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Step change of mobile phase flow rates to enhance protein folding in size exclusion chromatography

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Abstract

Minimal aggregation formation between injector and column inlet and enough residence time inside the column were identified to be the key for proper proteins refolding by size exclusion chromatography (SEC). Therefore, a step change of mobile phase flow rate strategy was developed. That is, during the injected denatured protein (sample) traveling from injector to column inlet, a higher rate of mobile phase should be applied to reduce aggregation. Then, a lower flow rate should be used to allow enough time for protein to refold inside the column. The refolding of denatured lysozyme in SEC by this method was shown to reduce the aggregation and thus resulting in good refolding yields, though not completely. Combining this method together with our previously proposed chaperon solvent plug strategy could obtain complete recovery of denatured protein, both mass and activity recoveries.

Keywords: Step change of mobile phase flow rates; Chaperon solvent plug; Size exclusion chromatography; Protein refolding; Lysozyme

1. Introduction

Proteins expressed by recombinant bacteria often result in protein aggregates inside the cells, i.e. inclusion bodies. Although additional downstream steps become necessary, inclusion bodies formation can be advantageous [1]. For example, the target proteins may be protected against proteolytic degradation as being embedded in the aggregates. The recovery of active protein from inclusion bodies involves several steps: inclusion body isolation and washing, solubilization of aggregated protein and refolding of the solubilized protein.

Refolding of soluble proteins is often accomplished by removing excess denaturants to provide a favorable environment for the inactive proteins to fold, for example, dilution [2], dialysis [3], ion exchange chromatography [4] and size exclusion chromatography (SEC) [5]. Inevitably, the aggregations of partially folded proteins due to their exposed hydrophobic amino acids significantly reduce the yield of the refolding process. Thus, a key to increase the efficiency of protein refolding/renaturation is to reduce the aggregation during the refolding process. There have been several methods to improve renaturation yield, such as addition of low molecular weights compounds to prevent aggregations [6], use of polyethylene glycol to stabilize refolding intermediates [7] and passage through a column with immobilized molecular chaperones [8]. On the other hand, refolding by SEC method is also an alternative buffer exchange method to promote protein renaturation. It has attracted some attention in recent years [5,9–14] because simultaneous refolding and purification is feasible. Although this technique has been successfully demonstrated with lysozyme, the effect of some process parameters has not been clearly elucidated. Particularly, the sample application conditions in SEC was found to strongly affect the efficiency of the refolding, probably because the undesired renaturation actually began immediately after the mixing of denatured protein and refolding buffer prior to the column inlet. The renaturation between injector and column inlet occurred without the aid of SEC packing (mechanism proposed in literature [5]). This refolding process prior to the column without proper SEC packing assistance could lead to significant aggregation and therefore reduce the yield. For example, Liu and Chang [15] designed

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a "chaperon solvent plug strategy" to escort the denatured lysozyme (injected sample) from the injector into column under "aggregation inhibition condition". Additionally, a linearly decreased urea gradient gel filtration refolding system [16,17] or a dual-gradient ion exchange chromatography with a descending urea concentration gradient combined with an ascending pH gradient [18,19] was developed to prevent from aggregation formation as it went through the column. Fahey and Chaudhuri [20] also noticed that aggregation was crucial in a SEC operation. Therefore, a step change of mobile phase flow rate is proposed in this report. The main goal is to suppress aggregation by reducing the traveling time of folding intermediates between injector and column inlet, thus hopefully to enhance protein folding yield. This method is a simple and effective strategy for protein refolding by SEC.

2. Materials and methods

2.1. Materials

Hen egg white lysozyme (HEWL; EC 3.2.1.17) was purchased from Merck. Dithiothreitol (DTT), *Micrococcus lysodeikticus* dried cells, oxidized glutatione (GSSG) and reduced glutathione (GSH) were from Sigma and urea from Hayashi Pure Chemical Ind. Co. Ltd. All other chemicals were analytical grade.

