JOURNAL OF CHEMICAL EDUCATION

Laboratory Experiment

pubs.acs.org/jchemeduc

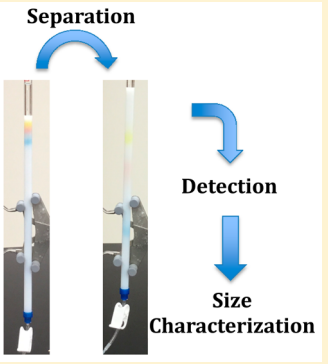
A Two-Week Guided Inquiry Protein Separation and Detection Experiment for Undergraduate Biochemistry

James P. Carolan*,† and Kathleen V. Nolta‡

†Program in Chemical Biology and ‡Department of Chemistry, University of Michigan, Ann Arbor, Michigan 48109, United States

Supporting Information

ABSTRACT: A laboratory experiment for teaching protein separation and detection in an undergraduate biochemistry laboratory course is described. This experiment, performed in two, 4 h laboratory periods, incorporates guided inquiry principles to introduce students to the concepts behind and difficulties of protein purification. After using size-exclusion chromatography to separate a mixture of proteins, students utilize a colorimetric enzymatic assay and an immunoassay to determine the location of individual mixture components. Students proceed to determine the molecular weight of each protein using gel electrophoresis and generated mass spectrometric data. Completing this experiment provides students with an opportunity to expediently separate proteins while learning about protein characterization.



KEYWORDS: *Upper-Division Undergraduate, Biochemistry, Laboratory Instruction, Hands-On Learning/Manipulatives, Inquiry-Based/Discovery Learning, Chromatography, Electrophoresis, Enzymes, Molecular Recognition, Proteins/Peptides*

Protein production, purification, and characterization form the focus of countless efforts in the biochemical research laboratory. Navigating these techniques often requires a keen understanding of concepts such as chromatography, protein−antibody recognition, or enzymatic catalysis, marking these techniques as ideal for introduction in an undergraduate biochemistry laboratory course. Accordingly, protein isolation and characterization have been the subjects of numerous educational laboratory experiments for this setting.[1−4] However, many of these efforts can often be difficult to adapt and integrate into curricula because they often require a full semester of instruction[5−10] or expensive instrumentation.[11−14] Here is described an alternative, adaptable experiment centering on protein separation and detection that aims to improve on student conceptual understanding using elements of guided inquiry.

In crafting this experiment, a guided inquiry approach was believed to be beneficial for students based on the demonstrated advantages associated with discovery-oriented learning.[15] Recent educational reforms have emphasized decreasing instructional-centered models of teaching in favor of enhancing the learning process of individual students.[16] Indeed, the standards and recommendations recently released by the National Research Council[17] highlight the importance of incorporating inquiry into student practices as a means of increasing scientific literacy and understanding of scientific process. Guided inquiry laboratories expand on the framework of traditional expository experimentation by enlarging the responsibility thrust upon the students.[15,18] While students are presented a problem and protocols to navigate toward a solution, it is largely up to the student to develop an overall method toward this destination and to determine an optimal method for communicating conclusions. With the student acting as the driver in his own learning, there is a greater opportunity to engage in higher level cognitive processes, such as analysis and evaluation.[19−21] Major critiques with this method center on the increased instructor effort necessary for development and implementation, demonstrating the need for robust, adaptable experiments that can be practically employed.[18]

The goals for this experiment center on succinctly introducing students to (1) the fundamental concepts and challenges associated with separating proteins and to (2) different biomolecular interactions, such as those observed in protein−protein or protein−substrate recognition, while gaining the benefits of a guided inquiry format.[22,23] In this exercise, students are provided a designed mixture of proteins and are asked to determine a way to isolate and ascertain the molecular weight of each component. To represent different protein classes and features, each component is distinguishable either visually, by enzymatic assay, or by immunoassay. Following size-exclusion chromatographic separation, students determine which of their collected fractions contains each protein using several different techniques. Each available method is tailored to provide rapid data collection for student analysis. Students utilize sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and convoluted mass spectra

Received: August 26, 2015
Revised: December 7, 2015

ACS Publications © XXXX American Chemical Society and Division of Chemical Education, Inc.

