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Purification effect of artificial chaperone in the refolding of recombinant ribonuclease A from inclusion bodies

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ABSTRACT

Artificial chaperone (AC) containing cetyltrimethylammonium bromide (CTAB) and β-cyclodextrin (β-CD) has been used to refold recombinant ribonuclease A (RNase A) from inclusion bodies (IBs). At low urea concentration (0.8 M), the AC could enhance the refolding yield of RNase A by effectively suppressing its intermolecular interaction-induced aggregation. As a result, 0.9 mg/mL RNase A could be 77% refolded, which was a 57% increase as compared to that without the AC. At high protein concentration range (0.9–2.3 mg/mL in total protein concentrations) and 1.6 M urea, CTAB selectively precipitated contaminant proteins distinctly, so a purification effect was achieved. For example, 1.5 mg/mL RNase A could be 62% refolded and recovered at a purity of 87%, which was a 34% increase in purity as compared to that in IBs (65%). The precipitation selectivity was considered due to the differences in the hydrophobicity of the proteins. The work indicates that by using the AC, RNase A could be efficiently refolded at low urea concentration and purified at high urea concentration from IBs at high protein concentrations.

1. Introduction

Prokaryotic expression systems, particularly *Escherichia coli* (*E. coli*), have been widely exploited for large scale production of valuable proteins [1,2]. However, high-level expression of recombinant proteins in *E. coli* often results in the formation of insoluble and inactive inclusion bodies (IBs). To recover functionally active proteins, it is necessary to adopt elaborate solubilization, refolding and purification procedures to deal with IBs [3]. Low-efficiency protein refolding is usually caused by aggregation of folding intermediates, especially in the case of refolding at high protein concentrations. Moreover, for the refolding of proteins from IBs, contaminants in IBs can lead to the aggregation of target proteins as well [4,5]. So inhibition of the aggregation is essential for high-efficiency protein refolding [6].

Artificial chaperone (AC) is a potential approach for refolding proteins on a large scale, considering its convenient, low-cost operation and applicability to a wide variety of proteins [7]. Different with other folding additives, AC mimicks the two-step mechanism of *in vivo* molecular chaperone-assisted protein folding [3,8–10]. So far, a few AC systems with various protein capturing and releasing agents have been reported [7,10–14]. However, previous researches have mainly focused on the refolding of denatured proteins from pure sources, and studies on practical refolding systems with IBs were limited [10,15,16], especially at high-concentration refolding conditions.

In the previous paper, we have reported that the redox system 4-mercaptobenzeneacetate (ArSH)/oxidized glutathione (GSSG) was more efficient than the traditional redox system reduced glutathione (GSH)/GSSG in assisting the oxidative refolding of ribonuclease A (RNase A) from IBs at a low concentration [17]. To make the refolding at elevated protein concentrations as efficient as that at low concentrations, the AC consisting of cetyltrimethylammonium bromide (CTAB) and β-cyclodextrin (β-CD) was introduced to assist the refolding. Besides the anti-aggregation effect, we have herein found for the first time a purification effect of the AC in the refolding of RNase A from IBs. Incorporating the purification capability, the AC system is considered to find more applications in the recovery of recombinant proteins.

2. Materials and methods

2.1. Materials

Bovine pancreatic ribonuclease A (type XII-A), cytidine 2′:3′-cyclic monophosphate monosodium salt (cCMP), bovine serum albumin (BSA) and ArSH were from Sigma (St. Louis, MO, USA). Reduced dithiothreitol (DTT), GSSG, CTAB and β-CD were from Bingguo Biotech (Beijing, China). Protein molecular weight markers were from TransGen Biotech (Beijing, China). Other chemicals

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were all commercially available reagents of analytical grade. All chemicals and reagents were used as received.

2.2. Preparation of denatured and reduced RNase A (DR-RNase A) IBs

The preparation of RNase A IBs was described previously [17]. Briefly, the recombinant *E. coli* BL21 (DE3) carrying pET28a–RNase A was grown in LB medium. The cell pellet was collected by centrifugation and disrupted by ultrasonication. The disrupted suspension was centrifuged and the precipitate was collected and washed to obtain RNase A IBs [15,17]. Finally, the RNase A IBs were dissolved and denatured using a solubilization buffer containing 18 mM DTT, 8 M urea, 0.1 M Tris–HCl and 1 mM EDTA (pH 8.0). The DR-RNase A IBs solution containing 18.5 mg/mL protein was used as the starting solution in the following refolding experiments. Analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (see below), the RNase A content in the IBs solution was 65% of the total protein, so the staring solution contained 12 mg/mL RNase A.

2.3. RNase A refolding

RNase A was refolded by two methods, namely, direct dilution and AC-assisted refolding. AC-assisted refolding was carried out following the method reported earlier with a minor modification [9,18]. The DR-RNase A was first diluted into the refolding buffer with predetermined concentrations of urea, CTAB, GSSG and ArSH. The solution was allowed to sit for 10 min, and then a certain volume of 16 mM β-CD solution was added to initiate the refolding. The final refolding solution contained 0.6 to 1.5 mg/mL RNase A (0.9 to 2.3 mg/mL in total protein concentrations), 0.9 to 2.25 mM DTT, 75 mM Tris–acetate, 1 mM EDTA and definite concentrations of urea, CTAB, β-CD, ArSH and GSSG (pH 8.3). For comparison, direct dilution refolding experiments were carried out by diluting the DR-RNase A solution with refolding buffer containing the same components except CTAB and β-CD. The direct dilution refolding at 0.6, 0.9 and 1.5 mg/mL RNase A are denoted as DD-0.6, DD-0.9 and DD-1.5, respectively. The corresponding AC-assisted refolding experiments are referred to as AC-0.6, AC-0.9 and AC-1.5, respectively.

