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Special Section: Innovative Laboratory Exercises

A Real-time and Hands-on Research Course in Protein Purification and Characterization*,†,‡

PURIFICATION AND CRYSTAL GROWTH OF HUMAN INOSINE TRIPHOSPHATE PYROPHOSPHATASE

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A new lecture/laboratory course to offer advanced biochemical training for undergraduate and early graduate students has been developed in the Department of Chemistry at the University of Nebraska at Omaha. This unique course offers students an opportunity to work hands-on with modern instrumentation not normally found in a predominately undergraduate institution, and to complete an entire research project in a realistic timeframe via a time-intensive curriculum as a special summer session. The course content gives a strong background in protein structure/chemistry, purification principles, protocol development, optimization strategies, use and programming of an automated chromatography instrument, and characterization strategies with an emphasis on X-ray crystallography. The laboratory portion offers students the chance to purify a protein (human inosine triphosphate pyrophosphatase) from start to finish, program and use an ÄKTA fast protein liquid chromatography instrument, and to grow and analyze their own protein crystals using their purified protein. This innovative laboratory experience gives the participating students the opportunity to complete a miniresearch project in real time and enhances their overall understanding of important biochemical research techniques and the instrumentation involved, fostering a better understanding of the research process all within a classroom setting. Evaluations and feedback concerning this course indicated a positive learning environment, a retention of knowledge and skills, a belief that the skill set learned continues to be useful in current endeavors, and a sense of accomplishment in the completion of an actual research project within the confines of a class setting.

Keywords: Active learning, curriculum design development and implementation, laboratory exercise, new course development, protein structure function and folding.

One major downfall in any undergraduate laboratory course is the disconnection between a series of 3-hour miniaturized experiments and the realistic nature of true bench work. In a typical undergraduate course, the experiments are written to introduce students to working at a bench, calculations, and use of equipment, but are all designed with maximal successful outcome ratios for the students. In an actual research laboratory setting, students must deal with experiments that take extended timeframes to complete, multiple trials for optimization, and work in collaboration to optimize equipment and reagent use. Students quickly learn that research results are not as easily obtained as their former laboratory manuals made it seem. Many of our science majors at the undergraduate level are hoping to join graduate programs or work forces which require applicants with research experience, who are detailed notebook writers, and who have developed solid critical thinking skills. Graduate students may find that their research project involves the need for protein isolation and/or purification, a skill set that may be outside of their laboratory's area of expertise, facilitating the need to work and think independently.

To address these issues of acquisition of experimental proficiency, equipment training, and critical thinking skills, a new course focused on protein purification and characterization was developed at the University of Nebraska at Omaha (UNO)[1] in collaboration with the Eppley Structural Biology Facility (ESBF) at the University of Nebraska Medi-

*Additional Supporting information may be found in the online version of this article.

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[1]The abbreviations used are: UNO, University of Nebraska at Omaha; ESBF, Eppley Structural Biology Facility; FPLC, fast protein liquid chromatography; UNMC, University of Nebraska Medical Center; TA, teaching assistant; His, histidine; ITPA, inosine triphosphate pyrophosphatase; IPTG, isopropyl-beta-d-thiogalactopyranoside; β-ME, β-mercaptoethanol; OD, optical density; PDB, Protein Data Bank.

cal Center (UNMC, both in Omaha, NE). Although courses on protein purification are not novel (see refs. 1–10 for selected recent examples), this course was developed to offer undergraduates or early graduate students from either university the opportunity to receive a unique laboratory training experience that would set them apart from other applicants to postbaccalaureate programs or provide them with the knowledge to improve their research projects by focusing on real-time experimentation and hands-on application involving modern instrumentation [such as the fast protein liquid chromatography instrument (FPLC)], which is not often found at primarily undergraduate universities. To present the realistic laboratory setting to our students, the course has always been given in an altered timeframe from the normal semester laboratory classes and is offered as an intensive special summer session course, where the duration matches the purification protocol required with the overall in-class hours adjusted to meet the credit hours.

As UNO is a primarily undergraduate institution, it was necessary to forge a collaboration with UNMC, a Research I institution located about 2 miles away to provide instrumentation and research opportunities for UNO students. To best teach a course on protein purification, we decided to partner with the structural biologists at UNMC, because they routinely purify milligram quantities of high-quality protein samples and thereby relatively easily provide an excellent research prospect for students. Several funding agencies were targeted to provide support for the instrumentation, equipment, and supplies required to purify different types of proteins. In the request for funding and support for this course, the focus of the grants concerned several principle items: 1) design of a new course that was not currently taught at either institution; 2) targeting of students from both UNO and UNMC to take the course, fostering intercampus ties between students and professors; 3) providing state-of-the-art instrumentation and research experience for undergraduates in a real-time, bench-like atmosphere with hands-on training but under classroom guidance, which was the major focal point in all of the proposals; 4) allowing undergraduates a further opportunity for a research internship at a Research I institution after completion of the course including a supply stipend; 5) the continued collaboration between UNO and UNMC investigators by offering to develop and implement a purification scheme for new proteins in each course offering; and 6) providing an academic training experience for graduate students interested in entering academia. This collaborative course has received funding from several sources at the state and national level (Nebraska Research Initiative, National Science Foundation, and the National Center for Research Resources/National Institutes of Health), which have provided UNO and UNMC with several large pieces of equipment (such as the ÄKTA FPLC), salary compensation, and various smaller equipments and supplies.

