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Preparative Protein Production from Inclusion Bodies and Crystallization: A Seven-Week Biochemistry Sequence

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Abstract

We describe how to produce and purify proteins from *E. coli* inclusion bodies by adapting versatile, preparative-scale techniques to the undergraduate laboratory schedule. This seven-week sequence of experiments fits into an annual cycle of research activity in biochemistry courses. Recombinant proteins are expressed as inclusion bodies, which are collected, washed, then solubilized in urea. Stepwise dialysis to dilute urea over the course of a week produces refolded protein. Column chromatography is used to purify protein into fractions, which are then analyzed with gel electrophoresis and concentration assays. Students culminate the project by designing crystallization trials in sitting-drop trays. Student evaluation of the experience has been positive, listing 5–12 new techniques learned, which are transferrable to graduate research in academia and industry.

Keywords

proteins; crystals/crystallography; electrophoresis; biochemistry; laboratory instruction; upper-division undergraduate research

Protein production and analysis projects have been successfully adapted to the undergraduate lab in many ways, making recombinant proteins in *Escherichia coli* as diverse as sperm whale myoglobin,¹ Taq polymerase,² xylanase,³ and RNAase One.⁴ Other projects have made green fluorescent protein in *E. coli*, either with student-designed mutations⁵ or four different types of chromatography.⁶ Student-directed inquiry has been introduced into the protein purification process either at the point of protein selection and purification design⁷ or using thermodynamic analysis to characterize unknown translation factors.⁸

These projects require proteins that are expressed as a soluble form in the host organism. Many proteins overexpressed in *E. coli* precipitate as insoluble inclusion bodies, which are useful because large quantities of protein from diverse organisms can be prepared in bacteria and partially purified by insolubility, but they must be refolded. For example, the Refold database⁹ includes refolding conditions for many proteins from inclusion bodies.

We describe how to produce and purify proteins from *E. coli* inclusion bodies by adapting versatile, preparative-scale techniques to the undergraduate laboratory schedule. These

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Supporting Information: A table showing a crystallization tray setup and student instructions and notes for the instructor. Answer keys to additional questions are available to instructors upon request from the corresponding author. This material is available via the Internet at <http://pubs.acs.org>.

techniques were developed in crystallography research to produce large quantities of a wide array of recombinant proteins, such as human immunoreceptors. In this article we show student results for producing pure MICA protein, a stress-induced single-chain activating ligand for natural killer cell receptors, which is one focus of our undergraduate research program.¹⁰ We adapt common research laboratory techniques to a group of 10–20 students working together in weekly three-to-four-hour lab sections.

The proteins produced in this sequence are used in other courses and independent projects. These experiments fit into an annual cycle of research activity in biochemistry courses so that proteins made in the winter support continued research momentum throughout the year (Figure 1).

Overview of the Exercise

Students work in pairs on all experiments (Table 1). We form groups based the room layout, which has 4 students per bench (Figure 2). Each of these 4-student “pair of pairs” is assigned the same protein and can use the same equipment for lysis, stepwise dialysis, and column chromatography, meaning only 4 instruments are necessary for a 16-student lab section. Students communicate their results through three written reports in the style of journal articles as described in the supplemental material.

Lab Period 1: Preparing Solutions

The first step is similar to beginning a project in independent research: preparation of growth media (including autoclave sterilization) and lysis solutions.

Lab Period 2: Protein Expression

Protein expression requires 8–10 hours, so the regularly scheduled 3-hour block of time is canceled for two weeks and students sign up for a day when equipment is available. Students set up an overnight saturation culture from previously made plates of an expression strain of *E. coli* such as BL21(DE3)RIL transformed with the pET21b expression plasmid containing the protein of interest. The next morning, students inoculate four 750 mL flasks of growth media containing antibiotics. The flasks incubate shaking at 37 °C 2–3 hours until they reach mid-log phase, and then the students add isopropylthio- β -D-galactoside (IPTG) to induce protein expression. After 3–5 hours of shaking (not requiring constant supervision) the cells are harvested by centrifugation and stored at –20 °C.

