JOURNAL OF CHEMICAL EDUCATION

Laboratory Experiment

pubs.acs.org/jchemeduc

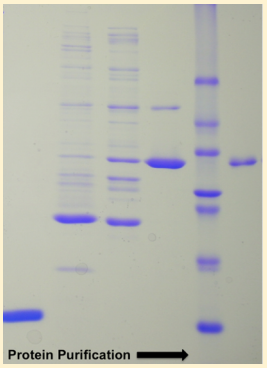
Puri cation and Electrophoretic Characterization of Lactate Dehydrogenase from Mammalian Blood: A Di erent Twist on a Classic Experiment

Linda S. Brunauer*

Department of Chemistry and Biochemistry, Santa Clara University, Santa Clara, California 95053, United States

* Supporting Information

ABSTRACT: A multiweek protein puri cation suite, suitable for upper-division biochemistry or biotechnology undergraduate students, is described. Students work in small teams to isolate the enzyme lactate dehydrogenase (LDH) from a nontraditional tissue source, mammalian blood, using a sequence of three column chromatographic procedures: ion-exchange, size exclusion, and a nity chromatography. Protein and enzyme activity elution pro les are determined by graphical analysis of assay data collected using rapid microplate spectrophotometric assays. Students perform quantitative assays on LDH pools and use these data to build a puri cation table for use in evaluating the protocol. The protocol typically generates nal overall fold-puri cations from 1500 to 2500 and activity recoveries of 45 60%. Electrophoretic separations in both denaturing and native-gel format are analyzed both visually and by use of commercial digital analysis software to assess the isoenzyme pattern of isolated LDH and to further evaluate the puri cation. Assessment of student work revealed a high level of achievement of course learning goals that include development of critical thinking skills required to (1) critically evaluate an experimental protocol and (2) draw conclusions based on a variety of forms of experimental data.



KEYWORDS: Upper-Division Undergraduate, Biochemistry, Laboratory Instruction, Hands-On Learning/Manipulatives, Bioanalytical Chemistry, Chromatography, Electrophoresis, Enzymes, Ion Exchange

INTRODUCTION

The development of a biochemistry laboratory course that introduces students to classical and modern biochemical techniques often involves overcoming a number of challenges, one being limited resources. Chief among the resources that are often in short supply are (1) funds for the purchase of materials and disposal of waste, (2) technical support to prepare large quantities of needed reagents, and (3) modern instrumentation adequate to allow students to complete the various tasks in a reasonable amount of time.

A second challenge is to design a suite of laboratory experiences that allows for a multiweek project that provides, when possible, protocols mirroring the materials and methods found in the primary literature rather than those found in a more cookbook style of laboratory manual. This ts well with the goal of the upper division laboratory course to provide a window into the world of research, requiring students to pursue tasks such as protocol optimization and timely analysis of the large amounts of data that such endeavors produce. This goal is of critical importance both to students who will eventually progress to graduate school and to students who will enter into industrial positions upon graduation.

A third challenge is that of providing an environment that stimulates and supports cooperative learning among the individuals in a student laboratory team, encouraging students to have joint responsibility for the success or failure of their projects.

In redesigning our Biochemical Techniques course, we sought to develop a project that would embrace these challenges. We chose the enzyme lactate dehydrogenase (LDH) as the target molecule for isolation and electrophoretic characterization. While previous papers[1 6] and laboratory manuals[7,8] have described the isolation of this protein from mammalian heart or skeletal muscle, we chose to do the isolation from mammalian blood, a nontraditional source that o ers a number of advantages. (1) Red blood cells (RBC) are easily isolated in pure form from whole blood by repeated centrifugation in isotonic saline; (2) the cytosolic fraction, or membrane-free lysate, is generated by simple hypotonic lysis followed by centrifugation; (3) the resulting lysate yields LDH activity that is stable for several weeks, allowing a measure of exibility with regard to the timing of its preparation for the class; (4) the lack of any signi cant protease activity in the lysate alleviates the need to add expensive and toxic protease inhibitors; (5) the fold puri cation and percentage recovery of activity are far superior to those typically reported for the isolation of LDH using mammalian muscle tissues.[2 4]

The puri cation protocol utilizes a set of three column chromatographic procedures that have found wide application in biochemistry: ion-exchange, size exclusion, and a nity

Received: August 31, 2015
Revised: March 1, 2016
Published: March 22, 2016

ACS Publications © 2016 American Chemical Society and Division of Chemical Education, Inc.

