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REVIEW

Solubilization and Refolding of Bacterial Inclusion Body Proteins

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Inclusion bodies produced in *Escherichia coli* are composed of densely packed denatured protein molecules in the form of particles. Refolding of inclusion body proteins into bioactive forms is cumbersome, results in poor recovery and accounts for the major cost in production of recombinant proteins from *E. coli*. With new information available on the structure and function of protein aggregates in bacterial inclusion bodies, it has been possible to develop improved solubilization and refolding procedures for higher recovery of bioactive protein. Inclusion bodies are formed from partially folded protein intermediates and are composed of aggregates of mostly single types of polypeptide. This helps to isolate and purify the protein aggregates to homogeneity before solubilization and refolding. Proteins inside inclusion body aggregates have native-like secondary structures. It is assumed that restoration of this native-like secondary structure using mild solubilization conditions will help in improved recovery of bioactive protein in comparison to solubilization using a high concentration of chaotropic agent. Analysis of the dominant forces causing aggregation during inclusion body formation provides information to develop suitable mild solubilization procedures for inclusion body proteins. Refolding from such solubilized protein will be very high due to restoration of native-like secondary structure. Human growth hormone inclusion bodies were purified to homogeneity from *E. coli* cells before solubilization and refolding. Pure inclusion bodies were solubilized at alkaline pH in the presence of 2 M urea solution. The solubilized proteins were refolded using a pulsatile renaturation process and subsequently purified using chromatographic procedures. More than 40% of the inclusion body proteins could be refolded back to the bioactive native conformation. Mild solubilization is thus the key for high recovery of bioactive protein from inclusion bodies.

[**Key words:** inclusion body, solubilization, native-like secondary structure, aggregation, protein refolding, human growth hormone]

Escherichia coli have been most widely used for the production of recombinant proteins that do not require post-translational modification such as glycosylation for bioactivity (1, 2). However, high-level expression of recombinant proteins in *E. coli* often results in accumulating them as insoluble aggregates *in vivo* as inclusion bodies (3, 4). Inclusion body proteins are devoid of biological activity and need elaborate solubilization, refolding and purification procedures to recover functionally active product (5, 6). In general, inclusion bodies are solubilized by the use of a high concentration of denaturants such as urea or guanidine hydrochloride, along with a reducing agent such as β-mercaptoethanol (5, 7, 8). Solubilized proteins are then refolded by slow removal of the denaturant in the presence of oxidizing agent (9, 10). Protein solubilization from the inclusion body using high concentrations of chaotropic reagents results in the loss of secondary structure leading to the random coil formation of the protein structure and exposure of the hydrophobic surface (11). Loss of secondary structure during solubilization and the interaction among the denatured protein molecules during refolding resulting in their aggregation are considered to be the main reasons for the poor recovery of bioactive proteins from the inclusion bodies. Many times, the overall yield of bioactive protein from inclusion bodies is around 15–25% of the total protein and accounts for the major cost in production of recombinant protein from *E. coli* (12). Thus, a major bioprocess engineering challenge has been to convert this inactive and insoluble protein more efficiently into soluble and correctly folded product (13, 14).

Although protein expression in the form of inclusion bodies is often considered undesirable, their formation can be advantageous, as their isolation from cell homogenate is a convenient and effective way of purifying the protein of interest. The major advantages associated with the formation of inclusion bodies are (i) expression of a very high level of protein; more than 30% of the cellular protein in some

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cases, (ii) easy isolation of the inclusion bodies from cells due to differences in their size and density as compared with cellular contaminants, (iii) lower degradation of the expressed protein, (iv) resistance to proteolytic attack by cellular proteases, and (v) homogeneity of the protein of interest in inclusion bodies (lesser contaminants) which helps in reducing the number of purification steps to recover pure protein. Thus, despite the expressed protein having no bioactivity, inclusion bodies facilitates straight forward purification of the protein of interest from the cell. In fact, because of these above advantages, recombinant proteins expressed as inclusion bodies in *E. coli* have been most widely used for the commercial production of proteins (15). The loss in the recovery is compensated by the very high level of expression of the desired protein in *E. coli*. There are two important issues in the recovery of bioactive protein from inclusion bodies; solubilization of the protein aggregates and refolding of the solubilized protein into a bioactive form. Both the steps need careful consideration for improved recovery of bioactive protein. The ultimate aim is to develop a high throughput protein recovery process for inclusion body proteins. In this review, new information about protein structure inside inclusion bodies along with physicochemical characteristics have been discussed. Based on this new information, a novel solubilization method without using high concentrations of chaotropic reagents has been described for the recovery of bioactive protein from inclusion bodies. The solubilization method was used for refolding of recombinant human growth hormone (r-hGH) from the inclusion bodies of *E. coli*.

CHARACTERISTIC OF PROTEIN AGGREGATES IN INCLUSION BODIES

Inclusion bodies are dense electron-refractile particles of aggregated protein found in both the cytoplasmic and periplasmic spaces of *E. coli* during high-level expression of heterologous protein (16). It is generally assumed that high-level expression of non-native protein (higher than 2% of cellular protein) and highly hydrophobic protein is more prone to lead to accumulation as inclusion bodies in *E. coli* (17). In the case of proteins having disulfide bonds, formation of protein aggregates as inclusion bodies is anticipated since the reducing environment of bacterial cytosol inhibits the formation of disulfide bonds. The diameter of spherical bacterial inclusion bodies varies from 0.5–1.3 μm and the protein aggregates have either an amorphous or paracrystaline nature depending on the localization (18). Inclusion bodies have higher density (~1.3 mg ml^{-1}) than many of the cellular components, and thus can be easily separated by high-speed centrifugation after cell disruption (18, 19). Inclusion bodies despite being dense particles are highly hydrated and have a porous architecture (18, 20). Inclusion bodies contain very little host protein, ribosomal components or DNA/RNA fragments (21, 22). They often almost exclusively contain the over expressed protein and aggregation in inclusion bodies has been reported to be reversible (23, 24). It has been suggested that inclusion bodies are dynamic structures formed by an unbalanced equilibrium between aggregated and soluble proteins of *E. coli* (25). There is a growing body of information indicating that formation of inclusion bodies occurs as a result of intracellular accumulation of partially folded expressed proteins which aggregate through non-covalent hydrophobic or ionic interactions or a combination of both. Aggregation analysis of the tail-spike trimer of *salmonella* phage P22 protein has provided valuable information about the intermediates of protein folding pathways and the nature of aggregation leading to inclusion body formation in *E. coli* (26, 27).

