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REVIEW

Large-scale Recovery of Recombinant Protein Inclusion Bodies Expressed in *Escherichia coli*

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The production of recombinant proteins in *Escherichia coli* often leads to the formation of an intracellular inclusion body. Key process steps that can determine the economics of large-scale protein production from inclusion bodies are fermentation, inclusion body recovery, and protein refolding. Compared with protein refolding and fermentation, inclusion body recovery has received scant research attention. Nevertheless, it can control the final product yield and hence process cost for some products. Optimal separation of inclusion bodies and cell debris can also aid subsequent operations by removing contaminant particulates that foul chromatographic resins and contain antigenic pyrogens. In this review, the properties of inclusion bodies and cellular debris are therefore examined. Attempts to optimise the centrifugal separation of inclusion bodies and debris are also discussed.

Escherichia coli is one of the most widely-used hosts for the production of simple recombinant proteins in industrial biotechnology. There are several reasons for this, including the accumulated knowledge base regarding its molecular biology and hence the availability of a range of easily-controllable promoters (42). Additionally, fed-batch fermentation techniques giving high yield are well established (45). Examples of industrial-scale production using *E. coli* include the somatotropins (32, 39, 44), growth factors (7, 15, 22), tissue plasminogen activator (6), and of course insulin (40).

When recombinant proteins are overexpressed in *E. coli*, a refractile body called an inclusion body typically forms. This inclusion body is predominantly the recombinant protein of interest with some other contaminants. However, the protein is in an inactive form, and must be processed to give native protein. In this review, I shall focus on large-scale inclusion body recovery methods, specifically centrifugation. First, I will define what an inclusion body is and give a brief review of key inclusion body characteristics. I shall then examine typical processing sequences for recovering active protein from inclusion bodies expressed in *E. coli*. We will see that fractionation of the inclusion bodies from cell debris is an important problem that has received little research attention. The remaining sections then review attempts to characterise cell debris and optimise the particle classification stage.

Key words: inclusion body, recombinant protein, centrifuge, *Escherichia coli*, protein refolding, cell disruption

What Is An Inclusion Body?

Fig. 1 shows recombinant *E. coli* cells containing inclusion bodies of porcine somatotropin, taken at Adelaide. The inclusion bodies are apparent as electron-dense spheroids within the cell's cytoplasm.

Excellent reviews concerning inclusion bodies are available (19, 26, 37, 41). Key points are that the likelihood of an inclusion body forming for a given protein expressed in *E. coli* is dependent on the nature of the protein as well as the environmental conditions. However, no common characteristic that causes an inclusion body to form has been identified (19). Wilkinson and Harrison (51) examined data for 81 proteins at 37°C in *E. coli* that do and do not form inclusion bodies. They found that charge average and turn forming residue fraction were strongly correlated with the tendency to form inclusion bodies. Cysteine fraction, proline fraction, hydrophobicity and total residues were weakly correlated.

It is worth noting that inclusion bodies are predominantly, but not exclusively, composed of the recombinant protein of interest. Valax and Georgiou (48) found that the expression system and growth conditions have a pronounced effect on inclusion body composition. The level of contaminating polypeptides ranged from 5 to 50%, while phospholipids constituted 0.5 to 13% of the inclusion bodies. Nucleic acids were a minor contaminant. They concluded that inclusion body formation was a highly specific process, and that in some cases contaminants may be present because of incomplete separation. Hart *et al.* (13) examined *Vitreoscilla* hemoglobin (VHb) production in *E. coli*, and identified two cyto-