COURSE RATIONALE

The pedagogical aspects of this project involve participation of approximately 10 students per offering in an advanced laboratory course to learn the techniques of protein purification and characterization by crystallography. The pedagogy of this course was chosen to provide students the training and knowledge required to become strong researchers and critical thinkers. The ability to offer a course that teaches these advanced topics with an actual research-based laboratory component is extremely advantageous to multiple audiences at both UNO and UNMC.

First, undergraduate students from several science disciplines (including biology, biotechnology, chemistry, and medicinal chemistry majors) are offered a unique opportunity to participate in a realistic laboratory experience while receiving upper-level science credit. Research experiences have been shown to be extremely beneficial to undergraduate students in various areas including professional development, skill acquisition, and help with deciding on career paths [11]. In the local scientific industry, there is a clear expectation that students need to be proficient with calculations, detailed note takers and note keepers, able to follow directions, and able to demonstrate critical thinking skills and problem-solving abilities. To aid towards helping our students be marketable in future industry positions, the course requires keeping a laboratory notebook of the experimentation and calculations for laboratory solution preparation, reagent preparation, and in the class exams. Perhaps more importantly, this course asks the students to perform an experiment following only a very basic outline for a laboratory manual, filling in important experimental considerations such as temperature, storage, time, calculating concentrations, and trouble-shooting any complications or errors that arise during the experiment when they are working in the laboratory.

The practical laboratory component, including hands-on training with the FPLC, some optimization of laboratory experiments, keeping a laboratory notebook, troubleshooting experimental pitfalls as the lab is running, and completing a full multistep experiment from start to finish in the timeframe of the course, are all rare experiences for undergraduates. For example, the students who are given a lecture concerning how to operate and program the FPLC, are allowed to assist in setting up the FPLC programs for the course chromatography runs, and are instructed how to read the resulting chromatographs. This instruction allows them to analyze how well the run has proceeded, decide which sample fractions to keep, and some modest ability to troubleshoot any issues with the column run. They are also given the opportunity to try modifying temperatures, incubation times, buffer concentrations, pH, and other experimental parameters during the purification scheme after having received instruction on what to consider when setting up a purification plan. As the students are monitoring protein purity and concentration throughout the purification, they are required to troubleshoot any experimental pitfalls during the procedure in the laboratory when the experiment is running and come up with modifications as they proceed to finish the purification within the given timeframe of the course. Several students who show an aptitude for protein biochemistry and/or desire for further research training are recruited to perform a semester-long or summer research internship with a UNMC

primary investigator that would tie their educational experience to a longer-reaching research project with actual laboratory bench exposure.

Second, introductory graduate students (in their first or second year of training or Master's students) are also candidates for this course. There is no course currently offered at UNO or UNMC that covers the topic of protein purification and crystallization in detail. However, the need to purify a protein or proteins as part of the scope of an overall research project is not uncommon. This course gives the students some solid background knowledge to design, optimize, and troubleshoot purification strategies, resources to find further guidance on protocol setup, and a hands-on chance at purifying a protein from start to finish while learning to use and program an FPLC, the traditional instrument used in protein purifications.

Third, if desired, this course offers an option for advanced graduate students, in the form of an in-depth teaching experience, for those interested in pursuing academia as a career path. The responsibilities of the graduate teaching assistant (TA) could include preparation of reagents for the laboratory, optimization of the current purification procedure, and supervising the laboratory portion of the course. The opportunity also exists for the assistant to participate in the lecture portion of the course as a lecturer, particularly in the lectures focusing on FPLC and/or X-ray crystallography.

Finally, flexibility of this course in scope/depth as well as timing allows for easy manipulation of lecture and/or laboratory content to fit students' interests. With the major instrumentation in place, in the future this course can be modified on an annual basis to meet the research needs of individual UNMC investigators by changing the protein purified, source material, and so forth, continuing to foster the collaboration between universities, and still fit into an appropriate educational experience for undergraduate and graduate students. Of the two times this course has been previously offered, the students have purified two different proteins from two different types of starting materials (first session: purification of profilin:actin from calf thymus [12]; second session: purification of recombinant histidine-tagged human inosine triphosphate pyrophosphatase (His-ITPA) from bacterial cells [13]; both of these proteins are part of ongoing research projects at UNMC). The second group also had the opportunity to crystallize their purified His-ITPA in the core laboratory at UNMC as well as to crystallize lysozyme, a protein that can grow crystals in an hour. By chance, the audience for the two offerings was quite different: first year consisted of all UNO undergraduates, second year happened to consist of all graduate students (from UNO and UNMC), also demonstrating the versatility of and the across-the-board need for the course.

Due to the pace and advanced level of the topics covered in this course, a full year of organic chemistry is required as a prerequisite and one semester of biochemistry is highly recommended for undergraduate students. As with any course, instructor discretion can be used. In the previous two offerings, one student completed the course having taken only fundamental organic and biochemistry courses (not upper-level courses), and one student completed the course having finished only organic chemistry and a molecular biology of the cell course with no prior biochemistry. Both students passed the course, but noted that they felt behind and struggled with the lecture content in comparison with their classmates.

If needed, this course could also be modified to fit into a traditional 16-week semester lecture/laboratory course or to fit into a modified course such as a twice per week lecture/laboratory for 8 weeks, as has been demonstrated by other institutions (e.g., see refs. 1, 3, and 7). As one of the major premises of this manuscript is to demonstrate the ability to offer a realistic research experience to undergraduates in terms of timing and rigor, the modification of this course to fit into a traditional university semester will not be a topic herein.