DOI: 10.1021/acs.jchemed.5b00713
J. Chem. Educ. 2016, 93, 1108 1114

chromatography. Rapid microscale analysis of protein concentration and LDH activity in column fractions, as well as the starting material and the three chromatographic puri cation "pools", is accomplished by use of a microplate reader and accompanying processing software. Electrophoretic behavior is investigated by native polyacrylamide gel electrophoresis (PAGE), denaturing SDS polyacrylamide gel electrophoresis (SDS PAGE), and Western blot analysis using visual examination as well as computer-assisted densitometric analysis. Students summarize their results in a formal written journal style report, orally defend a poster presentation of their work, or prepare a series of progress reports, one after each stage of the puri cation and a short summary analysis at the end of the multiweek project. The second half of the term students utilize the wide-range of skills they have developed by embarking on independent or small group research-based projects optimizing protocols from the literature.

Learning goals for this project are listed in Box 1. The current study describes the progress made in the development of the experimental protocol and provides an assessment of student success in attaining speci c learning goals and objectives.

Box 1. Learning Goals

Goal 1: Acquire an understanding of the general features of a variety of common chromatography resins and understand how intermolecular forces in uence the elution behavior of proteins during separation via column chromatography

Goal 2: Develop pro ciency in carrying out microscale analytical procedures and in utilizing appropriate software to do necessary data analysis

Goal 3: Gain experience in carrying out a variety of commonly encountered electrophoretic separations and in utilizing appropriate software to carry out necessary data analysis

Goal 3: Demonstrate the importance of deductive reasoning in using various pieces of analytical data to assess the e cacy of a puri cation protocol and to evaluate the quality of data generated by an experimental protocol

Goal 5: Gain experience in handling biochemical reagents in a safe manner

EXPERIMENTAL METHODS SUMMARY

A detailed description of the experiment is provided in the Supporting Information. Students worked in groups of 2 4 to complete this experiment over the course of 6 weeks, meeting for 4 h laboratory periods twice a week. A brief synopsis of the order in which the tasks are performed is given below.

Materials and Software

Rabbit blood was purchased from Hemostat Laboratories (Dixon, CA). Anti-LDH-H-subunit monoclonal primary antibody, chromatography resins, LDH standards, and electrophoresis reagents were purchased from Sigma-Aldrich (St. Louis, MO). Precision Plus prestained molecular weight standards for electrophoresis were obtained from Bio-Rad laboratories (Hercules, CA). Goat-anti-mouse-alkaline-phosphatase secondary antibody was purchased from Rockland Immunochemicals (Limerick, PA). All other reagents were reagent grade. UN-SCAN-IT-gel software (Silk Scienti c, Orem, UT) was used to perform densitometric analysis on stained electrophoresis gels. SoftMax Pro software (Molecular Devices, Sunnyvale, CA) was used to analyze microplate assay data.

Synopsis of the Experimental Suite

First Week. Students begin the puri cation by carrying out ion-exchange column chromatography. They obtain a sample of membrane-free lysate prepared by hypotonic lysis of washed rabbit RBC (Stage 1) and apply it to a column of Carboxymethyl-Sephadex (CM-Sephadex). The column is developed overnight at 4 °C by isocratic elution in phosphate bu er. The following day students perform spectrophotometric assays to assess elution pro les for protein and LDH activity. Protein elution is determined by mixing small aliquots of each column fraction with Bradford protein assay reagent in a 96-well plate. This reagent contains Coomassie Brilliant Blue G-250, a dye that exhibits an absorbance shift under acidic conditions to a blue form upon binding to proteins.[9] The increase in absorbance at 595 nm is proportional to the concentration of protein in the sample and may be readily determined in a microplate reader. The relative concentration of LDH activity in each column fraction is determined by mixing a small aliquot of the fraction with an assay "cocktail" containing phosphate bu er, sodium pyruvate, and NADH in a 96 well plate and then measuring the rate of decrease in absorbance at 340 nm, associated with oxidation of NADH,[10] in a microplate reader. Data are presented as the V_{max} or initial rate as determined by the use of SoftMax Pro analysis software. The data are used to prepare a graphical representation of the relative elution of protein (absorbance at 595 nm) and LDH activity (V_{max}); students consult with the instructor to determine which column fractions to combine to maximize recovery of LDH. The dilute combined pool is concentrated using centrifugal ultra ltration to prepare the nal CM-LDH pool (stage 2).

