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Integrative refolding and purification of histidine-tagged protein by like-charge facilitated refolding and metal-chelate affinity adsorption

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ABSTRACT

This work proposed an integrative method of protein refolding and purification by like-charged resin facilitated refolding and metal-chelate affinity adsorption. Hexahistidine-tagged enhanced green fluorescence protein (EGFP) was overexpressed in *Escherichia coli* as inclusion bodies (IBs), and then the protein was refolded and purified from urea-solubilized IBs by this method. A metal-chelating resin was fabricated by coupling iminodiacetic acid (IDA) to agarose gel (Sepharose FF). The anionic resin was used to facilitate the refolding of like-charged EGFP from IBs. After refolding, nickel ions were introduced for the affinity purification of the target protein by metal-chelating adsorption. It was found that the resin was effective in facilitating EGFP refolding. For 0.1 mg/mL EGFP IBs refolding, the fluorescence recovery (FR) by direct dilution was only 64%; addition of only 0.05 g/mL resin increased the FR to over 90%. Moreover, the FR increased with increasing resin concentration. Owning to the shielding effect of the oppositely charged impurities embedded in IBs on the surface charges of the IDA resin, more resin particles were required to exert an aggregation inhibition effect in the IBs protein refolding. Additionally, compared with direct-dilution refolding, inclusion of like-charged resins not only offered an enhanced FR of EGFP, but also bound some opposite-charged contaminant proteins, leading to a preliminary purification effect. Afterwards, the refolded EGFP was recovered by metal-chelating adsorption at an FR of 85% and purity of 93%. This work has thus extended the like-charge facilitated protein refolding strategy to the integrative protein refolding and purification.

1. Introduction

Over-expression of recombinant proteins in *Escherichia coli* (*E. coli*) often results in the formation of insoluble and inactive inclusion bodies (IBs), so refolding procedure is a critical step in the recovery of functionally active proteins [1,2]. A big challenge in protein refolding is the aggregation of folding intermediates, which is the main cause of decreased refolding yield [3,4]. In addition, the contaminants in IBs may also lead to the aggregation of target proteins [5,6]. So inhibition of the aggregation is the key to the high-performance preparative protein refolding. Moreover, the efficient separation and purification of refolded proteins is another important part limiting the large-scale production of recombinant proteins [7].

In protein refolding, one strategy to inhibit aggregation is to use folding additives in the refolding system. Additives such as urea [8], guanidine hydrochloride (GdmCl) [9], and arginine [10,11] are frequently used to eliminate the interactions that lead to intermolecular aggregation. Besides, some other additives such as glycerol [12], proline [13], and hydrophilic polymers [14] are also employed as stabilizers of native state proteins. Generally, these additives are effective in suppressing aggregation. But the intramolecular folding was also interfered to some extent at the same time [15], resulting in a decreased refolding rate [16]. What is more, these additives bring troubles of further separation.

In addition to refolding operations in bulk solutions, chromatographic techniques have also been widely investigated [17–21]. Protein refolding in a chromatographic column could not only effectively inhibit protein aggregation, but also have purification effect for target proteins [22]. However, it usually takes a long time to

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reach the refolding equilibrium state due to the protein-support adsorption, which interferes with the proper intra-molecular folding and thus reduces the refolding efficiency [20]. In addition, the adsorptive refolding could not solve the problem of massive aggregation caused by high local protein concentrations [22]. Moreover, refolding by chromatography often leads to a low-enriched target product [23]. The needs of pretreatment of cell lysate, long operation times, and solvent consumption also limited chromatographic techniques for refolding applications [24].

Recently, our group has found that like-charged resin particles in a refolding solution can greatly enhance the refolding yield [25]. The working mechanism was considered that the electrostatic repulsion between the like-charged resin and protein induced the oriented alignment of protein molecules near the charged solid surface, resulting in an inhibition effect on the intermolecular aggregation. Afterwards, effects of solid properties on like-charged lysozyme refolding were explored by using porous anion exchangers [26], non-porous microspheres [27,28] and charged polymers [29]. Compared with other methods, the use of like-charged resin enhanced the refolding yield at high protein concentrations without compromising the refolding rate. Furthermore, it is advantageous in easy separation and reusability.

As massive impurities are embedded in IBs, further separation and purification are required after refolding process. Affinity adsorption based on the specific interaction between metal ions and Histidine (His)-tagged proteins has been considered as one of the most efficient methods for the purification of recombinant proteins. Based on this, we have herein proposed an integrative refolding and purification method by using the like-charged resin facilitated protein refolding and metal-chelate affinity adsorption. Iminodiacetic acid (IDA), which is capable of chelating transition metal ions and possesses negative charges, was coupled to agarose gel (Sepharose FF) to fabricate a metal-chelating resin, IDA-Sepharose FF. The refolding and purification effects of hexahistidine-tagged enhanced green fluorescent protein (EGFP) from solubilized IBs with this new approach were explored and a recovery of target protein with high yield and purity was obtained.

