Unfolding the potential of proteins

New refolding technologies are essential for making useful recombinant proteins.

BY JOBY MARIE CHESNICK

The development of novel enzymes and proteins relies upon an understanding of their intrinsic structures. A protein's three-dimensional structure, with exposed reactive groups as well as hidden hydrophobic residues, defines its biological activity and can also confer properties such as protein trafficking on cellular organelles. It is well documented that several human diseases—including Alzheimer's disease, Parkinson's disease, and cystic fibrosis—have their origins in misfolded cellular proteins.

In vivo, small polypeptides often will fold spontaneously into their correct configuration. However, longer polypeptides have a greater likelihood of folding more slowly, with the potential to form partially folded intermediate structures and aggregates that are not functional. The timing of polypeptide folding—for example, whether it is concomitant with ribosomal synthesis or delayed until transport into the cytoplasm or to cellular organelles—can also influence the success rate of correct protein folding.

One of the largest problems encountered by scientists attempting to express recombinant proteins in bacteria is the formation of inclusion bodies, insoluble aggregates of misfolded polypeptides produced as bacteria quickly synthesize large quantities of the foreign protein. These misfolded protein aggregates must be unfolded and refolded in their correct three-dimensional structure before study or production can proceed. As research efforts continue to shift from the investigation of gene structure to the study of protein structure and function, the importance of studying recombinant proteins will only increase, and new methods for producing accurately folded structures will be essential.

Molecular chaperones

More than 40 years ago, Ferruccio M. Ritossa from the International Laboratory of Genetics and Biophysics in Naples, Italy, first discovered the heat-shock response of certain proteins through observation of a new puffing pattern in *Drosophila buschii* salivary gland polytene chromosomes (*1*). This puffing was indicative of increased gene expression of these proteins, which were later termed heat-shock proteins (HSPs). HSPs, and related proteins that have since been identified (e.g., DnaK, Hsp40, and GrpE; GroEL/S; trigger factor; prefoldin; CCT; SecB; and ClpA), are called "molecular chaperones" because they help direct the movements of polypeptides.

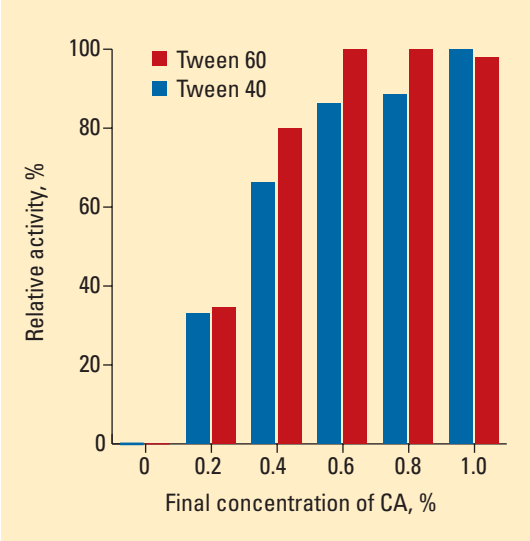

Figure 1. Comparison of citrate synthase activity following protein refolding using cycloamylose and Tween 40 or Tween 60. One-hundred percent activity is achieved for refolding of citrate synthase using as little as 0.6% cycloamylose with Tween 60. (Data courtesy of Takara Bio.)

One of the most important functions of chaperones is in aiding the protein-folding process. Because molecular chaperones coevolved with polypeptides—highly conserved sequence data for chaperones and representation of these molecules in every major taxon, including eukaryotes, eubacteria, archaea, and viruses, suggest that chaperones are ancient molecules—they provide for a controlled cellular mechanism by which proteins can reach their most thermodynamically stable three-dimensional conformation.

Chaperones accomplish controlled protein folding either by directly binding to conserved domains in a nascent polypeptide and preventing interactions with other adjacent protein domains or by providing, through their own three-dimensional structure, a space that allows controlled polypeptide folding (*2*). Folding typically occurs via several binding and release events with the polypeptide, which are mediated by the hydrolysis of ATP. But even with all of the chaperone machinery present in a cell, 30% or more of all synthesized polypeptides cannot fold correctly to form functional proteins (*3*).

Nonetheless, when there is the need to refold proteins to their correct form, molecular chaperones are essential tools.

Refolding strategies

The most common strategy currently used to recover active recombinant protein in vitro from isolated inclusion bodies consists of a three-step process: isolation and washing of the inclusion bodies, solubilization (i.e., denaturation and unfolding) of the protein aggregates, and correct refolding of the solubilized protein. The first two of these steps typically can be performed with high efficiency. However, misfolding and aggregation of the solubilized protein may complicate the last step.

A number of commercial kits are available for improving the refolding conditions of inclusion bodies, including Fold-It, developed by Hampton Research; Pro-Matrix Protein Refolding Kit from Pierce; and Novagen's Protein Refolding Kit. Their components include a denaturant to solubilize the inclusion bodies, one or more

small-molecule detergents that maintain an unfolded protein configuration and allow refolding through transient interactions with the protein, and various buffers that differ in parameters that support the refolding process, such as pH, redox concentration, and ionic strength. The degree of successful refolding obtained by the "dilution additive" strategy of these kits depends on the buffer properties, as well as protein concentration and temperature.