Aggregation leading to inclusion body formation has been reported to be due to specific intermolecular interaction among a single type of protein molecule (28, 29). The specificity of protein aggregation has also been reported *in vivo*, emphasizing the aggregating behavior of partially folded polypeptide chains (30). Significant features of protein aggregates in inclusion bodies are the existence of native-like secondary structure of the expressed protein and resistance to proteolytic degradation (31–33). Analysis of the secondary structure of β-lactamase inclusion bodies from *E. coli* by Raman spectroscopy indicated the presence of an amide bond similar to that present in the native protein molecules, thus indicating the existence of native-like protein structure in inclusion bodies (31). Structural characterization studies using attenuated total reflection-Fourier transform in frared (ATR-FTIR) have shown that the insoluble nature of the inclusion bodies may be due to their increased level of non-native β sheet content compared with native and salt-precipitated protein (32). As the inclusion bodies have high density (~1.3 mg ml^{-1}), they are easily separated by high-speed centrifugation after cell disruption. Sucrose gradient centrifugation can be used to obtain very pure inclusion body preparation from *E. coli* cell lysate (16). Purification of inclusion bodies can also be achieved by washing with detergents, low concentrations of salt and urea (8, 18, 34). With an appropriate isolation and washing process, an inclusion body preparation more than 95% pure can be obtained from *E. coli* (34). The formation of inclusion bodies thus facilitates the easy isolation and purification of the expressed proteins in denatured form. Solubilization and refolding of relatively pure inclusion body protein also reduces the number of chromatographic steps for the final purification of the expressed protein. As the presence of contaminating proteins reduces the refolding yield of denatured proteins (35), isolation and purification of inclusion bodies to homogeneity before solubilization improve the recovery of bioactive protein from inclusion bodies.

RECOVERY OF BIOACTIVE PROTEIN FROM INCLUSION BODIES

Refolding of inclusion body proteins into bioactive forms is cumbersome, requires many operational steps and most of the time results in very low recovery of refolded protein. In the cases where a high yielding recovery process has been developed for refolding of the aggregated protein, inclusion body formation provides a straight forward strategy for recombinant protein purification. The higher the amount of this partially folded protein that is converted into the bioactive form, the more therapeutic protein can be recovered with improved yield and at low cost from inclusion bodies

of *E. coli*.

Traditional method of protein recovery from inclusion bodies The recovery of bioactive therapeutic protein from the inclusion bodies involves four steps: isolation of inclusion bodies from *E. coli* cells; solubilization of protein aggregates; and refolding and purification of the solubilized protein (6–8, 10, 14). Among these steps, solubilization and refolding are the most crucial steps for high recovery of bioactive protein. Inclusion bodies are generally separated from the cell debris using low-speed centrifugation after cell lysis as they are denser than many of the cellular components. Semi-pure protein aggregates along with contaminants are then solubilized using high concentrations (6–8 M) of chaotropic reagents such as urea, guanidine hydrochloride (9, 10) and detergents such as SDS (36), *N*-cetyl trimethyl ammonium chloride (37) and sarkosyl (sodium *N*-lauroyl sarcosine) (38). Additional reducing agents like β-mercaptoethanol, dithiothreitol or cysteine are also often used for solubilization of inclusion body proteins (9). This helps to maintain cysteine residues in a reduced state and thus prevents non-native intra- or inter-disulfide bond formation in highly concentrated protein solutions at alkaline pH. Chelating agents like EDTA are frequently used in the solubilization buffer to prevent metal-catalyzed air oxidation of cysteines. Solubilized proteins are then refolded into their native state during the removal of chaotropic reagents (6, 9, 10, 14, 39, 40). Refolding followed by purification is generally preferable as some of the high molecular weight aggregates along with contaminants can be co-purified in a single step.

One of the reasons for the poor recovery of refolded protein from the solubilization mixture is its aggregation. Protein aggregation is a higher-order reaction while refolding is a first-order reaction. Thus, the rate of aggregation is more than the rate of folding at high initial protein concentration. Because of this kinetic competition, yields of correctly folded protein decrease at increasing initial protein concentration. Protein concentrations in the range of 10–50 $\mu g\ ml^{-1}$ are typically used during refolding. Dilution of the solubilized protein directly into the renaturation buffer is the most commonly used method for small-scale refolding of recombinant proteins. Refolding large amounts of recombinant protein using a dilution method needs a large refolding vessel, a huge amount of buffer and additional concentration steps after protein renaturation and thus adds to the high cost of protein production. Even though dilution has its own problems for large-scale operation, it is most conveniently used for refolding of small amounts of protein (6, 41).