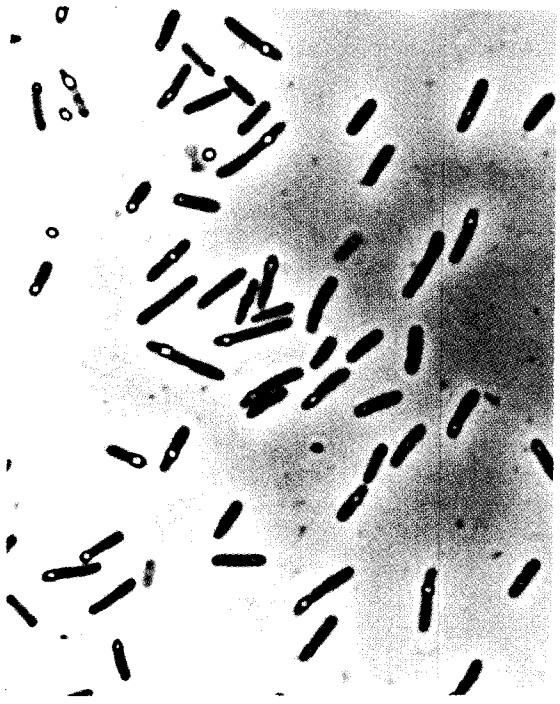

Fig. 1. *E. coli* containing recombinant protein inclusion bodies.

plasmic aggregates of different morphology. The granules differed in the relative fractions of VHb and pre-β-lactamase, the antibiotic resistance protein encoded on the same plasmid.

Taylor *et al.* (46) were the first to examine the physical characteristics of inclusion bodies in any detail. They employed a combination of centrifugal disc photosedimentation (CDS) and electrical sensing zone measurement (ESZ). The mean diameters of γ-interferon and calf prochymosin inclusion bodies were 0.81 and 1.28 μm, respectively. Inclusion body voidages of 70% for γ-interferon and 85% for prochymosin were also determined by matching data from the two sizing methods. Olbrich (38) determined the size of prochymosin inclusion bodies to be 0.85 μm using ESZ. By matching the ESZ data to CDS data, an apparent buoyant density of 1140-1160 kg/m^3 was obtained for the inclusion bodies after separation from debris by low-speed centrifugation. In later experiments Jin (17) measured prochymosin inclusion bodies by PCS and CDS and determined median diameters of 0.98 μm and 0.94 μm, respectively. Samples were fractionated prior to analysis using low speed centrifugation and the inclusion body density determined by Olbrich (38) was used for CDS measurements. At Adelaide, we have examined the physical characteristics of porcine somatotropin inclusion bodies (Fig. 1) and determined their median size by CDS to be approximately 0.4 μm. This is based on an inclusion body density of approximately 1260 kg/m^3, determined by caesium chloride gradients (Bresatec Ltd., Adelaide, Australia, personal correspondence). The measured size compares well with qualitative estimates by electron microscopy, which tend to indicate a closely-packed structure of approximately this size. We have also examined the inclusion body size for a range of insulin-like growth factor analogs expressed in *E. coli* (52, 53). Typical mean inclusion body sizes are 0.3-0.5 μm, based on a density of 1260 kg/m^3.

Typical Processing Sequences

As inclusion bodies are predominantly composed of the protein of interest, a significant purification can be effected by releasing the inclusion bodies from the cell and recovering them from the resulting mixture of soluble protein and insoluble cell debris (i.e. wall fragments). Recovery may be effected by differential centrifugation, which aims to partially separate the larger, denser inclusion bodies from the cellular debris (23). The resulting pellet will invariably contain cellular debris, a point that will become important subsequently. The protein in the inclusion bodies must then be converted to a native form and recovered from residual contaminants. It is generally believed that the protein contained in inclusion bodies is inactive and that it must be fully denatured and refolded to regain a native protein with activity. Inclusion bodies are therefore dissolved in strong denaturants such as urea or guanidine hydrochloride to give fully denatured protein. This can then be refolded to active protein by lowering the denaturant concentration. An excellent review of dissolution and refolding has recently been published (9). It is worth mentioning briefly that some reports indicate the possibility of some folded protein structure within the inclusion body. Hence, it may sometimes be possible to achieve dissolution with lower denaturant concentrations. This has been demonstrated by Hart and Bailey (14) for VHb and by Chang and Swartz (4) and Greenwood *et al.* (12) for insulin-like growth factors.

