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Review

Current status of technical protein refolding

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Abstract

The expression of heterologous proteins in microbial hosts frequently leads to the formation of insoluble aggregates. To fully exploit the production capacity of the cells, efficient strategies for further processing have to be developed. While in lab scale matrix assisted refolding techniques, especially of histidine-tagged proteins have become very popular, in production scale refolding by dilution is still predominant due to its simplicity. However scaling up dilution processes leads to large volumes and low protein concentration. This is a heavy burden both for liquid handling and for subsequent downstream processing steps. Process development aims to operate at uniform, reproducible conditions, to reduce costs to a minimum and to guarantee the required quality of the product. The general refolding kinetics, exploration of appropriate refolding conditions are reviewed. The major refolding operations such as dilution, matrix assisted refolding, pressure driven refolding or continuous refolding applications are discussed in view of industrial applicability.

Keywords: Inclusion bodies; *Escherichia coli*; Refolding; Large scale

Contents

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1. Introduction

Recombinant DNA technology allows the expression of valuable heterologous proteins at high expression rates. Particularly in *Escherichia coli* (*E. coli*) overexpression of proteins often leads to aggregation and deposition in dense, insoluble particles within the host cell, so-called inclusion bodies (IB). They are easily distinguishable from other cell components due to their refractile character (Fig. 1). Formation of inclusion bodies is heavily protein dependent, charge distribution and turn forming residues have a strong impact (Wilkinson and Harrison, 1991), also presence of cysteines may enforce tendency of aggregate formation (Rinas et al., 1992) but it may also be influenced by altering cell cultivation conditions (Panda et al., 1999). Decelerated cell growth achieved by lower temperature (Schein and Noteborn, 1988) or suboptimal pH (Kopetzki et al., 1989) can result in the production of soluble recombinant proteins which indicates that the cells are overburdened by the protein production at regular cultivation conditions. Still production of proteins as inclusion bodies is favored in several cases. Intracellular expression of proteins does have certain advantages over secretion of the product into the culture supernatant. Design heuristics of biotechnological processes recommend removing the most abundant impurities first. In a fermentation process this constitutes water. By a simple unit operation such as centrifugation the product can be concentrated by recovering the whole cells in the sediment. As outlined in Fig. 2 the attraction of inclusion body production compared to secretory systems is the simple primary recovery step. After cell harvest the cells have to be disintegrated and inclusion bodies have to be separated from cell debris and soluble cell components released into the homogenate.

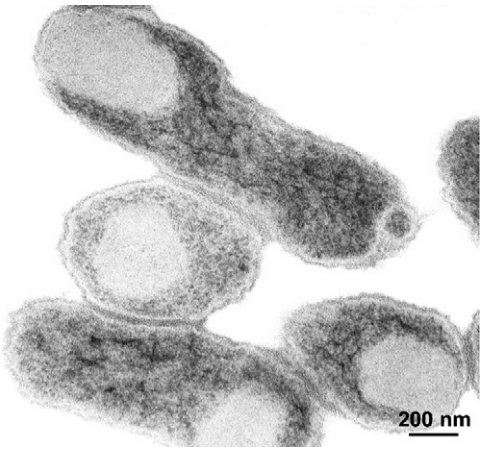

Fig. 1. Electron micrograph of *E. coli* cells containing cytosolic inclusion bodies.

Inclusion bodies consist nearly exclusively of recombinant proteins (Speed et al., 1996). Isolation of the desired product at already high purity is relatively easy due to density differences (Schoner et al., 1985) and high protein concentration can be achieved at the primary solubilization step. Although there are studies, that inclusion body protein is not the dead end deposit as believed earlier but inclusion bodies are dynamic structures subjected to permanent conversion (Carrio and Villaverde, 2002), the storage as aggregates still features distinct protection from protease degradation (Cheng et al., 1981; Kitano et al., 1987). Another advantage is the possibility to produce compounds, which are cell toxic in higher concentration. However, as a major disadvantage, the subsequently required refolding procedure poses a bottleneck in every downstream scheme. Protein aggregates have to be resolved and folded into their native structure. Various strategies have been employed to achieve an active compound refolded from inclusion bodies in reasonable yield. In this review special emphasis is taken on the scalability of a method and the use in industrial production processes.

