Laboratory Exercise

A Ten-Week Biochemistry Lab Project Studying Wild-Type and Mutant Bacterial Alkaline Phosphatase[S]

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Abstract

This work describes a 10-week laboratory project studying wild-type and mutant bacterial alkaline phosphatase, in which students purify, quantitate, and perform kinetic assays on wild-type and selected mutants of the enzyme. Students also perform plasmid DNA purification, digestion, and gel analysis. In addition to simply learning important techniques, students acquire novel biochemical data in their kinetic analysis of mutant enzymes. The experiments are designed to build on students' work from week to week in a way that requires them to apply quantitative analysis and reasoning skills, reinforcing traditional textbook biochemical concepts. Students are assessed through lab reports focused on journal style writing, quantitative and conceptual question sheets, and traditional exams. © 2016 by The International Union of Biochemistry and Molecular Biology, 00:000–000, 2016.

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Introduction

In the 2011 Vision and Change in Undergraduate Biology Education: A Call to Action report, it was reported that students feel disconnected from real-world science by doing traditional "canned" labs [1]. Instead, the call to action was to incorporate more inquiry-based labs where the answer is not known ahead of time and to afford the students the opportunity to deal with ambiguity that often comes along with real data. In an undergraduate biochemistry lab course, it is important for students to learn fundamental techniques that can be applied to a variety of research disciplines. The goal of the laboratory exercises described here is to expose students to such techniques in a 10-week, project-oriented laboratory course suitable for a one semester introductory biochemistry lab.

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[S]*Additional Supporting Information may be found in the online version of this article.*

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In designing the lab curriculum, the goal was to touch upon each of the ASBMB defined core concepts: evolution; matter and energy transformation; homeostasis; biological information; and macromolecular structure and function [2]. In addition, the design focused on highlighting quantitative and scientific reasoning skills in the experiments whenever possible. In many cases in the lab curriculum described in this article, lab exercises are included solely for the purpose of adding these types of problems for students to work through.

Alkaline phosphatase (EC 3.1.3.1) has been the subject of numerous biochemistry lab exercises due to its relative inexpensive cost and the simple, rapid, colorimetric assay used to measure its activity [3–5]. The enzyme exists as a dimer and requires the presence of two distinct cofactors for its activity in hydrolyzing phosphoric acid monoesters, releasing free phosphate. The laboratory curriculum described here is based on the study of the bacterial form of the enzyme, specifically a FLAG-tagged variant that can be purified by affinity chromatography. Given that the three-dimensional structure of bacterial alkaline phosphatase has been solved (PDB ID: 1ED8) [6] and the catalytic mechanism of *E. coli* alkaline phosphatase has been well-reported on [7–9], students can use these pieces of data in studying the enzyme during the project. Of note, the bacterial form of the enzyme is not as efficient as the mammalian homologs. While protein engineering to optimize the enzyme has been

TABLE I *Outline of the 10-week lab curriculum*

Lab	*Lab exercise*
1	Micropipetting and buffers
2	Protein structure lab
3	Purification of wild-type and mutant bacterial alkaline phosphatase
4	Analysis of purification using SDS-PAGE
5	Protein concentration determination of bacterial alkaline phosphatase purification fractions
6	Nucleic acids: purification, spectrophotometric analysis, restriction enzyme digestion
7	Nucleic acids: gel analysis
8	Determination of kinetic parameters of wild-type and mutant alkaline phosphatase
9	Inhibition of wild-type alkaline phosphatase
10	Data analysis of enzyme kinetics

For a more focused, shorter-term study, labs 6 and 7 (nucleic acids) could be omitted and the data analysis of enzyme kinetics could be performed outside a scheduled lab session.

previously performed [10, 11], the goal is to apply this same idea to a class setting to allow students to better understand enzyme structure-function relationships.

Course Synopsis

A typical section of this biochemistry lab course at The University of Tampa has between 8 and 12 students, and students work in lab pairs throughout the semester. This course is a required course for all Biochemistry majors and B.S. Forensic Science majors. Some biology majors (particularly pre-health students) take the class as an elective.

The class is designed for introductory biochemistry students, typically 3rd and 4th year students, depending on their major. While most of these students have some laboratory experience (either in laboratory classes or research experience), no technical experience is assumed in starting the class. For this reason, part of the first exercise is a micropipetting exercise, which can be omitted if students have been previously trained. This class is designed to be performed over the course of 9 to 10 weeks in 4 hr long laboratory periods (Table I). All the exercises can be fit into a 3 hr long lab session with little to no changes, particularly if students prepare their experimental plan before coming to lab.

