Hydrophobic interaction chromatography correctly refolding proteins assisted by glycerol and urea gradients

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Abstract

Chromatographic columns packed with commercially available hydrophobic interaction chromatography (HIC) media were found to be able to suppress aggregation and nevertheless had a tendency to promote the structural misfolding resulting in higher soluble protein recovery and lower specific activity than that by dilution when they were used to refold lysozyme, a model protein. Moreover, this misfolding effect was exacerbated with increasing hydrophobicity of media. A novel strategy involving the combination of glycerol, a typical osmolyte, a urea gradient and commercially available HIC media was introduced to facilitate protein refolding correctly as well as improve mass recovery by providing a gradual change of the refolding environment in the HIC column. In this process, unfolded lysozyme was bound to Poros PE HIC column at high salt concentration and was released by a urea gradient followed by elution with refolding buffer in the presence of 50% (v/v) glycerol, resulting in 86.3% activity yield and 85% mass recovery with the refolded product of native specific activity. For the absence of glycerol, only 50.9% activity yield and 59% specific activity recovery was obtained although mass recovery was close to that in the presence of glycerol. It was also discovered that glycerol addition during elution process was necessary for correct refolding compared to mixing of glycerol with post-column fraction. The possible mechanism for refolding with this system was proposed to be relevant to the formation of an on-pathway intermediate that could slowly reactivates.

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1. Introduction

In vitro refolding is a necessary and critical operation unit for processing recombinant proteins that are over-expressed and frequently form inactive inclusion bodies in Escherichia coli. [1]. During refolding process, aggregation that arises from assembly of kinetically trapped intermediates by intermolecular hydrophobic interaction often competitively occurs and dominates over on-pathway refolding at increasing protein concentration due to its higher reaction order than refolding resulting in the low yield of active protein of interest [2,3]. To minimize aggregate, classical dilution refolding way has to be performed at very low protein concentration, which is unfavorable for industrial process [4]. An efficient way that promotes refolding and simultaneously minimizes aggregation at high protein concentration will significantly improve the yield of target protein as well as reduce the cost of production [5].

Utilization of chromatographic columns for refolding is a good strategy because the solid media can provide inner particular environment able to separate molecules from each other efficiently during refolding, thereby minimizing the aggregation [6]. Size-exclusion chromatography (SEC)
A urea gradient was used to release bound protein followed by elution with refolding buffer containing glycerol of high concentration. The effects of various HIC media and the addition of glycerol on refolding efficiency were investigated. A possible mechanism of refolding with our strategy was proposed.

2. Materials and methods

2.1. Materials

Hen egg white lysozyme, Micrococcus lysodeikticus dried cells were obtained from Sigma. All chemical reagents were commercially available and were of analytical grade. Poros PE, a perfusion hydrophobic interaction support, was purchased from Applied Biosystems, USA. Hitrap Phenyl Sepharose HP (1 ml), Hitrap Butyl Sepharose FF (1 ml) and Hitrap Octyl Sepharose FF (1 ml), Superdex 75 HR 10/30 column (300 mm × 10 mm internal diameter (i.d.)), AKTA purifier 10, ultraspec 2000 UV–vis spectrophotometer were products of GE Healthcare, Uppsala, Sweden.

2.2. Denaturation of proteins

Ten milligrams lysozyme was dissolved in 1 ml unfolding buffer (6 M guanidine hydrochloride (Gdn-HCl), 100 mM Tris–HCl, 1 mM EDTA, 150 mM dithiothreitol (DTT), pH 8.5) and incubated for 3 h at 37 °C to confirm complete denaturation. And then denatured proteins of various concentrations were prepared by dilution of the protein described above with the same unfolding buffer.

2.3. Refolding by dilution

In order to compare with refolding by HIC, 20 μl denatured lysozyme was directly diluted into 1 ml refolding buffer (100 mM Tris–HCl, 1 mM EDTA, 3 mM GSH, 0.3 mM GSSG, pH 8.5) with or without the addition of glycerol.

