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Design and optimization of protein refolding with crossflow ultrafiltration

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HIGHLIGHTS

- Refolding of proteins in a crossflow ultrafiltration module was investigated.
- Efficiency of refolding by continuous and stepwise denaturant denaturant removal was analyzed.
- Mathematical model was used to predict and optimize refolding process.
- Higher productivity and lower buffer consumption could be achieved compared to batch dilution.

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ABSTRACT

This study analyzed the efficiency of protein refolding with crossflow ultrafiltration for two distinct types of model proteins: denatured bovine α-lactalbumin and a fusion protein that comprised green fluorescent protein coupled with an engineered N^{pro} autoprotease tag. A mechanistic model of the process dynamics that accounted for the refolding kinetics was developed and verified by comparing with experimental data. The model was used to quantify refolding performance under various operating conditions, including different denaturant removal rates, refolding durations, and protein concentrations. The performance of ultrafiltration with the fed-batch and stepwise operating modes for denaturant removal was analyzed and compared to the performance of the batch dilution method. Performance was evaluated in terms of productivity, yield, and buffer consumption. The superiority of one refolding method over another depended on the protein system. When a slow reduction in denaturant concentration suppressed protein aggregation, the best performance was achieved with the ultrafiltration system. For example, α-lactalbumin refolding with ultrafiltration achieved several-fold higher productivity and lower buffer consumption compared to refolding with the batch dilution method. A further reduction in buffer consumption was achieved with permeate recycling. Conversely, when rapid dilution of the denaturant was most efficient, a combination of batch dilution and ultrafiltration was recommended. The latter reduced the buffer consumption with permeate recycling; e.g., over 80% of the refolding buffer could be recycled during fusion protein refolding.

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1. Introduction

Over-expression of recombinant proteins in *Escherichia coli* often leads to the formation of inclusion bodies (IBs), which contain partially-folded proteins in an aggregated, inactive form (Kane and Hartley, 1998; Mitraki and King, 1989; Schein, 1989; Speed et al., 1996). Proteins are present in high concentrations in IBs. Moreover, because they can be washed with differential centrifugation, they are obtained in a rather pure form (Singh and Panda, 2005; Doglia et al., 2008). The IB proteins are partially unfolded and must be refolded to recover the active form (Kane and Hartley, 1988; Mitraki and King, 1989; Schein, 1989; Speed et al., 1996; Singh and Panda, 2005). Many refolding procedures have been developed previously (Jungbauer and Kaar, 2007; Eiberle and Jungbauer, 2010); nevertheless, there remains room for improvement.

After denaturing the IB proteins, the refolding process is realized by reducing the denaturant concentration in the presence

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of an appropriate refolding buffer, which contains oxidizing agents, stabilizers, and aggregate inhibitors. This buffer exchange rapidly induces the transformation of the denatured protein into a transient, partially refolded state. Then, it undergoes a slow refolding reaction that typically competes with inter-molecular aggregation. Intermediate steps of refolding include the so-called disulfide bond shuffling, which is catalyzed by reductants and oxidants. This step can produce both native and misfolded forms of the protein. The misfolded proteins can either convert to the correct conformation or interact with one another to form inactive aggregates (Singh and Panda, 2005; Doglia et al., 2008; Yamaguchi et al., 2013).

Fusion proteins, where a target protein or peptide is linked to a peptidic tag, are reported to undergo a similar mechanism of refolding (Kaar et al., 2009). The peptidic tags can be specifically designed to improve the efficiency of production, purification, and refolding (Gram et al., 1994; Davis et al., 1999; Haught et al., 1998). For example, the N^{pro} autoprotease tag can improve the yield of hard-to-express proteins, hormones, and peptides (Rumenapf et al., 2001; Stempfer et al., 2001). The N^{pro} autoprotease enzyme becomes active during refolding and it cleaves itself to release it from the fusion partner; the target protein remains with an authentic N-terminus. Therefore, the N^{pro} fusion protein system does not require chemical or protease treatments that might harm the protein or increase the cost of operation.

Recombinant proteins can be refolded with various methods. The simplest and most straightforward technique is to dilute the IBs in a solution of refolding buffer in a batch-stirred tank reactor. This simple production method is often used in industry (Jungbauer and Kaar, 2007). However, to avoid aggregation, which reduces the refolding yield, a very low protein concentration must be maintained in the reactor; i.e., 10–100 mg mL^{-1}. This low concentration requires a large reactor volume and significant consumption of the refolding buffer. Hence, to improve the process economics, fed-batch and continuous dilution methods have been suggested for different types of reactors (Katoh and Katoh, 2000; Buswell et al., 2002; Schlegl et al., 2005).

