7-2013

Purification and Characterization of Enzymes from Yeast: An Extended Undergraduate Laboratory Sequence for Large Classes

Kelly E. Johanson
Xavier University of Louisiana, kjohanso@xula.edu

Terry J. Watt
Xavier University of Louisiana, tjwatt@xula.edu

Neil R. McIntyre
Xavier University of Louisiana

Marleesa Thompson
Xavier University of Louisiana

Recommended Citation

Johanson KE, Watt TJ, McIntyre NR, Thompson M. Purification and characterization of enzymes from yeast: an extended undergraduate laboratory sequence for large classes. Biochem Mol Biol Educ. 2013 Jul-Aug;41(4):251-61. doi: 10.1002/bmb.20704.

7-2013

Purification and Characterization of Enzymes from Yeast: An Extended Undergraduate Laboratory Sequence for Large Classes

Kelly E. Johanson

Terry J. Watt

Neil R. McIntyre

Marleesa Thompson

Laboratory Exercise

Purification and Characterization of Enzymes from Yeast: An Extended Undergraduate Laboratory Sequence for Large Classes[S]

Kelly E. Johanson*
Terry J. Watt
Neil R. McIntyre
Marleesa Thompson

From the Department of Chemistry, Xavier University of Louisiana, 1 Drexel. Dr, New Orleans, Louisiana 70125

Abstract

Providing a project-based experience in an undergraduate biochemistry laboratory class can be complex with large class sizes and limited resources. We have designed a 6-week curriculum during which students purify and characterize the enzymes invertase and phosphatase from bakers yeast. Purification is performed in two stages via ethanol precipitation and anion exchange chromatography, and students perform both direct and coupled enzyme assays. By completion of the experimental series, students are able to identify which enzymes they have purified and have obtained kinetic parameters for one. This experimental series requires minimal instructor preparation time, is cost effective, and works with multiple sections of large groups of students. Students participating in this sequence showed increases in conceptual understanding of biochemical concepts as measured through in-class assessments and anonymous surveys. © 2013 by The International Union of Biochemistry and Molecular Biology, 41(4):251–261, 2013

Keywords: *protein purification; project-based curriculum; enzyme kinetics; protein identification*

Introduction

A junior-level biochemistry laboratory course should expose students to common biochemical techniques and illustrate how the techniques might be applied in a research laboratory setting. The use of a project-based curriculum fulfills both of these goals. The traditional biochemistry laboratory course taught at Xavier University of Louisiana (XULA) prior to 2010 was comprised of independent experiments, each designed to teach a specific concept rather than expose students to common biochemical techniques. As we began to modify our curriculum to focus on relationships between techniques, we searched for an existing project-based curriculum that could be adapted to our needs. Purification and characterization of a protein seemed to be the obvious choice, and several successful versions do appear in the literature [1–8]. However, these experiments were difficult to incorporate into our curriculum as written, because they required at minimum 4–6 hours of class time per week and were designed for a class size of 16 students or fewer. Our introductory 1-credit biochemistry laboratory course meets once a week, has between 18 and 24 students per section, and four to five sections are taught each semester. Many of the students at XULA live and work off-campus; therefore, the experiments must fit within the allotted class time. Our laboratory courses also do not have additional times scheduled for lecture, and the university does not supply teaching assistants; laboratory prep is generally the responsibility of one faculty member who receives a modest amount of course release.

We felt that there was a need for a project-based curriculum that could be used with a larger group of students, but that could be completed in 2 hour and fifty minute blocks, including the time necessary for pre-lab lectures and quizzes. We then set out to create our own set of experiments with several goals in mind. 1) The project should be modular in nature so that certain experiments can be removed if necessary due to unexpected class cancellations (e.g. severe weather events) or so that different modules can be swapped out in future semesters. 2) The protein or proteins chosen for these experiments should be robust so that they can remain active through a several week cycle. 3) The experiments should require minimal instructor preparation time and be cost efficient; they should also require minimal volumes of reagents per student. 4) There should be several portions of the project that are

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

**Address for correspondence to: Department of Chemistry, Xavier University of Louisiana, 1 Drexel. Dr, New Orleans, Louisiana 70125. E-mail: kjohanso@xula.edu.*

Received 11 April 2013; Accepted 15 April 2013
DOI 10.1002/bmb.20704
Published online in Wiley Online Library (wileyonlinelibrary.com)

student-driven or require students to think critically about the next stage of their experiment.

We began with a modification of the invertase purification by Timerman *et al.*, 2009 [7], and the result was a 6-week project in which the students simultaneously purified and characterized invertase and alkaline phosphatase from *S. cerevisiae*. Purification of invertase, alkaline, or acid phosphatase has been the basis for student laboratory exercises since the 1970s [9, 10]. However, the experiments described here are the first example of a combined purification strategy resulting in the isolation of two of these enzymes and the first designed to work with the class size and schedule we have at XULA.

The original purification of invertase described as a student experiment was a one-step procedure [9], later expanded to three steps to increase purity [7]. Our method includes only two steps and reduces the amount of starting material required for each student or student group by more than 20-fold. These modifications allow this project to work with a larger number of students and reduce the time needed to complete each purification step without a dramatic decrease in the purity of the final enzyme preparation.

