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Multi-variable operational characteristic studies of on-column oxidative protein refolding at high loading concentrations

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ABSTRACT

Chromatographic-based protein refolding techniques have proven to be superior to conventional dilution refolding methods, due to the higher loading concentration and simultaneous purification. Among these techniques, Size Exclusion Chromatography (SEC) has in particular been demonstrated as an effective method for refolding of variety of proteins. To date existing studies of protein refolding at high concentrations (>1 mg/mL) in SEC have primarily been conducted as single factor studies, in which a single parameter is varied to assess impact on operating performance, which does not allow for determination of the interactions of different operating parameters and optimized operating conditions. In this work a multi-variable investigation of size exclusion protein refolding at high protein concentration using lysozyme as a model protein was performed, in order to quantify the interaction of factors and optimize performance. It was observed when L-arginine is used as an additive the refolding yield becomes independent of the protein concentration and refolding buffer pH, providing that a redox couple is used to assist the reformation of disulfide bridges. Furthermore, the pore accessibility for small molecules was reduced at the presence of this additive particularly at higher protein concentrations indicating slower removal of these molecules and a possible additional mechanism of aggregation prevention. Using the subsequent optimized refolding buffer, a refolding yield of more than 90% was obtained for up to 40 mg/mL loading concentration of lysozyme which has only been reported for a urea gradient SEC (8–2 M) with lower equilibration and elution flow rates due to high viscosity of buffer containing high concentrations of urea.

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1. Introduction

Proteins are one of the most important biological compounds and beneficial to human health when used as therapeutic agents. Recombinant DNA technology continues to be one of the common methods in industry for production of many biopharmaceuticals, including proteins [1]. In particular, *Escherichia coli* (*E. coli*) is one of the most used microbial expression systems in biotechnology due to its well characterized genetics, very high expression level and ease of manipulation [2]. One of the primary issues resulting from protein expression in *E. coli* is the formation of inactive protein aggregates (inclusion bodies). These aggregates require solubilisation through providing an environment for protein chains to unfold, which may be accomplished by using denaturing and reducing agents. After unfolding and aggregate collapse, the refolding of proteins into their compact structures is critical in order to restore biological activity and functionality. Refolding by dilution is commonly practiced in laboratories and industry due to its simplicity in design and operation [3–5]. However the correct protein folding pathway often competes with misfolding and aggregation, particularly at high concentrations which substantially reduces refolding yield. Furthermore, the presence of aggregates in the final product as impurities provides health concerns for utilization as therapeutics [6]. Consequently, the dilution technique has serious drawbacks during scale-up due to requiring low product concentrations and purity in addition to large process volumes, which necessitate additional cost-intensive post-refolding concentration and purification steps. These challenges limit high throughput production of therapeutic proteins and the speed with which new protein drugs can be brought to market [3].

Recently, chromatographic based refolding [3,7–9] has drawn great attention to address the challenges associated with product dilution, by facilitating spatial isolation of protein molecules and unfolding agents based on different affinity for solid phase

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or molecular size. These methods allow for protein refolding at higher concentrations and simultaneous protein purification due to reduced intramolecular interactions in adsorptive chromatography methods and gradual separation of protein and unfolding agents waves in non-adsorptive size exclusion chromatography (SEC). Among various chromatographic methods, SEC offers many advantages and has been widely used at lab scale for protein refolding in either batch or continuous mode [3,10–15]. The performance of SEC in terms of refolding yield, protein recovery and purity depends on many parameters, such as: protein structure, protein concentration, loading state of non-native protein (e.g. denatured, denatured and reduced), column packing specifications (e.g. material, particle size, pore size), refolding buffer composition including its pH, redox potential, ionic strength and additives' concentrations [14–19]. However, majority of the research related to operational characteristics of SEC refolding pursued one-factor-at-a-time approach which cannot quantify the interactions of factors preventing determination of optimal operating conditions. In this work, a multi-variable study of key parameters on SEC refolding at high concentrations was carried out, using lysozyme as a model protein.