Lab Periods 3–4: Cell Lysis, Pellet Washing, and Pellet Solubilization

Cells are suspended in lysis buffer containing detergent, with repeated pipetting to disperse the pellet. Room-temperature incubation of lysozyme and DNase, and a glass-bead homogenizer, lyse the cells. Repeated cycles of pellet dispersal in fresh lysis buffer followed by centrifugation of cell debris in detergent wash away soluble elements, while insoluble inclusion bodies remain pelleted. A final wash with detergent-free buffer removes residual detergent, and the inclusion bodies are then dissolved in saturated urea with a glutathione redox buffer overnight. Because the lysis and washing steps involve repetitive pipetting and centrifugation steps, students have time during these two lab periods to prepare refolding buffers, column chromatography buffers, and protein crystallization solutions for the future lab periods.

Between Lab Period 4 and 5: Refolding by Stepwise Dialysis

After overnight incubation, the pellets are solubilized, and students place 100 mL of combined solutions from one 4-student “pair of pairs” in dialysis bags and transfer them to 2 L solutions of 4 M urea at 4 °C also containing buffer and 0.4 M arginine hydrochloride.

(Concentrated arginine prevents folding-intermediate aggregation and assists protein refolding by dilution.¹¹) Every 24 hours afterward, teaching assistants change the buffers to more dilute urea solutions, then to a solution of running buffer for column chromatography (Table 2). These 6 overnight steps produce refolded protein in solution and a large quantity of unfolded precipitate in the dialysis bag. Some proteins may require a protease blocker such as Pefabloc SC (AEBSF).

Lab Period 5: Column Chromatography

Unfolded precipitate is centrifuged from solution, and then the solutions are applied to chromatography columns for purification. Four peristaltic flow systems, one per 4-student “pair of pairs,” pump buffer solutions; these include conductivity and UV detectors that can produce elution profiles (Figure 3), but simpler systems such as pumps without detectors or gravity-flow columns may be used instead. For histidine-tagged proteins, we use nickel-NTA resin (Qiagen) in glass columns with flow adaptors (Bio-Rad); for untagged proteins, we use 5 mL ion-exchange prepacked Econocolumns (Bio-Rad). Loading a 100 mL solution onto a column takes about an hour. During this time each pair of students pours two acrylamide gels for use in lab period 6. Eluted protein fractions are stored at 4 °C.

Lab Period 6: SDS-PAGE and Bradford analysis

Students design two gels to investigate their protein fractions (Figure 4). They can run and stain the gels during a 3-hour lab period and then place the gels in destain solution overnight. While the gel is running, they analyze fraction protein concentrations with a quick assay adding microliter volumes of samples to Bradford reagent. The students can then synthesize and align information from three methods of analysis, each with strengths and weaknesses: SDS-PAGE, qualitative Bradford assay, and elution profile. A quantitative Bradford assay can be used at this point as well, and the students can use online tools to calculate an extinction coefficient from primary sequence.

Lab Period 7: Protein Crystallization

The students analyze the protein with crystallization trials. Protein samples are concentrated and then filtered with membrane-based devices in microcentrifuges. Sitting-drop crystallization techniques are simpler than hanging-drop because the post for each protein drop is part of the tray. Students design trays that mimic a common crystallization grid that varies two of three variables [pH, ammonium sulfate concentration, or polyethylene glycol (PEG) concentration] along the axes of the tray (see the table in the supplemental information for an example). The drops are later examined by microscopy. Crystals produced by this technique could be used for X-ray crystallography, but unless the proteins have undergone an additional size-exclusion chromatography purification step, students usually observe examples of pre-crystalline forms such as precipitate or needles.

Equipment Needed

The following expensive or unique pieces of equipment are needed: An autoclave large enough for 2 L flasks; a heated tabletop shaker for protein expression; a floor-standing centrifuge; instruments for *E. coli* lysis such as “bead beater” homogenizers; automated pipets for pellet dispersal; and SDS-PAGE equipment. The following are useful but not strictly necessary: a cold room for refolding (alternative: any refrigerated chamber, or room temperature for some proteins); and peristaltic pumps for liquid chromatography (alternative: gravity-flow columns).

