Application Note:
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Large Volume Pelleting Using the Thermo Scientific Sorvall Evolution RC Centrifuge and the Thermo Scientific FIBERLite F8S-6x1000y Carbon Fiber Rotor

Dan Lu and Wei-Jen Tang, Ph.D., Department of Neurobiology, Pharmacology and Physiology, University of Chicago, IL 60637
Cristina D. Guibao, Gaohua Liu and Jie Zheng, Department of Structural Biology, St. Jude Children's Research Hospital, Memphis, TN 38105
Stephanie Noles, Ph.D., Laboratory Equipment Division, Thermo Fisher Scientific, Asheville, NC 28804

KEY WORDS

- Large Volume Pelleting
- Bacteria Pelleting
- Protein Purification
- Proteomics
- Structural Biology
- Sorvall Evolution RC
- F8S-6x1000y
- Carbon Fiber Rotor

Summary

Batch centrifugation is an important tool for large volume cell culture processing in academia, pharmaceutical and biotechnology research environments. We offer a variety of large-volume rotors and centrifuges that allow for the efficient pelleting of yeast, bacterial, mammalian, insect, and plant cells. By using the high capacity Thermo Scientific FIBERLite F8S-6x1000y carbon fiber rotor in a Thermo Scientific Sorvall Evolution RC centrifuge, 6 liters of cell culture can be pelleted in as little as 10 minutes depending on the cell type. These two centrifugation products are useful for functional proteomics research involving protein-protein interaction studies, protein biochemical studies, large-scale protein folding, and 3-dimensional protein structure determinations.

Introduction

Proteomics research aims at analyzing the total protein profile of a given cell, organelle, or tissue. Currently this research is addressed by quantitative and functional approaches. While classical proteomics addresses quantitative differences between complete protein profiles of samples (e.g. normal vs. diseased) using 2-dimensional gel electrophoresis and mass spectrometry, functional studies are more focused on elucidating the important interactions between proteins. Functional proteomic studies focused on protein-protein interactions can take the form of "protein chip or arrays," biochemical characterizations, and x-ray crystallography-based or NMR spectroscopy protein structure determinations. All these methodologies provide valuable tools for clinical, pharmaceutical, and basic research environments. Although the end product, such as a protein chip, may only use a small amount (micrograms) of protein, initial testing and optimization of this technology requires large quantities of proteins to be purified. Centrifugation remains a vital tool for harvesting of cells expressing proteins of interest. This application brief will focus on the Sorvall® Evolution™ RC centrifuge and the F8S-6x1000y carbon fiber rotor for pelleting of bacterial cells for further protein purification and characterization.

Figure 1. Thermo Scientific Sorvall Evolution RC

Procedures

PROTOCOL 1: Pelleting of *Escherichia coli* for Purification of the Edema Factor, Adenylyl Cyclase

Dan Lu and Wei-Jen Tang, Ph.D., Department of Neurobiology, Pharmacology and Physiology, University of Chicago, IL 60637

Protein-protein and protein-ligand interactions form the molecular basis for complex cell-to-cell communication and intercellular signaling that control diverse physiological activities. Adenylyl cyclases produced by certain bacterial species (also referred to as edema factors) are an important class of proteins that regulate several cellular activities through protein-protein interactions. Interaction of the edema factor with host cellular proteins such as calmodulin can initiate a series of biochemical changes.[1] A comprehensive understanding of the bacterial toxin's interaction with cellular proteins can provide a wealth of information on how to intervene these processes chemotherapeutically. To achieve this, it is critical to identify the catalytic domain(s) of the edema factor and its interaction with calmodulin.

This procedure describes the use of our Evolution RC centrifuge and the F8S-6x1000y carbon fiber rotor to harvest large quantities of bacteria over-expressing adenylyl cyclase.

The pelleted cells can be processed to isolate the edema factor and this purified adenylyl cyclase can then be used for subsequent protein-protein interaction studies.