2.4. Refolding bypassing through HIC column

One hundred microlitres denatured lysozyme was directly applied to different HIC columns (including Packed Poros PE column (1 ml), Hitrap Butyl Sepharose FF (1 ml), Hitrap Octyl Sepharose FF (1 ml) and Hitrap Phenyl Sepharose HP (1 ml)), respectively pre-equilibrated with refolding buffer (100 mM Tris, 1 mM EDTA, 3 mM GSH, 0.3 mM GSSG, pH 8.5) and then was eluted with the buffer at 0.2 ml/min flow rate on AKTA Purifier.

Refolding was also carried out in Packed Poros PE column (128 mm × 10 mm i.d.) with the same buffer.

2.5. Refolding by adsorption–elution way in HIC column

One hundred microlitres unfolded lysozyme of 10 mg/ml was applied to the Poros PE HIC column (128 mm × 10 mm i.d.) with the same buffer.
i.d.) equilibrated with the buffer of high salt concentration (A: 3.6 M (NH₄)₂SO₄, 50 mM Tris–HCl, 1 mM EDTA, 3 mM GSH, 0.3 mM GSSG, pH 8.5) followed by 2 ml gradient of A to the denaturant (B: 8 M urea, 50 mM Tris–HCl, 1 mM EDTA, 3 mM GSH, 0.3 mM GSSG, pH 8.5) and subsequent 1 column volume (CV) washing with A resulting in adsorption as well as prevention of aggregation. After refolding buffer (C: 100 mM Tris–HCl, 1 mM EDTA, 0.4 M (NH₄)₂SO₄, 0.01% (v/v) β-mercaptoethanol ([β-ME], pH 8.5) continuously washed the column to decrease ionic strength, 4 ml gradient of increasing urea concentration from C to B and 4 ml gradient of decreasing urea concentration from B to C in turn was then introduced into the column to release bound lysozyme followed by elution with C to promote released protein refolding. Flow rate was 0.5 ml/min. All steps were carried out on AKTA purifier.

In the other experiments, 50% (v/v) of final glycerol concentration was added into the buffer C and then the steps described above were performed. The elution process by the gradient of urea and glycerol in the column and mobile phase is modified according to reference [17], as shown in Fig. 1. As a control experiment, glycerol was immediately mixed with pooled refolded lysozyme.

2.6. Enzyme activity assay

After incubation overnight at room temperature, refolded lysozyme obtained with above methods was adjusted to lower than 50 μg/ml of protein concentration with working buffer (0.06 M sodium phosphate, pH 6.2) to increase the sensitivity of reaction. The activity was measured according to the method described by Shugar [19]. Activity yield was the percentage ratio of the activity of refolded lysozyme to that of native lysozyme of the same mass. Specific activity was represented by the activity of refolded lysozyme per milligram. Specific activity recovery was the percentage ratio of the specific activity of refolded lysozyme to that of native lysozyme.

2.7. Protein concentration assay

The concentrations of proteins were determined according to Bradford method [20]. Pure native lysozyme was used as referenced protein instead of BSA. Mass recovery or soluble protein recovery of was the ratio of total mass of refolded lysozyme to that of loaded unfolded protein for HIC refolding. It was the ratio of protein mass in the supernatant upon centrifugation of refolded solution at 12,000 × g to remove sediment to adopted unfolded protein mass for dilution refolding.

2.8. SEC analysis

Fifty microlitres refolded lysozyme was applied to Superdex 75 HR 10/30 (300 mm × 10 mm i.d.) equilibrated with mobile phase (4 M urea, 20 mM Na₂HPO₄, pH 7.0, 0.16 M NaCl) and developed with above buffer at flow rate of 0.5 ml/min.