2. Refolding kinetics

The distinct folding pathway of a single protein is still case of many hypotheses. While debating the driv-

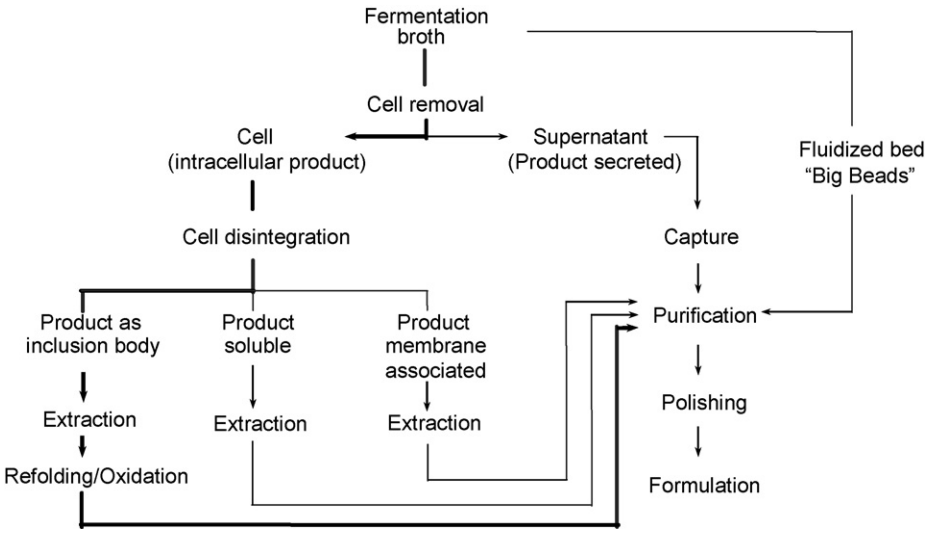

Fig. 2. Generalized processing scheme for the production of protein formulation from fermentation broth. Bold lines depict a usual inclusion body processing route.

ing force of the folding process they agree in the fact that a protein undergoes different more or less unstable conformations until it reaches its final native structure. At the absence of chaotropic agents these intermediates may exhibit intermolecular interactions, which leads to the major problems in a refolding procedure, aggregation and precipitation of the proteins. In refolding models estimating the final yield of a renaturation process, these competing side reactions are considered as of higher order while the folding reaction itself is approximated by a first order reaction (Zettlmeissl et al., 1979). A refolding reaction may therefore be described as

$$\frac{dU}{dt} = -(k_1 U + k_2 N U^n) \tag{1}$$

with k_1 the net rate constant of folding, k_2 the net rate constant of aggregation, U the concentration of unfolded protein, t time, N aggregation number and n the reaction order of aggregation, assuming that back reaction from folded or aggregated protein to unfolded protein is negligible and formation of possible folding intermediates is infinitely fast. Analytical solutions of this differential equation exist for second (Kiefhaber et al., 1991) and third order aggregation reactions (Hevehan and De Bernardez Clark, 1997) and are depicted in Eqs. (2) and (3), respectively.

$$Y(t) = \frac{k_1}{U_0 K_2} \ln\left[1 + \frac{U_0 K_2}{k_1}(1 - e^{-k_1 t})\right] \tag{2}$$

where $Y(t)$ is the yield of the refolding reaction, U_0 the initial concentration of the denatured protein and K_2 is the apparent rate constant of aggregation, combining aggregation number and rate constant of aggregation in $k_2 N$.

$$Y(t) = \Psi\left\{\tan^{-1}\left[(1+\Psi^2)\, e^{2k_2 t} - 1\right]^{1/2} - \tan^{-1}\Psi\right\} \tag{3}$$

with $\Psi = (k_1/k_2 U_0^2)^{1/2}$, where k_1 again is the net rate constant of folding, k_2 net rate constant of aggregation and U_0 is the initial concentration of unfolded protein.