This article will focus on the materials and methods used to purify His-ITPA. This purification requires about 8–10 days of experimentation from transformation to crystal formation, so the course was offered for a 2-week special summer session that met from approximately 9:30 AM to 3:30 PM Monday through Friday. Future offerings of this course will include the addition of a protein activity assay to further emphasize the importance of purification and characterization by means of structure and protein activity. Due to the new experiments, the number of days and the hours per day will be adjusted to appropriately match the credit hours assigned to this course.

EXPERIMENTAL PROCEDURES

Equipment

The laboratory is equipped to handle up to 10 students in a given session, primarily due to time constraints of the experiments rather than an equipment shortage. Larger equipment includes: a shaker/incubator, isotemp incubator, shaking/incubated water bath, ultraviolet (UV)–visible spectrophotometer, a monochromatic plate-reader, benchtop autoclave, refrigerated benchtop centrifuge with rotors capable of handling 1.7–50-mL tubes at low–medium spin speeds, refrigerated high-speed centrifuge with rotors for 50-mL tubes and 500-mL bottles, gel documentation system with analysis software, sliding-door refrigerator, Emulsiflex (UNMC), and an FPLC system with fraction collector and the necessary application/analysis software (see Supporting Information). At the end of the course, the students are given a tour of UNMC's X-ray crystallography core facility and use a liquid handling robot for loading reagents and protein into crystallization plates, and an automated microscope/camera system for documenting growth of their protein crystals. Chemicals and supplies were purchased from several companies as appropriate (see Supporting Information).

Course Schedule

The course was scheduled for approximately 6 hours per day for 9 days, including approximately 2–3 hours of lecture instruction and 3–4 hours of laboratory involvement per day. The students also met briefly for wrap-up and to check crystal growth on the 10th day. For a

TABLE I
Protein purification and characterization experiment timeline

Day 1	Transformation and solution preparation
Day 2	Starter and overnight cell cultures and solution preparation
Day 3	Cell induction, cell lysis, SDS-PAGE, staining/destaining, and program FPLC
Day 4	Affinity chromatography, SDS-PAGE, staining/destaining, and fraction pooling
Day 5	Thrombin cleavage of His tag, benzamidine treatment, dialysis, and program FPLC
Day 6	Ion-exchange chromatography, SDS-PAGE, staining/destaining, and fraction pooling
Day 7	Sample concentration, SDS-PAGE, and staining/destaining
Day 8	Crystal experiments setup
Day 9	Clean up and check out
Day 10	Check crystal growth

course laboratory outline, see Table I. The students work together to complete the purification protocol, sometimes working individually and pooling the best samples, and sometimes working as a unit to perform the more detail-oriented tasks.

The lecture portion of the course is intended to give students the background information necessary to think his/her way through the design and/or development of a protein purification scheme. Many of the graduate students who take this course are hoping to use the information learned to purify a protein for their own research projects. The following course outline matches the schedule for the most recent offering of the course. Depending on the protein purified and the steps required to accomplish that purification, the course schedule can be modified as long as it meets the requirements of hours to match the number of credits for the course.

Purification of Recombinant Human His-ITPA

ITPA is an enzyme that cleanses the cellular nucleotide pool of mutagenic ITP. The His tag on the recombinant protein made His-ITPA a good candidate for a quick and fairly straight forward purification protocol to be performed by students. The purification scheme made use of the affinity of His tags for nickel ions and the overall charge of the protein. The course protocol was adapted from Porta *et al.* [13], and details of the protein and techniques can be found in that article. The timeline and protocols were developed in conjunction with the TA for the course. The assistant optimized the experiments and wrote the original version of the protocol guide for the course.

Course Lecture and Laboratory Outline

Day 1—Lecture: Review of Amino Acids and Protein Structure/Function—A comprehensive review of protein structure and protein chemistry is necessary for the first 2 days of lecture. Although it is assumed that most of the students have already taken some introductory biochemistry, it is necessary to review topics such as amino acids, side chain chemistry, peptide bonds, protein structure (primary through quaternary), noncovalent interactions, water, and ionization. This section takes 3–4 hours to complete and is spread out over the first 2 days of the course. Any standard advanced biochemistry text would be sufficient [14].

Laboratory: Transformation and Stock Solution Preparation. Students set up a transformation using Escherichia coli Rosetta2(DE3) cells and the plasmid containing our gene of interest (pET-15b-His-ITPA) using calcium chloride competent cells in a standard heat shock protocol (Novagen). The transformations were grown overnight with the appropriate selection antibiotics for both E. coli host and plasmid. In addition to the transformation, students were asked to prepare several stock solutions that would be needed throughout the laboratory. All stock solutions are sterile filtered using 0.22-μm filters (see Supporting Information).

Day 2—Laboratory: Inoculation and Growth of Cultures and Preparation of Buffers—Starter cultures from the Day 1 transformations were inoculated for 5–6 hours with 37 °C aeration. One milliliter of the starter cultures was transferred to 500-mL cultures and grown overnight under conditions that allowed to reach appropriate cell densities to be reached by the next morning. In addition to the inoculation, several buffer solutions were prepared using the stock solutions from Day 1, including the FPLC buffers and a dialysis buffer.