Each week, lab handouts are posted online for students to read and prepare from before lab. These handouts give background information on the lab, instructions for completing the lab, and study questions for students to answer that relate to the topic in lab that day. These handouts are available as Supporting Information.

Laboratory Procedures

The descriptions below describe the highlights of the lab procedures performed each week, as well as assignments students are expected to turn in following each exercise. For more detailed descriptions, please see the individual lab handouts available in the Supporting Information.

Week 0 (Work Completed Before Beginning the Course)—Mutagenesis

Bacterial alkaline phosphatase mutants used in this study were made prior to initially teaching the class. Mutagenesis was carried out with the Q5 Site-Directed Mutagenesis Kit (New England Biolabs) using pFLAG-CTS-BAP (Sigma Aldrich) as a template. This vector contains the *E. coli phoA* gene (GenBank accession number M29664) as well as a C-terminal FLAG tag. In total, 10 mutations of this vector have currently been made: S102A, S102C, S102T, D153G, D153H, R166A, R166K, K167A, K167R, and H412N. All mutations described are available by contacting the author.

Week 1—Micropipetting and Buffers

Since no biochemical lab experience is assumed with students taking this introductory biochemistry lab, one of the goals of the first exercise is to ensure students can correctly use micropipettes. Once students have checked their pipetting skills (and calibrations of pipettes) by measuring the mass of different volumes of water, they prepare various buffers that will be used throughout the course. These buffers are 1.0 M Tris—pH 8.0; 2× alkaline phosphatase

reaction buffer (0.40 M Tris—pH 8.0; 10 mM $MgCl_2$); 10× Tris-buffered saline (10× TBS:250 mM Tris pH 7.5, 1.5 M NaCl); and 10× TBE (0.89 M Tris base; 0.89 M boric acid; 0.020 M EDTA, pH 8.0).

To stress the importance of quantitative skills, students are expected to use the Henderson-Hasselbalch equation to predict the amount of 6 M HCl that will be used to adjust the pH of the 1.0 M Tris solution before preparing the actual buffer. Additionally, the assignment for students associated with this lab is math-oriented. Students are assigned a handful of buffer problems (most taken and/or adapted from *Fundamental Laboratory Approaches for Biochemistry and Biotechnology, Second Edition* [5]) and must turn in a table of water measurements showing expected mass, actual mass, percent error, and standard deviation of replicate measurements.

Week 2—Protein Structure of Alkaline Phosphatase

This lab exercise has been adapted slightly from the exercise previously described by Grunwald and Kreuger [12]. Since enzyme catalysis is covered in the lecture component of the class much later, these details are only briefly mentioned in this exercise. Instead, students focus on more general structural characteristics and are guided to think about the effects certain mutations might have based on the chemical structure of different amino acids. Students also choose what mutant they want to study during this lab. The lab handout asks a number of questions regarding structure that students answer and hand in the following week.

Week 3—Purification of Wild-Type and Mutant FLAG Bacterial Alkaline Phosphatase

A few days before the lab, ensure a fresh plate of BL21(DE3) cells harboring wild-type and the various mutant alkaline phosphatase in the pFLAG-CTS vector is prepared. The day prior to lab, 5 mL of LB/Amp is inoculated with a single colony of the desired bacteria, with each lab group getting one wild-type and one mutant culture. Cultures were incubated to OD600 = 0.4–0.7 (approximately 3 hr at 37°C with shaking at 225 rpm) then induced with 50 μg/mL IPTG overnight. In pilot experiments, the timing of induction did not seem to matter significantly, and expression was observed in all conditions tested. The following morning (after ~16 hr of growth post-induction), students removed the overnight culture and began their purification procedure. While alkaline phosphatase can be purified from periplasmic space, this exercise involves creating whole cell lysates. By doing this, students can see a much more dramatic effect of purification when performing SDS-PAGE following purification.

Cell lysates were prepared from overnight cultures using the B-PER protein extraction reagent (Thermo Scientific). Cleared lysates were then purified using anti-FLAG M2 affinity gel (Sigma), using 100 μL of beads for each purification. Lysates were incubated with washed beads for 30 min at room temperature using an end-over-end rotator. Beads were then washed three times with TBS and eluted with 100 μg/mL FLAG peptide. Glycerol was added to the elution samples (final concentration 50%) to prevent freeze-thawing of the purified enzyme, preserving its activity for the kinetic experiments.

