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Refolding of Proteins at High Concentration by Size Exclusion Chromatography

Yixin Guan, Yonggui Gao, Shanjing Yao, Man-Gi Cho

Department of Chemical and Biochemical Engineering,
Zhejiang University, Hangzhou 310027, China
Engineering Research Center, Dongseo University, Busan, 617-716 Korea

Abstract

Renaturation of Lysozyme by size exclusion chromatography(SEC) to improve yield as well as the initial and final protein concentration has been studied in detail. Although urea decreases the rate of proteins refolding, it can suppress protein aggregation to sustain pathway of correct refolding at high protein concentration, and there existed an optimum urea concentration in renaturation buffer. Lysozyme was successfully refolded from initial protein concentration of up to 100mg/ml by SEC, the yield was more than 40%. And the refolding of Interferon-γ was further investigated.

Key words: Protein refolding, Lysozyme, Interferon-γ, Size exclusion chromatography

Introduction

The production of recombinant proteins from *E coli* is limited by expression of the cloned gene product as inclusion bodies. These aggregates have no

biological activity and there is a need to solubilise the inclusion bodies and refold the protein into its native structure. The process advantages of expressing the colned gene product as inclusion bodies can be exploited only if refolding yields are significant[1]. It is known that the refolding yield decreases with the increasing of the concentration of denatured proteins because of the kinetic competition between the protein aggregation and protein refolding[2]. However, refolding at low protein concentration often leads to the requirement of large refolding reactor and quantities of buffer, also the increasing difficulty in protein recovery. How to refold proteins with high recovery and at high initial protein concentration is valuable and challengable.

Size exclusion chromatography protein refolding system(SEPROS) was developed to increase the yield during the refolding of lysozyme and bovine carbonic anhydrase[2]. Using SEC, renaturaton of heterodimeric platelet-derived growth factor and urokinase plasminogen activator from inclusion bodies was also made a success[3,4]. Interferon-γ (IFN-γ) are secreted proteins which induce an antiviral state in their target cells and are universally accepted as a therapeutic agent.

Here we will present the results on refolding of denatured lysozyme, a popular system for evaluation of new refolding strategies[5] at high concentration by SEC with optimum urea in renaturation buffer as dilution additives. Then the refolding of IFN-γ by SEC was further studied.

Materials and Methods

Materials and apparatus

Hen egg white lysozyme, dried Micrococcus lysodeikticus cells, reduced glutathione(GSH), oxidized glutathione(GSSG),dithiothreitol(DTT) and Trizma base were purchased from Sigma. All other chemicals were of analytical grade

and obtained from commercial source.

Superdex 75 and colum(XK16/20) were obtained from Amersham Phamacia Biotech.. Chromatographic separations were performed using ÄKTA Explorer 100.

Denatured lysozyme and IFN-γ inclusion bodies was prepared in our lab.

Renaturation buffer

Renaturation buffer is 0.1 M Tris-HCl pH 8.0, containing 1 mM EDTA, 0.15 mM NaCl, 3mM GSH, 0.3 mM GSSG and a defined urea.

Refolding using SEC

Size exclusion chromatography refolding was performed using a XK16/20 column packed with superdex 75 gel media. Before the application of the denatured lysozyme and IFN-γ, the column was equilibrated with renaturation buffer, and the fractions were analyzed following sample injection for assays.

Assays

Lysozyme activity was determined by F.I.P method[6], protein concentration was determined by a modified Coomassie Brilliant Blue assay[7], IFN-γ was assayed by viral cytopathic assay that means human cells WISH were challenged with Vesicular Stomalitis Virus (VSV) in the presence or absence of rhuIFN-γ [8].

Results and Discussion

Refolding of Lysozyme

Effect of column height on resolution

In order to refold lysozyme, the key procedure is to slowly remove the

denaturant from the protein, this can be performed by SEC using a XK16/20 column packed with superdex 75 gel media. While the applied sample was 0.1ml (20 mg/ml lysozyme) and the volumetric flow rate was 0.3 ml/min, the separation performance of lysozyme and low molecular denaturant became better with the increasing of the column height. For 7 cm column height, the refolded lysozyme and the denaturant can be separated completely, the recovery yield was more than that operated at 3.5 cm and 5 cm column height, so the 7 cm column height was selected to refold lysozyme.

