Screening for Bacterial Clones Expressing Inclusion Body Proteins

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INTRODUCTION

High-level expression of recombinant proteins in *E. coli* can result in the formation of insoluble aggregates called *inclusion bodies*. The protein of interest found in these sub-cellular structures is often inactive, due to incorrect folding. The production rate of recombinant proteins stored in inclusion bodies is invariably higher than those synthesized as soluble proteins. The reason behind this is thought to be the resistance of insoluble proteins to proteolysis by cellular enzymes. In addition, separation of insoluble recombinant proteins in inclusion bodies is considerably easier than that of soluble proteins. These factors have been the major influences favoring scale-up of high-value proteins using bacterial fermentation.

Despite the unique advantages of producing proteins through bacterial inclusion bodies, there is currently no generalized method to ensure that proteins are expressed as insoluble aggregates. Studies have shown that gene origin, promoter usage, hydrophobicity index, and even protein transport signaling encoded in peptide sequences have little or no relationship with the production of a particular protein in either form. Therefore, the development of strategies for protein production in bacterial inclusion bodies often involves the consideration of several designs. Each design would then be evaluated for inclusion body production, a process that is often laborious.

Traditional methods for preparing recombinant proteins from inclusion bodies can be tedious and time-consuming. These methods require numerous solutions and lengthy centrifugation times. Although a few commercial methods have appeared recently, none are available in a kit format that allows rapid purification directly from cells and prepares the protein for analysis (e .g., SDS-PAGE).

In this study, the ProteoSpin™ Inclusion Body Isolation Micro Kit (P/N 10300) was evaluated for its capability to purify soluble recombinant proteins from bacteria using the protocols for acidic and basic proteins. The Inclusion Body Isolation Micro Kit is designed for rapid screening of clones that express a recombinant gene. Therefore, multiple colonies were individually grown in small cultures and processed accordingly to investigate their production of recombinant proteins in inclusion bodies.

The screening process consisted of three major steps, and can be accomplished in one hour:

1. Lysing the cells to free their insoluble inclusion bodies and capturing these protein aggregates by centrifugation.
2. Solubilizing the inclusion bodies to release the recombinant protein in soluble form.
3. Purifying the protein by chromatography using ProteoSpin™ spin columns.

All materials required to carry out each of the steps were conveniently provided in a single kit for the complete analysis of multiple bacterial clones. Purified proteins were analyzed directly by SDS-PAGE to evaluate the size of the protein, and to assess qualitatively the relative level of expression of each clone.

METHODS AND MATERIALS

The materials and methods used to evaluate the ProteoSpin™ Inclusion Body Isolation Micro Kit have been separated into discrete phases:

- Gene Cloning and Expression
- Isolation of Inclusion Bodies
- Isolation of Inclusion Body Proteins
- SDS-PAGE Analysis of Inclusion Body Proteins

Gene Cloning and Expression

Genes encoding recombinant proteins of sizes 25 kDa, 30 kDa, and 40 kDa (see properties in Table 1) were individually cloned in a vector for plasmid-directed expression in *E. coli*. The recombinant proteins used here are expressed from chimeric genes constructed from gene segments of various sources. Recombinant plasmids were isolated and transformed into competent *E. coli* hosts suitable for recombinant protein production. Colonies were picked using sterile wooden sticks and inoculated in test tubes with 2 mL of Luria broth that contained antibiotics for plasmid maintenance. The culture tubes were incubated in a 37°C waterbath shaker for 16 hours, after which 50 µL of

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culture was used to inoculate 2 mL of fresh media. These were then incubated for two hours. At this point, 20 µL of 100 mM IPTG (inducer) was added to each 2 mL culture. The tubes were incubated for another three hours before being harvested for analysis.

Table 1: Protein Masses and Theoretical Isoelectric Points (p*I*) of Recombinant Proteins

Protein	Theoretical p*I**	ProteoSpin™
25 kDa	7.54	Acidic
30 kDa	7.96	Basic
40 kDa	5.56	Acidic
* Theoretical p*I* determined from ExPASy Molecular Biology Server at www.expasy.org		

Isolation of Inclusion Bodies

To process cells for inclusion bodies, 1.5 mL of culture was removed from each test tube and the cells were collected by centrifugation in microcentrifuge tubes as specified in the Application Manual. The cell pellet was briefly frozen at -70°C and then thawed at room temperature. The cell pellet was then subjected to multiple rounds of lysis and centrifugation to obtain a pellet of inclusion bodies. To each tube containing an inclusion body pellet, 50 µL of the solubilization reagent was added. Pellets were dissolved by vigorous vortexing, then 50 µL of sterile deionized water was added to bring the total volume to 100 µL.

Isolation of Inclusion Body Proteins

From the preparation containing dissolved inclusion bodies, 50 µL was taken and mixed with 200 µL of sterile deionized water in a fresh tube. The pH of each sample was adjusted accordingly and the tubes were mixed by vortexing. The p*I* of the 30 kDa protein was nearly 8.0,

therefore the basic protocol was followed for this protein. The acidic protocol was then used for the 25 kDa and 40 kDa proteins. The procedures for binding proteins and washing columns were followed. The resulting bound proteins were eluted twice in 25 µL volumes from each column and collected in tubes containing the recommended amount of neutralizer.

SDS-PAGE Analysis of Inclusion Body Proteins

The eluted protein samples were analyzed in 12.5% polyacrylamide gels, which were run for 45 minutes at 200 V/6.5 cm. The protein bands were made visible by staining with Coomassie Blue R-250.

