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Review

Co-solute assistance in refolding of recombinant proteins

Susan Mir Najd Gerami[2], Safar Farajnia[1,2]* and Feridoun Mahboudi[3]

[1]Biotechnology Research Center, Tabriz University of Medical Sciences, Tabriz, Iran.
[2]Infectious and Tropical Disease Research Center, Tabriz University of Medical Sciences, Tabriz, Iran.
[3]Biotechnology Research Center, Pasteur Institute of Iran, Tehran, Iran.

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Prokaryotic expression system is the most widely used host for the production of recombinant proteins but inclusion body formation is a major bottleneck in the production of recombinant proteins in prokaryotic cells, especially in *Escherichia coli*. *In vitro* refolding of inclusion body into the the proteins with native conformations is a solution for this problem but there is a need for optimization of condition for each protein specifically. Several approaches have been described for *in vitro* refolding; most of them involve the use of additives for assisting correct refolding. Co-solutes play a major role in refolding process and can be classified according to their function as, aggregation suppressors and folding enhancers. This study presents a review of additives that are used in refolding process of insoluble recombinant proteins in small scale and industrial process.

Key words: Refolding, protein aggregation, low-molecular-weight additives, arginine.

INTRODUCTION

Recombinant expression of proteins is an essential method for producing target proteins and one of the most important unit processes for the production of therapeutic protein and structural study (Clark, 2001; Middelberg, 2002; Tsumoto et al., 2003; Salehi et al., 2010). There are different expression systems for producing recombinant proteins in biotechnology industry. Expression in mammalian cells produces active recombinant proteins that have post translation modification but expression by this system is time consuming and expensive. *E. coli* bacteria still represent a convenient expression system that is used for the production of many recombinant polypeptides (Montazam et al., 2009). But recombinant result in their accumulation as insoluble aggregates, proteins expressed in prokaryotic hosts undergo misfolding or incomplete folding processes that usually which are called inclusion bodies (Kafshnochi et al., 2010). *In vitro* refolding is required for obtaining active protein from inclusion body aggregates which consist of four main steps (Figure 1): Inclusion body isolation, solubilization, refolding and purification. Because of high density of IBs, they are separated easily by centrifuge. After isolation of the IBs, the proteins contained in the inclusion bodies need to be solubilized, usually by high concentration of denaturing agents such as urea or guanidine HCl (Gn-HCl). Then, solubilized IBs are subjected to refolding in specified conditions. Refolding of proteins from inclusion bodies is influenced by different factors including solubilization method, removal of the denaturant, and assistance of refolding by co-solute or additives. The refolding condition is critical in order to obtain acceptable amounts of active protein. It has been known that additives, especially low molecular weight compounds may significantly enhance the yield of the refolding process. In many cases, inclusion body protein can only be successfully refolded by making use of these

*Corresponding author. E-mail: farajnias@tbzmed.ac.ir.

Abbreviations: rPA, Recombinant plasminogen activator; **IB,** inclusion body; **Gn-HCl,** guanidine hydrochloride; **2-ME,** 2-mercaptoethanol; **PMSF,** phenylmethylsulfonyl fluoride; **BSA,** bovine serum albumin; **DTT,** dithiothreitol; **SDS-PAGE,** sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

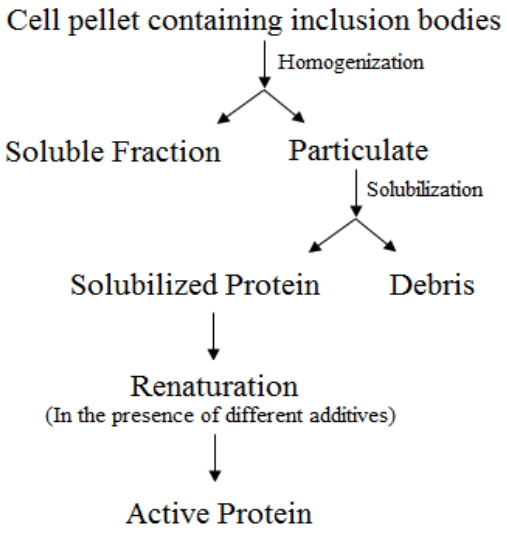

Figure 1. *In vitro* refolding steps of inclusion bodies.

effects.

FOLDING ADDITIVES

Low molecular weight additives are usually added for refolding buffer to promote the refolding process. In the case of refolding by dilution, there is a residual concentration of guanidine HCl or urea in refolding buffer. This low concentration of denaturants leads to the sustaining folding intermediates in soluble and flexible state, thus refolding yield efficiency improves. In addition to residual concentration of denaturants, additives should be applied for refolding buffer to decrease the degree of aggregates and misfolds. Co-solute may be classified into two groups: folding enhancer and aggregation suppressor. In principle, folding enhancer enhances protein-protein interaction, while aggregation suppressor reduces side chain interactions (Tsumoto et al., 2003; Wang and Engel, 2009).