Day 3—Lecture: Introduction to FPLC—As the laboratory component of Day 3 is the first time the students will be using the FPLC, this day of lecture is dedicated to understanding the history of chromatography and automated chromatography, the working components of the interior and exterior of the ÄKTA FPLC that we use, information on how to run and program the FPLC, and basic troubleshooting ideas. Generally, the TA who is helping to run the laboratory component of this course is trained in their own research laboratories to run the FPLC, so this lecture is written and given by the assistant. The end of the lecture involves having the students watch and participate in programming the FPLC for the affinity chromatography run that will be done on Day 4 of the laboratory procedure.

Laboratory: Induction and Lysis of Cells. On this day, the goal is to express the target protein in the growing cultures. As the cells used for this experiment contain the T7 lac promoter, isopropyl-beta-D-thiogalactopyranoside (IPTG) is added to the cultures to induce protein expression from the pET vector. The optical density (OD) at 600 nm is thereafter monitored by spectrophotometry until the cells reach the desired OD_{600} (0.6) before inoculation with IPTG and further incubation to allow for protein expression. The students were asked to run a sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) 12% minigel of collected induced versus uninduced samples and then to check protein expression by performing Coomassie staining and destaining steps.

For harvesting, the remaining induced cultures are centrifuged for 20 min at 13,000 × g in 500-mL culture bottles. After centrifugation, the cell pellets are aliquoted into 50-mL conical tubes with ~5 g of pellet per tube. Chemical lysis of the pellet was attempted using BugBuster following the manufacturer's protocol (Novagen, Calgary, ALB, Canada), but lysis was ineffective due to

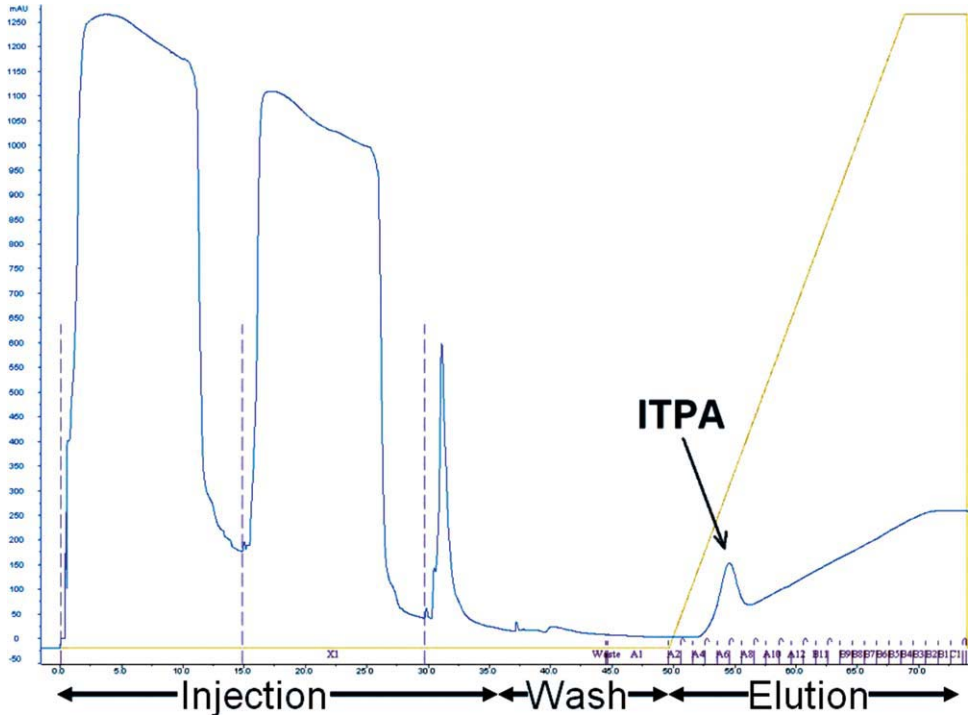

FIG. 1. **Chromatogram of primary purification of ITPA using Hi-Trap nickel charged affinity column.** Blue line, UV tracing; yellow line, concentration of elution buffer; and pink dotted line, injections. *Y* axis denotes UV absorbance at 280 nm and *X* axis denotes fractions collected. ITPA elutes in 16–35% elution buffer.

the large cell pellet mass. The students' cells were then lysed using an Emulsiflex. Lysis by other pressure/release methods or sonication is also possible. After lysis, students took aliquots of the supernatant and pellet for SDS-PAGE analysis.

Day 4—Lecture: Protein Purification—Days 4–7 of the lecture series involve information on protein purification techniques, handling of proteins from different starting materials, extract preparation, buffer selections, optimization of purification strategies, cost analysis of purification schemes, and other topics relevant to protein purification in general. These four lectures are the "heart" of the lecture portion of the course and the students are generally referred to a couple of protein purification texts [15–17] for further information. The intention is for students to walk away with a general understanding of how to use protein properties as the basis for separation strategies and how to think their way through a cost-effective purification process, including troubleshooting and optimization. The protein that is being purified during the course, in this case His-ITPA, is used as a standard when moving through the various topics so that the students might understand what goes into the purification process they are performing.