DOI: 10.1021/acs.jchemed.5b00697
J. Chem. Educ. XXXX, XXX, XXX−XXX

of each protein component as alternative methods for molecular weight determination and must rationalize any conflicts in their collected data. Throughout, students are tasked with utilizing collected data to determine how to approach subsequent experimental steps.

This guided inquiry experiment is designed to be concise and adaptable, allowing it to be easily incorporated into a biochemistry laboratory curriculum, providing a valuable method for introducing students to protein separation and detection.

EXPERIMENTAL DETAILS

Students work in groups of two to four depending on material limitations. This experiment was performed over two, 4 h periods following a 1 h lecture occurring during the week prior. The experiment is divided into two parts, outlined in Table 1, allowing for a break to occur between sessions after completing Part I.

Table 1. Experimental Workflow

Week	Lab Activity
Week 1	Students are provided a protein mixture
	Separation by column chromatography
	Visual analysis identifies fractions containing cytochrome c
	Analysis by ELISA immunoassay identifies fractions containing BSA
	Colorimetric assay identifies fractions containing β-galactosidase
Week 2	Students analyze samples from fractions with SDS-PAGE to aid in protein size determination
	Using collected data and provided MS spectra, students determine size of individual protein from mixture
	Students describe their results and justify their methodology, process, and conclusions

Part I: Separation and Identification of Proteins

Students are provided a mixture of proteins including cytochrome c (12 kDa), bovine serum albumin (BSA, 66 kDa), and *E. coli* β-galactosidase (460 kDa) along with a mixture of yellow riboflavin and blue dextran for determining maximum and minimum column elution volumes. Using a size-exclusion column, students collect fractions of eluent using the dyed molecules as beginning and end point indicators. Following separation, students are instructed to determine the size of each protein, potentially using provided techniques or information.

Students proceed to determine which fractions contain each individual protein. Fractions containing cytochrome c are readily identifiable by a visible red tint. To identify fractions containing β-galactosidase, students utilize a colorimetric assay fitted to β-galactosidase activity detection. Chlorophenol red-β-D-galactoside (CPRG) is a substrate for β-galactosidase that provides a quantitative and readily visible color change in buffer medium and is frequently utilized as a direct method of detecting the presence and concentration of β-galactosidase.

Additionally, an enzyme-linked immunosorbent assay (ELISA) was tailored for compatibility with a BSA-recognizing antibody to produce an immunoassay for BSA detection. This method allows for the detection of the presence of a nonenzymatic protein indirectly through the use of an antibody−enzyme conjugate capable of oxidizing the colorless substrate 3,3′,5,5′-tetramethylbenzidine (TMB) to a diimine form that produces blue color in solution. Students utilize these three methods (visual inspection, colorimetric assay, and immunoassay) to determine which fractions contain each component of their mixture. Students typically collect more fractions than there are wells available in a provided polyacrylamide gel for SDS-PAGE. As such, prior to continuing to Part II, students must process their data to determine which fractions to assess via SDS-PAGE.

Part II: Size Determination of Each Protein

Students prepare samples for loading on a precast polyacrylamide mini-gel by mixing with a provided loading dye and denaturing with high heat. Prepared samples, alongside a marker standard, are loaded on to a gel and subjected to electrophoresis. Following SDS-PAGE separation, students stain their gels in Coomassie solution and proceed to destain their gels for visualization. Students determine molecular weights by measuring migration distances of marker proteins, constructing a standard line, and comparing migration distances of their sample proteins.

Additionally, mass spectra of each purified protein were collected (or, when unavailable, a representative spectrum was constructed) by instructors. Students are provided these, with each spectrum labeled with the position in which the protein eluted (and no other identifying factors). Students are tasked with utilizing the differing (and potentially conflicting) data collected from assaying, electrophoresis, and mass spectrometry to determine protein elution order and molecular weight.