Materials and Methods

Escherichia coli (E. coli) BL21(DE3) cells were transformed with appropriate plasmids containing the gene coding for the edema factor. *E. coli* cells were grown in modified T7 medium with 50 mg/mL kanamycin at 25-30°C to A_{600} of 0.4, induced by adding IPTG and harvested 12-19 hrs post induction. Cells were harvested using the F8S-6x1000y rotor at 13,000 x g at 4°C for 15 min. The pelleted cells were collected, lysed and further processed by column chromatographic methods for isolating the edema factor.

Results

Figure 2 provides a basic scheme for overexpressing recombinant proteins in cells. To isolate active proteins overexpressed in cells, multi-liter cell cultures must be harvested rapidly at optimum conditions after induction of cells and processed quickly for protein purification. Harvesting delays due to centrifugation capacity constraints can result in protease-inactivated proteins or cause expressed proteins to "fall out" as undesirable inclusion bodies.

The *E. coli* cells obtained by centrifugation were then lysed and the edema factor(s) was isolated after processing through several chromatographic procedures. Three different truncated edema factor fragments were purified using the above methods. Figure 3 illustrates the three different purified Edema Factors (EF) 3, 3-N, and 3-C resolved on a SDS polyacrylamide gel.

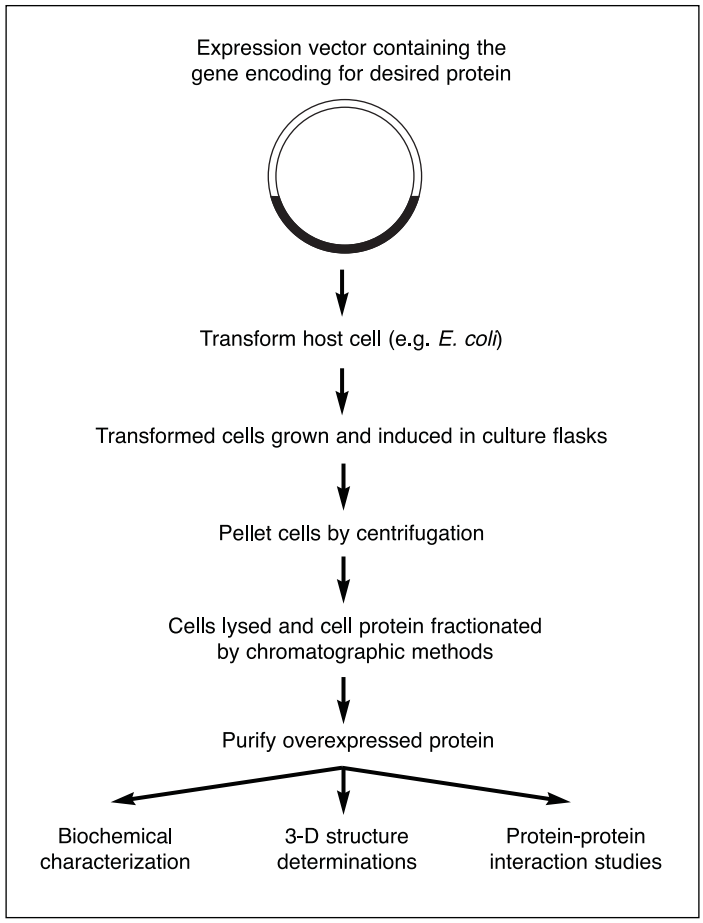

Figure 2. Overall Scheme for Protein Expression and Purification for Proteomics

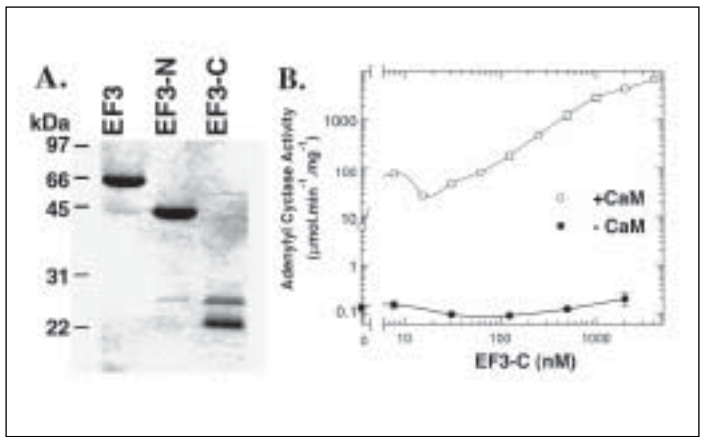

Figure 3. Characterization of the Edema Factor Fragments Expressed in *E.coli*
(reproduced with permission from the Journal of Biological Chemistry)