Laboratory: Nickel Affinity Column Chromatography. Lysates need to be centrifuged again or sterile filtered through a 0.22-μm Steriflip filter (Millipore) after overnight storage. Students need to prepare the column, column buffers, and FPLC for the run. This includes (but is not limited to) sterile filtration of the column buffers (0.22 μm), making sure that the buffers are at the appropriate temperature (for the column run), addition of any "day of" components to the buffers [such as 2 mM β-mercaptoethanol (β-ME)], and washing the ports and tubing lines of the FPLC with ethanol and water to flush the system before the run. As our protein was His-tagged, a 1 mL nickel-labeled resin HisTrap HP column was used with an increasing gradient of imidazole as our eluting agent (40 mM to 1 M gradient). The protein eluted at approximately 300 mM imidazole (see Fig. 1 for chromatograph). After analysis of the FPLC column run, aliquots were taken from all of the fractions of interest for SDS-PAGE analysis, including flow through, washes, and any peaks. After running the gel, stain and destain to monitor protein expression and the level of purification achieved. Pool all sample fractions that contain the protein of interest from the FPLC run.

Day 5—Laboratory: Thrombin Cleavage of His Tag and Dialysis—The pooled protein sample was replenished with fresh 2 mM β-ME (a necessary step for His-ITPA due to the large number of cysteines in the protein). Using a spectrophotometer, the concentration of protein in the sample was measured at 595 nm using the Bio-Rad protein assay with serum albumin as the standard. After determination of the protein concentration, thrombin was added to the sample (10 U/mg of protein). Thrombin is a serine protease that cleaves a specific site following the amino-terminal His tag of the His-ITPA. This removal of the tag cleaves an unstructured portion from the protein which improves the chance of success in the crystallization experiments.

After the incubation, thrombin must be removed from the sample before continuing with the purification. It was removed using benzamidine Sepharose 6B (about 200 μL resin/mL of solution) and incubating with rocking. The complex is poured through a drip column and the ITPA

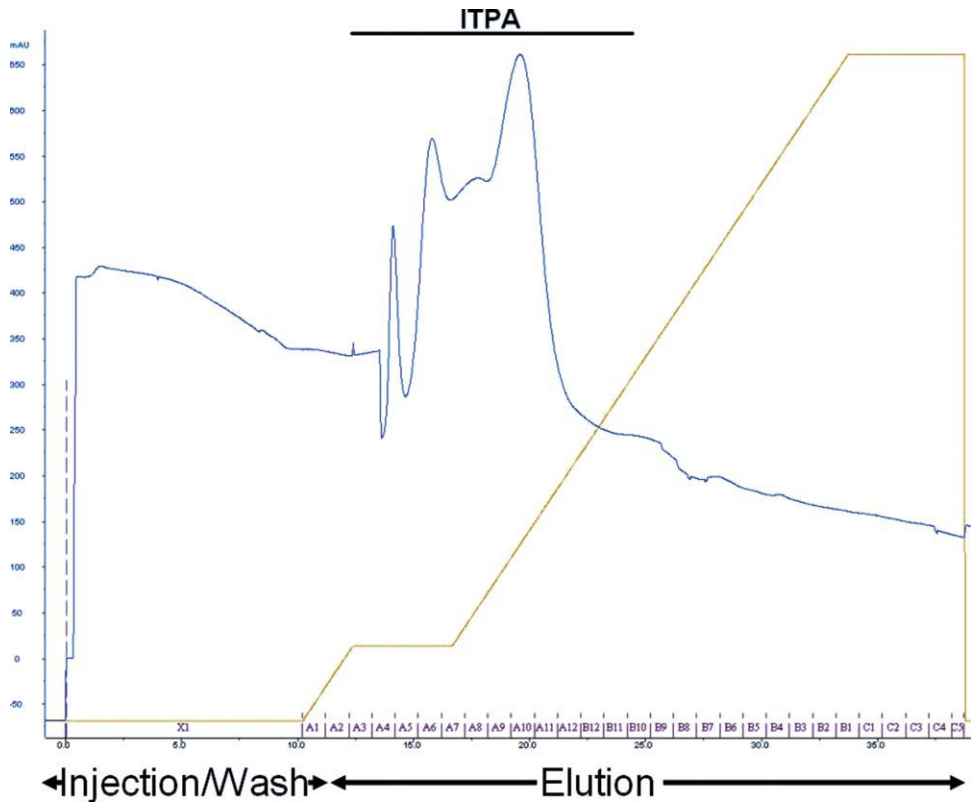

FIG. 2. **Chromatogram of secondary purification of ITPA using a Hi-Trap QFF ion-exchange column.** Blue line, UV tracing; yellow line, concentration of elution buffer; and pink dotted line, injections. *Y* axis denotes UV absorbance at 280 nm and *X* axis denotes fractions collected. ITPA elutes in 14–45% elution buffer.

protein is collected in the flow through. An aliquot of the flow through was kept to test for the effectiveness of the thrombin cleavage by gel analysis (His-ITPA is 23.6 kDa; ITPA without the His tag is 21.7 kDa).

After the flow through was collected, it was dialyzed in two sequential steps before being run on the ion-exchange column in Day 6. In our purification, we used Snakeskin dialysis tubing with the appropriate molecular weight cut-off (3,500) and dialyzed for 1 hour and then overnight at 4 °C in fresh buffer, both with stirring. Our dialysis buffer was the same as the first buffer solution that will be used in our ion-exchange chromatography run on Day 6 with fresh β-ME (20 mM BICINE, pH 8.5). Dialysis is a cost-effective technique for switching the buffer composition of an earlier purification step to one that is needed in later steps.