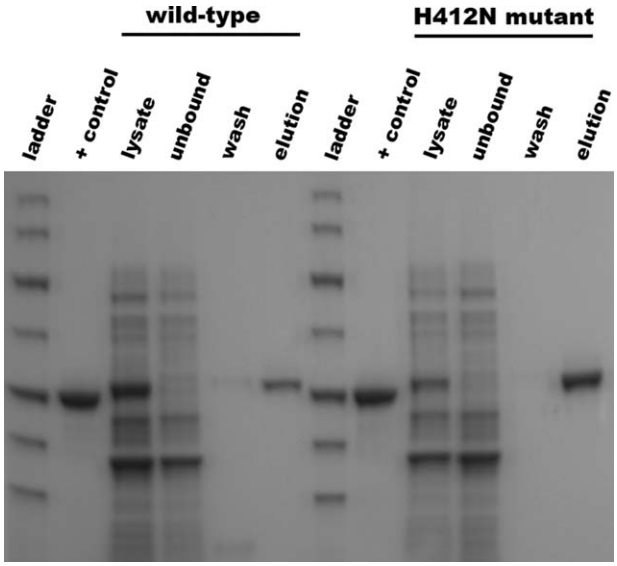

FIG 1 *Immunoaffinity purification of FLAG-tagged wild-type and H412N mutant bacterial alkaline phosphatase. Bacterial cells were lysed using B-PER reagent (Thermo Scientific), purified using FLAG M2 affinity beads (Sigma), and eluted using 100 μg/mL FLAG peptide. Shown is a 10% SDS-PAGE gel stained with GelCode Blue obtained by students.*

Week 4—Analysis of Purification Using SDS-PAGE

The main goal of this lab, in addition to gaining experience with the SDS-PAGE technique, is to ensure the purifications from the previous week worked properly. Students are reminded what samples should be run on the gel (total lysate, unbound, wash, and elution from both the wild-type and mutant purification along with a recombinant alkaline phosphatase control and protein ladder).

It takes most lab groups about 1.5 hr to gather their thoughts, prepare their samples and necessary buffers, and load their gel. This means that by the time they add their protein stain (GelCode Blue, Thermo Fisher), there is not enough time to destain and take a picture of the gel during the lab period. The instructor (or lab assistant or students) instead removes the stain the following day and destains with water, and the instructor takes pictures of the gels and distributes the image to students electronically. The physical gels were saved for a week for students to view after imaging, without significant loss of quality. A representative gel from a student lab group is shown in Fig. 1.

In analyzing their GelCode Blue stained gel, students should be able to estimate the purity of their elution,

determine the approximate molecular weight of the elution band, determine approximately what percent of the FLAG-alkaline phosphatase remained unbound from the beads, and estimate the concentration of the purified protein by comparison to a known amount of an alkaline phosphatase standard. These analyses supplement the quantitative analysis lab the following week.

Following this lab, students turn in a lab report combining their purification and SDS-PAGE lab work. See Teaching/Assessment methods for details on this assignment.

Week 5—Protein Concentration Determination Using BCA and A280 Assays

This lab serves the function of determining the concentration of FLAG-tagged alkaline phosphatase in the elution fractions. This is most simply done by measuring the A280 of elution samples (saved from the purification lab in the absence of glycerol) and calculating the concentration using the molar extinction coefficient (34,500 M^{-1} cm^{-1}, as determined using the ExPASy protein parameter tool), which is appropriately slightly higher than the reported value for untagged alkaline phosphatase in the literature due to the tyrosine present in the FLAG tag [13]. The calculation is made a little bit tricky due to the presence of the 100 μg/mL FLAG peptide solution used to elute the protein, primarily due to the tyrosine in the peptide. The peptide could be removed using a gel filtration step, but due to time constraints students instead record the A280 of a 100 μg/mL solution of FLAG peptide in TBS (same solution used to elute in week 3) and subtract that from the value of the elution fractions.

In addition to the A280 measurements, students also determine protein concentration on their cell lysate, unbound, wash, and elution fractions using the BCA assay. By calculating the concentration of the cell lysate and the amount of purified enzyme in the elution fraction, students can determine the percent of enzyme present in the original lysate. For the academic part of the lab exercise, students are given two different standards—bovine gamma globulin (BGG) and bovine serum albumin (BSA). Because the amino acid compositions of these standards differ, the calculated protein concentrations vary between the two standards. Students are also given the protein sequences of BGG, BSA, and FLAG-BAP and are assigned to analyze the sequences for determinant amino acids for the technique and discuss how the protein composition affects the results.

The assignment for this lab is to first determine the protein concentration (and total amount of protein) of the purification fractions. In particular, students are assigned to determine the concentration of the elution fractions three different ways—A280, BCA assay, and by visual determination of their SDS-PAGE gel in relation to a control. Students are required to explain which of the methodologies they feel gives the most accurate concentration of the elution.

