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Oxidative protein refolding on size exclusion chromatography at high loading concentrations: Fundamental studies and mathematical modeling

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ABSTRACT

Size exclusion chromatography has been demonstrated as an effective method for refolding a variety of proteins. However, to date process development mainly relies on laboratory experimentation of individual factors. A robust model is essential for high-throughput process screening and optimization of systems to provide higher productivity and refolding yield. In this work, a detailed kinetic scheme of oxidative refolding of a model protein (lysozyme) has been investigated to predict the refolding results in SEC. Non-reactive native, quenched and equilibrium studies were conducted to obtain the model parameters for the species formed during refolding of denatured/reduced lysozyme. The model was tested in various operating conditions, such as: protein loading concentration, injection volume, flow rate and composition of refolding buffer with and without the use of L-arginine additive. An apparent two-state mechanism was found adequate to describe refolding of lysozyme on SEC for the operating condition tested in this work. Furthermore, using low concentration of L-arginine combined with urea as common aggregation suppressor additives showed insignificant change in kinetics of refolding of lysozyme on SEC. However, addition of L-arginine changed mass transfer properties of some of the species formed in refolding reaction which was considered in the model to accurately predict the result of refolding on SEC.

1. Introduction

Conventional method of protein refolding, namely batch dilution refolding, requires working at low protein concentrations in order to prevent aggregation, which in turn results in low productivity impeding high-throughput protein refolding and downstream processing for production of many bacterially expressed recombinant proteins [1]. Size exclusion chromatography (SEC)-based protein refolding addresses this issue to some extent by facilitating gradual spatial isolation of protein molecules and unfolding agents, which can prevent aggregation and allow for application of higher protein loading concentrations and simultaneous purification compared with batch dilution refolding. For these reasons SEC has been widely used at lab scale for protein refolding in either batch or continuous mode using single or multiple column configurations (i.e. simulated moving bed) [1–9]. Existing work has also developed mathematical models for separation in SEC using various model proteins in their native forms [10,11]. For protein refolding in SEC (reaction–separation SEC), the refolding reaction is incorporated into the mathematical model as well as interactions of aggregating species in case they are formed under the operating conditions being studied and the model parameters must be obtained for kinetic species formed during these reactions [5,12]. Development of an experimentally verified and robust model helps minimizing the number of screening for a broader range of operation condition and is essential for systematic process optimization.

The mechanism of oxidative refolding reaction is commonly simplified to include only one intermediate involved in the rate limiting step based on dilution oxidative refolding kinetic data [13,14]. However, the ratio of kinetic constants of refolding steps is influenced by chemical composition of the environment [13,14] and this simplification may not be applicable in all cases. This is important in SEC as the refolding reaction is accompanied with protein size variation and a correct scheme of such variation along the column is necessary to accurately predict the elution profile of the

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protein. Furthermore, in case of aggregate(s) formation, the simplified mechanism is not able to capture the interactions of all intermediates that are prone to aggregation [5,14,15]. In this work, (1) a mathematical model for refolding of denatured/reduced lysozyme in SEC at high loading concentrations was developed to investigate a detailed reaction mechanism previously proposed for this protein which was selected as a model system due to importance of disulfide bond formation in considerable number of proteins [13]; (2) non-reactive native, quenched and equilibrium experiments were executed to find the model parameters for the final product and short-lived kinetic intermediates formed during unimolecular refolding reaction; (3) the model was tested by varying operating conditions, namely: protein loading concentration, injection volume, flow rate and composition of refolding buffer (with and without L-arginine additive); and (4) the effect of low concentration (0.2 M) of L-arginine on mass transfer and kinetic parameters in SEC was studied. L-Arginine has been extensively studied and used to increase the refolding yield in batch dilution refolding by suppressing aggregation [16–19] and protein mass recovery in chromatography methods by decreasing non-specific interaction with the matrix [20,21]. In terms of kinetics of refolding, Reddy et al. [19] have reported insignificant change in apparent kinetic constant of oxidative refolding of lysozyme by batch dilution using guanidinium chloride and low concentration of L-arginine (up to 0.5 M) to suppress aggregation. However in contrast to this Chen et al. [18] observed a considerable decrease when both urea (2 M) and L-arginine (0.5 M) were used in batch dilution refolding of recombinant human granulocyte colony-stimulating factor. Furthermore, Vagenende et al. [16] illustrated protein destabilization at the presence of low concentration of L-arginine (<0.5 M) by differential scanning calorimetric method which might in turn result in reduced folding kinetics. To the best of our knowledge there is no conclusive information available on the effect of L-arginine on mass transfer properties of various refolding species and the kinetics of this reaction in SEC, making it a topic worthy of investigation.

