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# Facilitated oxidative refolding of ribonuclease A from inclusion bodies with a new redox system

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ABSTRACT

Oxidative refolding is a crucial step in the bioseparation process of bioactive RNase A from inclusion bodies (IBs) overexpressed in *Escherichia coli*, and it has been well recognized that RNase A refolding suffers from the slow formation of correct disulfide bonds with traditional redox agents such as reduced glutathione (GSH) and oxidized glutathione (GSSG). Hence, there is demand of developing new redox systems that can accelerate the oxidative refolding of this protein. In this work, a new redox system composed of 4-mercaptobenzeneacetate (ArSH) and hexanoyl cystamine (HCA) was proposed. It was found that the oxidative refolding of RNase A reached a refolding yield of 94% in 2 h with ArSH/HCA at optimized concentrations, which was two times faster than that with GSH/GSSG. Moreover, the refolding yield of RNase A from IBs could also reach 89% in 3 h with ArSH/HCA, much better than the performance with GSH/GSSG, which was only 69% in 8 h refolding. Therefore, the use of the new redox system led to a significant increase of refolding yield and over 60% reduction in the refolding time in the inclusion body protein refolding. The results indicated that the new redox system was much more efficient in the oxidative refolding of RNase A than the traditional redox system.



## 1. Introduction

Bovine pancreatic ribonuclease A (RNase A, EC 3.1.27.5) is a typical ribonuclease catalyzing the cleavages of phosphodiester bonds in RNA [1,2]. RNase A is widely used in the removal of RNA contamination of plasmid DNA [3]. Besides, the oligomers of RNase A have antitumor activity, so the enzyme has clinical potential in antitumor therapy [4]. Hence, large-scale production of recombinant RNase A from inclusion bodies (IBs) overexpressed in *Escherichia coli* (*E. coli*) is of importance for obtaining high-purity RNase A without animal sourced contaminates [3]. However, an efficient refolding process is necessary for the recovery of the enzyme from IBs.

For disulfide-containing proteins like RNase A, redox system is essential for the refolding. It facilitates the formation of correct disulfide bonds. However, traditional redox system, for example, reduced glutathione (GSH)/oxidized glutathione (GSSG) usually suffers from low efficiency, leading to a long refolding time [5–8]. For example, Gough et al. [9] obtained a refolding yield of 80% in 5 h in the refolding of 0.34 mg/mL RNase A with 2 mM GSH/0.2 mM GSSG. Cabrele et al. [6] received a refolding yield of 83% in 10 h in the refolding of 0.34 mg/mL RNase A with 1 mM GSH/0.2 mM GSSG. Goto et al. [10] reached a refolding yield of 100% in 25 h in the refolding of 2.3 mg/mL RNase A with a reverse micellar system in the presence of 20 mM GSSG. Ono et al. [11] obtained a refolding yield of 100% in 40 h in the refolding of 2 mg/mL RNase A using a reverse micellar system incorporated with dialysis (inner phase: 0.3 M 2-mercaptoethanol, outer phase: 9 mM GSSG). Therefore, the development of new redox systems is necessary to accelerate the oxidative refolding of RNase A.

*In vivo*, efficient formation of disulfide bonds occurs by the catalysis of various oxidoreductases [12–15], such as protein disulfide isomerase (PDI) [16,17]. However, they are impractical for large-scale production *in vitro* [7,18]. As a result, several small-molecule thiols and disulfides were developed by the inspiration of oxidoreductases, which were proven to refold RNase A more efficiently than GSH/GSSG [19–21]. For example, the thiols CXXC and CXC peptides (C, cysteine; X, any residue) mimicking the amino acid sequence in the active site of oxidoreductases had PDI-like activity [6,19,20]. Another thiol, (±)-trans-1,2-bis(2-mercaptoacetamido)cyclohexane (BMC) could also increase the refolding yield [7]. In addition, aromatic thiols, including but not limited to 4-mercaptobenzeneacetate (ArSH), enhanced the rate constant of the oxidative refolding as compared to aliphatic thiols

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[9,19]. Pentapeptide RKCGC disulfide enhanced both the refolding rate and the refolding yield as compared to CGC disulfide [22]. Hexanoyl cystamine (HCA) refolded proteins more effectively than cystamine due to its hydrophobic alkyl tail [18].

Thus, it is considered that these small-molecule redox agents could be employed to construct new redox systems for accelerating the oxidative refolding of RNase A. In the present study, a new redox system composed of ArSH as the thiol and HCA as the disulfide has been proposed. As mentioned above, ArSH has excellent ability to reshuffle non-native protein disulfide bonds [9], while HCA has high oxidizing power [18]. Thus, this new redox system was first examined for the oxidative refolding of denatured RNase A from a commercial source. The operating parameters were optimized by dynamic refolding studies. Thereafter, ArSH/HCA at optimized concentrations was employed to the oxidative refolding of RNase A from IBs. Finally, the refolded RNase A from IBs was characterized for its enzymatic activity and secondary structures.

## 2. Materials and methods

### 2.1. Materials

Bovine pancreatic ribonuclease A (RNase A) (type XII-A), cytidine 2′:3′-cyclic monophosphate monosodium salt (cCMP), bovine serum albumin (BSA) and 4-mercaptobenzeneacetate (ArSH) were from Sigma (St. Louis, MO, USA). Restriction endonuclease (HindIII and BamHI) and T4 DNA ligase were from Fermentas Inc. (Hanover, MD, USA). Yeast extraction and tryptone were from Oxoid (Basingstoke, UK). Taq plus DNA polymerase, reduced dithiothreitol (DTT), reduced glutathione (GSH), oxidized glutathione (GSSG), tris(hydroxymethyl)aminomethane hydrochloride (Tris), isopropyl-β-D-thiogalactopyranoside (IPTG), kanamycin, imidazole, urea and sodium salt of ethylenediaminetetraacetic acid (EDTA) were purchased from Bingguo Biotech (Beijing, China). Protein molecular weight markers were obtained from TransGen Biotech (Beijing, China). Other chemicals were all commercially available reagents of analytical grade. All chemicals and reagents were used as received. HCA was prepared according to the literature [18]. An empty Tricorn 5/100 column (100 mm × 5 mm i.d.), an empty XK16/20 column (200 mm × 16 mm i.d.), Superdex 75 10/300 GL column (300 mm × 10 mm i.d.), Sephadex G-25 and Chelating Sepharose Fast Flow were from GE Healthcare (Uppsala, Sweden).