A. Coomassie Blue-stained SDS polyacrylamide gels of purified edema factor fragments. B. Activation of EF3-N in the presence or absence of calmodulin (CaM). Adenylyl cyclase activity was measured with 10 ng of EF3-N (+CaM) or 500 ng of EF3-N (-CaM).

PROTOCOL 2: Pelleting of *Escherichia coli* for the Purification of the FAK Focal Adhesion Target (FAT) Domain and Structural Determination by NMR Spectroscopy

Cristina D. Guibao, Gaohua Liu and Jie Zheng, Department of Structural Biology, St. Jude Children's Research Hospital, Memphis, TN 38105

NMR spectroscopy can be used to study the structure and function of proteins that are involved in intracellular signal transduction.[2-4] One such protein is focal adhesion kinase (FAK), which plays an important role in integrin-mediated signaling and in the modulation of processes such as cell growth, cell differentiation, wound healing, and tumor metastasis.[5,6] Although the precise function of FAK is currently undefined, some recent observations strongly suggest that

FAK suppresses apoptosis of certain types of cancer cells.[7] For this reason, FAK is considered a possible target for anticancer therapy.

This procedure describes the use of our Evolution RC centrifuge and the F8S-6x1000y carbon fiber rotor to efficiently produce large quantities of FAK's focal adhesion target (FAT) domain, whose structure was subsequently determined by NMR spectroscopy.[4]

Materials and Methods

Expression of the FAT domain of FAK

Chicken FAK-related non-kinase (FRNK) cDNA, which encodes the FAT domain, was kindly supplied by J. Thomas Parsons (University of Virginia). The cDNA encoding the FAT domain (residues 916 to 1053) was subcloned into a pET28a vector (Stratagene) and *E. coli* strain BL-21 pLysS cells (Stratagene) were transformed with the pET28a vector.[8] Cells expressing N-terminal His-tagged FAT domain were grown in Luria-Bertani (LB) broth containing the antibiotics kanamycin and chloramphenicol, overnight at 37°C with aeration. This culture was then diluted 1:100 in 1 L of LB broth and allowed to grow until the OD_{600} reached 0.7. Cells were induced to express the protein by incubation in LB broth containing 1 mM isopropyl-ß-D-thiogalactoside (IPTG) at 37°C with aeration for 4 hrs. The Evolution RC centrifuge was used to harvest the *E. coli* cells from 6 L of LB broth by spinning in the F8S-6x1000y rotor at 5,000 x g for 12 min.

For most NMR studies, the protein of interest must be isotopically labeled: the isotopes used were $^{15}NH_4Cl$ (1 g/L) and $^{13}C_6$-glucose (2.5 g/L) present in medium buffered with 3-(*N*-morpholino) propanesulfonate (MOPS).[9] After 10 mL of seed cells were grown overnight in LB broth, the resulting culture was used to inoculate 2 L of MOPS-buffered medium containing the isotopes and antibiotics, and the larger culture was then incubated at 37°C with aeration until the OD_{600} reached 0.6. Cells were induced to express protein by the addition of 1 mM IPTG and further incubation at 37°C with aeration, for 6 hrs. After induction, cells were harvested in the Evolution RC centrifuge as described above. Lysates of cells grown in the absence or presence of IPTG were separated by 10-20% SDS-PAGE (Figure 4).

Figure 4. Induction of FAT domain expression with IPTG. Lane 1: Bio-Rad Precision molecular weight standards (from top: 250, 150, 100, 75, 50, 37, 25, 15, and 10 kDa). Lane 2: Crude extract of *E. coli* strain BL-21 (pLysS) cells before induction with IPTG. Lane 3: FATdomain expression in crude extract of *E. coli* strain BL-21 (pLysS) cells after induction with IPTG.