3. Results and discussion

3.1. Effects of hydrophobicity of HIC media on refolding and aggregation

A series of columns packed with various commercially available HIC media were attempted to refold lysozyme by passing-through way. Unfolded lysozyme was loaded to the columns pre-equilibrated with the refolding buffer followed by elution with the same buffer. As the control experiment, sample was directly 50-fold diluted into refolding buffer at

On the other hand, pooled protein was diluted with 200 mM NaH₂PO₄ into about 30 μg/ml at different intervals and then was assayed to observe post-column refolding kinetics.

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HIC 3 In the eluent 86.3 85 100
HIC 2 In pooled fraction 58.8 80 68

room temperature and their activity and protein concentration were assayed.

rate on AKTA Purifier. The fractioned peaks were incubated overnight at

GSSG, pH 8.5) and then was eluted by refolding buffer at 0.2 ml/min flow

with the refolding buffer (100 mM Tris, 1 mM EDTA, 3 mM GSH, 0.3 mM

Hitrap Phenyl Sepharose HP column (1 ml)) respectively pre-equilibrated

plied to different HIC columns (including Packed Poros PE column (1 ml),

assisted refolding, 100

Tris–HCl, 1 mM EDTA, 3 mM GSH, 0.3 mM

For the dilution refolding, 20 µl denatured lysozyme at the protein concentra-
tion of 10 mg/ml was directly diluted into 1 ml refolding buffer (100 mM

concentration to the refolding buffer.

For dilution refolding, large amount of precipitation was

considerable amount of precipitation was

Dilution As an additive 32.9 36 91
Poros PE column 52 69.7 74.7
Butyl FF column 55.7 79.5 70
Octyl FF column 59.3 81.2 69.6
Phenyl HP column 55.9 89.2 62.7

For the dilution refolding, 20 µl denatured lysozyme of 10 mg/ml was directly diluted into 1 ml refolding buffer (100 mM Tris–HCl, 1 mM EDTA, 0.4 M (NH₄)₂SO₄, 0.01% (v/v) -mercaptoethanol, pH 8.5); HIC 1 , 100 µl denatured lysozyme of 10 mg/ml was adsorbed to the Poros PE HIC column (128 mm × 10 mm i.d.) at high salt concentration followed by elution with refolding buffer. Two ways was adopted to intro-
duce glycerol; for the first, glycerol was mixed with pooled fraction from HIC column; for the second, glycerol was added into eluent. In a control experiment, dilution refolding was conducted in the presence of glycerol. Table 2 shows the ef-
effect of glycerol addition on refolding efficiency.

For glycerol-assisted dilution refolding, precipitation was

Table 2

<table>
<thead>
<tr>
<th>Refolding ways</th>
<th>Glycerol addition ways</th>
<th>Activity yield (%)</th>
<th>Soluble protein recovery (%)</th>
<th>Specific activity recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dilution</td>
<td>As an additive</td>
<td>32.9</td>
<td>36</td>
<td>91</td>
</tr>
<tr>
<td>HIC</td>
<td>No</td>
<td>50.9</td>
<td>80</td>
<td>59</td>
</tr>
<tr>
<td>HIC</td>
<td>In pooled fraction</td>
<td>58.3</td>
<td>80</td>
<td>68</td>
</tr>
<tr>
<td>HIC</td>
<td>In the eluent</td>
<td>86.3</td>
<td>85</td>
<td>100</td>
</tr>
</tbody>
</table>

For dilution refolding, 20 µl denatured lysozyme at the protein concentration of 10 mg/ml was directly diluted into 1 ml refolding buffer (100 mM Tris–HCl, 1 mM EDTA, 3 mM GSH, 0.3 mM GSSG, pH 8.5), the addition of 30% (v/v) of glycerol. HIC 1 , 100 µl denatured lysozyme of 10 mg/ml was adsorbed to the Poros PE HIC column (128 mm × 10 mm i.d.) at high salt concentration. A urea gradient was used to release bound protein followed by elution with refolding buffer. Two ways was adopted to introduce glycerol; for the first, glycerol was mixed with pooled fraction from HIC column; for the second, glycerol was added into eluent. In a control experiment, dilution refolding was conducted in the presence of glycerol. Table 2 shows the ef-
effect of glycerol addition on refolding efficiency and protein molecules likely restrains intra-molecular hydrophobic association of some amino acids of proteins that is nec-