After completing the physical component of this exercise, students report the determined size of each protein in their mixture in a written laboratory report. This report includes a detailed explanation of how they came to each determination, how they reconciled and prioritized any potential conflicting data, and a discussion of the benefits and limitations of each technique that contributed to their final results. Included in this report is qualitative error analysis with comments on potential causes of discrepancies in each students' reported values.

HAZARDS

TMB is a mild irritant. CPRG and Coomassie reagent are not hazardous but will stain skin and fabrics. Unpolymerized acrylamide is hazardous and should not come into contact with skin. Methanol and acetic acid are skin and eye irritants and should be handled with caution. Students are required to wear personal protective equipment at all times including gloves, eyewear, and smock.

RESULTS

Student Results

This experiment was assessed during an implementation involving 87 students in three separate sections. The majority of students (69 of 87) attempting this experiment were able to successfully design a path to complete the exercise and provide an acceptable rationale for their determined protein molecular weights. Overwhelmingly, students constructed a path involving the utilization of both identification assays, SDS-PAGE analysis for confirmation of protein identity, and mass spectral data for higher accuracy size determination.

Students readily identified fractions containing β-galactosidase visually, noting clear color changes produced by the presence of galactosidase activity (Figure 1). Despite this being a time-sensitive assay, this portion of the laboratory proved remarkably robust, and few issues were reported.

DOI: 10.1021/acs.jchemed.5b00697
J. Chem. Educ. XXXX, XXX, XXX−XXX

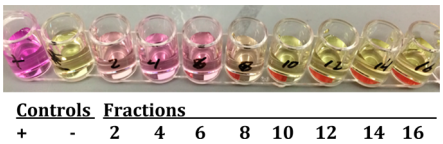


Figure 1. Detection of β-galactosidase activity. Control solutions (+,−) or samples from individual fractions of collected eluent are incubated with yellow CPRG. Cleavage of the galactoside bond results in the formation of red color in solution, indicating the presence of β-galactosidase, observed at highest concentration in fraction 6.

Similarly, students were able to identify fractions containing BSA by visual inspection through ELISA analysis (Figure 2). A

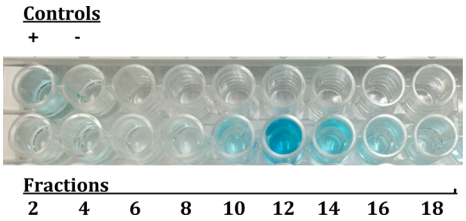


Figure 2. Detecting the presence of BSA. Samples from individual fractions of collected eluent are incubated in individual wells of a microplate, allowing for protein adherence. Wells are successively incubated with a BSA-recognizing antibody and a horseradish peroxidase-linked antibody. After removing unbound antibody, wells are incubated with TMB, where subsequent development of blue color indirectly indicates the presence of BSA (as in fractions 10, 12, and 14).

common technical issue reported by students was high background in color development, resulting in difficulty interpreting ELISA data; color development is time sensitive, and it is likely that slow reagent addition played a role, though incomplete washing of wells cannot be dismissed as a confounding factor.

The presence of individual proteins was confirmed through SDS-PAGE analysis (Figure 3). Despite a large size difference between β-galactosidase (460 kDa) and BSA (66 kDa), coelution in fractions was common; however, severe overlap was not as common in assay data (owing to lower detection

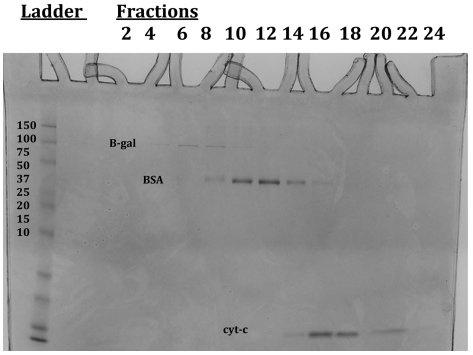


Figure 3. SDS-PAGE results displaying electrophoresed proteins. Samples from individual collected fractions of eluent were denatured and electrophoresed through a polyacrylamide gel. Following gel stain with Coomassie blue, proteins were visualized (depicted here in grayscale). The annotation by each set of bands indicates the protein identity. B-gal: β-galactosidase, 460 kDa (115 kDa subunits). BSA: 66 kDa. cyt-c: 12 kDa.

limits by Coomassie staining), and this did not frequently inhibit subsequent analyses.