FOLDING ENHANCER

The folding enhancers faciliate the interaction between proteins and thus increase the stability of proteins, whereas aggregation suppressor reduces side chain interaction of folding intermediates without interfering with refolding process (Tsumoto et al., 2003). Common additives used in refolding of recombinant proteins are shown in Table 1.

DENATURANTS

Urea, guanidine HCl, and strong ionic detergents are solubilizing agents that are most frequently used for initial solubilization of inclusion bodies. High concentration of denaturants unfolds almost all kind of proteins. Among denaturants, ionic detergents are the strongest denaturants in dispersing IBs into monomolecular structure due to strong electrostatic repulsion of detergent/protein complexes (Tsumoto et al., 2003), but removal of detergents from proteins is problematic in most cases. Therefore, high concentration of chaotropic denaturants such as urea and guanidine HCl is still the most used denaturants in solubilization processes.The proteins that are difficult to refold have also been successfully refolded in the presence of non-denaturing concentrations of denaturant (Jaenicke and Rudolph, 1989; Hevehan and Clark, 1997).

ARGININE (ARG)

Arginine is one of the most useful reagents that are effective in assisting refolding of recombinant proteins from inclusion bodies. It is an aggregation suppressor that improves refolding yield; however, the mechanism of arginine action during refolding is still unclear. Arginine was first used in refolding of human tissue type plasminogen activator (Buchner and Rudolph, 1991; Rudolph and Fischer, 1990). Since then, it has been used for refolding of a variety of proteins including casein kinase II, (Lin and Traugh, 1993), Fab antibody fragments (Buchner et al., 1992; Buchner and Rudolph, 1991), growth hormone (Hsih et al., 1997), human gamma interferon (Arora and Khanna, 1996), human matrix metalloproteinase-7human neurotrophins (Rattenholl et al., 2001) human p53 tumor suppressor protein (Bell et al., 2002), interleukin-21 (Asano et al., 2002), interleukin-

Table 1. Common refolding enhancers and aggregation supressors.

Parameter	Additive	Effect on protein structure	Effect on intra and inter molecular interaction
Denaturants	Urea Guanidine-HCL Strong detergent	Destabilized	Disrupted
Aggregation suppressors	Arginine Cyclodextrine Polyethylene glycol Proline	Neutral	Reduced
Folding enhancers	Amonium sulfate Magnesium chloride Glycine Polyols	Stabilized	Enhanced

6 receptor (Oneda and Inouye, 1999), lysozyme (Havehan and Clark, 1997), single-chain Fv fragments (Tsumoto et al., 1998) and single-chain immunotoxins (Brinkmann et al., 1992; Buchner and Rudolph, 1991). However, arginine may acts as a protein-denaturant, which limits the expansion of its applications. It was shown that the activity and stability of certain enzymes were perturbed by arginine and arginine is a protein-destabilizer (Yancey et al., 1982). Xie et al. (2007) have made a similar observation and showed that fluorescence properties of aminoacylase are perturbed by arginine. They interpreted the observed effects of arginine in terms of denaturing property of the guanidinium group, which makes guanidine hydrochloride (Gn-HCl) a strong denaturant. But arginine differs from Gn-HCl in the mode of interactions with proteins.

CYCLODEXTRINS (CDS)

Cyclodextrins have been reported to suppress aggregate formation during the refolding of recombinant proteins. These cyclic glucose macromolecules have hydrophobic cavities and functional groups that can interact with polypeptide chains by weak forces similar to those involved in protein folding. CDs may interact with proteins via hydrophobic forces, hydrogen bonding and Van der Waals interactions. Charged chemically modified CDs can also bind to the folding polypeptide chain via electrostatic interactions. CDs have been investigated in both dilution additive and artificial chaperone assisted modes. CDs have been used as stripping agents for the removal of detergents in proteins refolded with surfactants (Bajorunaite et al., 2009; Loretta and Ajit, 2001). CDs have been used for refolding of recombinant-human growth hormone (Yazdanparast et al., 2007) and α amylase (Yazdanparast et al., 2005).

POLYETHYLENE GLYCOL (PEG)

Polyethylene glycol is one of the water-soluble polymers that are used for refolding of recombinant proteins (Lee and Lee, 1987). PEG, a polyhydric alcohol, like other polyols are known to stabilize protein conformations as well as to increase the rate of *in vitro* protein refolding (Sato et al., 1996; Sawanoa et al., 1992).

It enhances the recovery of active protein by preventing aggregation during refolding (Cleland and Wang, 1990). PEG improves *in vitro* structure formation, which can block aggregation by combining with a molten globule folding intermediate. It stabilizes proteins by chemical modification (Harris and Kozlowski, 2001; Roberts et al., 2002). PEG binds an intermediate in the folding pathway of carbonic anhydrase, therefore prevent self-association and promote correct refolding (Cleland and Wang, 1990; Cleland et al., 1992). PEG interacts with the hydrophobic side chains exposed upon unfolding at high temperatures (Zielenkiewicz et al., 2006). PEG has been successfully used for refolding of interferon (Wang et al., 2006), xylanase (Rahimpour et al., 2007) and insulin like growth factor (IGF-1) (Hart et al., 1994) from inclusion bodies.

