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Glycerol-Assisted Hydrophobic Interaction Chromatography Improving Refolding of Recombinant Human Granulocyte Colony-Stimulating Factor

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Abstract Efficient refolding of recombinant proteins in the forms of inclusion bodies at higher concentration remains challenging. Here, we report a strategy of a dual-gradient hydrophobic interaction chromatography (HIC) mode to refold recombinant human granulocyte colony-stimulating factor from its inclusion bodies at high protein concentration. The strategy was taken to meet the demand of dynamic refolding proceeding by gradually decrease the denaturant (guanidine-HCl) concentration and gradually increase the hydrophilicity of media (column of Poros PE 20) with glycerol as additive to provide a mild refolding surroundings. Compared with dilution method, this dual-gradient HIC process gave about 8.5-fold of increase in specific activity and 30% increase in soluble protein recovery. Furthermore, much higher protein concentration could be obtained at the same time.

Keywords Hydrophobic interaction chromatography · Refolding · Glycerol · Recombinant human granulocyte colony-stimulating factor

Introduction

Overexpression of heterogeneous proteins in *Escherichia coli* usually leads to the formation of inactive inclusion bodies [1–3]. Refolding therefore becomes a crucial step in protein production. Aggregation and misfolding are often responsible for low refolding yield [4, 5]. Analysis of the kinetics of aggregation shows that the aggregation process exhibits an apparent reaction order ≥2, whereas the correct folding is first-order reaction [6, 7], suggesting low protein concentration favors refolding process. Dilution is a conventionally

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used method, but the protein concentration has to be carefully controlled as low as possible to prevent aggregation or misfolding [4–6]. As a consequence, large volumes of buffers and huge tanks are required [5, 8, 9].

Several chromatographic processes, including size exclusion chromatography [10, 11], ion exchange chromatography [12, 13], and immobilized metal affinity chromatography [14, 15] have been developed to improve the refolding efficiency. Compared to other chromatographic techniques, hydrophobic interaction chromatography (HIC) is also able to act as a practical and even more efficient tool for protein refolding [16–18]. Since hydrophobic interactions are the dominant forces for a given protein's folding and its structure stabilization [19], we therefore believe that HIC can be the artificial chaperone assisting the denatured protein to gain its native structure through interactions with the solid matrix. Furthermore, guanidine-HCl denatured proteins can be directly applied onto a HIC column, showing the advantage over ion exchange chromatography that cannot work at high salt concentration. Some researchers refolded a few proteins using hydrophobic interaction chromatography involving a silica-based hydrophobic media coupled with polyethylene glycol (PEG) as a hydrophobic end group [20].

However, one disadvantage for HIC is that too strong interaction between the hydrophobic core of the denatured protein and the ligand on the matrix may stop further folding of the peptide chain, leading to irreversible adsorption. A strategy to utilize HIC to correctly refold protein is to gradually increase the hydrophilicity of media with additives and promote intramolecular hydrophobic collapse of protein during refolding proceeding. Our previous study successfully used PEG as an additive for consensus interferon refolding on HIC [18]. Osmolytes can be considered as another ideal candidate in that they are able to strengthen the compactness of protein of inner hydrophobic moieties and prevent their exposure resulting in enhanced stability [21, 22]. In this paper, glycerol was purposefully selected as an additive to regulate the hydrophobic interaction.

Recombinant human granulocyte colony-stimulating factor (rhG-CSF) is an important cytokine used to treat neutropenia in clinic [23]. It is normally produced through heterologous gene expression in *E. coli* in inactive form of inclusion bodies. Dilution is often used for its refolding that gives less than 50% yield and 3.49×10^7 U/mg specific activity at the protein concentration of 181.6 μg/ml [24]. It is an attractive challenge to explore novel refolding methods that can effectively refold rhG-CSF at high concentrations. In the present work, a new refolding strategy, involving the combination of a commercially available HIC media of Poros PE and glycerol as additives, was developed for rhG-CSF refolding. All the results demonstrate that HIC with the dual gradient of guanidine-HCl and glycerol would be an efficient tool for protein refolding.

