Chemical Engineering Science 131 (2015) 91–100

Contents lists available at ScienceDirect

Chemical Engineering Science

journal homepage: www.elsevier.com/locate/ces

Engineering batch and pulse refolding with transition of aggregation kinetics: An investigation using green fluorescent protein (GFP)

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HIGHLIGHTS

- Aggregation transits from 2nd to 1st order as intermediate depletes during refolding.
- Better prediction in batch and pulse refolding using proposed transition model.
- Native model protein (sGFPmut3.1) does not aggregate with intermediates.
- Potential engineering tool to optimize in vitro refolding in bioprocess settings.

ARTICLE INFO

Article history:
Received 5 January 2015
Received in revised form
25 March 2015
Accepted 30 March 2015
Available online 6 April 2015

Keywords:
Inclusion bodies
Renaturation
Folding
Reaction order
GFP

ABSTRACT

Pulse refolding is a strategy to overcome concentration dependent aggregation, assuming that aggregation is significantly suppressed under diluted conditions. When a typical 2nd or higher order aggregation kinetics is assumed, kinetics over predicted yields at low refolding concentrations. Using GFP as our model protein, we found a transition in aggregation kinetics from 2nd to 1st order when intermediates deplete from 100 to 60 μg/ml. Taking this transition into account, the model can better predict refolding yields in batch and pulse refolding strategies. This model is suited for the design of refolding processes since this deviation from 2nd or higher order aggregation was also previously observed in other proteins.

1. Introduction

Pulse refolding is a strategy to overcome aggregation by feeding denatured proteins in discrete amounts over specific time intervals into refolding buffer. Previous work showed improved yields when performed in both batch and continuous reactors (Katoh and Katoh, 2000; Linke et al., 2014; Pan et al., 2014; Schlegl et al., 2005; Winter et al., 2002). Other strategies for improving yield and productivity include buffer optimization (Berg et al., 2012; Mannall et al., 2009; Ordidge et al., 2012), better mixing (Mannall et al., 2006), on-column refolding (Li et al., 2004; Schmoeger et al., 2009) and annular chromatography (Uretschlager and Jungbauer, 2002)

However, to accurately quantify process performance and optimize process of these refolding strategies, a robust kinetic model that satisfies different reactor formats is beneficial (Buswell and Middelberg, 2003). Moreover, a correct biomolecular reaction scheme would help facilitate product quality and acceptable variability of process parameters by serving as a mechanistic model support tool in Process Analytical Technology (PAT) (Glassey et al., 2011) as part of the Quality by Design (QbD) concept (Rathore and Winkle, 2009) in biomanufacturing.

The key criteria in developing rigorous kinetic models of biologics require knowledge of the simplest correct kinetic scheme (Buswell and Middelberg, 2003). For example, a kinetic scheme for lysozyme refolding and aggregation that involved a sequential polymerization with the folding intermediates and the native protein (Buswell and Middelberg, 2003) as well as the competition between aggregation and self-assembly during virus-like particle processing (Ding et al., 2010).

Similarly, our objective is to characterize and establish a simple but process-suited model to predict in vitro refolding yields. Current models proposing a fixed 2nd or higher aggregation order (Hevehan and De Bernardez Clark, 1997; Kiefhaber et al., 1991) overestimate yields at low protein concentrations. This was seen for lysozyme (Buswell and Middelberg, 2003), autoprotease EDDIE-pep6His (Kaar

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http://dx.doi.org/10.1016/j.ces.2015.03.054

et al., 2009) and insulin growth factors (Milner et al., 1995) where yields did not reach 100% at low refolding concentrations. This suggests a deviation from 2nd and higher order aggregation at dilute conditions.

Consequently, using the mutant sGFPmut3.1 (Franke et al., 2007) recovered from inclusion bodies (IBs) lacking the mature chromophore as our model protein (Reid and Flynn, 1997), we proposed a refolding model where aggregation transits from 2nd to 1st order aggregation as intermediates deplete below a critical concentration. Importantly, this study accounts for the protein concentration, denaturant and reducing agent concentrations during refolding. The effect on refolding due to the presence of native sGFPmut3.1 was also tested. To test the predictability of our model on other refolding strategies, pulse refolding experiments were also performed at different refolding conditions.

2. Materials and methods

2.1. Expression and inclusion body recovery

Unless stated otherwise, all chemicals were obtained from Sigma Aldrich (St. Louis, MO, USA). Recombinant protein sGFPmut3.1 was overexpressed in fed-batch cultivation was as described in Clementschitsch et al. (2005). Isolation of IBs was as previously described by Kaar et al. (2009).