Alkaline phosphatase is also a common enzyme used in student biochemistry laboratory exercises. The starting material for purification is usually *E. coli* grown in low phosphate media [10] or transformed with a plasmid expressing the protein [11], although a lyophilized mutant rich in alkaline phosphatase can be purchased and hydrated for student use [12]. Purification of alkaline phosphatase from yeast is described in the literature [13–15]; however, none of these experiments were written or designed for laboratory classes. Our procedures can be most directly compared to those starting from lyopholyzed *E. coli* [12], but again the purification described here has the advantage of requiring fewer purification steps, less class time, and less overall material and reagents per student.

The purification strategy is designed to specifically isolate the extracellular form of invertase (Suc2p) and the nonvacuolar form of alkaline phosphatase (Pho13p) from yeast. Yeast can contain up to seven sucrose fermentation (SUC) genes that code for invertase; however, all loci code for nearly identical forms of the protein [16–18] with SUC2 being the most commonly identified and therefore the most studied of the SUC genes [17]. Suc2p is expressed as both an intracellular and extracellular protein due to differential transcriptional start sites [19, 20]. The two forms can be distinguished by differences in molecular weight and the presence of glycosylation [20–22]. Yeast also have several genes that encode nonspecific acid or alkaline phosphomonoesterases, as well as several other related genes involved in phosphate metabolism (PHO) [14, 15, 23–26]. Two genes, PHO8 and PHO13, encode alkaline phosphatases [25, 26] that differ both in molecular weight and cellular location [14, 15, 27].

Invertase and alkaline phosphatase are both robust enzymes that can withstand repeated rounds of freeze/thaw and do not lose appreciable activity over the 6-week period. The dual purification of these enzymes has several benefits: (i) it allows for a discussion of the differences in reactivity among enzymes from the same class (i.e. hydrolase); (ii) it allows students to be exposed to both direct and coupled enzyme assays; and (iii) it mimics a real-world laboratory experience. The difference in the optimal pH and substrate specificity of the two enzymes allows the activity of each enzyme to be detected independently in the same sample, which helps students track their progress as the enzymes are first separated from cellular components and then from each other.

The project was presented to the students as an exploration. They were told that they would be purifying invertase and phosphatase from yeast, but they would have to determine which forms of the enzyme had been isolated (see Supporting Information). Any repetition of procedures over multiple weeks (i.e. enzyme assays) was done with the expectation that students would use their lab notebooks to write their own protocol for each subsequent lab. At the end of the series, the students were asked to write up their results as a manuscript following the guidelines for submission to Biochemistry. Here, we present an overview of the results obtained by 89 students in five sections completing this course in the fall semester of 2012. Student data shows that this experimental series was successful even with a large number of students. We also present survey data confirming that use of this project-based curriculum increased student comprehension of key biochemical concepts and understanding of skills required for biochemistry research.

Experimental Procedures

Equipment

Yeast Extract Preparation— The initial preparation of extract requires a temperature-controlled incubator (New Brunswick). Centrifugation was performed at 7,500 rpm in a refrigerated table-top centrifuge (Sorvall ST-16R); if equipment is available, the centrifugation speed can be increased to 10,000 rpm and the time shortened accordingly.

Ethanol Fractionation— Any standard refrigerated microcentrifuge is required for the ethanol precipitation, although a room temperature microcentrifuge can be used for the initial clarification of yeast extract.

Protein Activity Assays— All absorbance readings at 400 and 540 nm were performed using Bio-Tek HT plate readers. A Bio-Tek H1m was used in conjunction with a Take3 plate to read A_{280}, although any low-volume spectroscopic

system could substitute. A heat block capable of reaching 90 °C is also required for these assays.

Equipment for SDS-PAGE— A mini vertical electrophoresis system and the appropriate power supply is required.

Course Schedule and Experimental Procedures

Week 1: Ethanol fractionation of S. cerevisiae. Each group of two students is provided with *S. cerevisiae* cell extract prepared by the instructor using a modification of the method of Timerman *et al* [7]. Briefly, 35 g of dried yeast (Sigma YSC2) is suspended in 100 mL 0.100 M sodium bicarbonate solution. The suspension is capped and incubated at 35 °C overnight. The suspension is then centrifuged at 7,500 rpm for 30 min at 4 °C. The supernatant is removed and stored as aliquots of 1.5 mL for each group. This quantity of yeast was sufficient for 40 student groups with some remaining for the instructors to perform the experiment along with the students or to have additional extract in case of student errors. The yeast extract can be stored at 4 or 0 °C short term (1–2 weeks) and −20 °C for longer storage (up to 6 months). Each group is given a 1.5 mL aliquot of extract at the start of week 1.

The yeast extract is clarified by centrifugation for 2 min at 7,500 rpm. Ice-cold ethanol is added to the clarified yeast extract to produce a 29% solution in a volume under 2 mL, the sample is mixed by inversion, placed on ice for 5 min, then centrifuged at 10,000 rpm for 10 min at 4 °C. The supernatant is transferred to a new tube for the second precipitation. Ice-cold ethanol is mixed with the supernatant from the 29% solution to yield a solution that is 40% ethanol in a volume of less than 1 mL. The incubation and centrifugation steps are repeated. Both pellets are resuspended in 600 μL 5.0 mM Tris-Cl pH 7.4. The five samples produced are stored at −20 °C until the following week.