Common refolding techniques aim to inhibit these side reactions to enhance the final yield of correctly folded protein. Major attention has to be drawn to the chemical as well as physical environment during a refolding process since folding and aggregation kinetics are heavily influenced thereby. Kinetic constants of a refolding process are of importance for the design of operation parameters such as dilution rate, final protein concentration and refolding time. Once optimal refolding conditions are found, yield would be solely defined by protein concentration if an ideal dilution process could be applied, since aggregation is a concentration driven process. The determination of kinetic constants

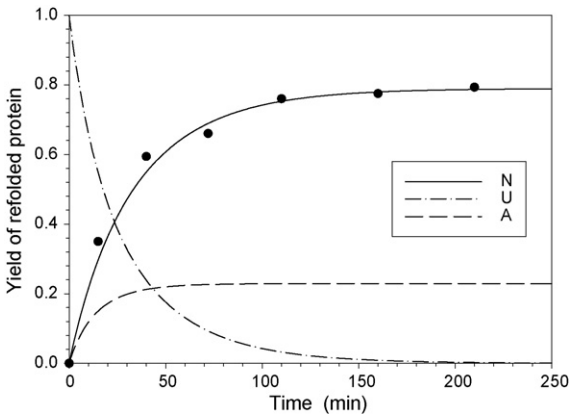

Fig. 3. Time course of the formation of native protein and aggregates during a refolding reaction.

may be accomplished by a direct fit of an appropriate refolding model such as shown in Eqs. (2) and (3) to data from dilution experiments at different protein concentration collected over refolding time until the endpoint is reached, or by an iterative approach when only data for one concentration are available over the whole time range, while endpoint data are available for different protein concentrations (Fig. 3). For a second order aggregation reaction yield at infinite time is described by

$$Y = \frac{k_1}{U_0 K_2} \ln\left(1 + \frac{U_0 K_2}{k_1}\right), \quad (4)$$

therefore kinetic constants can be easily extracted from the data set. However, kinetic constants heavily depend on refolding conditions and have to be determined for every buffer to be used.

The required scale of a refolding process influences choice of a distinct methodology.

3. Isolation of recombinant protein

While in lab scale cell lysis is often performed enzymatically yielding in almost complete degradation of cell walls, the industrial means of inclusion body isolation is mechanical disruption of cells followed by centrifugation. After high pressure homogenization, which results in a suspension of cell debris and the product, sedimentation of inclusion bodies has to be performed. The design of a centrifugal process requires knowledge of size distribution and density of the particles to be separated (Taylor et al., 1986). Monitoring separation in disc stack centrifuges based on different spectrophotometric properties of inclusion bodies and cell debris (Jin et al., 1994) supports centrifugal process design. Separation may prove critical if cell fragments and inclusion bodies have similar sedimentation properties. Pressure treatment for cell disintegration has to be optimized as has been studied in detail by Wong et al. (1997). They showed that repeated homogenizer passes resulted in better fractionation of inclusion bodies and cell debris leading to increased inclusion body purity. As a result of cell breakage, outer membrane components are released and may get adsorbed to the inclusion body surface (Hart et al., 1990). These compounds can be removed by several detergent washing steps, however, detergents sometimes cause problems in subsequent downstream processing and are therefore possibly avoided (Choe et al., 2006).

Chemical extraction of inclusion body protein as an alternative to mechanical means poses both advantages and disadvantages. Extraction directly from fermentation broths shortcuts unit operation steps, however release of high molecular weight DNA leads to increased viscosity of the solution which may cause severe problems for the capture of the product. Additionally high amounts of host cell proteins are contained in the extract. These problems were partly solved in different approaches. Removal of DNA could be achieved by precipitation with spermine (Choe et al., 2002) and also cheaper DNA-precipitants with comparable efficacy are available (Choe et al., 2006). A method for the selective extraction of recombinant proteins produced as inclusion bodies was described by Falconer et al. (1999) and successfully transferred to pilot scale. In a first step, membranes were permeabilized with a combination of urea and EDTA and host cell proteins were extracted, while inclusion bodies were kept insoluble by surface oxidation with the help of a disulfide bond promoting reagent. After removal of extracted compounds by diafiltration using a membrane with a high cut-off value, inclusion body protein was solubilized under chaotropic and reducing conditions. Compared with conventional extraction methods consisting of mechanical cell disruption, centrifugation steps and solubilization of inclusion bodies, similar protein extraction and purity could be reached. As a

drawback this method is only feasible for proteins with a high cysteine content. In a different approach it was aimed to disintegrate cells while maintaining inclusion bodies insoluble by treatment with a combination of Triton X-100 and EDTA (Lee et al., 2004). This method should be applicable for a wider range of recombinant proteins and might constitute a considerable shortcut in processing.

