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Getting ready for PAT: Scale up and inline monitoring of protein refolding of Npro fusion proteins

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ABSTRACT

Screening for optimal refolding conditions for recombinant protein overexpressed in *Escherichia coli* as inclusion bodies is often carried out on micro-scale in non-agitated reactors. Currently, scale up of refolding of N^{pro} fusion proteins is based on geometric similarity and constant *Re* number. Refolding/cleavage kinetics is recorded offline by HPLC and via fluorescence intensity. We show that the results for refolding obtained on the micro-scale can be transferred to the laboratory scale stirred tank reactor, with increases in scale up to a factor of 5000, with high agreement of kinetic constants and yield. Progress of refolding kinetics on the laboratory scale is monitored inline by attenuated total reflectance – Fourier transform infrared spectroscopy (ATR-FTIR). Addressing the demands for better process understanding, we demonstrate that ATR-FTIR enables the inline monitoring of refolding processes on the laboratory scale, replacing offline analysis which delivers the results with a time delay. Implementing inline monitoring will allow the integration of process control, thereby resulting in a more efficient and knowledge based production process.

1. Introduction

Currently, refolding processes of recombinant protein overexpressed in *Escherichia coli* (*E. coli*) as inclusion bodies (IBs) are performed on the industrial scale predominantly in batch reactors with fixed processing time and time-delayed off- or at line analysis of the product quality. The high productivity of the *E. coli* expression system affords proteins in high yield, but protein refolding is a bottleneck in the procedure and thus is a cost-driving factor in downstream processing [1]. The overall yield of protein recovery is specific for the individual recombinant protein and thus, proper conditions must be determined for each protein to ensure optimal yield [2–7]. Defining these conditions consumes time and materials and thereby raises costs. Screening for optimal refolding conditions is therefore often carried out on μ-scale such as with microtiter plates or HPLC vials [8–10]. Recently the concept of Quality by Design (QbD) has been applied for protein refolding by several investigators [11,12]. For rapid and efficient process development of protein refolding, reliable prediction from micro to the laboratory and industrial scale is required. Inline monitoring of the refolding progress would allow in process control and improve the overall productivity of protein recovery from IBs.

Results obtained from small scale investigations of high throughput systems can be transferred to achieve laboratory or industrial scale for the production of protein. Dimensionless numbers and geometric similarity of systems are the fundamental parameters used for scale up. Different systems that involve mixing in stirred reactors are characterized by the Reynolds number (*Re*) which is dependent on the stirrer diameter, the dynamic viscosity, and the agitation number. The influence of surface properties and surface to volume ratio must also be taken into account when comparing the small screening scale to laboratory and industrial scale.

Beside this, FDA introduced another requirement for design of bioprocesses, when they issued their guideline for process analytical technology for the pharmaceutical industry in 2004 [13]. Methods for on-, in- or at-line monitoring and control are of high interest for all bioprocesses since their use will "greatly reduce production cycle time and out-of-specification rates" [13]. Several analytical methods can be used for qualitative and quantitative characterization of protein structure and conformational changes throughout refolding such as size exclusion chromatography (SEC), SDS-PAGE, field flow fractionation, fluorescence spectroscopy, circular dichroism (CD), or static and dynamic light scattering. SEC and

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dynamic light scattering have recently been described as at-line methods for protein folding and aggregation [14,15]. So far a non-intrusive method for inline monitoring of refolding and aggregation formation applicable to an as wide range of proteins as possible has not been published. Therefore, one goal of the present studies has been to evaluate a method for inline monitoring of protein refolding in a stirred laboratory reactor which will also enable process control.

The infrared spectrum of a protein is a valuable tool for investigation of protein structure, molecular mechanisms of protein reactions, and protein folding, unfolding and misfolding [16–19]. Introduction of Fourier transform (FT) instruments to IR spectroscopy has enabled the detection of spectra with sufficiently high signal to noise ratios. Compared to secondary structure analysis by CD spectroscopy, FTIR spectroscopy is more tolerant to salt solutions and turbidity of samples [20]. The wavelength precision of FTIR spectroscopy over dispersive spectrometers is a great advantage that allows the subtraction of water which is a strong infrared absorber. Therefore, FTIR spectroscopy is most suitable for monitoring unfolding and refolding of the native secondary structure of recombinant protein as well as increases in aggregation throughout the protein recovery process [21]. By analysis of the amide I band in the 1600–1700 cm^{-1} region determination of secondary structure elements is possible. Insoluble protein aggregates are characterized by β-strand repeat units visible in ATR-FTIR in the region below 1630 cm^{-1}. In contrast, native β-sheets can be found between 1630 and 1643 cm^{-1}. In FTIR β-sheet contribution shows the highest absorption coefficient making it a well-suited method for analyzing β-sheet-rich proteins. Increase of aggregation can therefore be determined by increased absorbance in the region below 1630 cm^{-1} and the ratio of spectral contributions of aggregated and native β-sheet [4,5,22,23]. Beside this ability to determine native structure and aggregation formation in parallel, ATR-FTIR is applicable to all proteins without additional sample preparations or limitation to specific properties as it would be for determination of intrinsic fluorescence. So far, FTIR spectroscopy has not been widely used in analysis of protein folding [24,25].