Day 6—Laboratory: Ion-exchange Chromatography—The sample from the overnight dialysis tubing was centrifuged to remove particulates and then was ready for loading onto the FPLC column. Students participated in programming the FPLC for the ion-exchange chromatography run. For our protein, a 1 mL HiTrap Q-FF column was used to purify ITPA from other remaining proteins based on charge separation. As ITPA is negatively charged at our buffer pH (8.5), we used an anion-exchange column and a gradient of sodium chloride as the eluting reagent (0–1 M). The chloride anions replace the ITPA protein on the column and it will come off the column during elution. ITPA eluted at approximately 300 mM sodium chloride (see Fig. 2 for chromatograph). Aliquots were taken from the flow through, washes, and peak fractions of interest for electrophoresis and stored until Day 7.

Day 7—Laboratory: Gel Electrophoresis and Spin Concentration—Ion-exchange samples were electrophoresed and protein expression/quality of purification was visualized by staining and destaining. Samples with the protein of interest were pooled and concentration was determined using the Bio-Rad protein assay (a colorimetric Bradford assay) with serum albumin as the standard. The ITPA protein needed to be about 10 mg/mL for crystallization, so the sample was concentrated using spin concentrators (5,000 molecular weight cut-off), and protein concentration was monitored using a UV–visible spectrophotometer at 595 nm (the wavelength monitored to quantify protein concentration in Bradford assays) until the desired concentration was attained. The remaining sample (now less than 2 mL) was placed in a microcentrifuge tube and stored until Day 8 at 4 °C.

A final SDS-PAGE gel was run by the students that allowed them to monitor the overall purification process. This gel contained sample aliquots from the following: uninduced cells, induced cells, centrifuged cell lysate, the insoluble pellet, pooled protein peak from the affinity column, thrombin digested sample, pooled protein from the ion-exchange column, and a molecular weight standard. After electrophoresis, the gel was Coomassie stained to monitor protein expression and purification (see Fig. 3 for SDS-PAGE results).

Day 8—Lecture: Protein Crystallization—Since the culmination of this course's laboratory experience was an

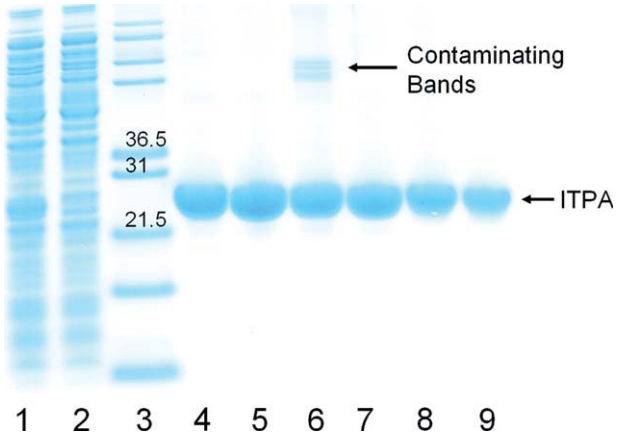

FIG. 3. **10% SDS-PAGE gel depicting the purity of ITPA after the primary round of purification via nickel affinity chromatography.** Lane 1, whole cell lysate; lane 2, flow through from column; lane 3, molecular weight marker (in kDa); and lanes 4 through 9 show fractions of relatively pure ITPA protein which were pooled and further polished using ion-exchange chromatography.

attempt at crystallizing our purified protein, the history, theory, and process of protein crystallization was discussed for this lecture period. This lecture was produced and given by the TA with the help of Dr. Gloria Borgstahl (director of the ESBF at UNMC).

Laboratory: Crystal Setup and Protein Crystallization Core Facility Tour. The equipment for growing and analyzing protein crystals is not available at UNO, where this course is taught, so the setup of crystallization experiments and subsequent analysis is done at UNMC's X-ray crystallography core facility. During this laboratory period, students are given a tour of the laboratories including an introduction to the various instrumentation, software, and so forth, which is part of the facility.

The purified ITPA is used in crystallography experiments using commercial screens and the Phoenix liquid handling robot. These experiments can be set up by hand, but use of robotics allows for more high-tech and extensive sampling of crystal conditions and smaller volumes. In each 96-well plate, 96 different chemical conditions are loaded into the reservoirs, then the solutions are mixed with the ITPA in a separate well and each well is sealed for the duration of the experiment. Our students performed 192 conditions selected for their similarity to conditions known to lead to crystal growth of ITPA in other laboratories. As actually getting an analyzable protein crystal can be difficult under the best of circumstances and often takes long incubation times for crystal seeding, each student also performed a simpler manual crystallization setup for the protein lysozyme [18]. This protein has a high success rate for crystal growth and the crystals generally start to grow within an hour, and can be visualized under a microscope. Due to the quick growth rate, the students can actually monitor the changes in crystal development over short periods of time.

Day 9—Final Exam (In-class Exam) and Take-home Exam Due, Clean-up, Instrumentation Preparation for Storage, and Check-out.

Day 10—Laboratory: Check on Crystal Growth and Turn in Laboratory Notebook—The students turned in their laboratory notebooks and met at the X-ray crystallography core facility to check on the progress of ITPA crystal growth. Crystal growth for a few of the reaction conditions within 24 hours was obtained (see Fig. 4). These crystals were not of diffraction quality, but gave information that could be used to optimize crystal growth. The conditions that led to crystal growth were explained to the students and they were instructed as to what the normal next phase would entail (analysis of the crystal and determination of structural information). If crystals are formed that are of diffraction quality in the future, Dr. Borgstahl's group would be responsible for the actual X-ray diffraction experiments as that is beyond the time-frame, scope, and cost of this class.