Week 2: Specific Activity I—In this lab period, students test their samples from ethanol fractionation for both invertase and phosphatase activity as well as determine protein content by A_{280}.

Invertase— Sucrose hydrolysis is measured by detecting the amount of 3-amino-5-nitrosalicylate present in the reaction, which results from the reduction of 3,5-dinitrosalicylate (DNS) by free glucose [7, 9]. For each ethanol fractionation sample, 10 μL of sample is incubated with 100 μL freshly diluted 20.0 mM sucrose for 5 min at room temperature. Next, 400 μL alkaline DNS is added to each sample, which is then heated at 90 °C for 5 min before the addition of 400 μL 50.0 mM acetate pH 4.8. An equal volume (200 μL) of solution is added into three wells of a 96-well plate and the absorbance read at 540 nm. A positive control of 100 μL of a mixture containing 20.0 mM each glucose and fructose ("hydrolyzed sucrose") and a negative control (sucrose and acetate buffer only) are treated in a similar manner.

Phosphatase— Enzyme activity is determined according to the amount of para-nitrophenol (PNP) produced from the hydrolysis of para-nitrophenol phosphate (PNPP). The reactions are assembled in a 96-well plate with each well containing 190 μL of 1 mM PNPP in 0.010 M Tris pH 9.0. 10 μL of each sample is added to a set of three wells and the absorbance detected after 5 min at 400 nm. A positive control of 0.100 mM PNP 0.010 M Tris pH 9.0 and a negative control of PNPP in buffer only are also assembled on the plate.

A_{280}— 2 μL of each sample is placed in the Take3 plate in duplicate and the A_{280} measured. The absorbance of 5.0 mM Tris-Cl pH 7.4 is determined to correct for background.

Data Analysis— The absorbance values of the reaction product detected at 400 and 540 nm are converted into the amount of product produced per unit time to give a value for the activity of each enzyme. First, the three absorbance values obtained from each set of samples are averaged and the average absorbance value of the negative control is subtracted from all to correct for background. Next, the molar absorptivities (ε) of the reaction products, 3-amino-5-nitrosalicylate and PNP respectively, are calculated using Beers Law ($A = \varepsilon cl$) with A = the corrected absorbance of the positive control sample; c = the final concentration of either PNP or hydrolyzed sucrose in the positive control sample; l = a standard path length (in cm) calculated by students in an earlier experiment. The activity of the enzymes in each sample is then determined using the equation:

[Corrected Abs × volume of 1 well (L)]/[ε ($M^{-1}cm^{-1}$) × path length(cm) × time (min)] = mol/min

The specific activity of each enzyme is determined by first calculating the mass of protein (mg) in each reaction is by multiplying the A_{280} value by 0.010 mL (the volume of sample tested in each reaction) under the assumption that a solution with $A_{280} = 1$ has a protein concentration of 1 mg/mL. The activity value calculated for each sample is then divided by the mg of protein in that sample to give a specific activity value (mol/min/mg), which is then adjusted to μmol/min/mg or nmol/min/mg where appropriate.

Weeks 3–4. Anion exchange chromatography and Specific Activity II—The next stage of purification is designed to separate invertase and phosphatase from each other using anion exchange chromatography. The weak anion exchange resin, DEAE cellulose (Sigma D6418), is prepared by the instructor according to the product instructions and equilibrated in 5.0 mM Tris-Cl pH 7.4/5.0 mM $MgCl_2$. DEAE Sepharose (Sigma DFF100) can be used as well, although the cellulose resin is significantly cheaper. Students pour their own columns using small, disposable plastic columns (Fisher) and 2.0 mL of DEAE-cellulose in 5.0 mM Tris-Cl pH 7.4/5.0 mM $MgCl_2$. The column is equilibrated with 4 mL of 5.0 mM Tris-Cl pH 7.4/5 mM $MgCl_2$. The protein sample selected at the end of Week 2 is diluted to 1 mL with 5.0 mM Tris-Cl pH 7.4/5.0 mM $MgCl_2$ and then loaded to the column by gravity flow. The elutant is collected in

500 μL fractions until the buffer is 1 cm above the resin. 2.5 mL of 5.0 mM Tris-Cl pH 7.4/5.0 mM $MgCl_2$/100 mM NaCl is then applied to the column and 400 μL fractions are collected until the buffer is again 1 cm above the resin. The process is repeated with 2.5 mL of 5.0 mM Tris-Cl pH 7.4/5.0 mM $MgCl_2$/300 mM NaCl. The resin is then resuspended in 2.5 mL of 5.0 mM Tris-Cl pH 7.4/5.0 mM $MgCl_2$/500 mM NaCl and transferred to a container for regeneration by the instructor. In most cases, the disposable plastic columns can be reused for several student groups, and the DEAE resin can be regenerated for use again the following semester. The fractions generated from chromatography are stored at −20 °C until the next week, at which time the enzyme assays and specific activity calculations are performed in the same manner as week 2.