Effect of urea concentration on refolding

SEC refolding with renaturation buffer addition of different amounts of urea was tested under the condition that the initial concentration of denatured lysozyme was 20mg/ml and the applied sample was 0.1ml, the elution profile was represented in Fig.1. As the concentration of urea increased from 1M to 6M, the elution volume for the refolded lysozyme decreased from 11.3 ml to 8.18 ml, it should be noted that for renaturation buffer without urea, there is no refolded lysozyme peak to be detected, the reason may be due to the increasing of the viscosity of the solution and the Stokes radius(Rs)[9]. The viscosity of the solution increased, the diffusive rate of protein into gel media decreased. During the refolding of the lysozyme, the protein molecule develops a more compact and native-like structure, called molten globule which is easy to refold. At this stage, the protein has a smaller Rs and can move further into the bead of the gel media. So the average partition coefficient of molten globule lysozyme was larger than that of denatured lysozyme, this contributed a lot to distinguish them. Transport of the lysozyme into the gel pores was diffusion-limited, so intermolecular aggregation can be reduced. The optimum urea in renaturation buffer was 4 M, accordingly the protein recovery and yield recovery were 95.4% and 62.5% respectively (see Table 1), the

concentration covered was up to 317.9 μg/mL. For urea concentration higher than 4 M, the denaturation effect was too strong to inhibit protein molecule go throng the high-energy transient state which is the free energy landscape for protein folding[10], hence the lysozyme refolding yield decreased, especially while the urea concentration was 5 M and 6 M.

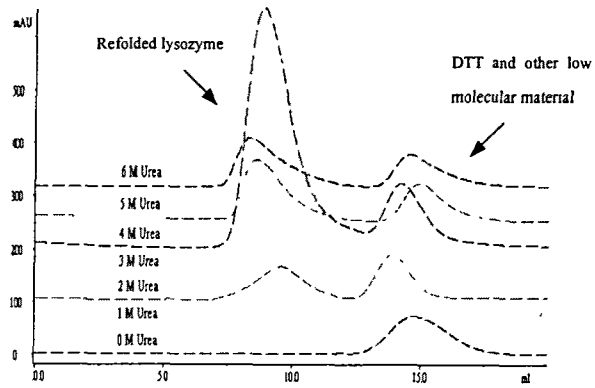

Fig. 1. Elution profile for SEC refolding lysozyme with renaturation buffer containing a defined urea

Table 1. Effect of urea on refolding lysozyme using size-exclusion chromatography

Urea concentration (M)	Concentration Recovered (μg/mL)	Elution Volume (ml)	Protein Recovery (%)	Yield (%)
0	0	0	0	0
1	32.2	11.13	6.4	4.9
2	259.4	9.50	55.9	33.1
3	298.9	9.16	89.7	44.6
4	317.9	8.84	95.4	62.5
5	270.3	8.55	81.1	30.2
6	184.6	8.18	43.8	21.7

Refolding at high initial concentration

Under above renaturation condition, refolding of lysozyme was investigated by SEC at initial protein concentration 20mg/ml, 50mg/ml and 100mg/ml respectively, the result was shown in Table 2. Protein refolding at high initial protein concentration and with high yield recovery is the focus for industrial process. The highest yield recovery was 62.5% at 20 mg/ml, although with the initial protein concentration increasing, the specific activity recovery and yield recovery decreased, at high concentration up to 100mg/ml, the yield recovery and the concentration recovered was as high as 34.8% and 1.06 mg/ml. This method is applicable to biological industry and is suitable for process scale-up.

Table 2. Refolding lysozyme using SEC at high initial concentration

Initial concentration (mg/mL)	Protein applied (mg)	Concentration recovered (mg/ml)	Specific activity recovery (%)	Yield recovery (%)
20	2	0.32	89.4	62.5
50	5	0.63	79.4	44.1
100	10	1.06	74.6	34.8

Refolding of IFN-γ

The elution profile of unfolding IFN-γ using SEC was shown in Fig.2., the first peak should be protein and second one low molecular weight DTT, with high conductivity. SDS-PAGE of fractions 3, 4, 5, 6 was presented in Fig.3. We can see that purified IFN-γ could be obtained after one step SEC refolding from inclusion bodies. The specific activity was 6.09×10^6 IU/mg with 0.24mg/mL concentration and 82.1% recovery.