AMMONIUM SULFATE ($(NH_4)_2SO_4$)

Salts can stabilize proteins through nonspecific electrostatic interactions at low concentration depending on the ionic strength of the medium (Tanford, 1961). At high concentrations, salts exert specific effects on proteins depending on the type and concentration of the salts, resulting in either the stabilization or destabilization of proteins, or even denaturation. Salts usually increase interfacial tension between the protein surface and solvent (Jarvis and Scheiman, 1968) that leads to incease in the solubility of proteins and aggregates.

Although, the stabilizing effect of salts on protein structure is closely correlated to the salting-out effect of salts.

Ammonium sulfate is widely used as a precipitant for protein purification and for crystallization as this salt can prevent aggregation. Ammonium salts have been reported to prevent heat-induced aggregation of lysozyme (Hirano et al., 2007). Also magnesium chloride is another example of salts that has been used in the refolding of recombinant proteins (Richter et al., 2002).

PROLINE (PRO)

The role of proline was studied for the first time in *in vitro* refolding of bovine carbonic anhydrase. Proline inhibited bovine carbonic anhydrase aggregation and enabled the protein to return to its native structure. It was proposed that proline acts as a protein-folding chaperone due to the formation of an ordered, amphipathic supramolecular assembly (Samuel et al., 2000). Later, proline was found to prevent aggregation during hen egg-white lysozyme refolding. Also, proline has been used during refolding of arginine kinase (Xia et al., 2007) and aminoacylase (Kim et al., 2006). Proline inhibits protein aggregation by binding to folding intermediates. Proline (1M) was also found to act as a creatin kinase (CK) folding aid (Meng et al., 2001). Results further indicate that proline possibly binds to and stabilizes folding intermediates and reduces the hydrophobic surface of CK, thus inhibiting protein aggregation and improving the final yield of CK. The unusual properties of proline may be due to hydrophobic stacking in aqueous solution and inter-molecular self-association (Rudolph and Crowe, 1985). The hydrophobic nature of proline covers hydrophobic region of the proteins, through which this amino acid effectively suppresses protein aggregation (Schobert and Tschesche, 1978).

POLYOLS

Polyols vary in the length of carbon chain and number of hydroxyl groups. Polyols are used as additives for enhancing refolding yield and stabilizing protein structure. The structure-stabilizing polyols are supposed to lead to compact folded state. It was demonstrated that the addition of certain osmolytes results in the compaction of RNase A (Bolen and Baskakov, 2001). Polyols have been shown to stabilize the acid-unfolded molten globule state of cytochrome *c* by enhancing hydrophobic interactions that overcome electrostatic repulsion at low pH between charged residues (Kamiyama et al., 1999).

Glycerol has long been known as a protein stabilizer, and its stabilization mechanism has been shown by Timasheff (2002). Glycerol leads to the enhancement of hydrophobic interactions as a result of an increase in the solvent ordering around proteins. Compared with the other polyols, glycerol is a mild stabilizer of protein conformation. Unlike other polyols, glycerol has unusual properties that decreases the surface tension of water but increases its viscosity (Gekko and Timasheff, 1981). Increasing glycerol concentrations is known to increase the stability of proteins at all concentrations (Jain and Ahluwalia, 1997; Timasheff, 2002) although, it decreases refolding at high concentrations due to its ability to considerably slow down the kinetics of refolding, which could result in off-pathway aggregation. An optimum glycerol concentration seems to be critical for striking a balance between its stabilizing effect governed by thermodynamic principles and its effect on the kinetics of folding. Glycerol is successfully used in refolding of lysozyme (Cao et al., 2002).

Sorbitol is another polyol that has been shown to reduce aggregation of nucleocapsid protein of rhabdovirus after its expression in *Escherichia coli* (Majumder et al., 2001). Sorbitol is likely to exert its effect on folding by altering the structure and properties of water around the folding protein molecule. Erythritol is also a protein structure stabilizer and studies showed that it stabilizes proteins to a lesser extents when compared with sorbitol (Gekko and Morikawa, 1981). The study on citrate synthase aggregation kinetics during refolding in erythritol and other polyols showed the ability of the polyols to stabilize proteins depending on the number of hydroxyl groups in them (Mishra et al., 2005).

CONCLUSION

Obtaining a good amount of active protein at low cost is the main objective in the *in vitro* refolding of recombinant proteins from bacterial inclusion bodies, but there is no single refolding technique or method that satisfies all protein refolding processes. With providing a good condition for *in vitro* refolding, bacterial system could be an excellent alternative to the mammalian expression system or other expression systems that can directly generate active proteins with a complex disulfide bond structures. In this process, the solubilized target protein is subjected to refolding using different methods. Low molecular weight additives are helpful agents for increasing protein concentration, especially in dilution method. Therefore, application and optimization of different additives and their concentration is a main step in protein refolding but should be performed for each protein individually.

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