2. Materials and methods

2.1. Materials

Sepharose 6 Fast Flow (Sepharose FF) was purchased from GE Healthcare (Uppsala, Sweden). Isopropyl β-D-thiogalactopyranoside (IPTG), bovine serum albumin (BSA), Tris(hydroxymethyl)aminomethane (Tris), dithiothreitol (DTT) and Urea were obtained from Bingguo Biotech (Beijing, China). Protein molecular weight markers were from TransGen Biotech (Beijing, China). Iminodiacetic acid (IDA), ethylenediaminetetraacetic acid disodium (EDTA), dimethyl sulfoxide (DMSO), epichlorohydrin (ECH) and other reagents were of analytical grade from Guangfu Fine Chemical Research Institute (Tianjin, China). All the materials were used as received.

2.2. Fabrication and characterization of IDA-Sepharose FF

IDA-Sepharose FF gel was fabricated by coupling IDA to Sepharose FF as described previously [24,30]. The effective porosity (ε_p) of the gel for EGFP and total porosity (ε_0) were determined following the method described earlier [25,26]. The ion exchange capacity of the resin was measured by acid–base titration [31].

The chelating capacity of the resin was measured using copper ion as probe by the method reported previously [32] with minor modifications. Briefly, 1 g drained resin was suspended and agitated in 10 mL of 0.1 M $CuSO_4$ for 15 min for chelating with Cu^{2+} ions. The resin was then washed with excess distilled water to remove any free copper ions. Next, the resin was added to 10 mL of 0.1 M EDTA and agitated for 30 min to release the adsorbed Cu^{2+}. The Cu^{2+} concentration in the supernatant was recorded at 735 nm with an UPV-900 detector (GE Healthcare, Uppsala, Sweden). Finally, the Cu^{2+} chelating capacity of IDA-Sepharose FF was calculated based on the copper concentration in the supernatant.

In the above measurements, each experiment was conducted in triplicate and the average value is presented.

2.3. Preparation of native EGFP and EGFP IBs

The recombinant *E. coli* harboring the vector Pet28a-EGFP expresses both soluble (native) EGFP and EGFP IBs. The native EGFP and EGFP IBs were prepared according to Dong et al. [22]. In brief, the recombinant *E. coli* was cultured for EGFP expression and the cells were harvested by centrifugation. After cell disruption by sonication, the homogenate was separated by centrifugation. The supernatant was used for the purification of native EGFP by immobilized metal affinity chromatography (IMAC), while the precipitate was collected and washed to obtain EGFP IBs. The IBs pellet was stored at 4 °C for further use. The purified native EGFP was used as a standard of EGFP for the determination of refolding efficiency and material for the following refolding experiments.

2.4. Denaturation and refolding of EGFP

The purified EGFP or IBs were solubilized in denaturing buffer (50 mM Tris–HCl, 10 M urea, pH 8.5) by incubation at 50 °C for 2 h. The IBs solution was clarified by centrifugation at 4 °C, 12 000 rpm for 15 min to remove any insoluble matter. EGFP content in the solubilized protein solution was about 70%, as estimated by the sodium dodecyl sulfate–polyacryamide gel electrophoresis (SDS-PAGE) (see below). The EGFP IBs solution containing 5 mg/mL total protein was used as the starting solution in the following refolding experiments. So the starting solution contained about 3.5 mg/mL EGFP. For purified native EGFP, the final concentration was 6 mg/mL.

In the refolding experiments, the IDA-Sepharose gel was pretreated by the method described earlier [25,26] before adding to the refolding system. By the procedure, the gel was equilibrated with the refolding buffer (50 mM Tris–HCl, pH 8.5), and drained on a G3 filter. The drained gel was added to the refolding system when necessary.

All refolding experiments were carried out in 1.5 mL microcentrifuge tubes placed in a shaking incubator at 25 °C and 170 rpm. Refolding was carried out by diluting denatured EGFP solution to the refolding buffer. For purified EGFP, the refolding system contained 0.4 or 0.6 mg/mL EGFP, 2 M urea, 20 mM DTT, 50 mM Tris–HCl and predetermined concentration of resin (pH 8.5). For EGFP IBs, it contained 0.1 or 0.2 mg/mL protein in total, including about 0.07 or 0.14 mg/mL EGFP, 0.2 or 0.4 M urea, 20 mM DTT, 50 mM Tris–HCl and predetermined concentration of resin (pH 8.5). The fluorescence recovery (FR) was measured after refolding for 12 h. In order for measuring the refolding kinetics, small aliquots were withdrawn from the reaction mixture at different time intervals for fluorometric assay to check the time course of FR. For the refolding with charged resins, the refolding suspension was centrifuged at 4000 rpm for 1 min, and the supernatant was collected for measurement.

In the above refolding studies, each experiment was conducted in triplicate and the average value is presented.