Proteins having multiple disulfide bonds need a more elaborate refolding process in the presence of optimal concentrations of both oxidizing and reducing agents for the formation of disulfide bonds (6, 9). Air oxidation in the presence of a metal catalyst is the simplest way of oxidizing protein but is highly empirical. Oxidation can also be achieved by adding a mixture of oxidized and reduced thiol reagents such as glutathione, cysteine and cystamine. The most widely used thiol reagents are reduced oxidized glutathione (GSH/GSSH), DTT/GSSH, cysteine/cystine, and cysteamine/cystamine at a total concentration of 5–15 mM with a molar ratio of reduced to oxidized compounds of 1 : 1 to 5 : 1, respectively (5, 6, 10, 39). Renaturation with mixed disulfide bond formation using oxidized glutathione also helps in the high recovery of disulfide-containing protein. This involves the formation of disulfide bonds between glutathione and the denatured protein followed by renaturation in the presence of a catalytic amount of reduced glutathione (42). The use of mixed disulfides increases the solubility of the protein during refolding and thus helps in lowering the extent of incorrect disulfide bond formation.

The use of additives (low molecular weight compounds) during the refolding process often helps in improving the yield of bioactive proteins from inclusion bodies (7). Additives such as acetone, acetoamide, urea, detergents, sugar, short-chain alcohols, DMSO and PEG have been used to enhance the yield of bioactive protein during refolding (42, 43). The most commonly used low molecular weight additives are L-arginine, low-concentration (1–2 M) urea or guanidine hydrochloride, and detergents. Among the additives, the positive effect of L-arginine/HCl in reducing aggregation has been demonstrated on various proteins (44–46). In general, 0.4 to 1 M arginine helps to reduce protein aggregation and thus improves the refolding yield of solubilized protein. The interaction of the guanidino structure of arginine with the tryptophan residues of proteins has been suggested as one way of reducing protein aggregation while using arginine as a folding additive (47). These additives influence both the solubility and stability of the unfolded protein, folding intermediate and the fully folded protein. They are easy to remove from solubilization buffer with the exception of detergents, which need special treatment after protein refolding.

Improved methods of protein refolding from inclusion bodies In recent years, many novel high-throughput protein-refolding methods have been developed for renaturation of inclusion body proteins (6, 41, 48). These include three methods such as dilution, dialysis or solid-phase separation for renaturation of inclusion body proteins (7). Different dialysis and dilution methods along with the use of additives have been reported for improved recovery of inclusion body proteins (41). Protein refolding using pulse renaturation processes (49), size exclusion chromatography (50) and adsorption chromatography (51) are most widely used for better recovery of the solubilized protein. These processes essentially involve physical separation of the partially folded protein molecules during buffer exchange, which helps in reducing the protein–protein interaction thereby lowering aggregation and improving recovery of the bioactive protein.

Pulse renaturation involves the addition of a small amount of solubilized protein to the renaturation buffer at successive time intervals (52, 53). The success of this process is based on the fact that once a small amount of denatured protein is refolded into the native from, it does not form an aggregate with the unfolded protein. By choosing the protein concentration and time of successive additions of solubilized protein, large quantities of proteins can be refolded in the same buffer tank. This helps to reduce the volume of buffer and improve the overall performance of the protein refolding process. Size exclusion chromatography facilitates the simultaneous removal of denaturant and renaturation of denatured protein (50). Use of an appropriately sized of gel filtration matrix facilitates in trapping the different forms of

folding intermediates depending on their hydrodynamic radius, thus physically separating the individual protein molecule. This reduces the protein–protein interaction of the folding intermediates, reduces intermolecular aggregation and thus improves the renaturation yield of denatured protein. Denatured lysozyme and carbonic anhydrase at a very high concentration have been successfully refolded using sephacryl S-100 columns (50). As size exclusion chromatography offers multiple advantages of buffer exchange, protein refolding and separation of monomer from aggregates, it provides an ideal method for the refolding of inclusion body protein at high concentration (54, 55).

Simultaneous buffer exchange, refolding and purification of inclusion body solubilized protein can be carried out using ion exchange chromatography where the denatured proteins of interest bind to the matrix (51, 56). Intermolecular interaction leading to aggregation is minimized as protein molecules are isolated through binding to the support matrix. Simultaneous use of denaturant-free buffer and optimization of elution conditions lead to the purification of protein in bioactive form (57). Using similar methodology, reduced lysozyme at very high concentration (9 mg ml^{-1}) has been successfully refolded to the bioactive form with almost 100% recovery using immobilized liposome chromatography (58). Chromatography based on immobilized mini chaperones has also been used for refolding of inclusion body proteins (59, 60).

NOVEL METHOD OF PROTEIN SOLUBILIZATION AND HIGH RECOVERY OF BIOACTIVE PROTEIN FROM INCLUSION BODIES OF *E. COLI*

Protein aggregation is one of the major problems associated with the refolding of inclusion body proteins. Aggregation results from intermolecular interaction that competes with intra-molecular reaction. Aggregates are mostly formed by non-native hydrophobic interactions between the folding intermediates in which the hydrophobic patches are exposed. Solubilization of protein aggregates in a high concentration of chaotorpic agents generates random coil structure of the protein where such hydrophobic amino acid stretches are exposed. This enhances the propensity of aggregation during refolding. One of the ways to reduce protein aggregation is to have a refolding process in which the intermediates are beyond the aggregated prone structure, *i.e.*, where the hydrophobic patches are not fully exposed. This can be achieved by mild solubilization of inclusion body proteins without generating the random coil configuration of the protein. Prevention of hydrophobic interactions during the initial stages of refolding is thus crucial for lower protein aggregation and improved renaturation of inclusion body protein.