Week 6—Nucleic Acids: DNA Purification and Spectrophotometric Analysis

This is the first part of a two-week sequence dealing with DNA, specifically purifying and analyzing the pFLAG-CTS-BAP plasmid DNA (containing wild-type alkaline phosphatase). It should be noted that this lab and the subsequent lab do not need to be performed in order to do the subsequent kinetics lab. These two labs are included to provide students with the opportunity to do DNA manipulations, which are skills important for them in many of our upper level classes. The labs also fall into place during a time that DNA techniques are being lectured about in our biochemistry lecture class, so these labs complement the lecture well in this position in our schedule.

Students perform a miniprep plasmid DNA purification (GeneJET kit, Thermo Fisher) and determine the concentration and purity of the purified DNA by measuring the absorbance of their purified solution and 260 and 280 nm. One downside of using kits is that students do not always understand the biochemistry behind how the kit works. To ensure students are understanding how the kit works, discussion questions in lab (see "Questions to think about" in the lab handout in the Supporting Information) revolve around the different components in the kit and their importance.

Using their purified DNA, students are then instructed to set up a series of restriction digests. They choose two enzymes for their digests—a single cutter (Group A: SalI, HindIII, KpnI, or NdeI) and a multi-cutter (Group B: NcoI, NaeI, BspHI, or EcoRI). A total of four reactions are performed: no enzyme, Group A digest, Group B digest, and double digest with Group A and B. In setting up their reactions, students are required to use the New England Biolabs website to determine the appropriate reaction conditions for each reaction. Following a 1 hr digest, samples are stored at −20°C until they are analyzed by agarose gel electrophoresis in the next lab session.

Week 7—Nucleic Acids: DNA Gel Electrophoresis

When surveyed, most of our students say they have performed DNA gel electrophoresis in other classes before taking this biochemistry lab. Upon further examination, this often meant pipetting pre-made samples into a pre-poured gel and pushing a button on a power supply and just looking at the gel. In this lab exercise, students are expected to perform everything themselves: preparing the 1% agarose gel, diluting the 10× TBE buffer they previously made, preparing their samples by addition of 6× loading buffer, running the gel, and obtaining a picture of the gel using a gel documentation system.

Students resolve a DNA ladder, uncut plasmid DNA, and their three digests using a 1% agarose gel (Fig. 2). For the uncut DNA and digestion reactions, students load 0.2 μg of DNA. This quantitative exercise often takes students a few minutes to figure out. Not only in terms of how much

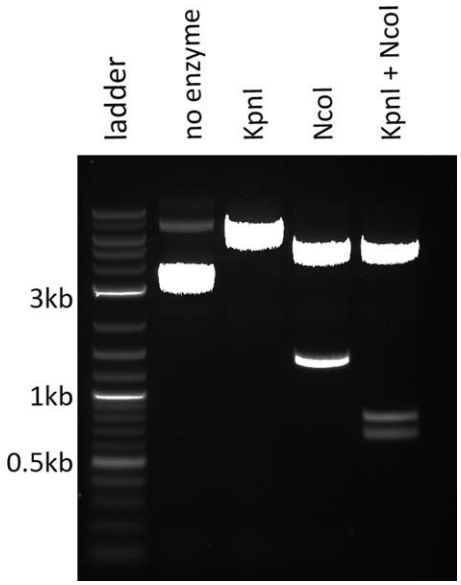

FIG 2 *Restriction digestion analysis of the pFLAG-CTS-BAP vector. Plasmid DNA was purified from bacterial cells harboring the pFLAG-CTS-BAP vector using a GeneJET miniprep kit. Purified plasmid (0.5 μg) was then digested by KpnI, NcoI, and KpnI + NcoI, separated on a 1.0% agarose gel containing 1× GelRed, and visualized under UV light.*

DNA relates to 0.2 μg, but also how much 6× DNA loading buffer to mix with the sample. Similar to SDS-PAGE, it usually takes lab groups approximately 1.5 hr to begin running their gel. The time when gels are running is used for students to draw their expected results. The work from both DNA labs is combined into a written lab report.

Week 8—Enzyme Kinetics: Determination of Kinetic Parameters of Wild-Type and Mutant FLAG Bacterial Alkaline Phosphatase

Students analyze their purified enzyme for activity using a standard kinetic assay for alkaline phosphatase with para-nitrophenyl phosphate (PNPP) as a substrate. This general reaction has been characterized in many places [4], and has been slightly modified for the purification fractions of bacterial alkaline phosphatase here. In short, PNPP is hydrolyzed to PNP (para-nitrophenol), which can be monitored via visible absorption spectroscopy at 410 nm.

