Purification of Bacterial Eukaryotic Fusion Proteins Using the Rotofor® Cell

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Expression vectors are plasmids that have been engineered to express eukaryotic proteins by fusing them in frame to inducible bacterial proteins. Gene fusions have proven to be particularly useful as immunogens and affinity purification reagents for the production of specific antibodies against the products of cloned genes.[1,2] Recently, they have also been used to directly study the functional properties of expressed proteins. For example, DNA binding proteins expressed as fusion proteins are able to bind specific DNA sequences with the same specificities as the native eukaryotic proteins.[3] Often the use of fusion proteins for other immunological or functional studies requires that the protein be both purified of contaminating bacterial proteins and in a native state.[1] In practice these two conditions are difficult to meet because the most efficient purification method, preparative polyacrylamide gel electrophoresis, requires the use of sodium dodecyl sulfate (SDS), an ionic detergent and powerful denaturant. In this bulletin we report a rapid and efficient means of purifying fusion proteins, without subjecting them to ionic detergents, by preparative isoelectric focusing using the Rotofor cell. The Rotofor cell was also useful for separating the full length fusion protein from its partial length breakdown products.

Materials and Methods

FUSION PROTEIN PRODUCTION

Fusion proteins were constructed using the pATH expression vector, in which exogenous coding sequences can be fused to the bacterial TrpE gene.[4] Two portions of the *Notch* gene from *Drosophila melanogastor*, a 0.99 Kb BstY1 fragment encoding epidermal growth factor (EGF)-like repeats in the extracellular domain, and a 2.1 Kb SalI/HindIII fragment encoding almost the entire intracellular domain,[5] were cloned into the appropriate pATH vector. *E. coli* strain HB101 was used as the host.

The TrpE fusions were expressed using a modification of procedures from Carroll and Laughlon[1] for beta-galactosidase fusions using the pUR expression vector, together with standard induction protocols for pATH vectors.[4] Overnight cultures, grown in M9 medium[6] plus 0.01 mg/ml thiamine B1, 5 mg/ml casamino acids, 20 µg/ml tryptophan, and 100 µg/ml ampicillin, were diluted 1:50 in the same medium less tryptophan. Cultures were then allowed to grow 4 hours at 37 °C before inducing fusion protein synthesis with 7.5 µg/ml beta-indoleacrylic acid (from 2 mg/ml stock in ETOH). Cells were lysozyme treated, sonicated, and the fusion protein precipitated with ammonium sulfate [$(NH_4)_2SO_4$] as previously described,[1] with the exception that 0.5 M NaCl was added prior to sonication to help solubilize the protein. Once precipitated, fusion proteins were redissolved in, and dialyzed overnight against, 6 M urea.

FUSION PROTEIN PURIFICATION IN THE ROTOFOR CELL

Rotofor runs were performed using 6 M urea, 2% Bio-Lyte® ampholytes, pH 3–10, and 5% (v/v) glycerol (to stabilize the larger fusion protein) in a total volume of 40 ml. To minimize the time that fusion proteins spent in the Rotofor cell, and to avoid pH extremes, the Rotofor cell was prefocused using 12 W constant power at 8 °C for 1 hour to establish the pH gradient before injecting the protein sample. The initial prefocus conditions were 450 V and 26 mA. At equilibrium the values were 690 V and 17 mA. The 3.0 ml protein sample, which had been dialyzed overnight against 6 M urea, was injected near the middle of the focusing chamber following prefocusing. Focusing of proteins was continued for another 2 hours and 45 minutes, at which time the voltage had reached 916 V. Typically, focusing was complete within 1–3 hours after addition of the proteins. Twenty fractions were harvested and their pH values measured. Fractions containing fusion protein were identified and analyzed by SDS-PAGE mini-gels followed by western blotting using anti-TrpE antibody. Fractions containing fusion protein were then pooled and dialyzed overnight against 100 volumes of phosphate buffered saline (PBS) plus 0.5 M NaCl to remove ampholytes and urea.

Results and Discussion

The major advantage of preparative IEF using the Rotofor cell is that it provides a rapid and efficient means of purifying fusion proteins without subjecting them to ionic detergents. After a 3 hour run at 12 W constant power, 20 fractions were collected. Figure 1A shows the pH profile and an SDS-PAGE analysis of crude $(NH_4)_2SO_4$ precipitate along with Rotofor fractions 1–12. The crude precipitate consists primarily of fusion protein with lower molecular weight contaminants. To remove these contaminants, the $(NH_4)_2SO_4$ precipitate was dissolved in 6 M urea and loaded into the Rotofor cell, which