Week 5. SDS-PAGE analysis—10% SDS-PAGE gels are prepared in advance by the instructor using a 10% acrylamide/buffer premix solution (Fisher EZ gel BP7710) poured as a continuous gel, which reduces instructor prep time without substantially increasing cost compared to traditional SDS-PAGE with stacking gel. The instructor also prepares Broad Range Marker (Bio Rad 161–0317) mixed with 2× Laemmli Sample Buffer and β-mercaptoethanol (prepared according to product instructions) and clarified yeast extract mixed 1:1 with 2× Laemmli Sample Buffer and β-mercaptoethanol. Both solutions are heated at 90 °C for 5 min then stored at −20 °C until needed. Students mix their protein samples 1:1 with prepared 2× Laemmli Sample Buffer and β-mercaptoethanol in a final volume of 30–40 μL, heat at 90 °C for 5 min, then electrophorese the samples at 110 V until the dye front runs off the gel (about 1.5 h). Gels are rinsed five times with distilled water (dH_2O), stained overnight in 10 mL Gelcode Blue, and destained with dH_2O for 2–4 h. Gels were photographed using a Gel Logic 220 PRO system (Carestream) and the images supplied to students as JPEG files.

We use the electrophoresis time for a dry lab to teach data analysis techniques for the kinetic experiments in week 6. Students are given a set of data similar to what they should obtain in week 6 and, with assistance from the instructor, are able to practice plotting the relationship graphically between absorbance versus concentration. Students are able to generate a linear trend line from this graphical relationship with the slope of the line equal to the velocity of the reaction (change in product absorbance over time). Using Beer's law and the volume of the reaction, the absorbance value can be converted into an amount of product (mol) product produced per time. The purpose for this dry lab is to familiarize students with the analysis of large data sets generated from the kinetic experiment. Without this dry lab many students become overwhelmed because they do not understand how to rearrange the data and generate the appropriate graphs.

Week 6. Kinetics of Phosphatase—Students first determine the optimum pH for the activity of their purified phosphatase using a stopped assay, which is a modification of the assay used to determine specific activity. 10 mM buffers are prepared in advance by the instructor that range in pH from 5 to 10. PNPP is diluted in each buffer to a final concentration of 1 mM. Each reaction is prepared in triplicate. As previously described in week 2, 180 μL of the PNPP/buffer mixture is added into a 96-well plate prior to the addition of 10 μL enzyme. For each pH, a set of three reactions is also assembled without enzyme to serve as a negative control. The reaction is allowed to incubate at room temperature for 5 min, then 10 μL 3 M NaOH is added to both stop the reaction and raise the pH of the acidic solutions to allow for the detection of PNP. To allow for adequate time to complete the kinetic experiments, students identify the pH optimum based on A_{400}. Their selection is later verified through calculation of the activity at each pH. Using the pH optimum, six solutions are made with PNPP concentrations from 0 to 1.0 mM. In order to determine the initial velocity of the enzyme reaction, students add enzyme to the plate immediately before the detection program is initiated. Absorbance readings are taken immediately after the addition of enzyme (time = 0) and then every 30 s for 3 min. Enzyme velocity is calculated using the activity calculation described in week 2. The substrate concentrations and velocity are used to determine the steady-state parameters K_M and V_{max} of the enzyme using either linear (double-reciprocal) or nonlinear methods.

TABLE I *Schedule of experiments*

Week	*Experiment*
1	Ethanol fractionation of yeast extract
2	Specific activity I
3	Anion exchange chromatography
4	Specific activity II
5	SDS-PAGE
6	Kinetics of phosphatase

Results and Discussion

Table I shows the schedule of experiments. During the first 2 weeks, students prepare the initial sample for purification by using ethanol to isolate the periplasmic fraction, and the presence of both enzymes in this fraction is then confirmed by a measurement of specific activity. In the next 3 weeks, the enzymes are further purified using anion-exchange chromatography, located by determining the specific activity in each fraction and visualized through SDS-PAGE. In the final week, students continue to characterize their

purified phosphatase. At the end of the semester, the raw data from three representative groups were used to generate the results for the specific activity, SDS-PAGE, and enzyme velocity experiments shown below.

The first week of this series is designed to be a short experiment to allow for time to discuss the overall project goals and expectations. Students are provided with a handout (Supporting Information) with background on the two enzymes and an explanation of the assays that will be used to test for activity. The experimental procedure in the first week is not difficult; the most common error is due to a miscalculation in the amount of ethanol required to bring the solution from 29 to 40%. Some guidance about this calculation is given in the protocols provided to students (see Supporting Information), but students are expected to work out the math without significant assistance from the instructor. As the experiment itself is short, there is plenty of time for students to work these calculations in class.

In the second week, students determine the specific activity of both enzymes in the five samples from ethanol fractionation. There should be measurable activity in nearly all samples, which allows for a discussion of the difference between activity and specific activity. Observing the activity alone would lead students to conclude that the original extract has the highest concentration of both enzymes.

The absorbance of the positive control is used to calculate the molar absorptivity for each reaction product using Beers law. For 3-amino-5-nitrosalicylate, ε values ranged between 1,400 and 2,000 $M^{-1}cm^{-1}$; for PNP, the range was 17,000–21,000 $M^{-1}cm^{-1}$, in agreement with literature values [7, 28]. The path length of the 96-well plate was determined by students in an experiment earlier in the semester and this value was used for all specific activity calculations. The molar absorptivity values were then used to convert product absorbance into units of activity and the A_{280} values used to estimate the amount of protein in each sample. Protein quantitation could also be done using a standard colormetric assay; A_{280} has the advantage of using very little sample (provided a spectrophotometer capable of reading microliter volumes is available) and requiring no sample preparation.