Theoretically, refolded product can recover its native ac-
tivity since normal protein folding is a spontaneous process

driven by Gibbs free energy between unfolded and native protein involving intra-molecular hydrophobic interaction


Table 1

<table>
<thead>
<tr>
<th>Refolding ways</th>
<th>Activity yield (%)</th>
<th>Soluble protein recovery (%)</th>
<th>Specific activity recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dilution</td>
<td>26.8</td>
<td>32.6</td>
<td>82.2</td>
</tr>
<tr>
<td>Poros PE column</td>
<td>52</td>
<td>69.7</td>
<td>74.7</td>
</tr>
<tr>
<td>Butyl FF column</td>
<td>55.7</td>
<td>79.5</td>
<td>70</td>
</tr>
<tr>
<td>Octyl FF column</td>
<td>59.3</td>
<td>81.2</td>
<td>69.6</td>
</tr>
<tr>
<td>Phenyl HP column</td>
<td>55.9</td>
<td>89.2</td>
<td>62.7</td>
</tr>
</tbody>
</table>

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Here, glycerol, a typical osmolyte, was attempted to assist commercially available HIC column in refolding lysozyme. Unfolded lysozyme was loaded to Poros PE HIC column (128 mm × 10 mm i.d.) at high salt concentration followed by elution with refolding buffer. Two ways was adopted to intro-
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observation, implying multiple-site hydrophobic interaction between the denatured peptide chains and the solid media. This kind of interaction is too strong for the peptide chains to refold freely. The introduction of urea gradient could efficiently release bound protein resulting in 80% mass recovery. Nevertheless, the activity yield was only 50.9% with 59% specific activity recovery, even lower than that of passing-through way with a short column. Compared to the absence of glycerol, immediate addition of glycerol into pooled fraction from the column leaded to slight improvement of activity yield. Under the optimum condition, it was 58.8% with 68% specific activity recovery at 40% of glycerol concentration, indicating that direct mixing of glycerol with pooled lysozyme could partially enhance refolding. Glycerol is thus considered to be unable to intrigue the re-arrangement of already misfolded structure and only strengthen compactness of local hydrophobic regions.

When glycerol acted as the eluent for HIC, activity yield continuously rose with increasing glycerol concentration. 86.3% activity yield and 85% mass recovery were obtained at 50% (v/v) of glycerol concentration. Furthermore, specific activity recovery reached 100% demonstrating the formation of native structure. Therefore, participation of glycerol into total refolding process is supposed to be necessary for the formation of final correct structure. In addition, a gradient of denaturant to glycerol provides a gradually strengthened hydrophilic environment to avoid a sudden shock to the protein. During elution process of bound protein, released and partially refolding protein that has many exposed hydrophobic groups can avoid of aggregation due to its hydrophobic interaction with HIC media. The successful refolding of lysozyme with our strategy lies in the combination of advantages of glycerol and HIC.

To further probe into the role of glycerol on refolding efficiency, refolded lysozyme by HIC column with or without glycerol addition was characterized with SEC. As Fig. 2 indicated, multiple lysozyme peaks were found in the profile of refolded lysozyme in the absence of glycerol indicating the presence of soluble aggregate and/or misfolded structure. In the case of 50% of glycerol in eluent, only one lysozyme peak of which retention volume was identical to the native was seen, which indicates that eluted lysozyme has been successfully refolded.