RESULTS AND DISCUSSION

The capability of the ProteoSpin™ Inclusion Body Isolation Micro Kit to obtain acidic and basic recombinant proteins sequestered in inclusion bodies was assessed using bacterial hosts that express either the acidic 40 kDa recombinant protein or the basic 30 kDa protein. These bacterial hosts were grown in 2 mL cultures. After the proper incubation time and following induction of protein synthesis, the recombinant proteins were purified according to the suggested protocol.

Figure 1 shows the recombinant proteins that were purified from bacterial clones expressing either the acidic or basic protein products. The Inclusion Body Isolation Micro Kit's capability to isolate inclusion bodies of high-quality is demonstrated on lanes marked "A," which contain solubilized inclusion bodies. On each "A" lane, a single predominant protein band is found to match the size of the basic protein (30 kDa) and that of the acidic protein (40 kDa). The presence of a single predominant band indicates that the desired proteins are intact. There are only a few other bands that can be identified on these lanes, suggesting that the amount of contaminating cellular proteins has been minimized.

The ProteoSpin™ Inclusion Body Isolation Micro Kit is also capable of further purifying the recombinant protein by spin column chromatography. This is demonstrated in Figure 1 on lanes marked "B". These lanes show a high degree of purification for the 30 kDa and 40 kDa proteins, which were column purified using the basic and acidic protocols, respectively. Their identification as acidic or basic was based only on theoretical calculations using their amino acid sequence, and these predictions were used to decide which protocol was appropriate for either one. It is evident from Figure 1 that the column purification of the proteins was successfully matched with the proper protocol; acidic for the 40 kDa protein, and basic for the 30 kDa.

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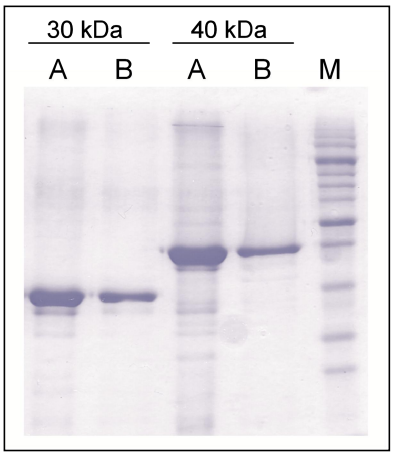

Figure 1: SDS-PAGE analysis of 30 kDa and 40 kDa proteins isolated from 1.5 mL of induced cultures. Five microliters of dissolved inclusion bodies (A) were loaded next to 5 µL from total elution (B). Lane M is the ProteoLadder 150 protein marker.

Next, the Inclusion Body Isolation Micro Kit's screening capability for bacterial clones expressing recombinant proteins in inclusion bodies was evaluated. An expression vector for plasmid-directed protein synthesis in bacteria was used to transform a bacterial host and allowed to form colonies on agar plates. The expression vector codes for an artificial protein with a predicted size of 25 kDa and a theoretical p*I* of 7.54.

Six colonies growing on agar plates were picked and propagated in 2 mL cultures. After inducing protein production, the cells were harvested and analyzed for production of the 25 kDa inclusion body protein using the ProteoSpin™ protocol for acidic proteins. The complete procedure for purifying the protein directly from freshly harvested cells was accomplished in 60 minutes.

The results of SDS-PAGE analysis (illustrated in Figure 2) shows that individual clones produced the desired recombinant protein based on the predicted size. It is also evident from the gel that there was a qualitative difference in expression levels as shown by varying intensities of the recombinant protein band, perhaps reflecting variability within a cell population. Therefore, as a screening method, the Inclusion Body Isolation Micro Kit was a convenient tool for rapidly processing candidate clones that express inclusion body proteins.

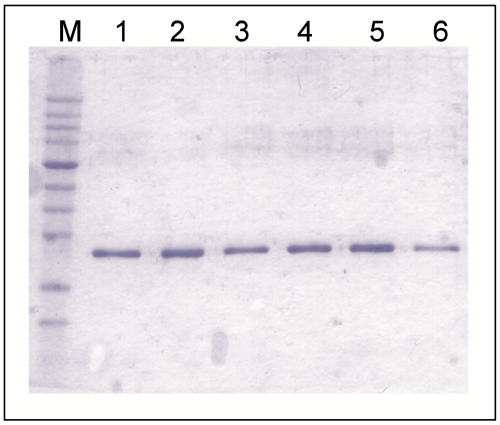

Figure 2: SDS-PAGE analysis of a 25 kDa protein isolated from 1.5 mL of induced cultures. Lanes 1-6 correspond to proteins isolated from candidate colonies originating from single colonies. Lane M contains the ProteoLadder 150 protein marker (P/N 12700).

CONCLUSION

The ProteoSpin™ Inclusion Body Isolation Micro Kit is useful in identifying clones that produce inclusion bodies because its cell lysis procedure allows cellular components to completely dissolve while keeping the inclusion bodies insoluble. The procedure for lysing bacterial cells does not involve sonication or enzymatic digestion of DNA. Upon resuspending the cells, complete cell solubilization is achieved by repeatedly passing the cell suspension through a needle fitted to a piston-type syringe. Centrifugation of the lysate enables the inclusion bodies to separate from dissolved cellular components. The presence of recombinant protein in the non-soluble fraction is a definitive indicator of a bacterial clone's ability to produce the protein in inclusion bodies.

The ProteoSpin™ Inclusion Body Isolation Micro Kit allows for the rapid assessment and identification of bacterial clones that express either acidic or basic proteins. For screening purposes, potential clones can be quickly assessed for expressing recombinant proteins in the form of inclusion bodies.

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