Materials and Methods

Materials and Equipment

The denatured rhG-CSF was stored at State Key Laboratory of Biochemical Engineering, Institute of Process Engineering, Chinese Academy of Sciences. Poros PE 20 was purchased from PE Company. Guanidine-HCl was purchased from Amresco and glycerol was purchased from Shanghai Chemical Reagent Co. All other chemicals were analytical grade. NFS-60 cells and standard rhG-CSF were purchased from National Institute for the Control of Pharmaceutical and Biological Products. Water used for the experimental work was ultrapure water from Milli-Q supplied by Millipore.

A Poros PE 20 column (82×12 mm i.d.) was connected with AKTA Purifier 10 (GE Healthcare, USA) to perform the chromatography process. A reverse-phase high performance liquid chromatography (HPLC) column Vydac 214 TP54C_4 (250×4.6 mm i.d.) was connected with Waters 600 HPLC system. A UV–visible spectrophotometer Ultrospect 2000 (GE Healthcare, USA) was used for assay of protein concentration.

Protein Refolding

Refolding by Dilution

Three milligrams denatured rhG-CSF protein at the concentration of 15 mg/ml was added slowly into a glass container filled with refolding buffer composed of 50 mmol/l Tris-HCl, 0.2 mmol/l ethylenediaminetetraacetic acid (EDTA)-Na, 2 mM β-ME, 2.0 mol/l guanidine-HCl (terminal concentration), and 20 μmol/l $CuSO_4$, pH 8.5. The solution was mixed thoroughly and incubated for 24 h at 4 °C. The solution was centrifuged at 12,000×*g* for 15 min to remove precipitated material. This was the optimal way of rhG-CSF dilution refolding on the basis of our observation (detail omitted).

Refolding by Dilution with Glycerol Additive

Glycerol was added into the above dilution refolding buffer to make its concentration at 10%, 15%, 200%, and 30% (*v*/*v*), respectively. Dilution refolding in the presence of glycerol was performed in the same way as 2.2.1, except the buffer containing glycerol at different concentration.

Refolding by HIC with Single Gradient of Guanidine-HCl

A Poros PE 20 column was first equilibrated with buffer A_1 (3.6 M $(NH_4)_2SO_4$, 50 mM Tris-HCl, pH 8.5). After 3 mg denatured protein solution at 0.324 mg/(ml gel) ratio was loaded and adsorbed on the column, elution was applied with buffer A_2 (50 mM Tris-HCl, 1 mM EDTA-Na, 2 mM β-mercaptoethanol, pH 8.5) for two column volumes. Then, the mobile phase was rapidly changed into buffer B_1 (6 M guanidine-HCl, 50 mM Tris-HCl, 1 mM EDTA-Na, 2 mM β-mercaptoethanol, pH 8.5). Gradient of guanidine-HCl elution was applied, which was from 100% buffer B_1 to 100% buffer A_2, at various gradient lengths of guanidine-HCl and different elution rates. Denatured rhG-CSF concentration was 15 mg/ml and the chromatography was done at room temperature.

Refolding by HIC Assisted by Dual Gradient of Guanidine-HCl and Glycerol

The three kinds of buffers (buffer A_1, buffer A_2, and buffer B_1) were the same as above. Buffer B_2 (buffer A_2 containing different concentrations of glycerol additive) was applied. After 3 mg denatured protein solution at 0.324 mg/(ml gel) ratio was loaded and adsorbed on the column, elution was applied with buffer A_2 for two column volumes. Then, the mobile phase was rapidly changed into buffer B_1. Dual gradient of guanidine-HCl and glycerol elution was applied, which was from 100% buffer B_1 to 100% buffer B_2 at different gradient of glycerol concentration and different elution rates. The fractions of peaks were collected and analyzed further by reverse-phase HPLC and nonreduced sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Reverse-Phase HPLC Analysis

Refolded rhG-CSF was applied to a Vydac 214 TP54C_4 reverse-phase HPLC and eluted with a linear gradient of 40% to 55% acetonitrile in 0.1% trifluoroacetic acid at 1 ml/min, monitoring the absorbance at 280 nm.