4. Determination of refolding conditions

As a starting point of each refolding reaction, the kind of solubilization strategy has to be considered. In case of chemical extraction at high denaturant concentration refolding procedures can directly be carried out. Starting from isolated inclusion bodies most techniques aim to reach complete unfolding, which is best accomplished by chaotropic agents such as guanidinium chloride (GdnCl) or urea at high concentration in combination with reducing agents. Depending on the protein to be refolded higher final yield could be obtained by retaining certain native-like secondary structure already present within the inclusion bodies. This could be achieved by using detergents (Puri et al., 1992), buffers at high pH (Khan et al., 1998; Singh and Panda, 2005), GdnCl or arginine at low concentration (Tsumoto et al., 2003a) or even sodium hydroxide (Mahmoud et al., 1998; Suttnar et al., 1994) as solvent reagents. However this strategy is very protein dependent and has no general applicability. As a second step refolding has to be initiated by removal of denaturant and providing conditions which allow intramolecular interaction and formation of correct structure.

To ensure optimal yield proper refolding conditions have to be found for every single protein. This is mostly done in an empirical approach based on former experience. If little is known about the protein of interest, this may result in a vast number of different experiments since a large number of refolding additives have been described in the past (De Bernardez Clark, 1998). This includes denaturants in low concentration, polyols such as sugars or sugar alcohols, ionic or non-ionic detergents and organic solvents. They may either promote folding of the protein or inhibit aggregation. The influence of GdnCl and L-arginine has been investigated in detail by Umetsu et al. (2003). They have studied the folding behavior of antibody fragments at the presence of GdnCl, L-arginine and redox systems in a stepwise dialysis system. Different chemical and spectroscopical means were applied to determine aggregation, formation of structure, exposure of hydrophobic patches and formation of disulfide bonds. Effects of various detergents and organic solvents on refolding yield of lysozyme were investigated by Yasuda et al. (1998) to find cheap refolding additives allowing processing at high protein concentration. Formation of aggregates was monitored by dynamic light scattering. Again it has to be emphasized that a positive effect of an additive on refolding of a certain protein may cause the opposite for another one since protein properties are extremely diverse.

In small scale refolding chaperones (Buchner et al., 1992), folding helper proteins, artificial chaperones (Machida et al., 2000; Rozema and Gellman, 1996) and redox pairs such as GSH-GSSG are used to improve yield. The use especially of chaperones is not practical in large scale, since they have to be available in a stoichiometric proportion to ensure efficacy. However, immobilization of folding aids allows a more efficient use of mostly expensive enzymes as shown with minichaperones (Altamirano et al., 1997), GroEL and GroES (Preston et al., 1999), oxidoreductases shuffling disulfide formation (Tsumoto et al., 2003b) or even a combination of chaperones (Altamirano et al., 1999). Reuse of artificial chaperones has been reported by Mannen et al. (2001). They used immobilized cyclodextrin to remove detergent from a denatured protein to allow refolding. Operation of the process in circulating expanded bed mode allowed the stripping of detergent in a refolding requirement comparable to a batch suspension system but had the advantage of scalability.

A fractional factorial design of the experimental setup significantly reduces the effort (Tobbell et al., 2002). Additionally automation of screening allows a first evaluation of different refolding conditions. Vincentelli et al. (2004) describe an automated screening system based on the detection of precipitation of proteins by turbidity measurements in a 96 well plate format. They associate solubility of the protein to native structure. Data provide valuable clues in terms of additive selection, however care has to be taken concerning soluble aggregates or stable misfolded species, therefore an activity assay is essential to draw further conclusions.

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