Detail analysis of protein aggregation using P22 tailspike protein has indicated that the protein aggregates are very specific in nature suggesting that the inclusion bodies are composed of mostly single polypeptide chains in denatured states (28). Aggregation is molecular-specific in nature: the partially folded intermediate of P22 does not aggregate with native protein or with the folding intermediate of another protein *in vitro* (26–28). The structure estimates of the protein expressed as inclusion bodies localized in different compartments of *E. coli* have been found to be similar, suggesting that inclusion body formation takes place at a later stage of the protein folding pathway, and thus proteins retain most of their secondary structure during aggregation into inclusion bodies (16, 31). This has been further confirmed by the presence of extensive native-like secondary structures of proteins in a number of bacterial inclusion bodies (31–33). All of this information suggests that the protein in the inclusion body already exists in an intermediate stage of the folding pathway and has a considerable amount of secondary structure. If protein from inclusion bodies could be solubilized without disturbing its existing native-like secondary structure, the extent of protein aggregation during refolding will be low and will result in high recovery of bioactive protein. Mild solubilization of inclusion body aggregates without generating random coil protein structure, which is more prone to aggregation, is thus the key for improved recovery of bioactive protein.

It is possible to determine the dominant forces that cause protein aggregation in inclusion bodies by analyzing their solubility behavior *in vitro* in different buffers (61). Such information can be used to develop mild solubilization buffers for inclusion body proteins without unfolding them to the random coil structure as experienced with high molar concentrations of chaotropes. This can be achieved by manipulating experimental conditions, such as pH and the use of different solubilizing agents in the presence of low concentrations of denaturants and detergents. This is supported by the research finding that mild solubilization leads to higher refolding yield of bioactive protein from inclusion bodies of *E. coli* (34, 38, 62, 63). Detergents such as SDS and CTAB also help in the solubilization of inclusion body protein with retention of secondary structure. However, their subsequent removal for the refolding of therapeutic protein becomes essential. Residual detergent not only hampers the downstream chromatographic operation for final purification but necessitates the use of extra process steps for final purification. It is thus more appropriate to solubilize protein aggregates using other agents by understanding the dominant forces that cause protein aggregation.

Thus, by exploiting the new information on inclusion bodies in terms of size, density, protein structure and dominant forces leading to aggregation, the use of mild solubilization procedures and novel refolding procedures, results in the recovery of bioactive protein from inclusion bodies being improved considerably. Such a protein refolding strategy from bacterial inclusion bodies is depicted in Fig. 1 and has been successfully applied for the recovery of human growth hormone (61), zona pellucida protein (64) as well as recombinant LHRH multimer (65) from inclusion bodies of *E. coli*. This essentially involves giving a pH shock to the protein aggregates distant from the isoelectric point of the protein, thus rendering them soluble in the presence of very low concentrations of denaturants. Once the inclusion body proteins are solubilized under such mild conditions, the subsequent refolding and purification are easier resulting in high recovery of the bioactive protein.

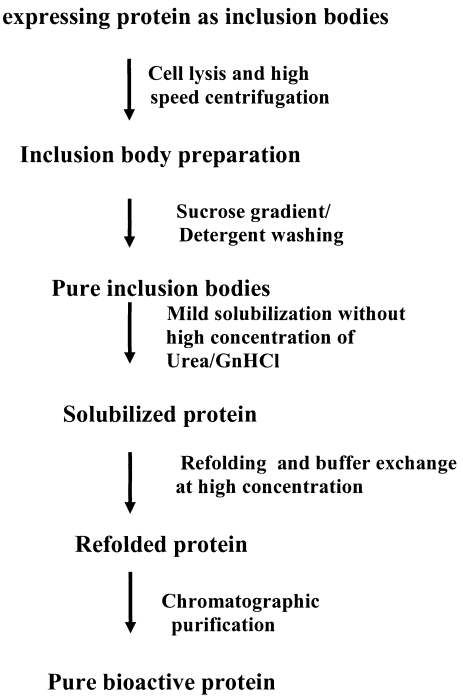

FIG. 1. Novel purification strategy for improved recovery of bioactive protein from inclusion bodies.

RECOVERY OF HUMAN GROWTH HORMONE FROM INCLUSION BODIES

Purification of inclusion bodies followed by solubilization without disturbing the native-like secondary structure and refolding was applied for the recovery of r-hGH from *E. coli*. Human growth hormone, a single chain polypeptide containing 191 amino acid residues, apart from stimulating cell growth, plays an important role in a variety of metabolic, physiologic and anatomic processes (66). The protein folds into a four-helix bundle structure with two disulfide bridges. Large-scale requirement of r-hGH necessitates its high-level expression in *E. coli* as inclusion bodies. However, expression of the protein along with a fusion tag and purification makes the overall process more complex and expensive as it lowers the yield of bioactive r-hGH. Human growth hormone was expressed as inclusion bodies without any additional tag in *E. coli* using the T5 promoter and was used for the recovery of bioactive protein using the above-described novel solubilization procedure (61).

Isolation of pure bacterial inclusion bodies from *E. coli* cells Human growth hormone was produced using fed-batch fermentation and the expression level was around 15% of the cellular protein (61). *E. coli* cells (1.5 gram dry cell weight) were used for inclusion body preparation and subsequent refolding. *E. coli* cells were lysed by a French press at 18,000 psi and the inclusion bodies were isolated by centrifugation at 8000 rpm. As the inclusion bodies have higher densities, high-speed centrifugation helps in separating them from contaminating cellular fragments/proteins. To prepare ultrapure inclusion bodies, they were separated by sucrose gradient centrifugation (18). However, for large scale preparation of purified inclusion bodies, they were