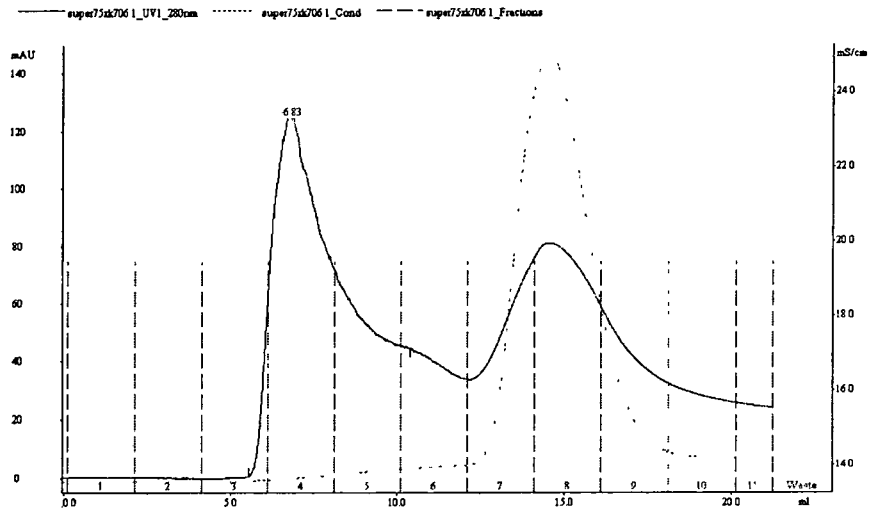

Fig. 2. Elution profile and collected elution samples of IFN-γ using SEC (Linear flow rate:3.3×10^{-5}m/s ; application sample:0.1mL×9.7 mg/mL, Elution buffer: 3Murea, 10mM EDTA, 0.15M NaCl, 0.05M PBS, pH 7.0)

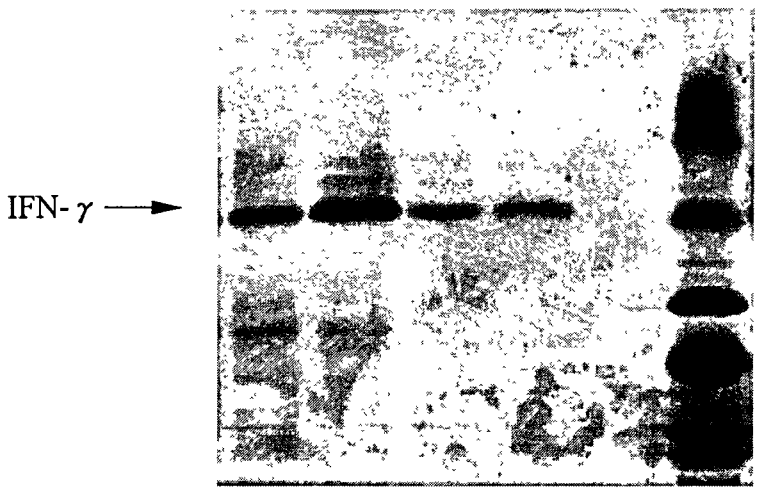

Fig. 3. Detection of elution samples of SEC by SDS-PAGE(3,4,5,6 denotes the collected sample shown in Fig.2; M:Marker, from top to button MW was 14400, 20100, 30000, 45000, 66000, 97000 Da respectively)

Conclusions

The results here illustrated that renaturation buffer additive of urea is a very useful strategy to improve activity recovery while refolding by SEC at high starting protein concentration, however, the optimum concentration of urea varies with the changing of starting protein concentration. After refolding by SEC, combining hydrophobic interaction chromatography or affinity chromatography (for recombinant protein with histidine tag), the refolded protein can be purified, this technique is also helpful for refolding inclusion bodies proteins at high protein concentration, for example endostatin inclusion bodies.

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