Analysis of provided mass spectral data was routine and did not produce significant issues. However, one common error arose when students attempt to rationalize differences in the β-galactosidase mass observed in SDS-PAGE analysis and in analysis of a mass spectrum for the protein. As β-galactosidase exists as a homotetramer, the denaturing conditions of SDS-PAGE yield a deceptively small mass; the majority of students were able to identify this and provide an attempted explanation, but a significant minority were unable to reconcile the mass differences. Further, many included this in error analysis, believing it to be a technical error.

Student Response

Students engaged in group retrospective analysis[24,25] to rate and rank the described protein isolation and assaying experiment in comparison to other traditional experiments in the course. Group retrospective analysis provides a method for small groups of students to collectively come to a consensus on assessments of each experiment, circumventing limitations that arise in individual retrospective analysis. Here, groups of students were tasked with rating each experiment in the course on its ability to accomplish a defined educational goal, relating to: the students' perceived utility and relevance of each experiment; how well completing each experiment emulated engaging in authentic research; and how well each experiment engaged students in the generation and critical evaluation of data (see Supporting Information for complete prompts). Subsequently, students ranked each experiment, relative to the others, in its ability to accomplish each goal. Eighty-seven students, working collectively in 15 groups of 5 or 6, provided feedback. Ratings were collected as numerical inputs correlating with how well student groups perceived that an education goal was achieved, from 'significantly accomplished' to 'not accomplished at all'. Rankings were collected and utilized to validate the data collected from rating questions, monitoring the reliability of the ratings by referencing each rating against the experimental rankings. The results of the experiment ratings, including standard error of the mean and a statistical p-value, are provided in the instructors' notes (Table S1).

On the basis of the feedback collected in this assessment, student groups indicated that they perceived this new experiment to have high utility and relevance and believed that this experiment was able to considerably prepare them for work in a research environment; significantly, this exercise was able to accomplish these goals as or more capably than other experiments in the course (rankings not shown). Additionally, student groups indicated that this exercise accomplished the goal of providing an opportunity to generate and critically evaluate data, a key benefit of many guided inquiry laboratories,[18] though not significantly moreso than other experiments in the course. One potential remedy to this would be to incorporate a quantitative component to each assay; for example, the use of a plate reader would allow for more precise determination of fractions containing each protein of interest. Taken together, this feedback indicates that student perception of the experiment was positive, especially in comparison to other experiments in the course.

To aid in determining if long-term cognitive gains may have been achieved, an end of semester assessment was conducted. In this written evaluation, students are prompted with an experimental issue centering on protein purification and are

DOI: 10.1021/acs.jchemed.5b00697
J. Chem. Educ. XXXX, XXX, XXX−XXX

provided several different hypotheses as to why the observed experimental issue has occurred. Students are tasked with providing written feedback that includes an evaluation of each hypothesis and a described plan of further experimental investigation (see Supporting Information for the written assessment). This assessment was constructed not as an attempt to elicit "correct" responses from students but rather to determine if students are able to recontextualize concepts from a laboratory experiment and apply them to new problems (as they would in a research setting). As such, detailed analysis of the assessment results is difficult. However, a course instructor independent to the design of this study rated each students' response using a self-designed rubric, determining a capability to provide complete evaluations for two of three hypotheses as above average and three of three as exceptional (see Supporting Information for the grading rubric). Encouragingly, it was found that 40% of students (35 of 88) were able to provide above average responses, and 19% (17 of 88) provided exceptional responses.

DISCUSSION

This experiment has been implemented three times. Typical course sizes include three sections of 25−35 students, primarily sophomores and juniors that have previously or are concurrently taking an introductory biochemistry lecture and have at least one semester of experience in a research laboratory (though this is not a requirement).