3.3. Proposed procedure for refolding lysozyme with HIC assisted by glycerol

As shown in Fig. 3a, when unfolded lysozyme passed through Poros PE HIC column (128 mm × 10 mm i.d.) filled with refolding buffer, two peaks were observable indicating two structures of different hydrophobic surfaces. Moreover, absorbance at 280 nm was higher than that at 254 nm for the first peak and nevertheless lower for the second peak. The second peak was thus assumed to be an abnormal protein implying refolding intermediate. Fig. 3a displays enzymatic reactivation as a function of time for eluted lysozyme passing through HIC column. A little activity of these two eluted peaks was immediately obtained accounting for the formation of partially active structure during elution. The two curves appeared two phases: activity yield of eluted peaks quickly increased within initial 10 min, but becoming flat during later incubation. The activity of the second peak was a little higher than that of the first.

Previous study demonstrated that refolding of lysozyme in solution was subject to two routes: the fast track involves the formation of near native structure containing both α and β domains; the slow track is responsible for the production of refolding intermediate containing α domain [24]. It can be speculated that the application of unfolded protein to the
Fig. 3. Curves for lysozyme to be refolded by HIC with passing and adsorption–elution way. Solid curves, monitoring at 280 nm; dotted curves, at 254 nm. (a) Refolding by passing-through way with the HIC column: 100 μl unfolded lysozyme of 10 mg/ml was applied to the Poros PE (128 mm x 10 mm i.d.) pre-equilibrated by refolding buffer (100 mM Tris, 1 mM EDTA, 3 mM GSH, 0.3 mM GSSG pH 8.5) followed by elution with the same buffer at 0.2 ml/min; (b) refolding by adsorption–elution way with the HIC column assisted by glycerol: 100 μl unfolded lysozyme of 10 mg/ml was adsorbed to the Poros PE HIC column (128 mm x 10 mm i.d.) at high salt concentration. A urea gradient was used to release bound protein followed by elution with the refolding buffer containing 50% glycerol. (*) Lysozyme peaks.

HIC column filled with refolding buffer leads to an immediate dilution process of denaturant and simultaneously initiates primary refolding. Thus, two initial refolding intermediates similar to those of dilution refolding were possibly produced. Our data suggests that HIC potentially functions as a tool to characterize refolding intermediates. These two refolding intermediates were likely dynamic-controlled and thus produced a slight initial activity due to quick hydrophobic collapse. However, they failed to form native structure due to the effect of hydrophobic environment of HIC in the end.

Fig. 3 b is the chromatogram for lysozyme refolding by HIC adopting adsorption–elution way in the presence of 50% glycerol in refolding buffer. One peak was eluted compared to above result. Furthermore, its absorbance at 280 nm was higher than at 254 nm, consistent with normal protein. This result suggests that refolded proteins with homogeneous hydrophobicity are likely produced unlike above case. On the other hand, the kinetic curve for its activity recovery versus time was distinctly different from that for passing-through way (Fig. 4 b). Initial post-column activity yield of eluted peak was almost absent. It continuously rose with time and reached 86% 10 h later. It can be assumed that enzymatic reactivation process of obtained lysozyme by adsorption–elution way with HIC mediated by glycerol is much slower than passing-through way. This strategy is speculated to involve the pre-formation of a productive refolding intermediate that can finally recover native activity during post-column incubation.

It is thus assumed that single refolding route distinct from dilution refolding was adopted resulting in a productive refolding intermediate that could reactivate. Furthermore, this process was possibly thermodynamic-controlled and relatively slow. Our refolding system is thus considered to vary the refolding pathway of lysozyme resulting in a decreased refolding rate. However, it does not interfere with the final formation of correct structure of target protein.

4. Conclusion

The combination of commercially available HIC media and glycerol was first introduced to refold denatured protein. Commercially available HIC columns were able to suppress aggregation and nevertheless prevented correct refolding resulting in non-native structure with low specific activity. The side-effect of misfolding was exacerbated with increasing hydrophobicity of media. Correct refolding could be achieved when glycerol was present in refolding buffer as the eluent for HIC column.

Two refolding intermediates of lysozyme were separated, suggesting that HIC can be used to a potent tool to monitor refolding process. Glycerol-HIC refolding system
was considered to produce a thermodynamic-controlled on-pathway refolding intermediate that can slowly recover native activity likely through a route different from that for dilution refolding.

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References