2. Design of experiments

A suitable refolding buffer is particularly critical in refolding of proteins. The refolding buffer reported to give highest refolding yield for lysozyme is comprised of 0.1 M Tris, 1 mM EDTA, 2 M urea, 3 mM cysteine, 0.3 mM cystine or the same concentration of glutathione redox couple buffered at pH 8.1 [15]. In this work, the reported refolding buffer (0.1 M Tris, 1 mM EDTA, 2 M urea, 3 mM cysteine, 0.3 mM cysteine buffered at pH 8.1) was initially used to identify protein concentrations at which aggregates are formed. This is followed by a buffer optimization process to minimize the aggregation and increase the refolding yield. L-Arginine is commonly used to increase the protein mass recovery in various liquid chromatography columns [20,21] and has been proven to be an effective aggregation suppressor due to its unique effects on protein association and folding [22–26]. Higher concentrations of L-arginine results in higher refolding yields, but it also slows down the rate of refolding [24,25]. Therefore, the concentration of L-arginine was selected as one of the key factors which affect the refolding yield of lysozyme by SEC. Apart from L-arginine concentration, refolding buffer pH, ionic strength and protein concentration are the other key factors which dictate the refolding yield of lysozyme [14,15,17,19]. A two-level full factorial design of experiment combined with replicated center point runs to test for curvature was executed in the current work to investigate the effect of the aforementioned operating parameters and the potential interactions between these factors. An empirical equation was developed to predict the refolding yield in the experimental space and search for optimum within the design space.

3. Materials and methods

3.1. Chemicals

Reagent grade L-arginine and urea, Ethylene Diamine Tetra Acetic acid (EDTA), lysozyme from chicken egg white, trizma® base (Tris-base), L-cysteine, L-cystine, BioUltra dithiothreitol (DTT) solution, *Micrococcus Lysodeikticus*, potassium phosphate monobasic and BioXtra sodium chloride were purchased from Sigma-Aldrich, Canada. Red 660™ protein assay reagent was purchased from G-Biosciences, USA. Superdex™75 prep grade resin (24–44 micron) was purchased from GE healthcare, Canada.

3.2. Feed preparation

Unfolding buffer (0.1 M Tris-base, 1 mM EDTA, 6 M urea and 32 mM DTT, pH 8.1) was used to prepare various concentrations of denatured and reduced lysozyme. The sample was incubated for 2–4 h at 37 °C to ensure loss of activity which was confirmed by enzymatic activity test as described below [15].

3.3. Refolding by size exclusion column

XK16/40 column (GE healthcare, Canada) was packed with Superdex™75 prep grade (Superdex 75 pg) resin. The total volume of column was 44 mL and the packing quality was tested by comparing the peak symmetry and number of theoretical plates per length of column with manufacturer recommended criteria using 2% (v/v) acetone injection. The packed column was installed on ÄKTA purifier 100, controlled by UNICORN 5.31 software equipped with online pH probe, UV detector and conductivity cell. The fractionation kit allows the collection of samples at desired volumes. The column was equilibrated with 2 column volumes (CV) of refolding buffer prior to protein injection. After equilibration, 0.5 mL of denatured and reduced lysozyme was injected and eluted for 1.5 CV with refolding buffer at 1 mL/min flow rate. During elution fractions of 7 mL were collected and stored at 4 °C before analysis which was conducted in less than 24 h. The stability of samples during storage was tested by comparing the enzymatic activity of samples analysed immediately and stored ones which showed no significant difference [27]. The fractions were pooled to measure total protein recovery (R), refolding yield (Y) and purity (P) as defined below.

$$R = \frac{M_{\text{total}}}{LV_{\text{inj}}} \tag{1}$$

$$Y = \frac{M_{\text{native}}}{LV_{\text{inj}}} \tag{2}$$

$$P = \frac{M_{\text{native}}^{\text{mono}}}{M_{\text{total}}^{\text{mono}}} \tag{3}$$

where M_{total} and M_{native} are total protein and equivalent native protein mass collected in pooled fractions associated with either all forms of protein or protein monomer which were measured by total protein and enzymatic activity assays as described in analytical methods, V_{inj} is injection volume, L is lysozyme loading concentration.

The column was then washed with 2 CV de-ionized water after elution. In case of in-column protein precipitation and flow blockage, the column was washed with 6 M urea, 32 mM DTT, 0.1 M Tris-base buffered at pH 8.1 at very low flow rates (< 0.2 mL/min) to dissolve the precipitated aggregates.

All the buffers were prepared fresh using ultra-pure water (Barnstead easy-pure RODI equipped with 0.2 μm filter, Fisher Scientific), filtered again with a 0.2 μm membrane and de-gassed prior to use.

3.4. Analytical methods

3.4.1. UV absorbance

The lysozyme powder was dissolved in 0.1 M potassium phosphate and 0.15 M NaCl buffer (pH 7) and the lysozyme content was determined by ultraviolet (UV) absorption spectroscopy (Shimadzu UV-3600) at 280 nm using extinction coefficient of 2.63 mL/mg/cm. The feed concentration (denatured and reduced lysozyme) was confirmed using extinction coefficient of 2.37 mL/mg/cm. Feed samples were diluted in 0.1 M acetic acid [27].

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