In crafting this experiment, a target was employed of effectively introducing students to techniques involved in protein separation and, importantly, to biomolecular interactions that allow for protein detection. Regarding the first point, students gain exposure to five different techniques involved in protein purification and characterization and utilize each collectively to pursue a solution to an experimental task. Second, in prior years where students were exposed to similar techniques but in disparate, expository laboratories, students often struggled to distinguish between the molecular events occurring while conducting different assays; for example, students commonly displayed difficulty comprehending how a colorimetric quantitation assay, such as a Bradford assay, is employed differently than a colorimetric detection assay such as the above-described ELISA. By completing this experiment, students are able to implement and juxtapose different protein detection techniques. In turn, students gain a greater understanding of how proteins interact with different molecules and, importantly, how these interactions can be taken advantage of to aid in protein identification.

Students responded positively to the guided inquiry format of this exercise. As noted above, students overwhelmingly adopted a near identical path to navigating this laboratory, indicating that the guiding provided is likely too explicit. Despite this, student-provided feedback demonstrated that the majority found this exercise, the only experiment in the course to adopt this format, to best emulate useful methodologies necessary in conducting biochemical research. Several students indicated that the opportunity to use different methods to solve a larger problem was helpful. Importantly, many students were able to later demonstrate their conceptual understanding of protein interactions by proposing methods for investigating a similar, but unrelated, experimental subject in a written assessment.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available on the ACS Publications website at DOI: 10.1021/acs.jchemed.5b00697.

List of materials and reagents, experimental preparation notes, instructors' prelaboratory lecture, student protocol, assessment questions (PDF, DOCX)

AUTHOR INFORMATION

Corresponding Author

*E-mail: jcarolan@umich.edu.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

Portions of this work were supported by a GAANN fellowship (P200A120196) awarded to J.P.C. The authors would like to thank Anna Mapp and Brian Coppola for important guidance during the design of this work, Ginger Shultz for providing valuable feedback during the preparation of this manuscript, and Mou-Chi Cheng and Shawn Eady for assistance in implementation.

REFERENCES

(1) Bowen, R.; Hartung, R.; Gindt, Y. M. A Simple Protein Purification and Folding Experiment for General Chemistry Laboratory. *J. Chem. Educ.* **2000**, *77*, 1456−1457.

(2) Winter, R. T.; van Beek, H. L.; Fraaije, M. W. The Nose Knows: Biotechnological Production of Vanillin. *J. Chem. Educ.* **2012**, *89*, 258−261.

(3) Karamanos, Y. Purification and Characterisation of Lactate Dehydrogenase: an Undergraduate Biochemistry Laboratory Experiment. *Adv.Biochem.* **2014**, *2*, 14−23.

(4) Hunnes, C. H. Isolation, Purification, and Characterization of Bovine Heart and Yeast Cytochromes *C*. *Biochem. Mol. Biol. Educ.* **2003**, *31*, 242−248.

(5) Knutson, K.; Smith, J.; Wallert, M. A.; Provost, J. J. Bringing the Excitement and Motivation of Research to Students; Using Inquiry and Research-Based Learning in a Year-Long Biochemistry Laboratory. *Biochem. Mol. Biol. Educ.* **2010**, *38*, 317−323.

(6) Peterson, M. J.; Snyder, W. K.; Westerman, S.; McFarland, B. J. Preparative Protein Production From Inclusion Bodies and Crystallization: a Seven-Week Biochemistry Sequence. *J. Chem. Educ.* **2011**, *88*, 986−989.

(7) Jewett, K.; Sandwick, R. K. Ribose 5-Phosphate Isomerase Investigations for the Undergraduate Biochemistry Laboratory. *J. Chem. Educ.* **2011**, *88*, 1170−1174.

(8) McKenzie, N.; McNulty, J.; McLeod, D.; McFadden, M.; Balachandran, N. Synthesizing Novel Anthraquinone Natural Product-Like Compounds to Investigate Protein−Ligand Interactions in Both an in Vitro and in Vivo Assay: an Integrated Research-Based Third-Year Chemical Biology Laboratory Course. *J. Chem. Educ.* **2012**, *89*, 743−749.