All the refolding experiments were carried out at 25 °C. During the refolding, small aliquots were withdrawn from the reaction mixtures at different time intervals to determine the enzyme activity. In 24 h of refolding operation, each refolding solution was centrifuged at 2000 rpm for 2 min. Then the soluble protein concentration and RNase A purity in the supernatant were determined using the Bradford method and SDS-PAGE, respectively. Besides, the collected precipitates were redissolved with the denaturation buffer and then subjected to SDS-PAGE analysis.

2.4. Analytical methods

Protein concentration was determined by the Bradford method using BSA as the standard. To determine RNase A purity, the software Gel-Pro Analyzer 3.1 (Media Cybernetics, MD, USA) was used to deal with the SDS-PAGE images. The Gel-Pro Analyzer 3.1 provided the calculated integrated optical density (IOD) value for each lane, as well as the IOD value for each band in a lane. As such, the ratio of RNase A band IOD to the entire lane IOD led to the calculation of RNase A purity. The SDS-PAGE was performed with 12% resolving gel and 5% stacking gel. Active RNase A concentration was measured spectrophotometrically by hydrolyzing cCMP [19,20], which was not affected by the presence of CTAB and β-CD (data not shown). The aggregation degrees in the direct dilution and AC-assisted refolding of RNase A at different protein concentrations were estimated by continuous in situ measurements of turbidity at 400 nm (A_{400}). In this study, the results are averages from triplicate experiments and the error bars represent standard deviations.

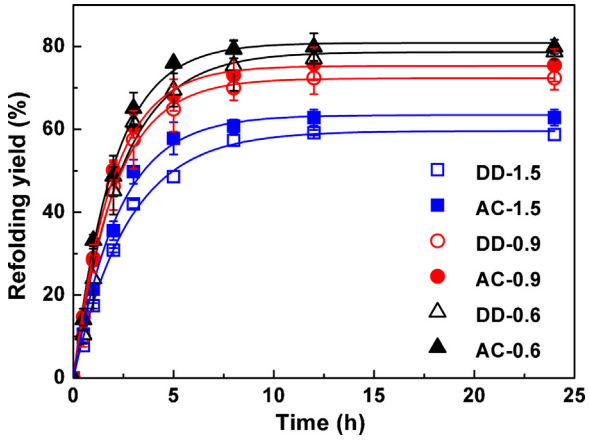

Fig. 1. Kinetics of direct dilution and AC-assisted refolding of RNase A at different initial RNase A concentrations. DD-0.6: 0.6 mg/mL RNase A, 0.9 mM DTT and 0.5 mM GSSG. DD-0.9: 0.9 mg/mL RNase A, 1.35 mM DTT and 0.8 mM GSSG. DD-1.5: 1.5 mg/mL RNase A, 2.25 mM DTT and 1.25 mM GSSG. Other condition: 4 mM ArSH, 1.6 M urea, 75 mM Tris–acetate pH 8.3 and 1 mM EDTA. AC-0.6, AC-0.9 and AC-1.5: 1.2 mM CTAB/4.8 mM β-CD, with other conditions the same as DD-0.6, DD-0.9, and DD-1.5, respectively. For the direct dilution refolding, time = 0 corresponds to the dilution of the solubilized IBs; for the AC-assisted refolding, time = 0 corresponds to the addition of β-CD to the RNase A-CTAB complex.

3. Results and discussion

3.1. AC precipitates contaminant proteins in IBs

In the RNase A concentration range studied (0.6 to 1.5 mg/mL), the AC just resulted in marginal improvements in refolding yields by comparison to the direct dilution refolding (Fig. 1). Similar results were obtained by trying the AC with other concentrations of CTAB and β-CD (data not shown). The inconspicuous efficiency of AC was considered due to the fact that 1.6 M urea had been sufficient to inhibit protein aggregation in the RNase A concentration range, where the refolding yield of RNase A was little compromised by aggregation. Previously, the effect of urea (denaturant) on protein refolding as well as the cooperative effects of urea and AC have been well studied [18,21], so we do not discuss more about the effect of urea. Instead, what we discuss below in detail is the phenomenon of AC-assisted protein refolding and contaminant protein precipitations, which has not been reported in literatures dealing with AC-assisted protein refolding [9,10,15,16].

According to the mechanism of AC-assisted refolding, a protein-detergent complex forms immediately after diluting a denatured protein into a refolding buffer containing the detergent, thus obtaining a clear refolding mixture [9]. However, in our study with RNase A IBs, we found a large amount of aggregates in the AC-assisted refolding, immediately after the DR-RNase A was diluted into the refolding buffer containing CTAB (1.7 mM after the dilution). This is obvious in Fig. 2. As can be seen, the initial A_{400} values for AC-0.6 and AC-0.9 were as high as 1.3 and 1.7, respectively, indicating the intensive aggregation of the proteins in the solubilized IBs. In contrast, the A_{400} values for DD-0.6 and DD-0.9 were only 0.2, implying there were a low level of aggregation immediately after the direct dilution. Ten minutes later, the addition of β-CD could not remove the aggregates, but just resulted in a decrease in the A_{400} values by the dilution. These aggregates kept growing over the course of time, and finally got settled to the vessel bottom in about 2 h. As a result of this sedimentation, the A_{400} values for the