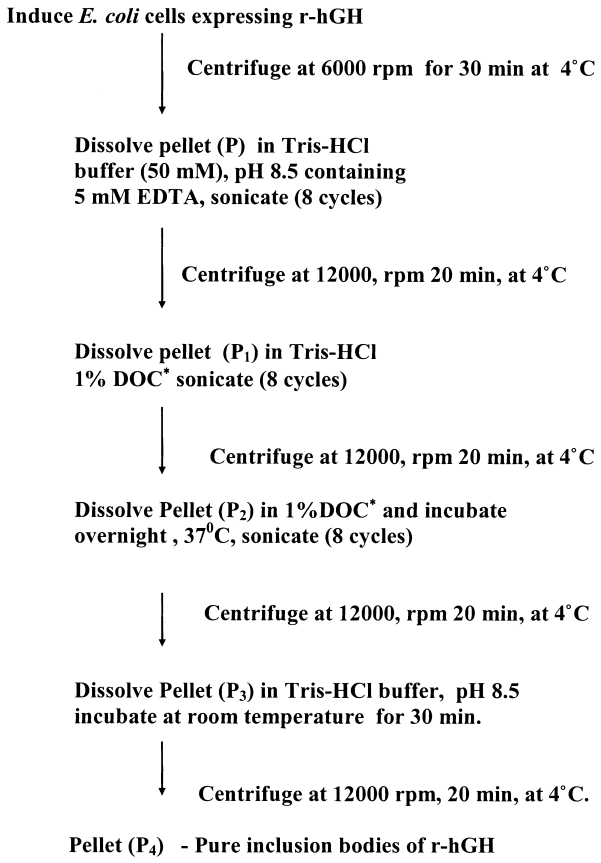

FIG. 2. Scheme for the purification of pure inclusion bodies from *E. coli* cells using detergent washing.

extensively washed with detergent as described in Fig. 2. Using an appropriate centrifugation and washing process, more than 95% pure inclusion bodies containing r-hGH were isolated from *E. coli* cells (61). The notable difference in the purification of inclusion bodies of human growth hormone was that they were always associated with high molecular aggregates. Pure inclusion bodies were found to have a regular shape having a diameter varying from 0.5 to 0.8 μm as observed in the scanning electron micrograph (Fig. 3). Such purified inclusion bodies were used for subsequent solubilization and refolding for the recovery of bioactive protein.

Solubilization of r-hGH from inclusion bodies Pure r-hGH inclusion bodies were solubilized at different pHs in 100 mM Tris buffer (pH 3–13) and percent solubilization of r-hGH was monitored. Solubilization of r-hGH from inclusion bodies was observed by increasing the pH from 6 to 12.5. Higher solubilization of r-hGH from inclusion bodies was observed by incorporating 2 M urea in 100 mM Tris buffer at pH 12.5 (Table 1). Further addition of urea in 100 mM Tris buffer at pH 12.5 did not further increase solubilization of r-hGH from the inclusion bodies. In 100 mM Tris buffer at pH 12.5 containing 2 M urea, a maximum of 6 mg ml^{-1} of r-hGH were solubilized from the inclusion bodies. Use of 2 M urea did not unfold the protein completely and preserved the native-like secondary structure.

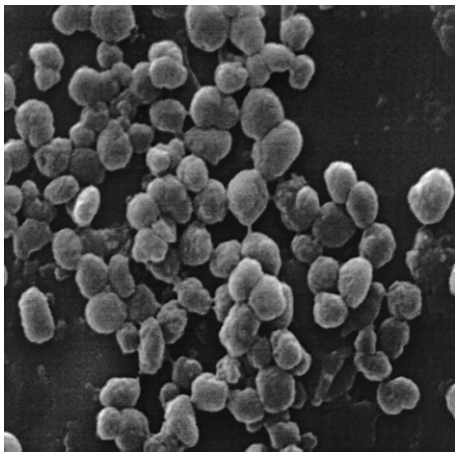

FIG. 3. Scanning electron micrograph of pure hGH inclusion bodies. Diameter of spherical size particle is around 0.5–0.8 μm.

Use of high pH facilitates in better solubilization as it was distant from the isoelectric point of human growth hormone which is 4.9. A combination of alkaline pH and 2 M urea destabilized both the ionic and hydrophobic interactions which are the major cause of protein aggregation in inclusion bodies of human growth hormone.

Purification and refolding of r-hGH The solubilized r-hGH was diluted in a pulsatile manner in the refolding buffer (50 mM Tris–HCl, 0.5 mM EDTA, 2 M urea, 10% glycerol, 5% sucrose, 1 mM PMSF at pH 8) at 4–6°C with constant stirring. This lowered the pH of the buffer from 12.5 to around 8. No aggregation of the solubilized r-hGH was observed during dilution and buffer exchange. Solubilized r-hGH was filtered through a 0.22-μm filter and the clear solution was passed through a DEAE–Sepharose column for purification (61). r-hGH which eluted between the conductivity ranges of 14 to 16 mS/cm was found to be homogeneous and represented 40% of the total protein. However, some of the r-hGH was co-eluted with r-hGH dimer in the conductivity range of 22 to 25 mS/cm, which constituted about 25–30% of the total protein. Pure r-hGH containing dimers/oligomers was passed through a size exclusion chromatography column for further purification. The dimeric or higher forms of the proteins were removed through gel filtration. The overall yield of the purified refolded r-hGH from the inclusion bodies of *E. coli* was >40% (Table 2).

Authenticity of the purified r-hGH was further confirmed from the N-terminal analysis of r-hGH and from spectroscopic analysis. The UV spectrum of the purified r-hGH showed an absorbance maximum at 276.8 nm, and a shoulder at 283 nm, which was comparable to that of native human growth hormone (61). The fluorescence spectrum of refolded r-hGH was found to be identical to that of native human growth hormone, which gave a peak at 340 nm. Growth of Nb2 cells in the presence of different concentrations of r-hGH was found to be comparable to that observed for the commercial human growth hormone indicating the bioactivity of the preparation (61). The overall yield of the r-hGH from the inclusion bodies was >40% in comparison to 15% to 20% achieved by solubilizing the inclusion bodies in high concentrations of chaotropic reagents. Solubilization of the r-hGH from inclusion bodies, while retaining the native-like secondary structures lowered protein aggregation during buffer exchange and dilution. Despite the presence of two disulfide bonds, extensive protein aggregation during refolding due to incorrect disulfide bond formation was not observed for r-hGH. High recovery of bioactive human growth hormone from the inclusion bodies of *E. coli* further substantiated the usefulness of the novel mild solubilization procedure.