Second Week. Students continue the puri cation by applying 75% of their CM-LDH pool onto a Sephacryl S-200 gel ltration column, developing the column overnight at 4 °C in phosphate bu er supplemented with ammonium sulfate. The following day, they perform relative protein concentration[9] and LDH activity[10] microplate assays on column fractions as noted above and prepare a graphical representation of the data. They then consult with the instructor to determine which fractions to combine to maximize the recovery of enzyme activity and minimize contamination from closely eluting proteins. The desired fractions are combined to prepare the S-200-LDH pool (stage 3).

Third Week. Students continue the puri cation by applying 75% of their S-200-LDH pool onto a Cibacron Blue a nity chromatography column, using NADH-containing phosphate bu er to e ect a nity elution from the resin. They perform relative protein concentration[9] and LDH activity[10] microplate assays on column fractions as noted above, prepare a graphical representation of the data the same day and combine fractions containing LDH. The dilute pool is then concentrated using centrifugal ultra ltration to prepare the Cibacron-LDH pool (stage 4).

Fourth Week. Students perform quantitative LDH activity microplate assays[10] to determine the activity in units per milliliter (units/mL) for each pool. V_{max} data, as well as information regarding the volume of each pool used, dilution factors, well path length, and the millimolar extinction coe cient of NADH at 340 nm, are used to determine the concentration of LDH activity in each pool. In this experiment, a "unit" of LDH activity