Large-scale processes for inclusion body recovery are based on the laboratory-scale processes, and have been described in several publications (21, 28, 36, 47). In typical industrial processes, cells may be harvested and are then disrupted by mechanical shear methods such as high-pressure homogenisation. The mixture following homogenisation resembles a lysis mixture and contains soluble proteins and other cell constituents, insoluble cell debris, and the target inclusion bodies. Differential separation of the inclusion bodies and cellular debris is then achieved using continuous disc-stack centrifuges. A washing step may often be introduced to further reduce the contaminant concentration in the inclusion body paste. Solubilisation and renaturation are then achieved using essentially the same processes as at laboratory

scale, but obviously with a view to minimising the use of expensive chemicals such as redox couples for disulfide formation.

There are several variations on the above processing schemes. Membranes have been used in place of centrifuges for inclusion body collection and washing (10, 27). In one case a threefold increase in yield compared with batch centrifugation was achieved, by incorporating a washing step into a membrane-based process (27). The method was not compared with a disc-stack centrifuge which is more effective at debris and inclusion body classification (see later). Bailey *et al.* (3) have introduced detergent and chaotrope prior to homogenisation and have demonstrated that this significantly enhances the disruption of cells. Hart *et al.* (15, 16) have achieved the *in situ* dissolution of periplasmic inclusion bodies with subsequent recovery of the soluble protein by aqueous two-phase extraction. We have recently extended this scheme at Adelaide to achieve the *in situ* dissolution of cytoplasmic inclusion bodies using combinations of chaotrope (urea) and EDTA (8). Recently, Walker and Lyddiatt (50) have commenced investigating aqueous two-phase systems for direct recovery of inclusion bodies. Other studies have focussed on improving the protein refolding operations (5, 36, 49).

The Critical Effect of Classification: the Cell Debris Problem

One of the key steps in the large-scale process described above is particle classification by centrifugation, or the separation of inclusion bodies from cellular debris. The separation is invariably imperfect because of the grade-efficiency characteristics of centrifuges and the closeness of the inclusion body and debris size distributions.

Residual cellular debris in the inclusion body paste can cause extreme problems in processing. *E. coli* contains a range of peptidases and proteases (24), and several of these are associated with the cell wall (11, 20, 24, 43). Consequently, if particle classification is not effective, significant protease concentrations may be carried into the dissolution and solubilisation stages. As an example of this, Babbitt *et al.* (2) examined the recovery of creatine kinase from inclusion bodies in *E. coli*. Significant proteolysis of the product was detected during inclusion body solubilisation and refolding. This was not reduced by strong protease inhibitors, but could be removed by introducing a washing step using detergent-containing buffer (5% Triton X-100). A 100x increase in yield resulted following introduction of the washing step. Similar problems have been identified for bovine pancreatic trypsin inhibitor (2). However, the cost of introducing a washing step utilising detergent can be expensive at a large-scale. Petrides *et al.* (39) examined the economics of large-scale porcine somatotropin manufacture from inclusion bodies in recombinant *E. coli*. A washing step with Triton X-100 was incorporated into the process. Economic analysis showed that the annual cost of Triton was $2 M, or 57% of the annual consumables cost. Some processes employ a wash with low-concentration denaturant (e.g. urea) to reduce contaminant loads. This has the disadvantage of again adding to consumable costs and also to waste treatment costs because of the high biological oxygen demand of the waste urea. More importantly, some proteins unfold in low urea concentrations (4, 12, 14) giving a reduced product yield and thus countering the benefits of reduced proteolysis.

In addition to the protease problem, carry-over of cellular debris from the particle classification step can cause further downstream problems. Residual debris in the refolded protein stream has a high fouling potential for downstream chromatography columns. This can reduce the effective life of resins. It also leads to variations in the resin binding capacity due to irreversible fouling, making process validation difficult. The residual debris can often be removed after solubilisation or refolding using centrifuges (47) or filtration (39). Again, this increases the overall process cost, particularly because of the small debris size following dissolution. It should be noted that all pyrogens must eventually be removed from the product as these cause immunological reactions when administered. These pyrogens are lipopolysaccharide components associated with the cell wall. Good removal of cellular debris at the classification stage will therefore reduce pyrogen burden at an early stage in the process.

