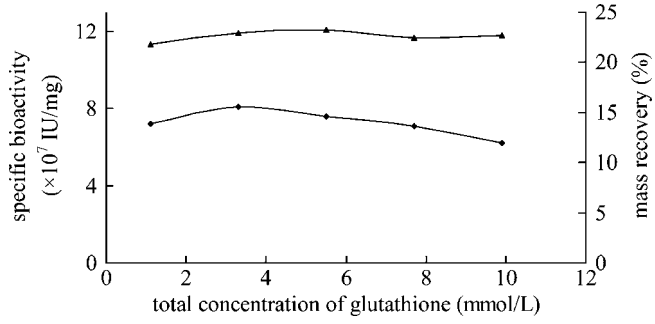


***Figure 5.*** Effect of total concentration of glutathione on refolding of rhG-CSF by SEC. Chromatographic conditions: $3.0\,mol \cdot L^{-1}$ urea, ratio of GSH to GSSG is $10/1$, other conditions are the same as those in Figure 1 except for concentration of glutathione. ◆, specific activity; ▲, mass recovery.



Refolding is a net oxidative process, but the highest refolding yields are often obtained in the presence of GSH, the usual ratio of GSH to GSSG is $1/10$,[34,35] so the ratio of GSH to GSSG was limited in the range between $1/1$ and $10/1$ in the present work. As seen in Figure 6, the mass recovery of rhG-CSF did not change significantly with increasing the ratio of GSH to GSSG in the range from $1/1$ to $10/1$. There is a peak for the specific bioactivity of rhG-CSF when the ratio of GSH to GSSG is $3/1$, while any lower or higher ratio than it decreases the specific bioactivity. As a result, total concentration of $3.3\,mmol \cdot L^{-1}$ for glutathione and the redox ratio of $3/1$, i.e., $2.5\,mmol \cdot L^{-1}$ GSH and $0.8\,mmol \cdot L^{-1}$ GSSG in the mobile phase were used for the following SEC refolding experiments.

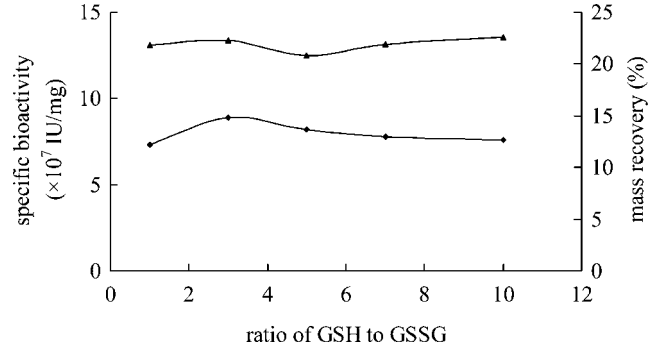


***Figure 6.*** Effect of ratio of GSH/GSSG in mobile phase of SEC on refolding of rhG-CSF. Chromatographic conditions: $3.0\,mol \cdot L^{-1}$ urea, total concentration of GSH/GSSG is $3.3\,mmol \cdot L^{-1}$, other conditions are the same as those in Figure 1 except for ratio of GSH to GSSG. ◆, specific activity; ▲, mass recovery.

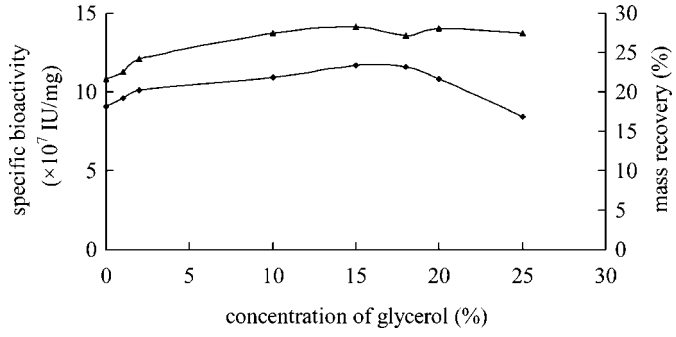


***Figure 7.*** Effect of concentration of glycerol on refolding of rhG-CSF by SEC. Chromatographic conditions: $3.0\,mol \cdot L^{-1}$ urea, $2.5\,mmol \cdot L^{-1}$ GSH and $0.8\,mmol \cdot L^{-1}$ GSSG, other conditions are the same as those demonstrated in Figure 1 except for adding glycerol in the mobile phase. ◆, specific activity; ▲, mass recovery.



## Glycerol Concentration

Aggregation is probably the major process competing against correct refolding. Therefore, a reasonable strategy to improve protein refolding is to prevent protein aggregation by adding small molecules to interfere with unwanted protein-protein interactions. It was reported that glycerol can increase protein stability, thus improving protein refolding.[36] Figure 7 shows the effect of glycerol concentration on the specific activity and mass recovery of rhG-CSF during SEC refolding. The results indicate that the intermediate concentration of glycerol really increases the specific bioactivity of rhG-CSF with the concentration of glycerol being 15% (v/v), but any lower and higher concentrations than it decreases the specific bioactivity. Mass

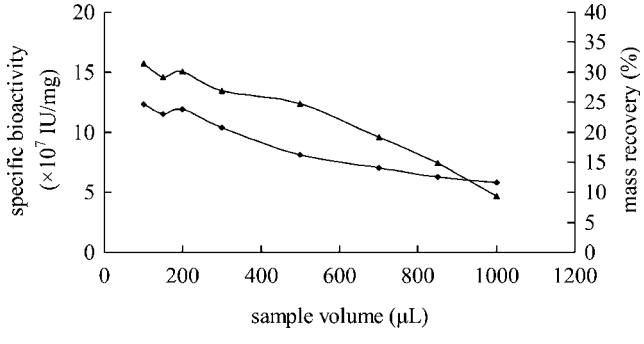


***Figure 8.*** Effect of sample loading volume on refolding of rhG-CSF by SEC. Chromatographic conditions: $3.0\,mol \cdot L^{-1}$ urea, 15% glycerol (v/v), other conditions are the same as those shown in Figure 7 except for sample loading volume. ◆, specific activity; ▲, mass recovery.