TABLE 1. Solubilization of purified human growth hormone (hGH) inclusion bodies at different pHs

pH	Percent solubilization of hGH inclusion body	
	Tris buffer (no urea)	Tris buffer with 2 M urea
6	–	5
7	–	5
8	5	10
9	5	10
10	12	15
11	20	25
12	40	85
12.5	50	95

Inclusion body proteins (2 mg/ml) were solubilized at different pHs and the percent solubility was calculated by measuring the solubilized protein concentration by a Micro BCA assay.

TABLE 2. Purification efficiency of human growth hormone from inclusion bodies

Steps	Total protein (mg)	Step yield (%)	Overall yield (%)	Purity (%)
Pure inclusion body	104	100	100	90
Solubilization and refolding	83	80	80	93
Ion exchange chromatography	57	70	57	95
Gel filtration chromatography	45	75	43	99

Inclusion bodies were purified from 1.5 g dry cell weight of *E. coli* cells produced using fed-batch fermentation.

CONCLUSION

The main objective of protein refolding from bacterial inclusion bodies is to recover a good amount of bioactive protein at low cost. Although understanding of inclusion body protein structure, novel solubilization and improved refolding methods has increased recently, a single straight forward method which satisfies all protein folding requirements remains the desired objective. Protein aggregation during different refolding step is the major bottle neck in recovering high amounts of protein from inclusion bodies. It is thus necessary to reduce the extent of protein aggregation at each step of refolding starting from isolation to final purification. Completely unfolded protein is more prone to aggregation than a partially folded intermediate polypeptide chain. It is essential to restore the native-like secondary structure of inclusion body protein during solubilization which renders protein less prone to aggregation. It is expected that a novel solubilization process which reduces the propensity of protein aggregation followed by improved refolding will help

in the high recovery of recombinant protein from inclusion bodies. Human growth hormone in the form of inclusion bodies was separated and purified to homogeneity from *E. coli*. Inclusion body aggregates were solubilized at alkaline pH without disturbing the existing secondary structure and subsequently refolded and purified to the bioactive form. As both ionic and hydrophobic interactions are the dominant forces resulting in protein aggregation in inclusion bodies, a pH shock in the presence of a low concentration of urea facilitates solubilization while retaining native-like secondary structure, which reduces protein aggregation during refolding and purification. Even though it is similar to solubilization of growth hormones using detergents (37), such novel solubilization facilitates improved recovery of the bioactive protein without requiring any additional steps for removal of detergents. The use of a mild solubilization process is the key for the high recovery of bioactive protein from inclusion bodies. Once a mild solubilization process is developed for a particular inclusion body protein, subsequent refolding will lead to high recovery of bioactive protein.