DOI: 10.1021/acs.jchemed.5b00713
J. Chem. Educ. 2016, 93, 1108 1114

(1) Katchevich, D.; Hofstein, A.; Mamlok-Naaman, R. Argumentation in the Chemistry Laboratory: Inquiry and Confirmatory Experiments. Res. Sci. Educ. , 43, 317 345
(2) Lunetta, V. N.; Hofstein, A.; Clough, M. P. Learning and Teaching in the School Science Laboratory: An Analysis of Research, Theory, and Practice. In Handbook of research on science education; Lederman, N., Abl, S., Eds.; Lawrence Erlbaum: Mahwah, NJ, 2007; pp 393 441.
(3) Hofstein, A.; Kind, P. M. Learning in and from Science Laboratories. In Second International Handbook of Science Education; Fraser, B. J., Kenneth, T., McRobbie, C. J., Eds.; Springer International Handbooks of Education; Springer: Netherlands, 2011; Vol. 24, pp 189 207.
(4) Fay, M. E.; Grove, N. P.; Towns, M. H.; Bretz, S. L. A Rubric to Characterize Inquiry in the Undergraduate Chemistry Laboratory. Chem. Educ. Res. Pract. , 8 (2), 212 219.
(5) Coppola, B. P. The Distinctiveness of Higher Education. J. Chem. Educ. , 90, 955 956.
(6) Dillner, D. K.; Ferrante, R. F.; Fitzgerald, J. P.; Schroeder, M. J. Integrated Laboratories: Laying the Foundation for Undergraduate Research Experiences. J. Chem. Educ. , 88, 1203 1209.
(7) Dasgupta, A. P.; Anderson, T. R.; Pelaez, N. Development and Validation of a Rubric for Diagnosing Students' Experimental Design Knowledge and Difficulties. CBE Life Sci. Educ. , 13, 265 284.
(8) Association of American Medical Colleges. Scientific Foundations for Future Physicians; Association of American Medical Colleges: Washington, DC, 2009.
(9) American Association for the Advancement of Science. Vision and Change in Undergraduate Biology Education: A Call to Action; American Association for the Advancement of Science: Washington, DC, 2011.
(10) Lang, P. L.; Miller, B. I.; Nowak, A. T. Introduction to the Design and Optimization of Experiments Using Response Surface Methodology: A Gas Chromatography Experiment for the Instrumentation Laboratory. J. Chem. Educ. , 83 (2), 280 282.
(11) Van Ryswyk, H.; Van Hecke, G. R. Attaining Optimal Conditions: An Advanced Undergraduate Experiment That Introduces Experimental Design and Optimization. J. Chem. Educ. , 68 (10), 883 882.
(12) Strange, R. S. Introduction to Experiment Design for Chemists. J. Chem. Educ. , 67 (2), 113 115.
(13) Krawczyk, T.; Slupska, R.; Baj, S. Applications of Chemiluminescence in the Teaching of Experimental Design. J. Chem. Educ. , 92 (2), 317 321.
(14) Bouzidi, N.; Gozzi, C. Experimental Design and Optimization: Application to a Grignard Reaction. J. Chem. Educ. , 85 (11), 1554 1557.
(15) Domin, D. S. A Review of Laboratory Instruction Styles. J. Chem. Educ. , 76 (4), 543 547.
(16) Weaver, G. C.; Russell, C. B.; Wink, D. J. Inquiry-Based and Research-Based Laboratory Pedagogies in Undergraduate Science. Nat. Chem. Biol. , 4 (10), 577 580.
(17) Iskander, M. N.; Jones, P. A. Synthesis of an Enkephalinase Inhibitor, Gly-Gly-Phe-Leu, by Continuous-Flow Peptide Synthesizer. J. Chem. Educ. , 67 (2), 170 172.
(18) Truran, G.; Aiken, K.; Fleming, T.; Webb, P.; Markgraf, J. Solid Phase Organic Synthesis and Combinatorial Chemistry: A Laboratory Preparation of Oligopeptides. J. Chem. Educ. , 79 (1), 85 86.
(19) Taralp, A.; Turkseven, C.; Cakmak, A.; Cengel, O. Introducing Freshmen Students to the Practice of Solid-Phase Synthesis. J. Chem. Educ. , 79 (1), 87 89.
(20) Kirin, S. I.; Noor, F.; Metzler-Nolte, N.; Mier, W. Manual Solid Phase Peptide Synthesis of Metallocene-Peptide Bioconjugates. J. Chem. Educ. , 84 (1), 108 111.
(21) Bockman, M. R.; Miedema, C. J.; Brennan, B. B. A Discovery-Oriented Approach to Solid-Phase Peptide Synthesis. J. Chem. Educ. , 89 (11), 1407 1413.
(22) Albin, T. J.; Fry, M. M.; Murphy, A. R. Synthesis, Characterization, and Secondary Structure Determination of a Silk-Inspired, Self-Assembling Peptide: A Laboratory Exercise for Organic and Biochemistry Courses. J. Chem. Educ. , 91, 1981 1984.
(23) Horowitz, G. The State of Organic Teaching Laboratories. J. Chem. Educ. , 84 (2), 346 351.
(24) CDC. Antibiotic Resistance Threats in the United States, 2013. http://www.cdc.gov/drugresistance/threat-report-2013/ (accessed Mar 21, 2016).
(25) Brogden, K. A. Antimicrobial Peptides: Pore Formers or Metabolic Inhibitors in Bacteria? Nat. Rev. Microbiol. , 3, 238 250.
(26) Sharma, R. K. R.; Sundriyal, S. S.; Wangoo, N. N.; Tegge, W. W.; Jain, R. R. New Antimicrobial Hexapeptides: Synthesis, Antimicrobial Activities, Cytotoxicity, and Mechanistic Studies. ChemMedChem , 5, 86 95.
(27) Amblard, M.; Fehrentz, J.-A.; Martinez, J.; Subra, G. Methods and Protocols of Modern Solid Phase Peptide Synthesis. Mol. Biotechnol. , 33, 239 254.
(28) Bacsa, B.; Desai, B.; Dibo, G.; Kappe, C. O. Rapid Solid-Phase Peptide Synthesis Using Thermal and Controlled Microwave Irradiation. J. Pept. Sci. , 12 (10), 663 668.
(29) Sarin, V. K.; Kent, S. B. H.; Merrifield, R. Properties of Swollen Polymer Networks. Solvation and Swelling of Peptide-Containing Resins in Solid-Phase Peptide Synthesis. J. Am. Chem. Soc. , 102 (1), 1748 1757.
(30) Letters in italics represent the D isomer of the indicated amino acid.
(31) Haug, B. E. B.; Stensen, W. W.; Stiberg, T. T.; Svendsen, J. S. J. Bulky Nonproteinogenic Amino Acids Permit the Design of Very Small and Effective Cationic Antibacterial Peptides. J. Med. Chem. , 47, 4159 4162.
(32) Mileusnic, R.; Rose, S. P. R. The Memory Enhancing Effect of the APP-Derived Tripeptide Ac-ER Is Mediated through CRMP2: APP-Related Tripeptides, Memory and CRMP2. J. Neurochem. , 118, 666 675.

DOI: 10.1021/acs.jchemed.5b00912
J. Chem. Educ. XXXX, XXX, XXX XXX