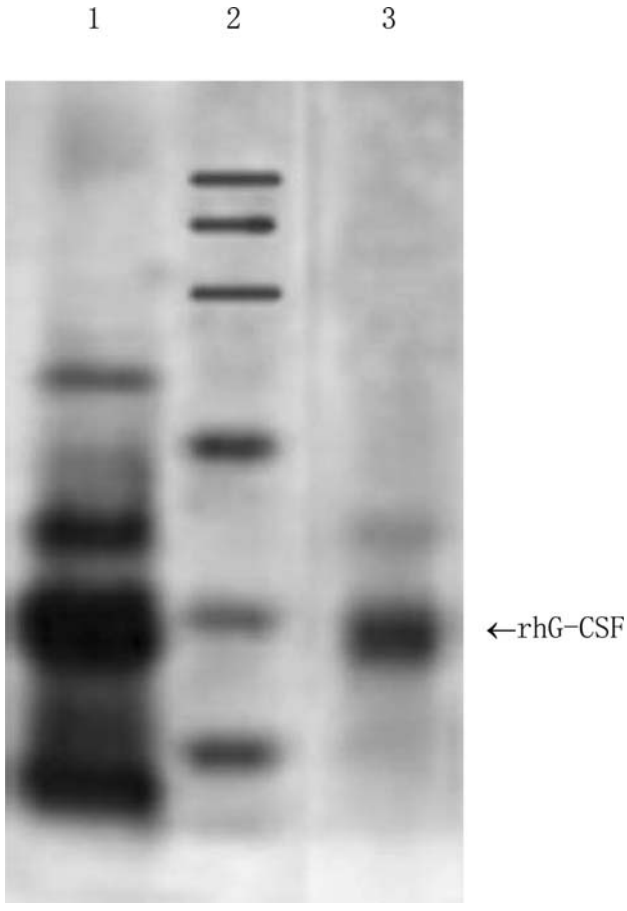


*Figure 9.* SDS-PAGE analysis of rhG-CSF. Lane 1, rhG-CSF inclusion body extract; 2, molecular weight marker (from bottom to top 14,400; 20,100; 31,000; 43,000; 66,200; 97,400 Da); 3, rhG-CSF refolded with simultaneously partially purified by SEC.

recovery of rhG-CSF was also found to increase somewhat with the increase in the concentration of glycerol.

## Sample Volume

It was reported that volume loading of the sample affects the protein resolution and also protein refolding by SEC to a large extent.[37–39] Figure 8 shows the effect of volume loading of a sample on the refolding of rhG-CSF by SEC. From Figure 8, both specific bioactivity and mass recovery of rhG-CSF decreased with increasing the volume loading of the sample. This is

because with the increase in the volume loading of the sample, the concentration of denatured rhG-CSF increases during the refolding process; so does the total mass of rhG-CSF. As is well known, aggregation increases with the increasing of the initial protein concentration or the total mass; also, as pointed out above, the resolution between refolded rhG-CSF and its aggregates or intermediates, as well as that between refolded rhG-CSF and the denaturants or contaminants decrease.

From the above discussions, the optimal composition of the mobile phase for refolding of rhG-CSF by SEC at a flow rate of $2.0\,mL \cdot min^{-1}$ is $0.15\,mol \cdot L^{-1}$ sodium chloride, $0.1\,mol \cdot L^{-1}$ Tris (pH 8.0), $1.0\,mmol \cdot L^{-1}$ EDTA, $2.5\,mmol \cdot L^{-1}$ GSH, $0.8\,mmol \cdot L^{-1}$ GSSG, $3.0\,mol \cdot L^{-1}$ urea, 15% glycerol (v/v). With the selected chromatographic conditions, such as sample size of $200\,\mu L$ of denatured/reduced rhG-CSF and Superdex 75 column, the refolded rhG-CSF with specific bioactivity of $1.2 \times 10^8$ $IU \cdot mg^{-1}$, purity of 83% (shown in Figure 9) and mass recovery of 30% could be obtained.



## CONCLUSIONS

The denatured rhG-CSF expressed in *E. coli* was successfully refolded by SEC. Advantages of the SEC refolding method are: the prior dilution and concentration steps for chromatographic purification are not needed, aggregates can be suppressed during the refolding, the refolded form of rhG-CSF was separated from its denatured and aggregated forms simultaneously, and thus rhG-CSF was partially purified. These points are very important for large scale production of rhG-CSF. Moderate concentration of urea and appropriate pH are essential for this SEC refolding process. In addition, other parameters investigated in this work, such as flow rate, glutathione concentration, volume loading of sample are also important.

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