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REFERENCES

1. **Baneyx, F.:** Recombinant protein expression in *Escherichia coli*. Curr. Opin. Biotechnol., **10**, 411–421 (1999).
2. **Swartz, J. R.:** Advances in *Escherichia coli* production of therapeutic proteins. Curr. Opin. Biotechnol., **12**, 195–201 (2001).
3. **Kane, J. F. and Hartley, D. L.:** Formation of recombinant protein inclusion bodies in *Escherichia coli*. Trends Biotechnol., **6**, 95–101 (1988).
4. **Fahnert, B., Lile, H., and Neubauer, P.:** Inclusion bodies: formation and utilization. Adv. Biochem. Eng./Biotechnol., **89**, 93–142 (2004).
5. **Rudolph, R. and Lilie, H.:** *In vitro* folding of inclusion body proteins. FASEB J., **10**, 49–56 (1996).
6. **Vallejo, L. F. and Rinas, U.:** Strategy for recovery of active protein through refolding of bacterial inclusion body proteins. Microb. Cell Fact., **3**, 2–12 (2004).
7. **Clark, E. D.:** Refolding of recombinant proteins. Curr. Opin. Biotechnol., **9**, 157–163 (1998).
8. **Lilie, H., Schwarz, E., and Rudolph, R.:** Advances in refolding of proteins produced in *E. coli*. Curr. Opin. Biotechnol., **9**, 497–501 (1998).
9. **Fischer, B., Sumner, I., and Goodenough, P.:** Isolation, renaturation and formation of disulfide bonds of eukaryotic proteins expressed in *E. coli* as inclusion bodies. Biotechnol. Bioeng., **41**, 3–13 (1993).
10. **Rudolph, R., Böhm, G., Lilie, H., and Jaenicke, R.:** Folding proteins, p. 57–99. *In* Creighton, T. E. (ed.), Protein function, a practical approach. IRL-Press, Oxford University Press, Oxford (1997).
11. **Dill, K. A. and Shortle, D.:** Denatured states of proteins. Annu. Rev. Biochem., **60**, 795–825 (1991).
12. **Datar, R. V., Cartwright, T., and Rosen, C. G.:** Process economics of animal cell and bacterial fermentations: a case study analysis of tissue plasminogen activator. Bio/Technology, **11**, 349–357 (1993).
13. **Panda, A. K.:** Bioprocessing of therapeutic proteins from the inclusion bodies of *Escherichia coli*. Adv. Biochem. Eng./Biotechnol., **85**, 43–93 (2003).
14. **Clark, E. D.:** Protein refolding for industrial processes. Curr. Opin. Biotechnol., **12**, 202–207 (2001).
15. **Walsh, G.:** Biopharmaceutical benchmarks-2003. Nat. Biotechnol., **21**, 865–870 (2003).
16. **Bowden, G. A., Paredes, A. M., and Georgiou, G.:** Structure and morphology of protein inclusion bodies in *Escherichia coli*. Bio/Technology, **9**, 725–730 (1991).
17. **Mitraki, A., Fane, B., Haase-Pettingell, C., Sturtevant, J., and King, J.:** Global suppression of protein folding defects and inclusion body formation. Science, **253**, 54–58 (1991).
18. **Taylor, G., Hoare, M., Gray, D. R., and Marston, F. A. O.:** Size and density of protein inclusion bodies. Bio/Technology, **4**, 553–557 (1986).
19. **Georgiou, G. and Valax, P.:** Isolating inclusion bodies from bacteria. Methods Enzymol., **309**, 48–58 (1999).
20. **Carrio, M. M., Cubarsi, R., and Villaverde, A.:** Fine architecture of bacterial inclusion bodies. FEBS Lett., **471**, 7–11 (2000).
21. **Valax, P. and Georgiou, G.:** Molecular characterization of β-lactamase inclusion bodies produced in *Escherichia coli*. 1. Composition. Biotechnol. Prog., **9**, 539–547 (1993).
22. **Rinas, U. and Baily, J. E.:** Protein composition analysis of inclusion bodies produced in recombinant *E. coli*. Appl. Environ. Microbiol., **59**, 561–566 (1992).
23. **Carrio, M. M. and Villaverde, A.:** Protein aggregation as bacterial inclusion bodies is reversible. FEBS Lett., **489**, 29–33 (2001).
24. **Carrio, M. M. and Villaverde, A.:** Construction and deconstruction of bacterial inclusion bodies. J. Biotechnol., **96**, 3–12 (2002).
25. **Villaverde, A. and Carrio, M. M.:** Protein aggregation in recombinant bacteria: biological role of inclusion bodies. Biotechnol. Lett., **25**, 1385–1395 (2003).
26. **Betts, S. and King, J.:** There's a right way and a wrong way: *in vivo* and *in vitro* folding, misfolding and subunit assembly of the P22 tailspike. Struct. Fold. Des., **7**, R131–R139 (1999).
27. **Kreisberg, J. F., Betts, S. D., Haase-Pettingell, C., and King, J.:** The interdigitated beta-helix domain of the P22 tailspike protein acts as a molecular clamp in trimer stabilization. Protein Sci., **11**, 820–830 (2002).
28. **Speed, M. A., Wang, D. I., and King, J.:** Specific aggregation of partially folded polypeptide chains: the molecular basis of inclusion body composition. Nat. Biotechnol., **14**, 1283–1287 (1996).
29. **Fink, A. L.:** Protein aggregation: folding aggregates, inclusion bodies and amyloid. Fold. Des., **3**, R9–R23 (1998).
30. **Rajan, R. S., Illing, M. E., Bence, N. F., and Kopito, R. R.:** Specificity in intracellular protein aggregation and inclusion body formation. Proc. Natl. Acad. Sci. USA, **98**, 13060–13065 (2001).
31. **Przybycien, T. M., Dunn, J. P., Valax, P., and Georgiou, G.:** Secondary structure characterization of β-lactamase inclusion bodies. Protein Eng., **7**, 131–136 (1994).
32. **Oberg, K., Chrunyk, B. A., Wetzel, R., and Fink, A. L.:** Native-like secondary structure in interleukin-1 β inclusion bodies by attenuated total reflectance FTIR. Biochemistry, **33**, 2628–2634 (1994).
33. **Umetsu, M., Tsumoto, K., Ashish, K., Nitta, S., Tanaka, Y., Adschiri, T., and Kumagai, I.:** Structural characteristics and refolding of *in vivo* aggregated hyperthermophilllic archaeon proteins. FEBS Lett., **557**, 49–56 (2004).
34. **Khan, R. H., AppaRao, K. B. C, Eshwari, A. N. S., Totey, S. M., and Panda, A. K.:** Solubilization of recombinant ovine growth hormone with retention of native-like secondary struc-

