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Integrated continuous dissolution, refolding and tag removal of fusion proteins from inclusion bodies in a tubular reactor

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ABSTRACT

An integrated continuous tubular reactor system was developed for processing an autoprotease expressed as inclusion bodies. The inclusion bodies were suspended and fed into the tubular reactor system for continuous dissolving, refolding and precipitation. During refolding, the dissolved autoprotease cleaves itself, separating the fusion tag from the target peptide. Subsequently, the cleaved fusion tag and any uncleaved autoprotease were precipitated out in the precipitation step. The processed exiting solution results in the purified soluble target peptide. Refolding and precipitation yields performed in the tubular reactor were similar to batch reactor and process was stable for at least 20 h. The authenticity of purified peptide was also verified by mass spectroscopy. Productivity (in mg/l/h and mg/h) calculated in the tubular process was twice and 1.5 times of the batch process, respectively. Although it is more complex to setup a tubular than a batch reactor, it offers faster mixing, higher productivity and better integration to other bioprocessing steps. With increasing interest of integrated continuous biomanufacturing, the use of tubular reactors in industrial settings offers clear advantages.

1. Introduction

The large amounts of therapeutic proteins efficiently expressed in *Escherichia coli* as inclusion bodies (IBs) (Marston, 1986) in highly pure form (Jungbauer and Kaar, 2007; Speed et al., 1996) have created a bottleneck in downstream processing, which may eventually be solved by process integration (Cho et al., 2001; Choi et al., 2005; Hedhammar et al., 2006; Machold et al., 2005b) and continuous processing (Freydell et al., 2010; Machold et al., 2005a; Schlegl et al., 2005; Wellhoefer et al., 2013b). Although the large scale recovery of IBs can be easily performed through repeated centrifugation (Jin et al., 1994; Taylor et al., 1986), homogenization (Wong et al., 1996), and detergent washing steps (Lee et al., 2004), subsequent dissolution and refolding of IBs at high dilution folds have resulted in the need of large batch reactors that are economically expensive (Datar et al., 1993) and have long mixing times (Doran, 1995), compromising process quality and yield. Large stirred tanks also increases holding times between process steps and is less flexible to any process changes. Furthermore when proteins are expressed as fusion proteins, an additional step is required to remove the tag after cleavage of the fusion construct (Einhauer and Jungbauer, 2001).

Continuous processing is one way to alleviate the bottleneck in the downstream processing of recombinant proteins. Alternative protein refolding reactors were previously developed to circumvent these problems (Eiberle and Jungbauer, 2010). These include protein refolding in a continuous stirred tank reactor (Schlegl et al., 2005), a flow type packed column reactor (Terashima et al., 1996), and a membrane tube reactor equipped with paddles, partitioning disks (Katoh and Katoh, 2000) and continuous matrix assisted refolding (Park et al., 2005, 2006). While previous continuous reactors focus only on one process step, current study extended and integrated this continuous strategy to three processing steps in a tubular reactor. The operations include dissolution, refolding and tag removal by acid precipitation. This simplified system would allow three process steps to be performed in one continuous flow, eliminating any holding times between steps. This strategy could be adopted into typical purification processes of IBs as shown in Fig. 1.

IBs can be fed at slow rates into tubular reactor inlets for dissolution, reducing tubular reactor volume and the pressure to refold dissolved IBs all at once. This brings about greater smaller equipment size, flexibility and mobility (Warikoo et al., 2012). While keeping residence times constant, flow rates, tube diameter, tube

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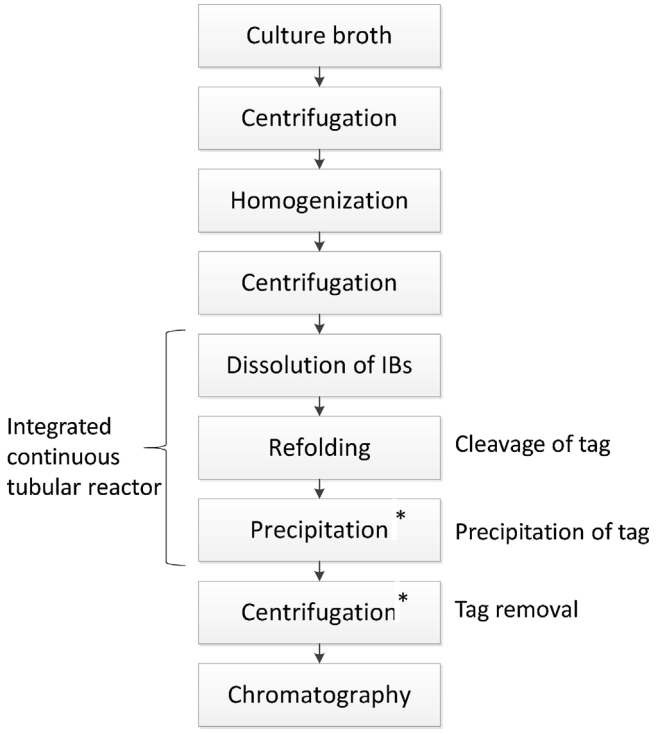

Fig. 1. Process flow in the tag removal from fusion proteins that were expressed as IBs. Dissolution, refolding and precipitation steps were performed in an integrated continuous tubular reactor. * Steps are optional in a typical purification process of IBs.

lengths can be changed easily suiting different process conditions. If disposable materials are used for tubular reactor, less cleaning is needed at lower upfront investment costs (Ho et al., 2010). The tubular reactor also offers direct connection to capture systems such as expanded bed chromatography (Ferre et al., 2005).