Students are expected to identify the second resuspended pellet (sample 4) from ethanol fractionation as having the highest specific activity for both enzymes. Figure 1 shows representative specific activity data from the three student groups. As seen in Fig. 1*a*, the normalized specific activity for invertase is clearly highest in the second resuspended pellet (sample 4). Figure 1*b* shows a similar result for phosphatase. Although there is variation in the value of the specific activity calculated, normalizing each group's values to their highest specific activity clearly reveals the trend and students are easily able to identify the correct sample for further purification (note that normalization is only necessary when comparing between groups; because individual groups see only their own data, simply identifying the highest value is sufficient). Even with variation in the activity values, 82% of students identified the correct sample (4) as having the highest specific activity for both enzymes. The remaining 18% either identified an additional sample as having a specific activity equal to that of sample 4 or identified the highest specific activity of invertase and phosphatase in separate samples. Groups that did not identify the correct sample were advised to either pool samples or retest their samples before proceeding with the next stage of purification. Common errors during the

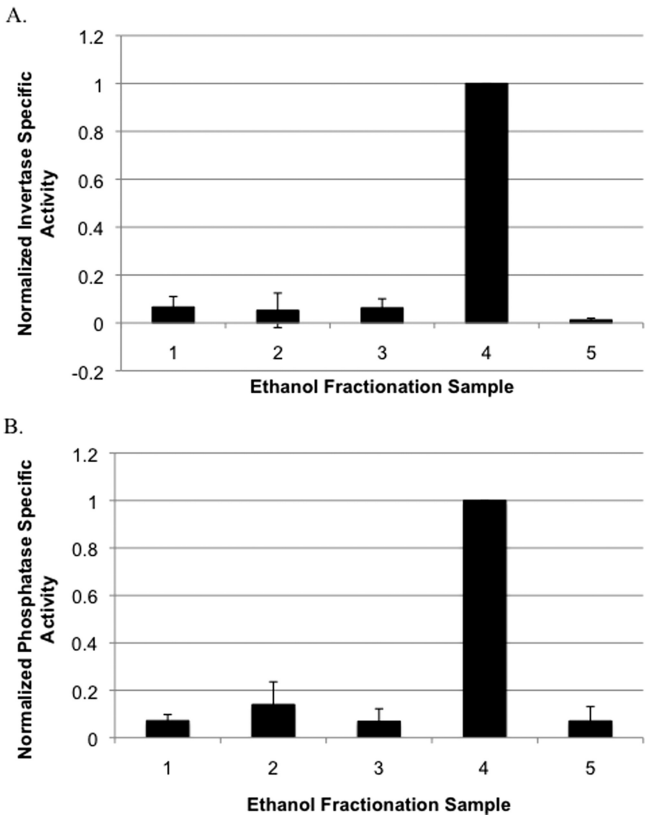

FIG 1 Specific activity of invertase and phosphatase in representative student samples after ethanol fractionation. Student samples were incubated with the appropriate substrate for 5 min and the product absorbance detected at 540 nm (invertase, A) and 400 nm (phosphatase, B) in triplicate. Specific activity was calculated using a molar absorptivity of 1435 $M^{-1}cm^{-1}$ for 3-amino5-nitrosalicylate (*a*) and 17,000 $M^{-1}cm^{-1}$ for para-nitrophenol (*b*) with a path length of 0.80 cm. Values shown are the average of three independent student experiments after normalization. Values are normalized to the highest specific activity observed by each group to more clearly illustrate the consistency of the trend seen by each group. Actual specific activity values varied approximately twofold between the groups with the highest and lowest values.