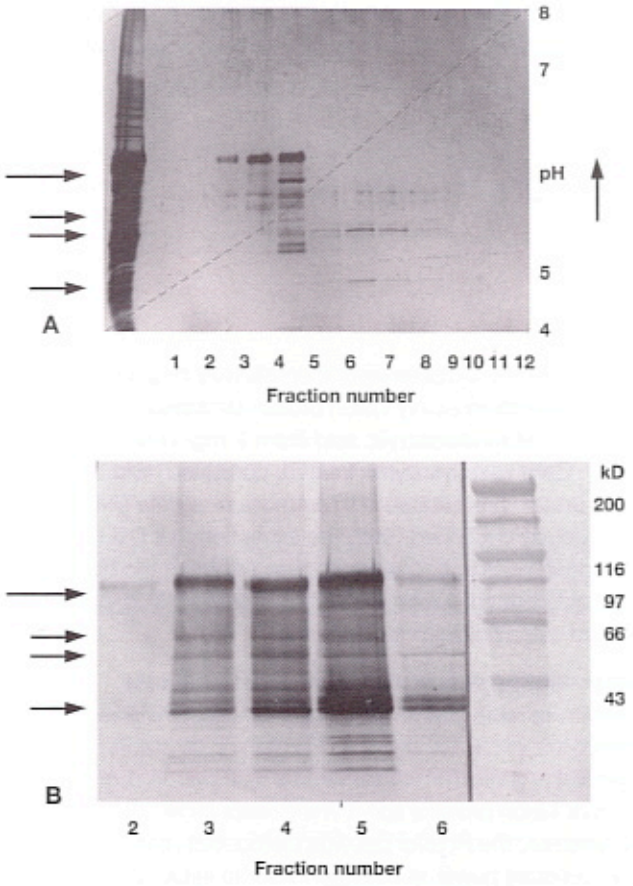

Fig. 1. Analysis of Rotofor run of 0.99 Kb BstY1 fusion protein by pH, SDS-PAGE, and western blot analysis. Panel A shows the pH profile and a 3–15% polyacrylamide gel of the fusion protein before focusing (lane 1) and Rotofor fractions 1–12 after focusing in the Rotofor cell. Proteins were visualized by silver staining (Bio-Rad). Arrows at the left correspond to arrows in panel B. Panel B shows a western blot of lanes 2–6 visualized using anti-TrpE antibody. Large arrow at left marks the full length fusion at ~98 kD. Small arrows show proteolytic fragments of the fusion protein, visible in the silver stained gel in A. Molecular weight markers shown at the right are 200,116, 97, 66, and 43 kD.

had been prefocused as described above. Fusion protein focused in three Rotofor fractions, centering at approximately pH 5.1. While much of the fusion protein remained soluble under these conditions, a sizable precipitate that contained both fusion protein and contaminants always formed near the peak of soluble protein. This precipitate was removed by centrifugation following harvesting. Preparative isoelectric focusing in the Rotofor cell substantially removed low molecular weight contaminants (see Figure 1). Western blot analysis using anti-TrpE revealed that many of protein bands still remaining were degradation products of the fusion protein (see Figure 1B).

A second advantage of isoelectric focusing in the Rotofor cell was apparent from the purification of the 2.1 Kb Sal I/Hind III fusion protein (approximately 115 kD). This fusion protein breaks down during expression, resulting in a mixture of full and partial length proteins in the $(NH_4)_2SO_4$ precipitate. Preparative IEF in the Rotofor cell resulted in a significant enrichment of full length fusion protein in a single fraction due to its having a slightly higher isoelectric point than the major breakdown product (see Figure 2).

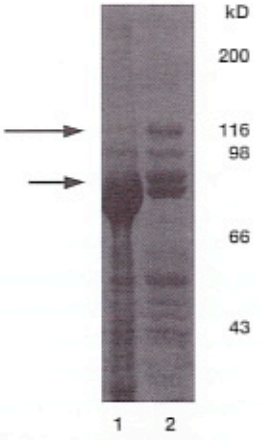

Fig. 2. Coomassie® blue stained gel of 2.1 Kb fusion protein showing enrichment of full-length fusion protein by Rotofor purification. Lane 1, urea solubilized sample of fusion protein preparation before focusing in the Rotofor cell. Lane 2, three pooled fractions (7–9) from the Rotofor cell run described in Methods. The heavy band (large arrow) is the major proteolytic fragment of this large, unstable fusion protein. The small arrow marks the full length protein, which while barely visible in lane 1, is highly enriched in lane 2. This enrichment occurred because the pI of the full length product is slightly higher than that of the proteolytic fragment. Molecular weight markers are as shown in Figure 1.

Conclusion

The Rotofor cell is an effective tool for purifying TrpE fusion proteins. This method should also be useful for purifying other fusion proteins, such as those that use the bacterial β-galactosidase gene (pUR expression vector and lambda gtII libraries). Because this method uses urea, a mild denaturant, rather than SDS or any other ionic detergent, it produces fusion proteins with a more native structure than those produced by preparative SDS-PAGE. Thus, preparative IEF in the Rotofor cell should be an advantageous purification method for using fusion proteins as antigens or in studies of protein structure and function.

References

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