(9) Johanson, K. E.; Watt, T. J.; McIntyre, N. R.; Thompson, M. Purification and Characterization of Enzymes From Yeast: an Extended Undergraduate Laboratory Sequence for Large Classes. *Biochem. Mol. Biol. Educ.* **2013**, *41*, 251−261.

(10) Murthy, P. P. N.; Thompson, M.; Hungwe, K. Development of a Semester-Long, Inquiry-Based Laboratory Course in Upper-Level Biochemistry and Molecular Biology. *J. Chem. Educ.* **2014**, *91*, 1909−1917.

(11) Counterman, A. E.; Thompson, M. S.; Clemmer, D. E. Identifying a Protein by MALDI−TOF Mass Spectrometry. *J. Chem. Educ.* **2003**, *80*, 177−180.

DOI: 10.1021/acs.jchemed.5b00697
J. Chem. Educ. XXXX, XXX, XXX−XXX

(12) Mascotti, D. P.; Waner, M. J. Complementary Spectroscopic Assays for Investigating Protein−Ligand Binding Activity: a Project for the Advanced Chemistry Laboratory. *J. Chem. Educ.* **2010**, *87*, 735−738.
(13) Southard, J. N. Protein Analysis Using Real-Time PCR Instrumentation: Incorporation in an Integrated, Inquiry-Based Project. *Biochem. Mol. Biol. Educ.* **2014**, *42*, 142−151.
(14) Johnson, R. J.; Savas, C. J.; Kartje, Z.; Hoops, G. C. Rapid and Adaptable Measurement of Protein Thermal Stability by Differential Scanning Fluorimetry: Updating a Common Biochemical Laboratory Experiment. *J. Chem. Educ.* **2014**, *91*, 1077−1080.
(15) Coppola, B. P. Laboratory Instruction: Ensuring an Active Learning Experience. In *McKeachies Teaching Tips Strategies, Research, and Theory for College and University Teachers*; Svinicki, M., McKeachie, W. J., Eds.; Cengage Learning: Belmont, CA, 2010; pp 280−291.
(16) Barr, R. B.; Tagg, J. From Teaching to Learning—A New Paradigm for Undergraduate Education. *Change* **1995**, *27*, 12−26.
(17) National Research Council. *A Framework for K-12 Science Education*; National Academies Press: Washington, D.C., 2012.
(18) Domin, D. S. A Review of Laboratory Instruction Styles. *J. Chem. Educ.* **1999**, *76*, 543−547.
(19) Bloom, B. S.; Engelhardt, M. D.; Furst, E. J.; Krathwohl, D. R. *Taxonomy of Educational Objectives: The Clasification of Educational Goals*; David McKay Company: New York, 1956.
(20) Horowitz, G. A Discovery Approach to Three Organic Laboratory Techniques: Extraction, Recrystallization, and Distillation. *J. Chem. Educ.* **2003**, *80*, 1039−1041.
(21) Conway, C. J. Effects of Guided Inquiry Versus Lecture Instruction on Final Grade Distribution in a One-Semester Organic and Biochemistry Course. *J. Chem. Educ.* **2014**, *91*, 480−483.
(22) Spencer, J. N. New Approaches to Chemistry Teaching. 2005 George C. Pimental Award. *J. Chem. Educ.* **2006**, *83*, 528−533.
(23) Weaver, G. C.; Russell, C. B.; Wink, D. J. Inquiry-Based and Research-Based Laboratory Pedagogies in Undergraduate Science. *Nat. Chem. Biol.* **2008**, *4*, 577−580.
(24) Poggio, J. P.; Miller, M. D.; Glasnapp, D. R. The Adequacy of Retrospective Judgments to Establish Instructional Validity. *Educ. Psychol. Meas.* **1987**, *47*, 783−793.
(25) Moy, C. L.; Locke, J. R.; Coppola, B. P.; McNeil, A. J. Improving Science Education and Understanding Through Editing Wikipedia. *J. Chem. Educ.* **2010**, *87*, 1159−1162.

DOI: 10.1021/acs.jchemed.5b00697
J. Chem. Educ. XXXX, XXX, XXX−XXX