Given the above problems, it seems clear that optimisation of the centrifugation step is a critical part of process scale-up. Nevertheless, this area has received scant attention in the literature compared with other areas such as molecular expression and protein refolding. Optimisation of the classification stage requires an understanding of the properties of the cell debris, and how this is affected by upstream operations such as homogenisation.

Properties of Cell Debris

A range of sizing techniques are available for characterising cellular debris, including photon correlation spectroscopy (PCS), centrifugal disc photosedimentation (CDS), and electrical sensing zone measurement (ESZ). Olbrich (38) examined the properties of cell debris generated from recombinant *E. coli* containing prochymosin inclusion bodies, and from host cells (i.e. non-transformed). Debris following repeated homogenisation was sized by a combination of PCS and CDS. The CDS data were fitted to the PCS data giving an effective cell debris density of approximately 1060 kg/m^3. The median size of the debris from PCS data ranged from 0.43 to 0.28 μm as the number of homogeniser passes increased from one to five at an operating pressure of 55 MPa. Jin (17)

reports a median debris size of 0.23 μm after five homogeniser passes at 55 MPa using PCS, compared with 0.26 μm after three passes. Using a stable CDS method (29), we have confirmed a significant shift in the settling velocity distribution of cell debris free of inclusion bodies as the number of homogeniser passes is increased from two to four (33). Olbrich (38) reported no significant reduction in debris size as the number of passes was increased further (from five to seven). It also appears that the host strain is more difficult to disrupt than the recombinant strain (32, 38).

Agerkvist *et al.* (1) examined the size of *E. coli* debris using PCS following disruption in a high-pressure homogeniser, a Microfluidizer homogeniser, and a bead mill. Samples were filtered (1.2 μm) before analysis, and treated with nucleases to reduce interference from RNA and DNA. The high-pressure homogeniser yielded a single debris peak at 0.19 μm after three passes. A second peak at approximately 0.45 μm was apparent after one and two passes through the homogeniser. The Microfluidizer gave larger debris, centred at 0.45 μm after three passes. The bead mill also yielded larger debris with a bimodal distribution having peaks at 0.47 and 0.79 μm.

Bailey *et al.* (3) examined the size distribution of *E. coli* debris following homogenisation, using scanning electron microscopy. Average particle size was 0.16-0.18 μm, considerably smaller than previous studies using PCS and CDS. This may be due to the low sensitivity of these methods at low sizes, or to shrinkage of the debris samples during preparation for electron microscopy. Interestingly, cells treated with 4 M guanidine hydrochloride prior to homogenisation at 62 MPa gave debris with a very small particle size (75% of particles < 0.19 μm).

Unfortunately, each of the sizing methods discussed above has significant disadvantages for debris sizing. As PCS is a low resolution technique, extensive sample preparation is required prior to size analysis. For example, Olbrich (38) centrifuged homogenate samples at 2000× g for 26 minutes, and took the supernatant as the cell debris sample and the pellet as the inclusion body sample. This is necessary as PCS cannot resolve the inclusion body and debris size distributions. Jin (17) notes that the problem with this method is that cell debris will co-sediment with the inclusion bodies and some inclusion bodies will remain in the supernatant. In fact, studies by Jin (17) demonstrate that up to 47% of the cell debris is co-sedimented with the inclusion bodies using Olbrich's (38) fractionation scheme, while 14% of inclusion body proteins remain in the supernatant (i.e. the debris sample). It is clear that the apparent size distribution is affected by the pretreatment employed. Other methods also have disadvantages. ESZ is prone to orifice blocking if high sensitivity is desired, limiting the size range that this technique can be employed for. It is typically unsuitable for sizing *E. coli* cell debris. It is also limited for non-spherical particles such as cell debris. CDS has inherent disadvantages because of low sensitivity below approximately 0.2 μm (29), where much of the cell debris is located. Results from this technique are prone to baseline drift and errors in extinction coefficient (29). Results from electron microscopy are also prone to error because of sample preparation techniques, including drying and plating, prior to analysis (3).

At Adelaide, we have recently developed a new method for the analysis of cell debris size (Wong *et al.*, manuscript in preparation). The technique permits the sizing of samples without the need for pretreatment, and can size debris in the presence of inclusion bodies and whole cells. Initial results suggest that debris from recombinant cells is slightly smaller than that from the uninduced host when processed under the same conditions. We have also observed a significant decrease in median debris size as the number of homogeniser passes is increased from two to five and ten.