To improve the dilution method, several alternative approaches have been developed. One of them is based on matrix-assisted refolding (MAR). MAR includes adsorptive techniques such as ion-exchange chromatography, hydrophobic interaction chromatography, affinity chromatography (Langenhof et al., 2005; Machold et al., 2005; Freydell et al., 2010b; Geng and Chang, 1992; Wang et al., 2004; Jungbauer et al., 2004; Chen and Leong, 2010; Basu and Leong, 2012; Ryś et al., 2015), and non-adsorptive methods, such as size exclusion chromatography (Gu et al., 2001; Freydell et al., 2010a; Ryś et al., 2015). The MAR process integrates protein refolding with chromatographic purification, which can enhance the process efficiency in terms of yield, productivity, and buffer consumption (Jungbauer et al., 2004; Ryś et al., 2015). However, often, the denatured proteins aggregate in the course of the adsorption–desorption cycle, which impairs the yield in refolding processes assisted by adsorptive chromatographic matrices (Yamaguchi et al., 2013; Ryś et al., 2015). In turn, the non-adsorptive chromatographic method is characterized by low selectivity in the separation process and extensive dilution of the bands, due to the slow linear velocity required for this method (Ryś et al., 2015). Moreover, the refolding efficiency depends on a number of operating variables that must be properly selected, determined, or controlled, including the matrix type, the adsorption properties of the protein forms involved in refolding, and the chromatographic separation conditions, which define the residence time of the protein and the denaturant distribution along the column (Ryś et al.2015; Fahey et al., 2000; Middelberg, 2002).

Dialysis with ultrafiltration is an alternate approach to protein refolding (West et al., 1998; Yoshii et al., 2000; Umetsu et al., 2003; Tsumoto et al., 2010; Zhao et al., 2014). It provides a controlled exchange of the denaturants for the refolding buffer to induce protein refolding. When the buffer exchange rate is properly altered, protein aggregation can be suppressed and the refolding yield is improved. Ultrafiltration also allows protein refolding at high concentrations, which considerably reduces the buffer consumption (Yoshii et al., 2000; Umetsu et al., 2003; Tsumoto et al., 2010). The crossflow mode of operation is typically recommended, because it allows reduction of boundary layer resistances and achievement of higher filtration rates compared to dead-end systems.

Efficient utilization of crossflow ultrafiltration systems requires the selection of appropriate operating conditions, such as the denaturant removal rate, the process duration, and the protein concentration. Nevertheless, to date, the influence of the operating parameters on the process performance has only been described qualitatively, and empirical methods have been used to design and perform protein refolding in ultrafiltration modules (Yoshii et al., 2000; Umetsu et al., 2003; Tsumoto et al., 2010).

In this study, we aimed to determine the operating conditions based on the mathematical predictions compared to others, who used a high throughput approach (Treier et al., 2012). We developed a mechanistic model of the process dynamics. We used this model to calculate the process efficiency, find optimal process parameters, and rank different operating modes. We studied two model proteins. One was bovine α-lactalbumin, for which refolding kinetics were quantified in a previous study (Ryś et al., 2015). The second was a fusion protein, which included a green fluorescent protein mutant, GFPmut3.1 (Reischer et al., 2004), fused to the engineered N^{pro} variant, termed EDDIE. In its native active form, GFP exhibits bright green fluorescence when exposed to light in the blue to UV range; this property allows in situ monitoring of the refolding process. Both proteins were refolded with crossflow ultrafiltration and with batch dilution.

2. Theory

2.1. Refolding kinetics

A simplified mechanism for the refolding process is illustrated in the scheme shown in Fig. 1.

Formation of the intermediate state, *I*, and the correctly folded protein, *N*, is typically described as a first order reaction. The aggregate, *A*, is formed in a higher order reaction (Kiefhaber et al., 1991) or by sequential polymerization (Freydell et al., 2010a; Speed et al., 1997; Buswell and Middelberg, 2003). The disulfide bond shuffling step can be represented with a simplified reaction pathway, which leads to the formation of the misfolded protein, *M* (Ryś et al., 2015).

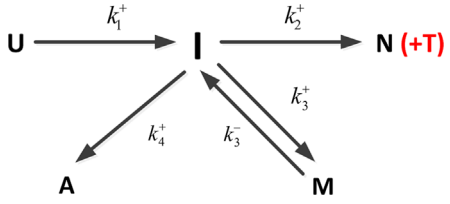

Fig. 1. Reaction scheme for protein refolding. U: unfolded (denatured) protein, I: intermediate form; M: misfolded form; A: aggregate; N: correctly folded protein, T: fusion tag (only for the fusion protein); k^+: forward rate constant; k^-: backward rate coefficient.

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