Typical Student Pitfalls and Opportunities for Troubleshooting

In this course, students are required to analyze the progress of their purification at each step. To move on if a problem is detected, the students must be able to come up with alternative techniques and/or determine where their error in the procedure occurred. Several examples of student errors or experimental pitfalls from the first two offerings of the course include: leaky ports and pressure problems with the FPLC, clogged chromatography columns (especially when purifying a protein from a tissue source), incorrect calculation of the amount of thrombin that should be added during the ITPA purification, setting the spectrophotometer at the incorrect wavelength to correctly read protein concentration, and use of the incorrect staining/destaining technique for the commercial gels that were used in the course. In each of the above-mentioned situations, the students as a group worked together to diagnose the issue, come up with reasons for the problem and potential resolutions, and,

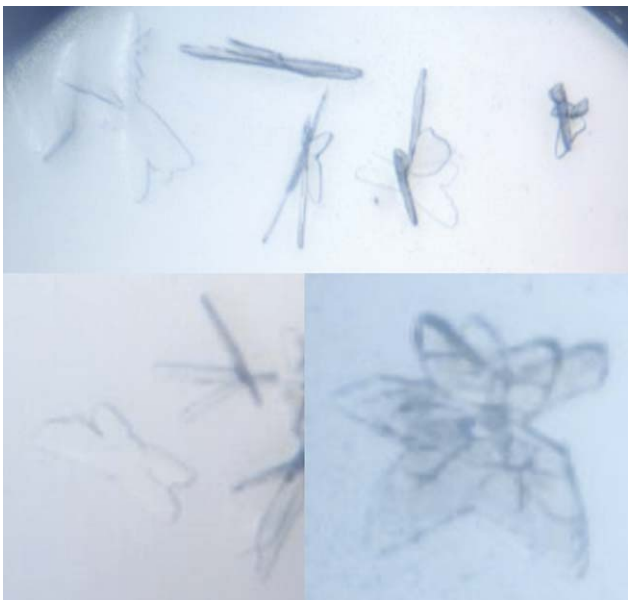

FIG. 4. **Protein crystals grown from the students' purified ITPA.**

only if they were not on the right track, were able to seek instructor or TA guidance to resolve the problems.

The chromatography runs offer the most chance of error. In the case of our course, having a graduate TA who is experienced in running the FPLC proved to be a nice asset in the overall success of the course. Problems with the computer, the collection phase, and with air or pressure in the ports are all possible during the chromatography phase of the experiment. Student calculations should be double-checked, especially if they are important in the experiment. This laboratory requires attention to clean equipment and glass- or plasticware. Solutions must be sterile filtered, and degassing is sometimes required for chromatography. Obtaining crystals in the last phase of the course will always be a potential pitfall, because crystal formation is difficult. As the class produced several hits for crystal growth on the first purification of ITPA, we are optimistic that the conditions are worked out well enough to at least optimize our chances of forming crystals, though they might not be of diffraction quality.

COURSE COMPLETION

This course has a very short timeframe, but as an upper-level course it needs to have a fairly stringent grading policy. Therefore, a combination of exams, papers, laboratory practice, and laboratory notebook points have been used as assessment tools.

Exams

Two exams are assigned. One is handed out as a take-home exam and the other is given in class. The in-class exam was given on Day 9 of the course, so it included all lectures and laboratory materials. The test covered protein purification techniques, calculations, amino acids/protein structure and chemistry, specific technique questions about their protein purification protocol from the laboratory portion of the course, development of a protein purification scheme, protein crystallization questions, and an open-laboratory notebook section that examined the students' ability to keep a good laboratory notebook throughout the course. This exam was worth one-third of their final grade.

The take-home exam was meant to be a journal searching exercise. Students were given the Protein Data Bank (PDB) identification number of a protein, or graduate students were allowed to come up with their own protein to research. They were to find the identity of the protein (or the PDB identification number if choosing their own protein) and asked to answer a series of questions about that protein. The exercise required students to use PubMed, the PDB, and the local university libraries to answer the questions fully, and references were required. The questions ranged from basic to detailed. They included PDB topics such as protein function, size, number of amino acids and polypeptides, % secondary structure, method of structural determination and to what accuracy, species of purified protein, amino acid sequence, and determining the source article. The students were also required to tell about any further structural advances that have been made for that protein if applicable, and to find a recent research article or review article and write about the current advances or scope of research for that protein. As an exercise in showing their comprehension of the course material, the students must find an article concerning purification of the protein and then describe how and why they would have purified the protein differently. In answering those questions, the students must comment on the parameters affected by changing the purification scheme, such as time, money, efficiency of purification, and retention of protein activity. The point of the exercise was to compel the students into a literature search and to read articles for information and comprehension. (The take-home exam was due the same day as the in-class exam.) This exam was worth one-third of their final grade.

Purification Paper

Students were asked to pick a purification technique (other than FPLC, which is featured in the course) and write a synopsis. This paper needed to include the basis of the purification, the methodology, types of proteins that are good candidates for this type of purification with an explanation of why, and references. This assignment was worth one-sixth of their final grade and was intended to be about five pages in length.

Laboratory Practice/Laboratory Notebook

Student behavior in the laboratory was expected to be respectful, conscientious, motivated, and efficient. The notebooks were to be maintained as if they were working on independent projects within a research laboratory. Student attitudes, work ethics, and their notebooks were evaluated throughout the 10 days, and were worth one-sixth of the final grade.