Protein Bioactivity Assay and Protein Concentration Assay

Protein bioactivity assay was based on NFS-60 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide method [25] and the protein concentration assay was according to the Bradford method [26].

Results and Discussions

Refolding of rhG-CSF by HIC with Single Gradient of Guanidine-HCl

The denatured rhG-CSF, which was adsorbed on the matrix, could be desorbed and eluted out at a high protein concentration of 0.6 mg/ml (as shown in Table 1) when the denaturant concentration was gradually decreased, indicating HIC method could efficiently minimize precipitation. This was because the exposed hydrophobic region of denatured or partly refolded peptides could interact with hydrophobic ligand of the media, decreasing the chances of intermolecular association. So, a high protein concentration could be obtained. As shown in Fig. 1, the eluted rhG-CSF has obtained certain bioactivity, suggesting the denatured protein could simultaneously refold when released from the matrix, although less than one tenth specific activity was recovered. The reason is that the protein matrix hydrophobic interaction, maybe multiple-site interactions, would compete against intramolecular hydrophobic collapse of protein, which was the main force for protein folding,

Table 1 Comparison of rhG-CSF refolding results among different methods of dilution and HIC.

Refolding ways	Initial protein concentration (μg/ml)	Final protein concentration (μg/ml)[g]	Specific activity ($\times 10^7$ U/mg)	Protein yield (%)	Total activity ($\times 10^7$ U)[j]
Dilution[a]	200	106	2.10	53	3.34
Dilution[b]	200	115	2.21	57.5	3.81
HIC[c]	–	602[h]	1.06	84	2.67
HIC[d]	–	602[h]	9.02	89	24.08
Dilution[e]	181.6[f]	no	3.49[i]	19.5[i]	2.04[f]

[a] Dilution refolding

[b] Dilution refolding with 15% glycerol additive

[c] HIC refolding

[d] HIC assisted by a dual gradient of guanidine-HCl and glycerol

[e] The data are calculated or obtained from [24]

[f] The data are calculated from [24]

[g] Final protein concentration meant the concentration of soluble refolded protein

[h] Final protein concentration meant the concentration of protein which was eluted off from HIC column

[i] The data are obtained from [24]

[j] The feed for refolding every time was 3 mg denatured protein in solution of 15 mg/ml

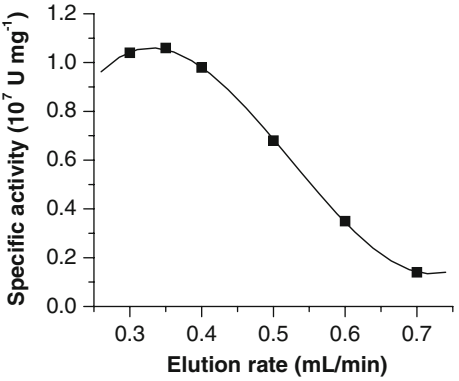

Fig. 1 Influence of elution rate on the specific activity of rhG-CSF refolded on HIC by guanidine-HCl gradient. Column: Poros PE 20; column volume 9.27 ml. Elution way is from 100% buffer B_1 (6 M guanidine-HCl, 50 mM Tris-HCl, 1 mM EDTA-Na, 2 mM β-mercaptoethanol, pH 8.5) to 100% buffer A_2 (50 mM Tris-HCl, 2 mM β-mercaptoethanol, 1 mM EDTA-Na, pH 8.5). Protein loading 3 mg, room temperature

resulting in incorrect refolding and low specific activity when denatured rhG-CSF was eluted from HIC.

Figure 1 also showed the flow rate had a great influence on the specific activity of refolded rhG-CSF. The specific activity was 1.06×10^7 U per mg protein when the flow rate was 0.35 ml/min. The specific activity was decreased significantly with flow rate decreasing from 0.35 to 0.7 ml/min. It was only 0.2×10^7 U per mg protein at the flow rate of 0.7 ml/min, indicating too low flow rate was not suitable for rhG-CSF refolding.