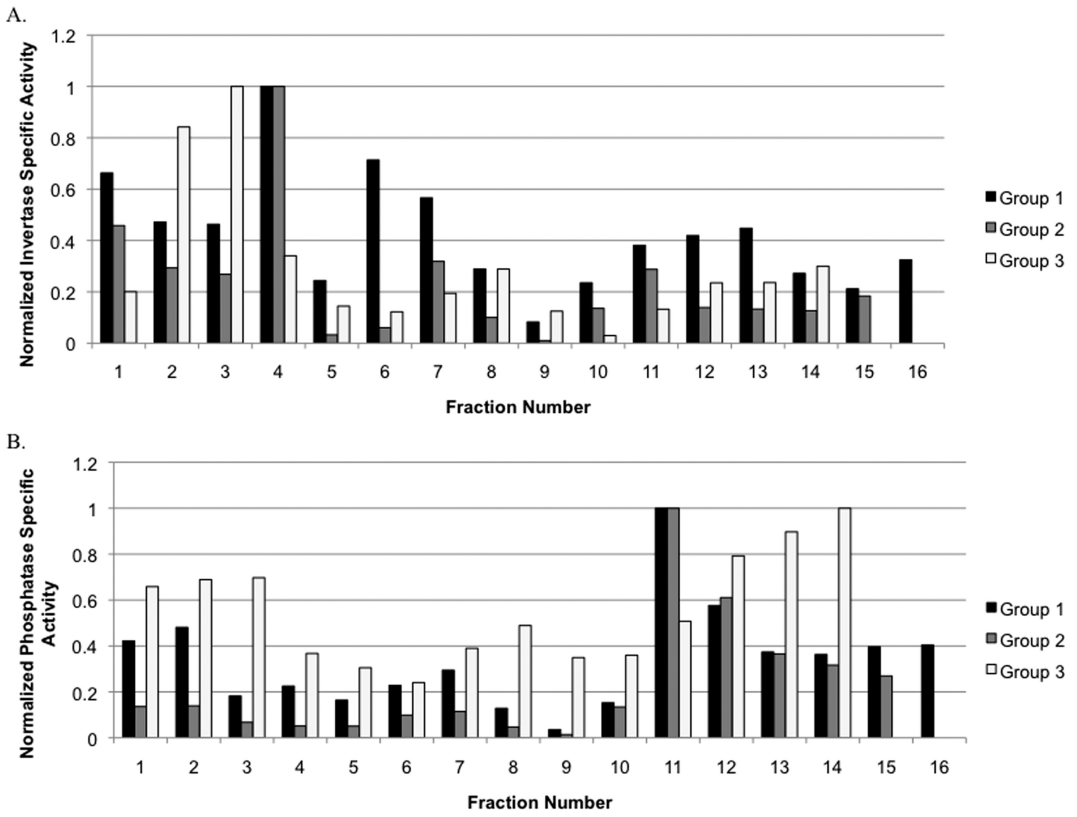

FIG 2

Specific activity of invertase and phosphatase in representative student samples after anion exchange chromatography. Student samples were incubated with the appropriate substrate for 5 min and the product absorbance detected at 540 nm (invertase, A) and 400 nm (phosophatase, B) in triplicate. Specific activity was calculated using a molar absorptivity of 1435 $M^{-1}cm^{-1}$ for 3-amino5-nitrosalicylate (*a*) and 17,000 $M^{-1}cm^{-1}$ for para-nitrophenol (*b*) with a path length of 0.80 cm. Values are normalized to the fraction with the highest specific activity for each enzyme for each group to emphasize the trend in specific activity.

enzyme assays included switching the positive and negative control and using the wrong pH buffer. Errors such as these are generally identifiable by the presence of similar absorbance values in all samples. Instructors monitored students as they collected data, and several students were advised to repeat their experiments based on these errors.

After identifying the sample with the highest specific activity for both enzymes, the purification is continued in week 3 using anion exchange chromatography to separate the two enzymes from each other, and then identification of the fractions containing each enzyme using specific activity measurements in week 4. After loading their sample on the column, invertase is eluted with the addition of 100 mM NaCl and phosphatase follows with the addition of 300 mM NaCl; each group is expected to collect 14–16 fractions. The small-scale nature of the column allows students to experience pouring their own columns and collecting numerous fractions while still leaving time for a quiz at the beginning of the lab period and a short pre-lab lecture. During the pre-lab lecture, students are advised that they will be required to write their own protocol to measure the specific activity of both enzymes in each fraction during the next lab period.

It is expected that the invertase will elute in fractions 3–6, and 69% of students identified a fraction in this range as having the highest specific activity for invertase as seen in the sample student data collected from the three groups (Fig. 2*a*). A small number of students (15%) identified invertase as eluting before the addition of NaCl and the remainder (16%) detected a high specific activity for invertase in one of the later fractions after the addition of 300 mM NaCl. Those detecting invertase in the later fractions may have either made an error during chromatography or mislabeled their samples while performing assays. It is also possible that more than one form of invertase is contained in the original sample (e.g. Fig. 2*a* group 1 is suggestive of two invertase elutions); however, there was not enough student data available to test this hypothesis.

Phosphatase is expected to elute in fractions 9–12, and 80% of students identified a fraction in this range as having the highest specific activity for phosphatase as seen in the student sample data (Fig. 2*b*, groups 1 and 2). The

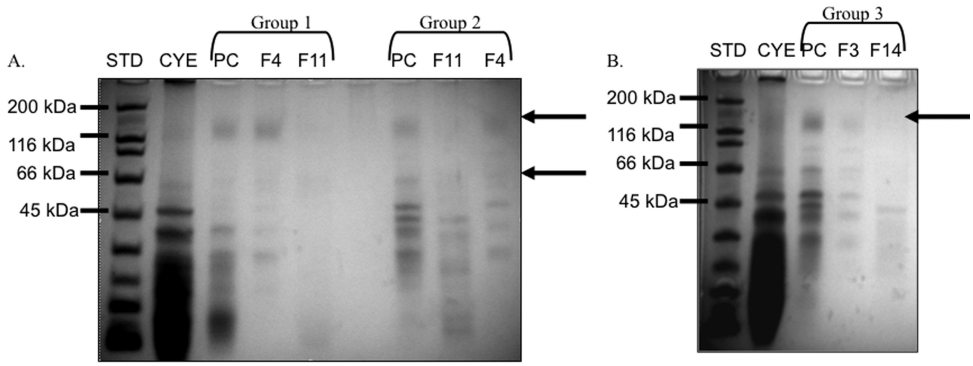

FIG 3 SDS-PAGE analysis of student samples. Gels shown are from three independent student groups. Group 1 and 2 data is shown on (*a*); Group 3 data on (*b*). All gels contain BioRad broad Range marker (STD) with the band sizes indicated on the sides of the gels. Clarified yeast extract (CYE) was prepared and loaded by the instructor. Each group loaded a sample collected prior to chromatography (PC), the sample with the highest invertase (Groups 1 and 2, F4; Group 3, F3) and phosphatase (Groups 1 and 2, F11; Group 3, F14) specific activity. A band representative of extracellular invertase is indicated by the top arrows in (*a*) and (*b*). A band representative of alkaline phosphatase is indicated by the lower arrow on (*a*). All samples were electrophoresed on a 10% SDS-PAGE gel for 1.5 h at 110 V, stained in Gelcode Blue, and the image captured using the Gel Logic camera.

remaining 20% identified phosphatase in one of the last two fractions (Fig. 2*b*, group 3).