Attendance

Because of the shortened timeframe of the course, attendance was a critical component to successful completion. At this level, it would be somewhat unsuitable to actually include attendance as part of the point total, so instead it is written into the syllabus that one absence would be tolerated with a valid excuse and two absences meant an automatic failure for the course.

EVALUATION

In terms of assessment, the exams and paper demonstrated the students' ability to understand all facets of protein purification. However, the actual goal for the course was to provide students with an opportunity to work in a supervised laboratory setting that was run with modern instrumentation and realistic timing. The students themselves programmed the equipment, ran the experiments, helped with optimization and troubleshooting as the course progressed. Ultimately, this course was intended to offer advanced biochemistry credit and to provide students with

a unique set of research skills that would provide them with the hands-on experience that future employees or graduate programs are looking for in their applicants.

Student Evaluations

Six students participated in the course in each of the two offerings, including six students who took the course for undergraduate credit and six who took it for graduate credit. With such a small student population, it is difficult to draw large conclusions from the evaluation tools. However, here is an overview of the student response to the course.

The 12 students completed the normal teaching assessment tool, namely course evaluations. In the second offering, the professor and the graduate TA were both evaluated separately. The evaluations for the course both times have been very positive. The overall trend has been supportive of the pace, information gained, and the belief that the laboratory experience was truly unique and offered a more genuine research experience. The students have commented that the laboratory portion is particularly engaging and helpful and have suggested that it could be modified to become a laboratory-only course. That suggestion is being considered seriously as it would make the course potentially more "offerable" in a normal semester setting, although it would be important to maintain the real-time feel of the course. Most likely, it would have to be offered in a special format or under an altered timeframe of some sort.

The students also support the use of a TA, particularly in the laboratory setting. They felt that the TA was easy to approach, ask questions, and interact with. They liked having more of a senior student than a "professor" feel to the experimental environment. In the lecture, the only negative comment was that the TA delivered the lectures less efficiently and effectively. This is to be expected based on experience and knowledge of subject alone (hopefully), and the students agreed that the opportunity to give a lecture as a graduate student was an important experience and put forth no large complaint.

Survey Answers

In addition to the typical course evaluations, the students were asked to fill out a survey concerning the course either several months or a year after taking the course. This timing allowed the students to really gauge what they had learned and maintained from the course. The survey was completed by three of the undergraduates and four of the graduate students. All of the students indicated a belief that both the lecture and laboratory portions of the course were beneficial, one for the foundation of knowledge, the other for acquisition of bench experience and training. Even though the course has been offered to undergraduates and graduates, all of the students also indicated that the course was taught at an appropriate pace and level of difficulty. Of the six students who took the course for undergraduate credit, five are pursuing graduate educations of some type and the other student's current status is unknown. Of these students who completed the survey, all of them indicated that the course was very beneficial in showing them laboratory techniques, experimental development and planning, and other laboratory-based skills that made them excellent candidates for graduate programs and laboratory positions. Three of the undergraduate students started as laboratory technicians before starting graduate programs, and stated that the skill-base learned from the course was instrumental in receiving and succeeding at the positions granted.

Future Improvements

Probably the biggest downfall to the course as outlined above was the lack of the completion of an overall protein purification/activity table for the whole procedure. In future offerings where ITPA will still be the protein purified, an ITPA enzyme activity assay, in combination with calculations of fold purification at each step of the purification protocol, will be used for the students to complete this table. One possibility is use of a relatively simple colorimetric malachite green assay to measure ITPA activity (see ref. 19 for details on this assay).

The other issue is that the tight timeframe of the course allows little to no room for student error during the procedure. The addition of the activity assay to the sequence was tried in Summer 2010 and the course days were extended from 9 to 14 days, allowing completion of the assay, monitoring of crystal growth, and room for fixing small problems encountered along the procedure without losing the only opportunity to have a successful purification run. If the course runs completely on schedule with no major experimental flaws, the purification, crystallization, and activity assays can be completed in 10 laboratory days. As an example, the 3-week timeframe of the 2010 course allowed time for experimental pitfalls, optimization, and the addition of a new activity assay, with the extra laboratory time only being used if needed to not detract from the real-time pace of the experiment. The experiment ran on schedule with the only problem being a pressure issue on the first FPLC step, which required the students to complete additional centrifugation runs to clear the supernatant more thoroughly of particulate and extra buffer to be run through the FPLC tubing to clear the lines. These steps required the students to stay late on this day of laboratory, but they were able to resolve the problems within an extra hour and proceed with the experiment. As no major experimental issues were encountered, the students used the "extra" days to write their final papers, study for the in-class exam, and finish their laboratory notebooks.

SUMMARY

The purpose of this course is to give students a solid foundation and unique advantage as they prepare for the next step in their scientific careers or in their graduate training. It is beneficial regardless of the next phase for the students, whether it be graduate school, professional school, or a scientific industry position. The material presented and the laboratory content is meant to give stu-

dents a solid background in the field of protein purification and characterization and to provide adequate bench and instrumentation training to produce proficient laboratory personnel, including critical thinking skills, experimental troubleshooting and optimization, and notebook writing skills. All of these traits are important to potential employers and for postbaccalaureate programs. It is our intention that students walk away from this experience with an understanding of protein purification and characterization, a firm set of research skills, and a positive perspective toward experimentation and instrumentation that is different from the atmosphere in conventional undergraduate laboratories. Also, for the TA, the course offers an opportunity to build a strong profile for an academic position.

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