2.2. Purifying soluble sGFPmut3.1 expressed in Escherichia coli for spiking experiments

Cell broth was centrifuged with the Contifuge Stratos Heraeus (Thermo Fisher Scientific, Waltham, MA, USA) to gain the cell pellet. This was suspended in a chilled (+4 °C) solution of 10 mM Tris, 0.1 M NaCl and 0.1% Tween 20 at pH 7.5. Cell disruption was done with the Ariete 2-stage high pressure homogenizer (GEA Niro Soavi, Parma, Italy) at 80/800 bar in two passages. Subsequently, homogenate was cleared (Contifuge) and filtrated with Sartopure PP2, Sartoguard GF and Sartobran P at 1.2, 0.65 and 0.45+0.2 μm respectively. Buffer exchange to 10 mM Tris at pH 7.5 was done with a Sartoflow advanced system with a 10 kDa Hydrosart Sartocoon (Satorious, Göttingen, Germany) in 7 volume changes. After, 3 chromatography steps were performed with the Äkta Pilot System (GE Healthcare, Buckinghamshire, UK). For capturing, CaptoQ ion-exchange resin was used. Purification was made with Butyl-Sepharose, a hydrophobic interactions chromatography. Polishing was done by size exclusion, Superdex 75. All resins were obtained from GE Healthcare. Quantification of impurities were analyzed by Superdex 75 column at the Äkta Explorer System and by SDS-PAGE, BioRad PowerPack basic (Bio-Rad Laboratories, Hercules, USA).

2.3. Denaturing and reducing sGFPmut3.1 IBs

From IBs, sGFPmut3.1 was lyophilized, weighed and suspended in 50 mM Tris (pH 7.3) overnight. Suspended IBs were denatured and reduced by 1:10 ratio in dissolution buffer containing 10 M urea, 50 mM Tris and 100 mM α-monothioglycerol (MTG) at pH 7.3 for 0.5 h. Protein concentration stock was measured on a Cary 50 Bio UV–vis Spectrophotometer (Varian, Palo Alto, USA) at a theoretical extinction coefficient of 0.813 (mg/ml protein) cm^{-1} at 280 nm. Stock was further diluted to the desired concentrations using buffer containing 9 M urea, 50 mM Tris, 100 mM MTG and pH 7.3.

2.4. Determining rate constants of sGFPmut3.1 at different residual urea

Refolding was initiated in 5 ml eppendorfs containing 0.3 M L-arginine/HCl (SERVA, Heidelberg, Germany), 1 M Tris, 0.25 M sucrose, 2 mM EDTA, 20 mM MTG and pH 7.3 refolding buffer in predefined urea concentrations. Solution was vortexed immediately and inserted onto laboratory rotator (SB3, Stuart) (10 rpm). All refolding in this study took place at 23 ± 1 °C. At specified times over 7 h, 100 μl samples were drawn and measured for fluorescence yield. Refolding concentrations of sGFPmut3.1 were 25, 38, 56, 114, 158, 190 μg/ml in residual urea concentrations of 0.24, 0.50, 0.90, 1.12, 1.32, 1.52, 1.80 M. Using Table Curve3D (SPSS, Erkrath, Germany), kinetic constants for concentrations 25, 38, 56 μg/ml at each residual urea were calculated by fitting data sets into Eq. (4) while the higher concentrations 56, 114, 158, 190 μg/ml were globally fitted with Eq. (3) at each residual urea condition.

2.5. Batch refolding at different reducing agent concentrations

Refolding at 0.2 mg/ml sGFPmut3.1 was initiated as previously described at 1:10 ratio dilution where 0.5 ml dissolved IBs were added to 5 ml eppendorfs containing 4.5 ml refolding buffer previously described but at predefined MTG concentrations. The resultant MTG concentration ranges between 10 and 100 mM. Samples were drawn and measured for fluorescence yield over refolding time.

2.6. Establishing refolding simulation

Using fourth-order Runge–Kutta method, Eq. (1) (2) and (5) and rate constants that were experimentally derived with increasing residual urea, batch and pulse refolding simulations were established using Microsoft® Office Excel 2013. Simulations were verified with analytical solutions of Eqs. (3) and (4) at different refolding conditions. Additionally, the total mass balance of intermediates, native and aggregate species were always 100% over refolding time. This simulation was then used to predict the refolding experimental results.

2.7. Batch refolding with presence of native GFPmut3.1

Refolding was performed in 50 ml beakers at different refolding conditions of 46, 49, 62, 95 μg/ml at a residual urea concentration of 0.90, 0.69, 0.90, 0.90 M respectively containing specific amounts of pure native sGFPmut3.1. As a control, identical refolding conditions were also performed without pure native sGFPmut3.1. Yields were then calculated after accounting for fluorescence due to native pure sGFPmut3.1.

2.8. SEC analysis of refolded sGFPmut3.1

Analytical SEC analysis was performed with Agilent 1290 Infinity UHPLC instrument (Agilent, Waldbronn, Germany) together with Agilent Bio SEC-5 Column (300 mm × 4.6 mm i.d.) with particle size 5 μm and pore structure of 100 Å. Samples analyzed were purified sGFPmut3.1 and refolded sGFPmut3.1 from IBs. Each analysis took 15 min where running buffer was 1 × phosphate saline buffer. Flow rate was 0.5 ml/min, column temperature was 25 °C and injection volume was 10 μl. Absorbance was measured simultaneously at 214 nm to detect peptide bonds and 485 nm to detect fluorescence chromophore. To determine molecular weight of soluble aggregates, a high molecular weight kit of 5 proteins (GE Healthcare, Buckinghamshire, UK) between 44 and 669 kDa was analyzed.