Hazards

HCl, NaOH, imidazole, and Bradford reagent cause burns and are corrosive. Solid sodium azide is fatal if absorbed through the skin, ingested, or inhaled. Dithiothreitol, arginine, and urea cause skin, eye, and respiratory tract irritation. Chloramphenicol, nickel(II) sulfate hexahydrate, and isopropyl- β -D-thiogalactopyranoside may cause cancer in humans. Ampicillin may cause allergic skin and respiratory reactions. Tert-pentyl alcohol is flammable and may cause CNS depression. Unpolymerized acrylamide is harmful if inhaled, swallowed, or absorbed through the skin, may cause cancer, and CNS damage. TEMED is flammable and causes eye and skin burns.

Results and Discussion

Students have followed this sequence of laboratory exercises to produce milligram quantities of different proteins and mutants for seven years, using two 16-student, 3-hour lab sections to make as many as eight different proteins per year. Undergraduate students have produced more than 40 different mutants of the MICA immunoprotein for structural study and have published subsequent protein analysis.¹² Dozens of students have graduated from this course to employment involving protein production and to graduate school laboratories.

Subsequently, these proteins have been analyzed by surface plasmon resonance, isothermal titration calorimetry, circular dichroism, fluorescence, and temperature-dependent second-derivative absorbance spectroscopy.¹³ Some protein has been crystallized through collaboration with a local research center using a mosquito crystallization robot (TTP Labtech). In semester courses these analyses could be integrated into the course or students could design proteins for investigation, producing expression plasmids through gene synthesis.

Students positively evaluate the course at the end of the term (85% average response rate). To the question “What new things did you learn how to do in the lab?”, most students listed 5–12 specific techniques, such as liter-scale recombinant protein expression, glass-bead cell lysis, centrifugation and washing of inclusion bodies, SDS-PAGE, His-tag affinity chromatography, and protein crystallization. Other comments include “The laboratory module is well-designed to integrate real-world lab experience with the classroom”, “The lab is an excellent way to learn. This quarter’s protein purification and crystallization lab helped us to understand the type of work that goes into research”, and “[It was] an efficient use of resources and shows a level of trust between student and professor.”

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

The “Research Cycle” of adapting research to a sequence of three undergraduate courses. During the fall quarter, an initial biochemistry course teaches general research techniques. During the winter quarter in a second biochemistry course, protein production is taught as described in this article. During the spring quarter in a physical chemistry survey course, students analyze protein-binding thermodynamics and kinetics with surface plasmon resonance. During summer quarter, students apply these techniques to independent projects.

Lab Layout: 16 students = 4 “pair of pairs”
consisting of two pairs of two students each

Figure 2.
Assigning combined groups to reduce bottlenecks.

Figure 3.

Elution profile produced by application of MICA protein and imidazole solutions to a nickel-NTA column. Events marked by triangles indicate changing solutions or fraction collection after 75 minutes. The UV increase showing elution of protein coincides with the increased conductivity of high-imidazole elution buffer.

Figure 4. SDS-PAGE gel showing purity and concentration of different fractions of refolded MICA protein and two protein ladders.

Table 1

Sequence of Experiments

Experiment	Problems	How to Resolve Problems
Lab 1: Check-in and Prepare Solutions and Media		
Lab 2: Protein Expression	shaker space	sign up for time outside class
Lab 3: Cell Lysis and Inclusion Body Wash	lysis instruments	pair of pairs for instrument lysis
Lab 4: Inclusion Body Wash and Solubilization		
Between Lab 4 and 5: Refolding by Dialysis	large dialysis volume	pair of pairs, TA outside of class
Lab 5: Purification by Affinity or Ion-Exchange Chromatography	pump equipment	use peristaltic pumps, pair of pairs
Lab 6: Analysis by SDS-PAGE and Bradford Assay		
Between Lab 6 and 7: Possible FPLC SEC Purification		done by TA outside of class
Lab 7: Protein Crystallization Trials	hanging-drop cover slips	sitting-drop

Table 2

Scheme for Week of Refolding by Dialysis

Day	[Urea] in Solution/M
1 (day of lab 4)	8
2 (set up dialysis)	4
3	2
4	1
5	0
6	0 (1/4 strength)
7	Column-compatible buffer
8 (day of lab 5)	(ready for chromatography)