Within recent decades, a number of systems have been developed to enhance the *E. coli* expression systems. N^{pro} fusion technology is based on the autoprotease N^{pro} from classical swine fever virus which cleaves itself from the target, leaving the peptide/protein with an authentic N-terminus [26]. No further additives are needed. The autoprotease can be removed from the target peptide/protein by acidic precipitation. When using N^{pro} fusion technology, the dominant part of recombinant protein is deposited in IBs which helps to overcome limitations of peptide degradation or cell toxicity. We showed that this system is efficient for many recombinant peptides/proteins with expression yields of up to 30% of biomass, purity of target protein in IBs, and cleavage yields up to more than 90% [26,27].

The design of a process for protein recovery from IBs requires an understanding of the characteristics of the expression technology, the scale-up criteria, and with respect to the requirements of the US FDA, a method for in-process monitoring and control. Since the characteristics of the N^{pro} fusion technology have already been discussed in detail [26–28], in the present studies we have focused on scalability and process control for refolding of peptides and proteins expressed by this fusion technology. We compared cleavage and refolding kinetics of diverse N^{pro} fusion proteins in small scale (200 μL to 1 mL) and at laboratory scale up to 1000 mL, a scale up factor of up to 5000. The possible impact of mixing was also taken into account as well as differences in material and surface-to-volume ratio of the different scales. Protein refolding was monitored by an ATR-FTIR probe in the laboratory scale reactor and compared to offline measurements to evaluate this method for in process monitoring/control.

2. Materials and methods

2.1. Chemicals

Acetonitrile was purchased from J.T. Baker (The Netherlands). Ammonia solution, KH_2PO_4, $C_6H_5Na_3O_7{\cdot}2H_2O$, $MgCl_2{\cdot}7H_2O$, $CaCl_2{\cdot}2H_2O$, glucose mono-hydrate, $FeSO_4{\cdot}7H_2O$, $AlCl_3{\cdot}6H_2O$, $ZnSO_4{\cdot}7H_2O$, $Na_2MoO_2{\cdot}2H_2O$, $CuCl{\cdot}2H_2O$ and H_3BO_3 were obtained from MERCK (Germany). All other reagents were of analytical grade from Sigma (USA), if not otherwise indicated.

2.2. Proteins

The proteins were produced by N^{pro} fusion technology in *E. coli*. Target molecules were over-expressed in fusion with a tailor-made mutant of the N^{pro} autoprotease from classical swine fever virus as described previously [26]. Upon refolding, the fusion partner is released by self-cleavage from the C-terminal end of the autoprotease, leaving the target protein with an authentic N-terminus. This process enables high-level production of recombinant peptides and proteins in *E. coli* without chemical or enzymatic removal of the fusion tag [26–28]. These autoprotease fusion proteins are deposited predominantly in IBs. Achmüller et al. [26] introduced a mutant of the wildtype autoprotease N^{pro} called EDDIE, which has proven to provide higher solubility and better cleavage properties compared to the wildtype. For the present study, experiments are shown for EDDIE fusion proteins containing the peptides pep6His, and Hepcidin, as well as green fluorescent protein (GFP) (Table 1).

2.3. Recombinant protein expression and IB isolation

The recombinant proteins were over-expressed in *E. coli* BL21 (DE3) cells by a pET30a plasmid (Novagen, Madison, WI, USA) containing the corresponding gene [26]. Fermentation and IB isolation were carried out as previously described by Walther et al. [29].

2.4. Protein solubilization

IB solubilization was carried out in either a laboratory scale reactor (EasyMax™, Mettler Toledo, Switzerland) or in microtiter plates (MTPs) (transparent flat bottom, low absorbance, Greiner, Germany) depending on the scale of the experiment. To dissolve the IB proteins, lyophilized IBs were resuspended in water for 1 h. The IB suspension was dissolved at a ratio of 1:10 in the corresponding solubilization buffer. The concentration of buffer ingredients was adjusted taking the dilution factor of IB suspension into account to achieve resulting urea concentrations of 8 M or 4 M. After 2 h, solubilization in the reactor was stopped by centrifugation at 14,000 rpm at 21 °C for 10 min (JLA 25.50 rotor, Avanti J25, Beckman Coulter, USA) while solubilization in MTPs was terminated by centrifugation at 13,200 rpm at 21 °C for 5 min (Centrifuge 5415R, Eppendorf, Germany) and consecutive filtration through 0.22-μm filters (Millipore, Billerica, MA, USA).

Table 1
Autoprotease fusion proteins used for solubilization kinetics.

Fusion protein	Amino acids	MW[a] (Da)	pI[a]	References
EDDIE-pep6His	184	20,970.5	6.63	[23]
EDDIE-Hepcidin	193	21,928.9	7.54	P81172[b]
EDDIE-GFP	405	46,663.5	6.19	[1]

MW, molecular weight; pI, isoelectric point.
[a] Estimated using the Expasy ProtParam tool (http://ca.expasy.org/tools/protparam.html).
[b] UniProtKB/TrEMBL (http://www.uniprot.org/uniprot).

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