We tested our system with an N^{pro} fusion protein, EDDIE-pep6His. N^{pro} is an autoprotease derived from the classical swine fever virus (Stark et al., 1993), which are fused to peptides/proteins and overexpressed as inclusion bodies in *E. coli* (Durauer et al., 2010). Under refolding conditions, it releases the fused target protein with an authentic N-termini (Achmüller et al., 2007). The variant of N^{pro}, EDDIE named after the newly generated sequence motif E53-D54-D55-I56-E-57 (Achmüller et al., 2007), when fused to a target protein, showed improved solubility and faster refolding and cleavage (Kaar et al., 2009). Studies have shown that the artificial peptide, pep6His (a short 16 amino acid artificial peptide consisting of 10 randomly chosen amino acids and a C-terminal polyhistidine tag) was cleaved from EDDIE-pep6His when refolded, where yields were concentration independent between 0.26 and 3.88 mg/ml (Kaar et al., 2009). Additionally a low pH of 4–5 can precipitate out EDDIE and EDDIE-pep6His, isolating the cleaved pep6His dissolved in solution (Schmoeger et al., 2009).

A strategy to purify the target peptide can thus be implemented, where isolated IBs after fermentation is first dissolved in chaotropic buffers before refolded in kosmotropic buffers to cleave peptide from fusion protein (Achmüller et al., 2007; Kaar et al., 2009; Ueberbacher et al., 2009). Subsequently, appropriate precipitation conditions are used to precipitate out cleaved EDDIE and uncleaved EDDIE-pep6His, while isolating pep6His in solution.

Due to the many advantages of continuous processes in biopharmaceuticals (Godawat et al., 2012; Jungbauer, 2013; Konstantinov, 2011; Ottow et al., 2011; Rosa et al., 2013), the objective of this study is to test the viability and robustness of an integrated continuous tubular reactor encompassing the dissolution, refolding and precipitation step. Continuous process was initially performed where refolding and acid precipitation of pre-dissolved EDDIE-pep6His is continuously performed in a tubular reactor over 3 and 21 h. Later, the additional dissolution step was also included and performed over 5 and 20 h. The refolding kinetic studies at the start and end of process were compared and pep6His yields were collected from tubular reactor outlets over process time. Different tubular dimensions, flow rates and refolding protein concentrations were performed for each process run. Refolding kinetics and pep6His yields obtained in batch reactor were also compared to the tubular reactor in similar conditions. To establish complete process purification, the soluble fusion peptide (pep6His) after acid precipitation was further purified and concentrated using reverse-phased chromatography and subsequently lyophilized. The final quality of pep6His was further analyzed with mass spectroscopy.

2. Materials and methods

2.1. Chemicals

All chemicals were purchased from Merck (Darmstadt, Germany) and Sigma (Steinheim, Germany), respectively, if not indicated otherwise.

2.2. Recombinant protein expression and IBs isolation

The recombinant protein EDDIE-pep6His were overexpressed in *E. coli* BL 21 with a pET30a plasmid (Novagen, Madison, WI, USA) containing the corresponding coding gene (Achmüller et al., 2007). *E. coli* fed-batch cultivation was performed with a semi-synthetic medium on a 5-L scale according to Clementschitsch et al. (2005). Isolation of IBs was performed with an APV 2000 lab homogenizer (Invensys, Albertslund, Denmark) as described previously (Kaar et al., 2009).

2.3. Screening of precipitation conditions of refolded EDDIE-pep6His

1 M acetic acid was titrated with refolding buffer conditions containing 5 ml of 8 M urea, 50 mM Tris and 100 mM MTG (pH 7.3), and 45 ml of 1 M Tris, 0.25 M sucrose (Acros Organics), 2 mM EDTA, 20 mM MTG (pH 7.3). Using titration curve, precipitation was performed at pH 4.4 on 24 h refolded EDDIE-pep6His in final concentrations of 0.4, 0.8, 1.2 and 1.6 mg/ml. Precipitation was initiated by pipetting the appropriate 1 M acetic acid volumes into cuvettes containing 3 ml of refolded EDDIE-pep6His without additional mixing. Kinetic measurements at 600 nm using Cary 50 UV–vis Spectrophotometer were performed with Cary WinUV scanning kinetics application software.

Using optimal pH and precipitation time found with 1 M acetic acid, 1 M citric was also tested on refolded EDDIE-pep6His at 1.3 mg/ml. In addition, salting out method using 30% v/v, 50% v/v 3.75 M ammonium sulphate, and 50% v/v 1.72 M sodium sulphate was also tested on refolded EDDIE-pep6His. All screened precipitants were tested in triplicates.

2.4. Batch dissolution, refolding and acid precipitation of EDDIE-pep6His

EDDIE-pep6His IBs were dissolved by 1:5 ratio dilution dissolution buffer containing 10 M urea, 50 mM Tris and 100 mM MTG (pH 7.3). To measure protein concentration, dissolved samples were filtered and measured on a Cary 50 UV–vis Spectrophotometer using the theoretical extinction coefficient 1.098 (mg/ml protein)/cm at 280 nm.