Figure 5. Ribbon diagram of the solution structure of the FAT domain. The FAT domain (dark) is bound to the LD2 motif of paxillin (light). The image was generated with MOLMOL software.[9]

Purification of the FAT domain of FAK

E. coli lysate was incubated (4°C, overnight) with 6 mL of nickel-chelating resin. The resin was harvested and packed into a chromatography column through which an imidazole gradient was run to elute the FAT domain. The protein was digested with thrombin purified by size exclusion chromatography. The FAT domain comprises four helices in an elongated "right-turn" bundle that is stabilized mainly by hydrophobic interactions. The presence of a bound peptide derived from paxillin further stabilizes the structure (Figure 5).[4]

Conclusion

Higher throughput in batch centrifugation is achieved using the high capacity F8S-6x1000y rotor with the Evolution RC centrifuge. Comparing the processing capabilities in one batch run, the use of the F8S-6x1000y rotor is 2 to 4 times more productive than conventional 6 x 500 mL or 6 x 250 mL rotors. For the above described applications, a 15-minute run at 13,000 x g, 4°C resulted in efficient pelleting of *E. coli* cells expressing the edema factor and a 12-minute run at 5,000 x g was sufficient to pellet *E. coli* cells expressing the FAT domain of FAK. Overall, the Evolution RC centrifuge and F8S-6x1000y rotor meets the need for high throughput, efficient processing of samples for proteomics applications. The well-designed Evolution RC centrifuge is easy to use and offers reliability, state-of-the-art technology and delivers optimal separation performance.

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References

1. Drum C.L., Yan, S-Z., Sarac, R., Mabuchi, Y., Beckingham, K., Bohm, A., Grabarek, Z, and Tang W-J. 2000. An extended conformation of calmodulin induces interactions between the structural domains of adenylyl cyclase from *Bacillus anthracis* to promote catalysis. *J. Biol. Chem.* **275:** 36334-36340.
2. Wong, H. C., J. Mao, J. T. Nguyen, S. Srinivas, W. Zhang, B. Liu, L. Li, D. Wu and J. Zheng. 2000. Structural basis of the recognition of the Dishevelled DEP domain in the Wnt signaling pathway. *Nat. Struct. Biol.* **7:** 1178-1184.
3. Wong, H. C., G. Liu, Y.-M. Zhang, C. O. Rock, and J. Zheng. 2002. The solution structure of acyl carrier protein from *Mycobacterium tuberculosis*. *J. Biol. Chem.* **18:** 15874-15880.
4. Liu, G., C. D. Guibao, and J. Zheng. 2002. Structural insight into the mechanisms of targeting and signaling of focal adhesion kinase. *Mol. Cell. Biol.* **22:** 2751-2760.
5. Cary, L. A. and J. L. Guan. 1999. Focal adhesion kinase in integrin-mediated signaling. *Front. Biosci.* **4:** D102-D113.
6. Friedl, P. and E. B. Brocker. 2000. The biology of cell locomotion within three-dimensional extracellular matrix. *Cell. Mol. Life Sci.* **57:** 41-64.
7. Ilic, D., E. A. Almeida, D. D. Schlaepfer, P. Dazin, S. Aizawa, and C. H. Damsky. 1998. Extracellular matrix survival signals transduced by focal adhesion kinase suppress p53-mediated apoptosis. *J. Cell Biol.* **143:** 547-560.
8. Moks T., L. Abrahmsen, E. Holmgren, M. Bilich, A Olsson, M. Uhlen, G. Pohl, C. Sterky, H. Hultberg, S. Josephson, A. Holmgren, H. Jornvall, and B. Nilsson. 1987. Expression of human insulin-like growth factor I in bacteria: use of optimized gene fusion vectors to facilitate protein purification. *Biochemistry* **26:** 5239-5244.
9. Koradi, R., M. Billeter, and K. Wuthrich. 1996. MOLMOL: a program for display and analysis of macromolecular structures. *J. Mol. Graph.* **14:** 29-32, 51-55.

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