### 2.2. Denaturation and reduction of commercial RNase A

Commercial RNase A was reduced and denatured following the method reported by Konishi and Scheraga [23]. Native RNase A was dissolved in denaturing and reducing buffer (0.1 M Tris–HCl, 8 M urea, 65 mM DTT and 1 mM EDTA, pH 8.0) at a final concentration of 20 mg/mL. The solution was incubated at 25 °C overnight. Denatured and reduced RNase A (DR-RNase A) was isolated with a XK 16/20 column packed with Sephadex G-25 on an ÄKTA Basic liquid chromatography system (GE Healthcare). The column was equilibrated with 0.6% acetic acid. Finally, the DR-RNase A was lyophilized and stored at −70 °C. Before use, the DR-RNase A stock solution was prepared by dissolving the lyophilizate into the denaturing buffer without DTT to a final concentration of 6 mg/mL.

### 2.3. Expression vector construction

The RNase A coding gene was amplified from the genomic DNA of bovine pancreas with the upstream primer (5′-ACGGATCCAAGGAAACTGCAGCAGCCAAG-3′) and the downstream primer (5′-CCAAGCTTAGACCTACACTGAAGCATC-3′) containing recognition sequences for the BamHI and HindIII restriction enzymes (underlined). As the result of DNA sequencing, the PCR product (395 bp) showed 100% identity with the gene sequence (NCBI accession no. x07283.1) encoding RNase A (Fig. S1 in the Supplementary data). Then, the fragment digested with BamHI and HindIII was cloned into the pET28a vector. The construction strategy of the pET28a recombinant plasmid is shown in Fig. S2. The constructed recombinant plasmid pET28a–RNase A (5685 bp) was transferred into *E. coli* BL21 (DE3) to express RNase A. The pET28a–RNase A encodes a protein of 158 amino acids with a calculated molecular mass of 17.1 kDa. The N-terminal hexahistidine tag in the recombinant RNase A was brought in by the pET28a. The carboxylic end of the recombinant RNase A was the same as that of the native RNase A. The amino acid sequence of the recombinant RNase A is given in Fig. S3.

### 2.4. Expression of RNase A and preparation of DR-RNase A IBs

The recombinant *E. coli* BL21 (DE3) carrying pET28a–RNase A was grown in the LB medium containing kanamycin (50 μg/mL) by shaking culture (170 rpm) at 37 °C until the optical density at 600 nm reached 0.4. Protein expression was induced by the addition of IPTG (1 mM) and the cultivation was continued for additional 3 h. The cell pellet from 100 mL of the fermentation broth was harvested by centrifugation at 4000 rpm at 4 °C for 10 min. The cell pellet was then resuspended in 10 mL of PE buffer (20 mM $NaH_2PO_4$, 20 mM $K_2HPO_4$, 1 mM EDTA, pH 7.2), and sonicated at 300 W for 4 s in ice-water. This disruption was repeated in 6 s intervals for 60 cycles. The disrupted cell broth was centrifuged at 12,000 rpm for 15 min at 4 °C to collect the precipitate containing IBs. The precipitate was washed to purify the IBs by a routine procedure described elsewhere [24], and the purified IBs pellet was stored at 4 °C for further use.

The purified RNase A IBs were solubilized in 500 μL denaturation buffer with 10 mM DTT, and incubated at room temperature for 1 h. It was then purified by centrifugation (12,500 rpm for 15 min) to remove any insoluble matter. The concentration of RNase A in the supernatant was determined following the methods reported previously [25]. According to the concentration of RNase A, a definite amount of DTT was added. After reduction and denaturation, the solution contained 6 mg/mL DR-RNase A and 18 mM DTT. It was used as the starting solution for refolding experiments.

### 2.5. Oxidative refolding of RNase A

Tris–acetate buffers of pH 7.0–9.0 were prepared according to Gough et al. [9]. Stock solutions of 5 mM GSSG, 20 mM GSH, 20 mM ArSH and 5 mM HCA were prepared separately with the Tris–acetate buffers of different pH values. The refolding buffers were prepared by mixing the stock solutions with the Tris–acetate buffer of the same pH value.

All refolding experiments were carried out in 1.5 mL microcentrifuge tubes incubated at 25 °C. Refolding was carried out by diluting DR-RNase A with refolding buffer. The refolding system contained 0.3 mg/mL RNase A (in the case of RNase A IBs, it contained 0.4 mg/mL protein in total, including 0.3 mg/mL RNase A and 0.1 mg/mL other proteins), 1 mM EDTA, a definite concentration of disulfide (GSSG or HCA), a definite concentration of thiol (GSH or ArSH), a definite concentration of urea, and Tris–acetate (different pH values). During the refolding, small aliquots were withdrawn from the reaction mixture at different time intervals, and enzyme activity was measured immediately (see Section 2.7).

### 2.6. Purification of refolded RNase A and DR-RNase A IBs

To obtain the purified samples for the measurement of far-UV circular dichroism (CD) spectra and for the analysis of enzymatic