Optimising Particle Classification

There are few reports of rational optimisation attempts for large-scale centrifugation. The key limitation has been accurate information on debris properties, as discussed above.

Olbrich (38) used the debris size distributions determined above to estimate the theoretical classification of debris and inclusion bodies in a disc-stack centrifuge, using grade-efficiency curves generated by Mannweiler (25) for polyvinylacetate particles. At low centrifuge flowrates a high inclusion body recovery was predicted, but at the expense of low paste purity. Conversely, high feedrates gave poor centrifuge recovery but highly effective removal of cell debris. The results were not confirmed experimentally. More recently, Jin (17, 18) conducted experimental trials into the separation of inclusion bodies and cellular debris using a disc-stack centrifuge. Deviations between the predicted and theoretical performance were observed, although general trends were correct. A method for on-line control of inclusion body recovery using turbidity was also developed. The work demonstrates that the ratio of two absorbance measurements (OD_{600nm}/OD_{400nm}) is a good correlator of the amount of inclusion body material in the centrifuge supernatant. The robustness of the method to changes in inclusion body size and other feed properties has not been defined. Clearly, a separate empirical equation will be required for each different feed stream.

We have conducted extensive analysis of the centrifuge problem at Adelaide. An experimental study of inclusion body and debris size was conducted using differential sedimentation (28). The important effect of homogenate viscosity was demonstrated. Experimental

study of the recovery of inclusion bodies was then undertaken, allowing the fractional recovery of inclusion bodies under a range of conditions to be defined (31). The data were regressed to an empirical curve for collection efficiency, and used to simulate the classification performance of an industrial centrifuge (30, 33). The simulation results suggest that overall process cost is determined to a large extent by debris size reduction in the homogeniser. The results also suggested that a given inclusion body purity could be attained at lower cost using multiple centrifuge passes (i.e. washing steps) without the need to add chaotropes or detergents. This is simply a consequence of the centrifuge grade efficiency characteristics. A subsequent study defined the impact of proteolysis on refolding yield, where proteases are carried through the system with cell debris because of sub-optimal particle classification (34, 35). It was shown that two distinct operational regimes for the centrifuge could be identified. First, for products that are resistant to outer-membrane proteases the minimum cost strategy is to collect a large fraction of the inclusion bodies with little reference to the resultant purity. Conversely, when products are highly protease sensitive, an optimal approach is to aim for low fractional inclusion body recovery which yields a paste with high purity. Proteolysis is then minimised, giving an overall cost optimum. The simulation results also suggest that multiple centrifuge passes will provide an overall reduction in process cost. Confirmation of these results has recently been obtained experimentally. Wong *et al.* (52, 53) examined the impact that centrifugation operation has on overall product yield for a proteolytically-sensitive analog of insulin-like growth factor. It was shown that overall protein yield following dissolution and refolding was relatively insensitive to centrifuge feedrate. This confirmed the simulation findings that a loss of inclusion bodies at high feedrate is offset by reduced proteolysis downstream. Multiple centrifuge passes without using chaotrope or detergent (i.e. washing steps with buffer) significantly improved protein yield following dissolution. Under the standard dissolution scheme, a twofold increase in recoverable protein was achieved by introducing two washing steps, even though this increased the inclusion body loss from 25% to 42%. This work is continuing, with the aid of the new debris analysis method discussed above.

Concluding Comments

The large-scale processing of proteins produced as inclusion bodies in *E. coli* presents a unique set of optimisation problems that have largely been ignored. In particular, the separation of inclusion bodies and cellular debris is a critical problem that can control final product yield, and hence process cost. Progress in optimising the large-scale classification operation has to date been slow, mainly because of a lack of information on the properties of cellular debris. The response by many researchers at small scale has been to introduce expensive washing steps using detergents and chaotropes. Hopefully, further rational optimisation of the classification step will be possible as we gain further understanding of the physical characteristics of inclusion bodies and cellular debris.

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