Students are given a 1 mM stock solution of PNP (the colored product of the reaction) in order to prepare a standard curve (1, 5, 10, 20, and 40 μM) for which the molar absorption coefficient can be calculated by plotting absorbance versus concentration (slope = molar absorption coefficient). Students are also provided with a 50 mM stock solution of PNPP and use their already prepared 2× reaction buffer (Lab 1) and purified enzyme solutions that have been stored in glycerol (Lab 3).

Students are instructed to set up a table for 6 reactions (Table II). Each reaction contains 10 nM alkaline phosphatase and is prepared with 2× reaction buffer (0.4 M Tris, pH 8.0; 10 mM $MgCl_2$) in a final volume of 2 mL. The concentration of PNPP is varied (0.10, 0.25, 0.50, 1.0, and 2.0 mM). Once their table setup is confirmed by the instructor, they begin the kinetic analysis in duplicate with both wild-type and mutant elution fractions from the purification (a total of 20 reactions, recording spectrophotometer data at 410 nm every 15 sec for 2.5 min each reaction). An important reminder to students in this lab (as is the case with many experiments) is the importance of properly mixing the samples. In this experiment, mixing the tubes after adding enzyme is critical in obtaining consistent data. Students are instructed to cover the tubes with a piece of Parafilm and invert five times before taking their initial reading.

Week 9—Enzyme Kinetics: Inhibition of Wild-Type Alkaline Phosphatase

The second part of the study of enzyme kinetics looks at the effect of two different inhibitors (sodium phosphate and L-phenylalanine) on the kinetics of bacterial alkaline phosphatase. Ideally, the purified wild-type enzyme from Lab 3 would be used in this experiment, but in most cases there is not enough of it remaining for this lab (see Laboratory Pitfalls and Potential Modifications). In theory, additional wild-type enzyme could be purified in week 3, but it is more cost-effective to simply purchase recombinant enzyme for this lab. One benefit of using a commercial enzyme is the discussion that can be made with students as to why their purified enzyme might have different kinetic parameters than the commercial enzyme. For instance, the FLAG tag could cause the purified form to fold slightly differently, altering its activity. In our experiment, we use commercial bacterial alkaline phosphatase from Takara Bio.

The experimental setup is very similar to the experiment performed in week 8. Students prepare a standard curve to determine the extinction coefficient of PNP and then set up a table of reactions with their inhibitors using a final enzyme concentration of 10 nM. Students are given stock solutions of 50 mM PNPP, 10 mM sodium phosphate, 100 mM L-phenylalanine, and 100 nM recombinant alkaline phosphatase. In this experiment, a more narrow range of PNPP (20, 50, 100, 300, and 500 μM) is used since we are dealing with only wild-type enzyme. For each PNPP concentration, five different reactions are performed: no inhibitor, 0.1 mM sodium phosphate, 0.5 mM sodium phosphate, 5.0 mM L-phenylalanine, and 10.0 mM L-phenylalanine. With there being 25 total reactions, only one replicate is performed for each condition. As with the previous lab, data is collected at 15 sec intervals for 2.5 min and is saved for analysis the following week.

Week 10—Enzyme Kinetics: Data Analysis

The data collected in the last two labs are analyzed during this final lab session. Calculations of K_M, V_{max}, k_{cat}

TABLE II *Sample student table showing reaction setup for kinetics with wild-type purified enzyme*

Tube	Final [] of PNPP in mM	Vol of 50 mM PNPP (μL)	Vol of 2× reaction buffer (mL)	Vol H_2O (μL)	Vol of enzyme for 10 nM (μL)
1	0.1	4	1.0	955	41
2	0.25	10	1.0	949	41
3	0.5	20.0	1.0	939	41
4	1.0	40.0	1.0	919	41
5	2.0	80.0	1.0	879	41

Example of a student table showing the setup for a kinetics experiment for wild-type alkaline phosphatase. In practice, students would have a second table for the mutant enzyme, which would differ only in the volume of enzyme (assuming the wild-type and mutant enzymes have different concentrations). All reactions in this experiment were performed in duplicate.

(turnover number) are performed for the purified wild-type and mutant enzymes. For the inhibited enzyme, K_M, V_{max}, k_{cat} (turnover number) are also determined along with K_i and the type of inhibition for both sodium phosphate and L-phenylalanine.

First, students plot absorbance versus time for each reaction. This slope gives the initial velocity, which can be converted to concentration/min using the extinction coefficient determined with the slope of PNPP. Students are instructed to view the graphs carefully and determine the linear range; if the graph plateaus at later time points, students are instructed to not factor those time points into their initial velocity determination. The initial rates are then used to make both Michaelis-Menton and Lineweaver-Burk graphs, the latter from which the K_M and V_{max} are determined (Fig. 3). For the inhibition studies, the data was manipulated the same way except that each inhibitor condition was treated individually (Fig. 4). Thus, five different lines were made from which the kinetic parameters and K_i could be calculated.