Writing the protocol for this measurement of specific activity emphasized the importance of keeping a good laboratory notebook and understanding how the assays work. Although students had some initial difficulty in modifying the initial protocol (from week 2) for a larger number of samples, the majority were able to successfully complete the lab with little to no input from the instructor. The most common error in the student protocols was failing to scale up the amount of substrate for the required number of reactions. Students realized this error while they were preparing reagents and it did not have a significant impact on their ability to complete the lab or on their final results. A few students still struggled with differentiating between the positive and negative control solutions.

In week 5, the two fractions with the highest specific activity for invertase and phosphatase respectively were further examined using SDS-PAGE. The instructor loaded a sample of clarified yeast extract and a molecular weight marker (Figs. 3*a* and 3*b*, lanes 1 and 2), and students were asked to load three samples: a sample reserved prior to anion exchange chromatography and their two chromatography fractions with highest specific activity. Limiting students to three to four samples reduces the number of gels that must be poured and run for each section, which minimizes instructor prep time and requires the use of only three vertical electrophoresis systems per class. Determining the specific activity prior to SDS-PAGE gives students the experience of using their data to choose which samples should be further examined, although the order of the SDS-PAGE and specific activity experiments could be reversed so as to only perform activity measurements on samples suspected of containing the correct protein.

As seen in the representative student sample gels (Figs. 3*a* and 3*b*) containing samples from the student groups described above, the extracellular form of invertase is observed at the expected molecular weight of 125 kDa [7] in the sample before chromatography (Fig. 3*a*, lanes 3 and 7; Fig. 3*b*, lane 3) and in fraction 4 (Fig. 3*a*, lanes 4 and 9; Fig. 3*b*, lane 4). 74% of student samples contained this same form of invertase visible on the SDS-PAGE gel. Of the remaining students, 21% observed the invertase band in the pre-chromatography sample but not in the fraction identified as having the highest specific activity after purification and 4% did not observe any bands in their SDS-PAGE samples.

Phosphatase was more difficult to observe by SDS-PAGE with only 63% of students having a visible ~60 kDa band expected from the isolation of Pho13p [15] present in the fraction collected during chromatography. As seen in Fig. 3*a*, this band was observed in the pre-chromatography sample (lanes 3 and 7), and a very faint smudge can be seen at this same location in the phosphatase fraction (lanes 5 and 8). A faint band is observed in the pre-chromatography sample shown in Fig. 3*b* (lane 3) but no visible band is observed near 60 kDa in the phosphatase fraction (lane 5).

With these results, students are able to conclude that they have purified the extracellular form of Suc2p based on the molecular weight and presence of glycosylation, which gives the protein band a diffuse appearance [7]. However, they were not able to conclusively identify the form of phosphatase present. Several students had inferred by this point in the lab that the phosphatase was alkaline due to the pH of the buffer used for the phosphatase assays in weeks 2 and 4. In week 6, students confirmed that the phosphatase was alkaline by testing the activity of their purified enzyme in a range of buffers (pH 5–10). They then chose the