2.2. Preparation of denatured lysozyme

Native lysozyme (HEWL) was denatured by incubating the protein (5 g/l) in 0.1 M Tris–HCl, pH 8.6 containing 8 M urea and 0.01 M DTT for 24 h at room temperature. No activity of denatured lysozyme was found after incubation.

2.3. Refolding of lysozyme using size exclusion chromatography

2.3.1. Step change of mobile phase flow rates strategy

The high performance liquid chromatography (HPLC, Waters) was equipped with a dual λ absorbance detector (Waters 2487) and a binary HPLC pump (Waters 1525). All eluents were filtrated by nitrocellulose membrane (0.2 μm) and degassed beforehand. A 20 μl sample of denatured lysozyme (5 g/l) was injected onto a Superdex 75 HR 10/30 column (Pharmacia Biotech) previously equilibrated with refolding buffer (0.1 M Tris–HCl, pH 8.2, 3 mM GSH, 0.3 mM GSSG, 0.15 M sodium chloride, 1 mM EDTA and 2 M urea) and eluted with various elution profiles at room temperature. All the tested elution profiles are illustrated in

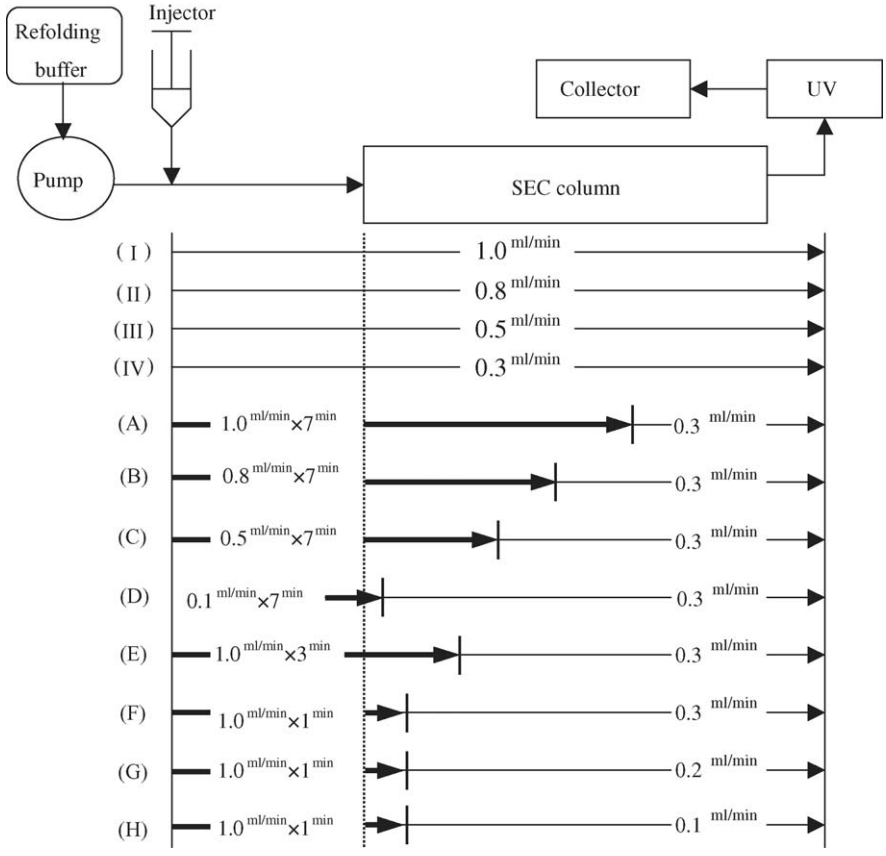

Fig. 1. Experimental operations for constant flow rate (I–IV) and step change of mobile phase flow rate strategy (A–H). The bold arrow and the thin arrow indicated the flow rate of first step and the flow rate of second step, respectively.