A new kit from Takara Bio, the Refolding CA Kit, offers a novel approach to the dilution additive method. By using an artificial chaperone—highly polymerized cycloamylose (CA)—the kit provides high efficiency and correct refolding of aggregated proteins.

CA aids refolding through its structure, a single helical V-amylose conformation containing an anhydrophilic channel-like cavity (*4*). Inclusion complexes with other molecules, such as detergents, can be formed in this space, preventing protein aggregation. In contrast to the conventional dilution additive method, CA supports stable interactions between the protein and detergent, and then strips the detergent from the protein–detergent complex to initiate refolding (*5*). This scenario results in greater refolding efficiency.

CA can be used in conjunction with many different types of detergents, and it is compatible with redox reagents. In addition, CA can interact with peptides of multiple sizes, it is highly soluble and has a long shelf life in aqueous solution, and it accomplishes protein refolding in a short time (i.e., a few hours to overnight). Figure 1 shows the results of citrate synthase activity following refolding using CA. In this application, full enzyme activity was obtained using 0.6% CA with Tween 60 detergent (polyoxyethylene sorbitan monostearate) or 1% CA with Tween 40 (polyoxyethylene sorbitan monopalmitate).

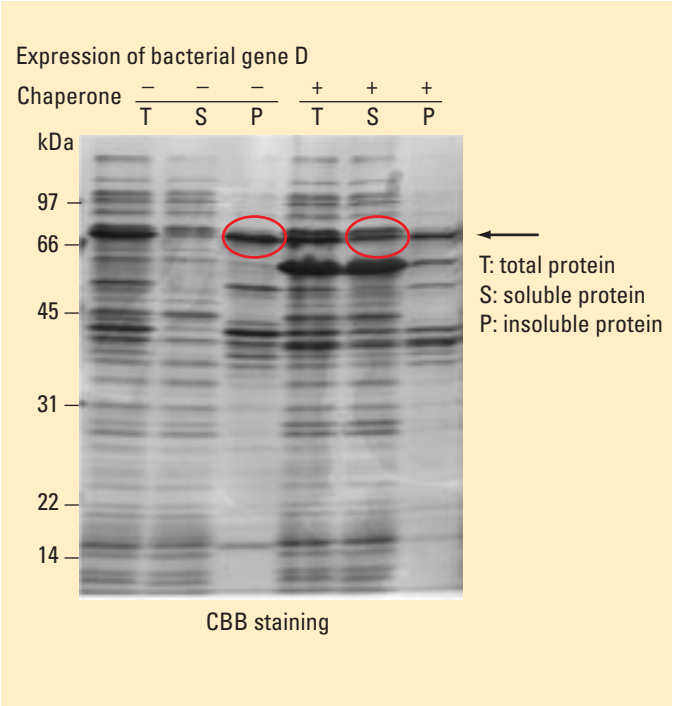

Figure 2. Increase in soluble protein with coexpression of chaperone genes from Takara's chaperone plasmid vectors with a bacterial gene. (Data courtesy of Takara Bio.)

A new alternative in vivo method for obtaining increased soluble expressed protein recruits the use of chaperone-containing plasmid vectors. In this case, a "team" of chaperone genes, such as *DnaK-DnaJ-GrpE* or others, is engineered into a plasmid vector and placed under control of either an *araB* or *Pzt-1* promoter, with induction by L-arabinose or tetracycline. Chaperones are then coexpressed with the target protein of interest in *E. coli*, and separate expression of chaperones and target proteins can be accomplished if the target gene is placed under the control of a different promoter (e.g., *lac*).

The Chaperone Plasmid Set, developed by HSP Research Institute and introduced into the market by Takara Bio in 2002, contains five chaperone-team-containing plasmids for increasing yields of soluble foreign proteins in vivo. These plasmids carry a pACYC origin of replication plus a chloramphenicol resistance gene, which allows their use with ColE1-type plasmids containing an ampicillin resistance gene, and provides for selection of clones containing both a chaperone plasmid and a plasmid containing a target gene of interest. Figure 2 illustrates increased amounts of soluble target protein (bacterial protein D), with a concomitant decrease in insoluble protein, obtained by coexpression of chaperones from Takara's pG-KJE8 chaperone plasmid vector.

For obtaining high yields of recombinant proteins, the latest technology utilizes the cold-shock protein A (cspA) gene promoter system for gene expression. This system induces protein expression at low temperatures (15 °C), which suppresses the synthesis of most other proteins—up to 60% of all of the expressed cellular protein is the desired target—and lowers potentially destructive cellular protease activity. Additionally, high-efficiency metabolic labeling of the protein is possible for structural analysis. Large-scale culture and purification of the proteins using affinity chromatography (up to 400 mg of protein per liter of culture) is achievable, making cold-shock protein expression suitable for commercial applications.

Takara Bio offers custom protein production using cold-shock technology, and later this year the company will introduce its line of pCold Vectors allowing insertion of a foreign target gene into a vector for expression using the cspA promoter. In the near future, the combination of chaperone plasmid and cold-shock vector technologies should provide a system by which high-specificity–high-yield production of soluble expressed foreign proteins is routinely possible at affordable costs.

The availability of new and modified proteins and enzymes for medical and biological research will depend to a large extent on the development of quick, reliable, and cost-effective refolding methods.

References

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