2. Modelling

The protein refolding in size exclusion column was modeled using the "transport-dispersive" and "solid-film linear driving force" models formulated from differential mass balances for solutes in the bulk-fluid phase and the particle–solid phase respectively [5]. The governing equations are

$$\frac{\partial C_{b,i}}{\partial t} = D_L \frac{\partial^2 C_{b,i}}{\partial x^2} - u\frac{\partial C_{b,i}}{\partial x} - Pk_{ov,i}(C_{eqS} - C_s) + r_{b,i} \quad (1)$$

$$\frac{\partial C_{s,i}}{\partial t} = k_{ov}(C_{eqS,i} - C_{s,i}) + r_{s,i} \quad (2)$$

where $C_{b,i}$ and $C_{s,i}$ are the concentration of solute i (unfolded, intermediates and native configurations) in bulk-fluid phase and solid phase respectively. t is the time, x is an axial distance along the column, D_L is an axial dispersion coefficient, u is an interstitial velocity, $k_{ov,i}$ is the solute overall mass transfer coefficient, P is the phase ratio, $C_{eqS,i}$ is the solid phase concentration in equilibrium with the bulk concentration. $r_{b,i}$ and $r_{s,i}$ are the net concentration change due to refolding reaction in bulk and solid phases with details presented in Section 2.2.

The solute solid phase concentration in equilibrium with the bulk concentration was treated as a linear equilibrium relationship with a fixed equilibrium constant [10]:

$$C_{eqS,i} = K_{eq,i}C_{b,i} \quad (3)$$

where $K_{eq,i}$ is the equilibrium constant.

The boundary and initial conditions used to solve Eqs. (1) and (2) are:

$$C_{b,i}(t, 0^-) = \begin{cases} C_{f,i} & 0 < t < t_{pulse} \\ 0 & t > t_{pulse} \end{cases} \quad (4a)$$

$$\frac{\partial C_{b,i}}{\partial x}(t, L_c) = 0 \quad (4b)$$

$$C_{b,i}(0, x) = 0 \quad (4c)$$

$$C_{s,i}(0, x) = 0 \quad (4d)$$

where $C_{f,i}$ is solute concentration in feed, t_{pulse} is the duration of sample injection, and L_c is the column length. The assumption that the sample is introduced into the column as a rectangular pulse of length t_{pulse} may not be valid in most practical applications. However, as the injection time is very small compared to retention time, such simplification still seems to be applicable [22].

To solve the above system, the first and second spatial derivatives were discretized using fourth-order finite difference equations except for boundary points for which second order forward and backward finite difference approximations were used. The resulting method of lines system of ODEs was solved numerically in MATLAB.

2.1. Determination of model parameters

The axial dispersion coefficient was calculated based on the definition of the Peclet number, which was estimated using the correlation of Chung and Wen for small Reynold numbers as follows [5]

$$D_L = \frac{uL_c}{Pe} \quad (5a)$$

$$Pe = \frac{0.1L_c}{R_p\varepsilon_b} \quad (5b)$$

where R_p is particle radius and ε_b is bed void volume fraction which was measured using thyroglobulin from bovine thyroid as a test probe

$$\varepsilon_b = \frac{V_0}{V_c} \quad (6)$$

where V_0 and V_c are the elution volume of thyroglobulin from bovine thyroid and column volume respectively, correcting by subtracting the system volume such as tubing and valves. The phase ratio (void to non-void volume) is

$$P = \frac{1 - \varepsilon_b}{\varepsilon_b} \quad (7)$$

The mass transfer and equilibrium constants ($k_{ov,i}$ and $K_{eq,i}$) were found by minimization of the deviation of measured concentration vs. calculated in a least squares sense with an additional constraint for recovery ($R_i \leq 1$) which was defined as

$$f(x) = \sum_{j=1}^{j=n} \left(C_{b,i}^{exp,j} - C_{b,i}^{fit,j}(x)\right)^2 \quad (8)$$

$$R_{i=} \frac{M_i}{C_{f,i}V_{pulse}} \quad (9)$$

where x is a vector of mass transfer and equilibrium constants, $C_{b,i}^{exp}$ is the vector of experimental solute concentration at the column outlet, n is the number of elements in concentration vector, M_i is the sum of mass of solute collected in fractions and V_{pulse} is an injection