While students are familiar with the determination of K_M and V_{max} from Lineweaver-Burk graphs from their introduction to enzyme kinetics in the classroom, determining these parameters using non-linear regression is often preferred. Non-linear regression is a better true fit to the Michaelis-Menton equation than the linear regressions, given that linear regressions (such as Lineweaver-Burk) often skew data points. Specifically, deviations at low substrate concentrations are compounded in the linear regression. Students are given an Excel template (based off [14]; see Supporting Information) where they can input substrate concentrations and calculated v_0 values and determine both K_M and V_{max} using non-linear regression. This allows students to compare the linearly-derived kinetic values to that from the non-linear regression and report their best value in their lab report.

The data for the K167A mutant shown in Fig. 3 were particularly interesting as it was a mutant that has not been previously characterized. While the figure shows data from a single experiment, the results suggest the K167A mutant displays a lower V_{max} and a higher K_M than the wild-type enzyme. This lysine is oriented such that the side chain projects into the active site. While one might expect the lysine would stabilize the P_i via electrostatic forces, the data suggests otherwise. One possible explanation for this is the K167A mutation allows the P_i to interact stronger with the enzyme via the Mg^{2+} and Zn^{2+} cofactors within the active site, by not pulling the P_i towards the lysine. This increased affinity would explain both the lower K_M and higher V_{max}, as the increased binding would facilitate more rapid turnover.

Table III shows representative student data for kinetic calculations from one student group. It should be noted that L-phenylalanine did not effectively inhibit bacterial

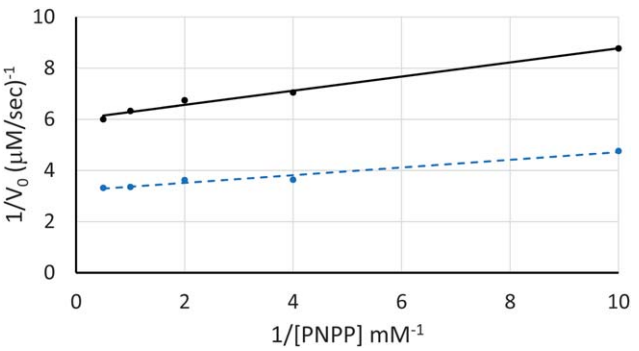

FIG 3 *Lineweaver-Burk (double reciprocal) plots for purified wild-type (solid black line) and K167A mutant (dashed/blue line) FLAG-tagged bacterial alkaline phosphatase. Assays were performed using 2 nM enzyme using the reaction setup shown in Table II.*

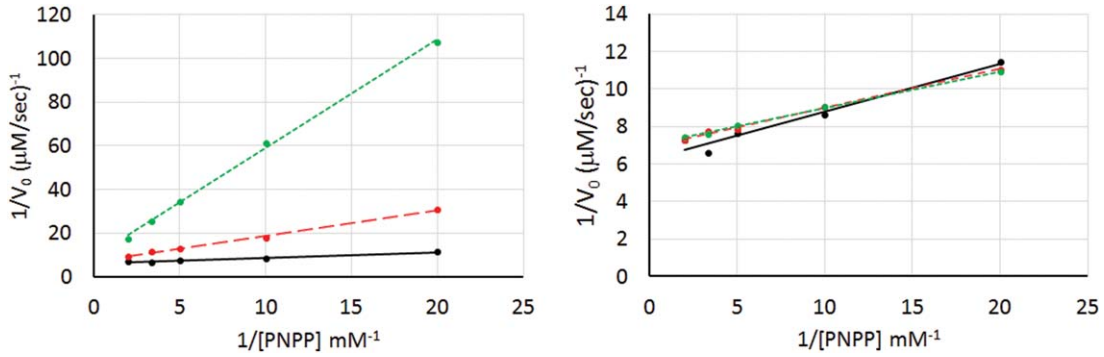

FIG 4

Lineweaver-Burk (double reciprocal) plots for inhibition of bacterial alkaline phosphatase using sodium phosphate and L*-phenylalanine. Kinetic assays were performed with 10 nM recombinant* E. coli *alkaline phosphatase in the presence and absence of sodium phosphate and* L*-phenylalanine. (*a*) Solid/black line—uninhibited; long-dashed/red line—0.1 mM sodium phosphate; shorted-dashed/green line—0.5 mM sodium phosphate. (*b*) Solid black line—uninhibited; long-dashed/red line—5.0 mM* L*-phenylalanine; short-dashed/green line—10.0 mM* L*-phenylalanine.*

alkaline phosphatase at the concentrations tested; however, K_i was still calculated as an academic exercise.