ture and its refolding from the inclusion bodies of *Escherichia coli*. Biotechnol. Prog., **14**, 722–728 (1998).
35. **Maachupalli-Reddy, J., Kelly, B. D., and De Bernardez Clark, E.:** Effect of inclusion body contaminants on the oxidative renaturation of hen egg white lysozyme. Biotechnol. Prog., **13**, 144–150 (1997).
36. **Stockel, J., Doring, K., Malotka, J., Jahnig, F., and Dornmair, K.:** Pathway of detergent-mediated and peptide ligand-mediated refolding of heterodimeric class II major histocompatibility complex (MHC) molecules. Eur. J. Biochem., **248**, 684–691 (1997).
37. **Cardamone, M., Puri, N. K., and Brandon, M. R.:** Comparing the refolding and reoxidation of recombinant porcine growth hormone from a urea denatured state and from *Escherichia coli* inclusion bodies. Biochemistry, **34**, 5773–5794 (1995).
38. **Burgess, R. R.:** Purification of overproduced *Escherichia coli* RNA polymerase sigma factors by solubilizing inclusion bodies and refolding from sarkosyl. Methods Enzymol., **273**, 145–149 (1996).
39. **Misawa, S. and Kumagai, I.:** Refolding of therapeutic protein produced in *Escherichia coli* as inclusion bodies. Biopolymers, **51**, 297–307 (1999).
40. **Mayer, M. and Buchner, J.:** Refolding of inclusion body proteins. Methods Mol. Med., **94**, 239–254 (2004).
41. **Tsumoto, K., Ejima, D., Kumagai, I., and Arakawa, T.:** Practical considerations in refolding proteins from inclusion bodies. Protein Expr. Purif., **28**, 1–8 (2003).
42. **De Bernardez Clark, E., Schwarz, E., and Rudolph, R.:** Inhibition of aggregation side reactions during *in vitro* protein folding. Methods Enzymol., **309**, 217–236 (1999).
43. **Yasuda, M., Murakami, Y., Sowa, A., Ogino, H., and Ishikawa, H.:** Effect of additives on refolding of a denatured protein. Biotechnol. Prog., **14**, 601–606 (1998).
44. **Arora, D. and Khanna, N. J.:** Method of increasing the yield of properly folded recombinant human gamma interferon from inclusion bodies. J. Biotechnol., **52**, 127–133 (1996).
45. **Arakawa, T. and Tsumoto, K.:** The effect of arginine on refolding of aggregated proteins: not facilitate refolding, but suppress aggregation. Biochem. Biophys. Res. Commun., **304**, 148–152 (2003).
46. **Umetsu, M., Tsumoto, K., Hara, M., Ashish, K., Goda, S., Adschiri, T., and Kumagai, I.:** How additives influence the refolding of immunoglobulin-folded proteins in a stepwise dialysis system. Spectroscopic evidence for highly efficient refolding of a single-chain Fv fragment. J. Biol. Chem., **278**, 8979–8987 (2003).
47. **Tsumoto, K., Umetsu, M., Kumagai, I., Ejima, D., Philo, J. S., and Arakawa, T.:** Role of arginine in protein refolding, solubilization, and purification. Biotechnol. Prog., **20**, 1301–1308 (2004).
48. **Middelberg, A. P. J.:** Preparative protein folding. Trends Biotechnol., **20**, 433–437 (2004).
49. **Mark Buswell, A., Ebtinger, M., Vertes, A. A., and Middelberg, A. P.:** Effect of operating variables on the yield of recombinant trypsinogen for a pulse-fed dilution-refolding reactor. Biotechnol. Bioeng., **77**, 435–444 (2002).
50. **Batas, B. and Chaudhuri, J. B.:** Protein folding at high concentration using size exclusion chromatography. Biotechnol. Bioeng., **50**, 16–23 (1996).
51. **Li, M., Su, Z., and Janson, J. C.:** *In vitro* refolding by chromatographic procedures. Protein Expr. Purif., **33**, 1–10 (2004).
52. **Katoh, S. and Katoh, Y.:** Continuous refolding of lysozyme with fed-batch addition of denatured protein solution. Process Biochem., **35**, 1119–1124 (2000).
53. **Valejo, L. F. and Rinas, U.:** Optimized procedures for renaturation of recombinant human bone morphogenic protein-2 at high protein concentration. Biotechnol. Bioeng., **85**, 601–609 (2004).
54. **Fahey, E. M., Chaudhuri, J. B., and Binding, P.:** Refolding and purification of urokinase plasminogen activator fragment by chromatography. J. Chromatogr. B, **37**, 225–235 (2000).
55. **Gu, Z., Su, Z., and Janson, J. C.:** Urea gradient size exclusion chromatography enhance the yield of lysozyme refolding. J. Chromatogr. A, **918**, 311–318 (2001).
56. **Schlegl, R., Iberer, G., Machold C., Necina, R., and Jungbauer, A.:** Continuous matrix assisted refolding of proteins. J. Chromatogr. A, **1009**, 119–132 (2003).
57. **Li, M. and Su, Z.:** Refolding of superoxide dismutase by ion exchange chromatography. Biotechnol. Lett., **24**, 919–923 (2002).
58. **Yashimoto, M. and Kubio, R.:** Oxidative refolding of denatured/reduced lysozyme utilizing the chaperone-like function of liposomes and immobilized liposome chromatography. Biotechnol. Prog., **15**, 480–487 (1999).
59. **Altamirano, M. M., Golbik, R., Zahn, R., Buckle, A. M., and Fersht, A. R.:** Refolding chromatography with immobilized mini-chaperones. Proc. Natl. Acad. Sci. USA, **94**, 3576–3578 (1997).
60. **Gao, Y-G., Guan, Y-X., Yao, S-J., and Cho, M-G.:** On-column refolding of recombinant human interferon-γ with an immobilized chaperone fragment. Biotechnol. Prog., **19**, 915–920 (2003).
61. **Patra, A. K., Mukhopadhyay, R., Mukhija, R., Krishnan, A., Garg, L. C., and Panda, A. K.:** Optimization of inclusion body solubilization and renaturation of recombinant human growth hormone from *Escherichia coli*. Protein Expr. Purif., **18**, 182–192 (2000).
62. **Puri, N. K., Crivelli, E., Cardamone, M., Fiddes, R., Bertolini, J., Ninham, B., and Brandon, M. R.:** Solubilization of growth hormone and other recombinant protein from *E. coli* inclusion bodies using cationic surfactant. Biochem. J., **285**, 871–879 (1992).
63. **Tsumoto, K., Umetsu, M., Kumagai, I., Ejima, D., and Arakawa, T.:** Solubilization of active green fluorescent protein from insoluble particles by guandine and arginine. Biochem. Biophys. Res. Commun., **312**, 1383–1386 (2003).
64. **Patra, A. K., Gahlay, G. K., Reddy, B. V. V., Gupta, S. K., and Panda, A. K.:** Refolding and structural transition of recombinant bonnet monkey zona pellucida glycoprotein-C expressed in *Escherichia coli* that binds to spermatozoa. Eur. J. Biochem., **267**, 7075–7081 (2000).
65. **Raina, K., Panda, A. K., Ali, M. M., and Talwar, G. P.:** Purification, refolding and characterization of recombinant LHRH-T multimer. Protein Expr. Purif., **37**, 8–17 (2004).
66. **Isaksson, O. G., Eden, S., and Jansson, J. O.:** Mode of action of pituitary growth hormone on target cells. Annu. Rev. Physiol., **47**, 483–499 (1985).