Refolding of rhG-CSF by HIC Assisted by Dual Gradient of Guanidine-HCl and Glycerol

To solve the problem of HIC with single gradient of denaturant, one strategy is to gradually shield exposed hydrophobic ligand of media and to promote refolding of released protein by some additives. Some chaperone chemicals are expected to promote protein surface hydration and thus enhance formation of correct structures. We successfully refolded lysozyme by HIC using a dual gradient of glycerol and urea [17]. Figure 2 showed the

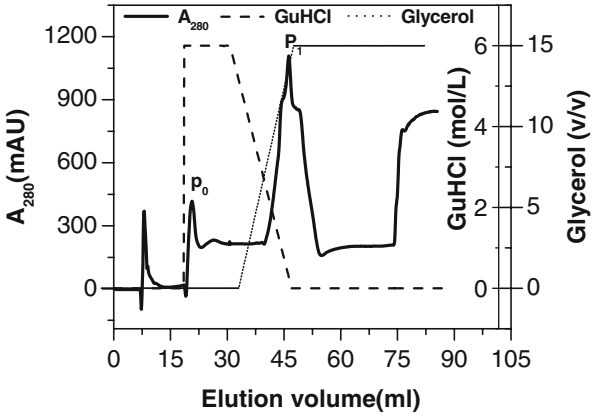

Fig. 2 Chromatogram for refolding of rhG-CSF by glycerol-assisted hydrophobic interaction chromatography. Elution gradient is from 100% buffer B_1 (6 M guanidine-HCl, 50 mM Tris-HCl, 1 mM EDTA-Na, 2 mM β-mercaptoethanol, pH 8.5) to 100% buffer B_2 (15% glycerol, 50 mM Tris-HCl, 1 mM EDTA-Na, 2 mM β-mercaptoethanol, pH 8.5), glycerol concentration gradient is from 0% (*v*/*v*) to 15% (*v*/*v*) in a gradient of glycerol concentration increasing 0.36% (*v*/*v*) per min, protein loading is 3 mg, and flow rate is 0.35 ml/min, room temperature. From *left* to *right*: peak P_0, impurity; peak P_1, rhG-CSF

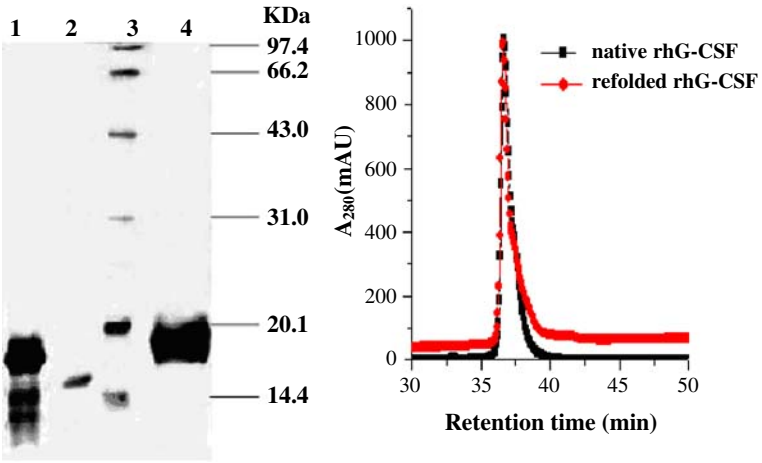

Fig. 3 *Left*: Nonreduced SDS-PAGE and reversed phase chromatography analysis of rhG-CSF elution peaks on HIC. For nonreduced SDS-PAGE, a 5% stacking gel with 15% running gel was used. From *left* to *right*: *lane 1* denatured rhG-CSF, *lane 2* fraction from peak P_0 of Fig. 2, *lane 3* marker, and *lane 4* fraction from peak P_1 of Fig. 2; *right*: reversed phase chromatography analysis of P_1. Elution way: a linear gradient of 40% to 55% acetonitrile in 0.1% trifluoroacetic acid; elution rate 1 ml/min