Teaching and Assessment Methods

In designing assignments and assessing students for this course, the focus was on three major goals: quantitative skills, presentation and explanation of data, and understanding of the techniques used. Quantitative skills were a large part of most of the labs, from using the Henderson-Hasselbalch equation to predict the amount of acid needed to make a buffer in the first lab, to calculating enzyme parameters and inhibitor dissociation constants in the final lab. The protein quantitation lab is the most quantitation heavy of all the labs, and the lab students struggled with the most. A key teaching point during this lab is not only the quantitative skills, but the scientific reasoning that goes along with it. For instance, why measure the concentration of the alkaline phosphatase three different ways? Once a concentration is calculated, how can that be translated for use in kinetics assays? What is the explanation behind the differences in calculated protein concentration using two different protein standards in the BCA assay? One of the biggest frustrations of students was the amount of math they had to do in order to complete many of the labs. For many lab groups, the time it took to figure out how to load 0.2 μg of DNA from a 50 μL restriction digest (including diluting the sample with 6× gel loading buffer) took far longer than loading and running the gel. Since this type of math is not taught in textbooks but is used often in research environments (particularly using stock solutions that are 5×, 6×, 10×, etc.), it is important for students to be exposed to and gain confidence in doing these types of calculations.

Another important skill that students often become aware of while trying to navigate through the labs is the

TABLE III

Representative student data of bacterial alkaline phosphatase kinetic parameters in the presence of inhibitors

	V_{max} *μM/sec)*	K_M *(μM)*	K_i *(mM)*	K_{cat} *(sec^{-1})*
No inhibitor	0.159	42.8	–	15.9
0.1 mM Na_3PO_4	0.138	162	0.034	13.8
0.5 mM Na_3PO_4	0.110	555	0.040	11.0
5 mM L-phenylalanine	0.144	29.9	48.0 (from V_{max}^{app}) 15.0 (from K_M^{app})	14.4
10 mM L-phenylalanine	0.142	27.9	83.5 (from V_{max}^{app}) 11.7 (from K_M^{app})	14.2

While L*-phenylalanine did not seem to inhibit bacterial alkaline phosphatase for the class as a whole,* K_i *was still calculated based on the minor changes to both* K_M *and* V_{max}.

importance of keeping a detailed lab notebook. In classes where the experiments do not carry over from week-to-week, the lack of detail in a lab notebook often has no negative consequences. However, when students go back and try to remember what their elution volume was in a purification protocol to calculate the total mass of purified enzyme, for example, it becomes imperative. While the lab notebook is not directly graded as part of the overall class assessment, part of the final exam (20%) is a lab notebook section where students answer questions relating to procedures and data using their lab notebook.

Another of the primary goals of the course is to teach students how to present data in a way comparable to that in a peer-reviewed journal. While most students may never submit a paper for peer-review, the importance of succinct and efficient writing is important for everyone. And while students may never submit a paper, all students should be reading peer-reviewed journal articles at some point during their education. Over the course of this class, students are required to turn in three separate lab reports: protein purification and SDS-PAGE, DNA purification and digest, and enzyme kinetics and inhibition. Since this is taught as only a 1 credit class, these lab reports were pared down to allow students to focus on very specific aspects of reporting their work.

Students are instructed to find a peer-reviewed journal article where the technique they used was performed and where similar type data was obtained. Students can then use the paper as a resource to model the detail to include in the various sections. The sections that students are to turn in are *Experimental Procedures*, a journal-quality figure (or figures) with figure legend(s), and *Results*. The main focus of the writing is to ensure that the correct amount of material is provided. For example, detail about how much loading buffer was mixed with sample before loading onto an SDS-PAGE gel is not appropriate details for a peer-reviewed paper. When assessing these assignments, the ability to write in the style of their reference paper and using the appropriate amount of detail was the main criteria for grading. Significant feedback was given when handing back the first of these reports, and subsequent assignments improved dramatically.

Laboratory Pitfalls and Potential Modifications

Experimentally, there are a few places where optimization of the lab protocols or modifications could improve the overall course. In the enzyme purification, while the purification is simple and efficient, the use of a FLAG tag is rather expensive and does not lend itself to large-scale purification. For instance, some lab groups did not obtain enough purified enzyme to enable them to use the suggested 10 nM amount in their kinetic assays. In these cases, lab groups were instructed to scale down the amount of enzyme as necessary. Students acquired data using as little as 2 nM enzyme (Fig. 3). Subcloning the mutants into a different expression vector with a different tag (6×-His or GST, for instance), would allow for larger levels of expressions. If this were to be done, the inhibitor studies could be carried out on the purified enzyme instead of the commercial enzyme.

Another drawback of the experiment as it is designed is the fact that bacterial alkaline phosphatase is not sensitive to L-phenylalanine at the concentrations tested (Fig. 4*B*, Table III). While the uncompetitive nature of inhibition of L-phenylalanine has been well documented for mammalian forms of the enzyme, the bacterial form does not seem to be susceptible to this inhibition [15]. There are multiple ways to modify the course to allow for inhibition analysis outside of the competitive inhibition by phosphate. First, millimolar concentrations of EDTA could be used, which should inhibit the reaction by chelating the cofactors required for catalysis [16]. This would tie in nicely with the protein structural analysis that was performed by pointing out the importance of cofactors in catalysis. Alternatively, the entire experiment (or just the inhibitor lab) could be carried out using phosphatase from a mammalian species in which the enzyme would be sensitive to L-Phe.

In the initial offering of the course, some of the mutants had no measurable activity. While there is still benefit to students in understanding why a particular mutation completely abolishes activity (or at least makes it unmeasurable in the assay conditions used), it does take away from the kinetic analysis of the mutant. In future course offerings, mutations lacking activity will not be studied again. Instead, additional mutagenesis of mutants that have increased or comparable activity to the wild-type enzyme will be studied.

In terms of student struggles and pitfalls, one unexpected area in which students struggled in this lab is in diluting and working with stock solutions that are 10× (10× TBS or 10× TBE) or 6× (SDS-PAGE loading buffer or DNA loading) buffers. Many students have never worked with stock solutions that have an "×," and while they pick up on these types of dilution problems relatively quickly, they often need a little coaching to understand the idea.

Student Evaluation

Students were asked to complete a brief survey following the completion of this 10-week lab curriculum. The main goal of the survey was to determine student perceptions of the course format and their perceived learning. While only a small number of students participated in the course (n = 17), the survey data showed that students enjoyed the semester-long course project and felt their scientific skills improved as a result of the design of the lab (Table IV). Most notably, the focus on quantitative skills seems to have paid off, as all the students who responded perceived their quantitative skills improved as a result of the lab. The effort to tie together lab concepts with lecture material

TABLE IV ***Student survey data (n = 17) of the course design and their perceived learning following completion of the 10-week laboratory course***

	Average Response (std dev)
I prefer the semester-long project compared to separate, individual labs.	3.94 (1.16)
I am more confident in being able to design and perform an experiment independently.	3.82 (0.86)
My scientific reasoning skills improved as a result of the experimental design of the lab.	4.29 (0.67)
My quantitative skills improved as a result of the experimental design of the lab.	4.29 (0.67)
The labs helped my understanding and reinforced the concepts that were introduced in lecture.	4.44 (0.70)

Responses were on a scale from 1 (strongly disagree) to 5 (strongly agree).

showed dividends as well, with all reporting students feeling that the material in the lab reinforced those concepts.

In the survey, students were also asked to report their favorite and least favorite labs of the semester. While there was no clear favorite lab (both the DNA labs and enzyme kinetic labs seemed popular), the least favorite lab was clearly the protein quantitation lab. As mentioned earlier, students struggled with the numerous calculations in that lab, particularly if relying on data from insufficiently complete lab notebooks to perform the calculations. However, given the perceived improvement in quantitative skills, the struggle did not take away from the positive overall effect on learning. Additionally, multiple students reported that the overlap between the lecture and lab was very helpful for their learning, as shown in the quotes from student comments below:

> "Labs strongly correlated with lecture material and even though they were difficult they helped the understanding of material."
>
> "All the labs during this course greatly helped me better understand the Biochemistry content in lecture really helped with following concepts in class as well."
>
> "The data analysis days were awesome, I wouldn't have been able to do all of that without them. The way that the labs were written also helped a great deal and helped tie together topics from lecture."

Summary

In summary, this work describes an effective way to introduce many biochemical methods in an introductory biochemical lab course. This lab provides students a semester-long experience where they purify, quantitate, and characterize the enzymatic activity of wild-type and mutant bacterial alkaline phosphatase. In the process, students gain confidence in their quantitative and scientific reasoning skills and gain experience in presenting their data in a scientifically appropriate manner. The data generated by students in the lab could also be expanded upon by motivated students as independent undergraduate research projects, where students would have already learned many of the requisite techniques needed to carry out the research.

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