chromatogram of denatured rhG-CSF refolded by HIC assisted by a dual gradient of glycerol and guanidine-HCl. As shown in Table 1, the specific activity of refolded rhG-CSF by this strategy was 9.02×10^7 U/mg, 8.5-folds increase compared with merely using denaturant gradient (Fig. 1). Peak 1 exhibited one band on SDS-PAGE analysis and gave one peak on HPLC, indicating that the impurities could be separated at the same time. The retention time of P_1 is in accordance with that of native rhG-CFS (Fig. 3), suggesting that all of the eluted rhG-CSF was correctly refolded. Possibly, glycerol was via regulating hydrophobic interactions between the HIC media and the protein to assist the denatured protein to gain its native structure.

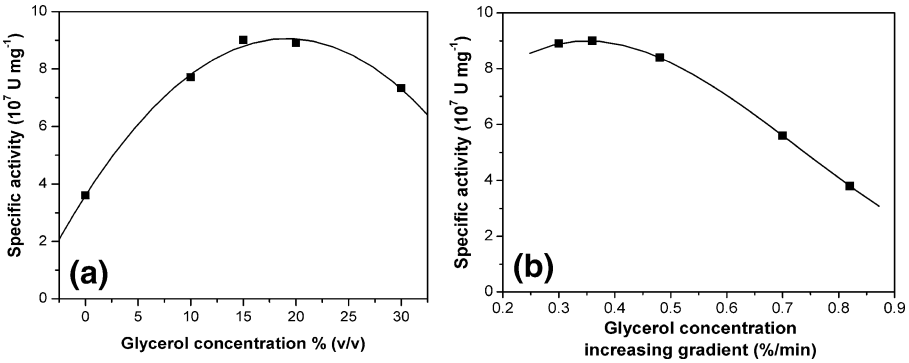

Fig. 4 **a** Specific activity of rhG-CSF refolded with different glycerol concentrations on glycerol-assisted HIC mode. Protein loading 3 mg. Gradient elution rate 0.35 ml/min. **b** Specific activities of rhG-CSF refolded with different gradient slopes of glycerol on dual gradient-assisted HIC mode. Final glycerol concentration 15% (*v*/*v*). Gradient elution is from 100% buffer B_1 (without glycerol) to 100% buffer B_2 (with 15% glycerol). Protein loading 3 mg. Gradient elution rate 0.35 ml/min

Comparison of the Four Refolding Processes

Table 1 is a comparison of rhG-CSF refolding results among different methods of dilution and HIC. It was obvious that the HIC strategy assisted by dual gradient of guanidine-HCl and glycerol gave the highest specific activity and total bioactivity recovery. Furthermore, a much higher protein concentration could be obtain, which would be very favorable for downstream production process.

Effects of Glycerol Concentration and Glycerol Gradient on the rhG-CSF Refolding

Our previous study has demonstrated that the final concentration and gradient slope of additives had great influences on HIC-assisted refolding process. Figure 4 showed the effect of final glycerol concentration and its gradient on rhG-CSF refolding by HIC system. It was found that the highest specific activity of rhG-CSF was obtained by eluting with glycerol concentration from 0% to 15%, a gradient of glycerol concentration increasing 0.36% (*v*/*v*) per min (Fig. 4a, b). Lower or higher concentration of glycerol and faster or slower gradient of glycerol concentration did not help its refolding (data not shown).

Conclusion

The HIC with dual gradient of the guanidine-HCl and the osmolytes (glycerol) has given higher specific activity and final protein concentration than the dilution for protein refolding. By this novel method, recombinant rhG-CSF expressed in the form of inclusion bodies has been efficiently refolded with a high specific activity of 9.02×10^7 U/mg, a high mass recovery yield of 89%, and a high protein concentration of 602 μg/ml. Another advantage is partial purification achieved simultaneously in the refolding process. This result would be a useful alternative method for rhG-CSF industry production.

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