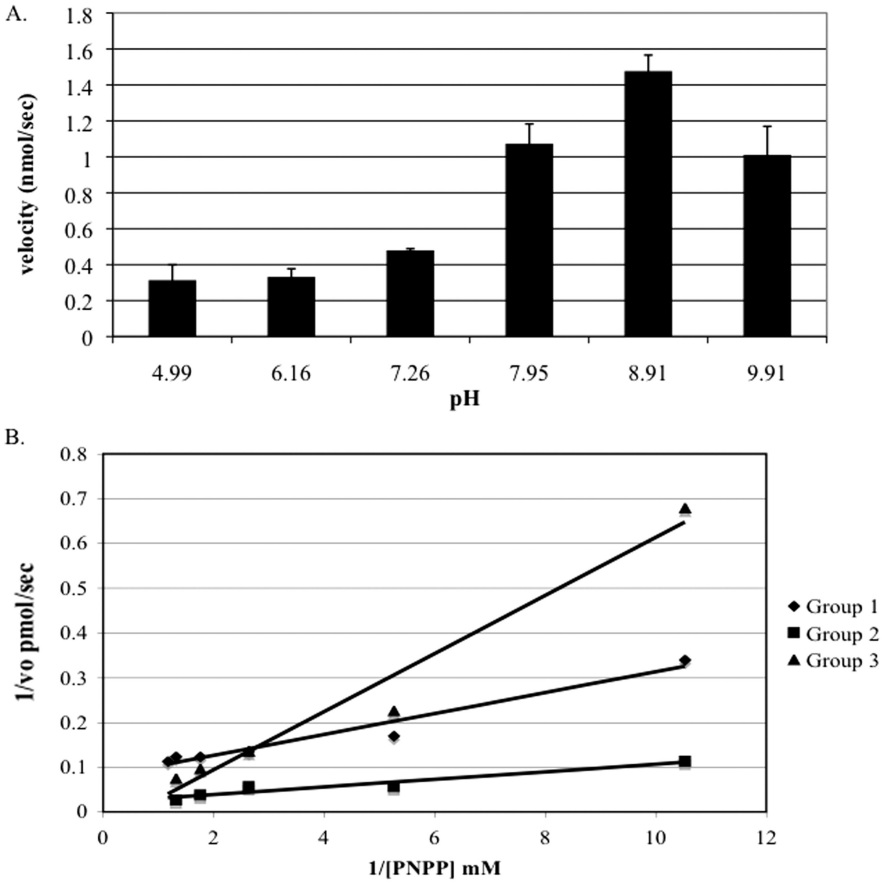

FIG 4

Kinetics of alkaline phosphatase. (*a*) Student purified Pho13p was incubated with 10.0 mM PNPP in 10.0 mM buffers: acetate pH = 4.99, citrate pH 6.16, phosphate pH 7.26, tris pH 7.95, glycine pH 8.91, and glycine pH 9.91. After 5 min, 3 M NaOH was added to stop the reaction and the absorbance read at 400 nm in triplicate. Enzyme activity was calculated using a molar absorptivity of 17,000 $M^{-1}cm^{-1}$ for para-nitrophenol with a path length of 0.80 cm. Values shown are the average of three independent student experiments. (*b*) Student-purified Pho13p was incubated with PNPP (0–1 mM) in 10.0 mM glycine pH 8.91. Absorbance readings in triplicate were collected at 400 nm every 30 s for 3 min. Velocity was calculated using a molar absorptivity of 17,000 $M^{-1}cm^{-1}$ for para-nitrophenol with a path length of 0.80 cm. The double reciprocal plot was obtained by graphing 1/initial velocity versus 1/[PNPP]. Each line represents the results from an independent student group.

optimal buffer and determined the initial velocity at several concentrations of PNPP to calculate the K_M and V_{max} of their enzyme. Altering the pH conditions also allowed for a modification of the phosphatase assay to include the addition of 3 M NaOH both to stop the reaction and raise the pH to a constant value above the pK_a of PNP so the reaction product could be detected at 400 nm.

Figure 4*a* shows sample student data collected from the three representative groups confirming that the purified phosphatase is most active at a pH near 9. 81% of students identified this as the optimal pH for their enzyme, 2% identified a pH above 9 as optimal, and the remaining 18% had no detectable activity in their phosphatase preparations. It is possible that the students with no enzyme activity in their samples identified and saved the wrong fraction in week 4. It is also possible that some of the samples with relatively low enzyme concentration from the beginning may have degraded at this point as the samples were 5 weeks old and had been subjected to several rounds of freeze/thaw.

After identifying the optimal pH for activity, the activity of the enzyme at various concentrations of PNP was determined to characterize the steady-state kinetics of this enzyme. Figure 4*b* shows the double reciprocal plot obtained from the three sets of student data. Again, while there is variation in the actual kinetic values obtained, students are clearly able to observe a change in the velocity of their enzymes over time and with increasing substrate concentrations. All students that observed measurable enzyme activity when determining the optimal pH were also able to collect enough kinetic data to calculate both a K_M and V_{max} for their enzymes. Student values of K_M were in the range of 10^{-3}–10^{-4} M, which is higher than the literature value of 3.6×10^{-5} M for Pho13p [15]; however, this is not

TABLE II *Student survey results*

How would you describe the gain you made in:	*No gain*	*Little gain*	*Moderate gain*	*Good gain*	*Great gain*
General technical lab skils	3	10	19	29	17
Understanding of protein purification methods	4	11	18	30	15
Understanding of the logical flow of steps in purifying and characterizing a molecule	4	14	22	27	11
Understanding of assays for measuring enzyme activity	2	11	18	28	19
Understanding of enzyme kinetics and inhibition	6	14	21	25	12
Understanding of how to communicate the results of scientific experiments	3	9	17	32	17
Numerical problem-solving skills	3	10	22	31	12
Conceptual problem-solving skills	6	9	25	30	8
Ability to develop a logical, effective lab procedure	3	13	23	29	10
Ability to interpret experimental results	4	7	24	29	14
Ability to use a critical approach when analyzing data and arguments in daily life	6	15	23	30	4

unexpected given the limited sample number and the assay conditions (e.g. room temperature vs. 37 °C, variable purities, and several freeze-thaw cycles).

TABLE III *Student assessment results*

Question	*Pre-test (%)*	*Post-test (%)*
Calculation of molar absorptivity of control	26	93
Meaningful presentation of data in a figure	55	89
Determination of protein amount from A_{280}	4	85
Relationship between specific activity and enzyme purity	1	71

All differences significant at $p < 0.001$ *by paired* t*-test.*

An anonymous survey was used to collect student opinions about the experimental series and was completed by 88% of the students. The linked series of experiments was not overwhelmingly popular, as 54% of students of students responded "strongly agree" or "agree" to the statement "I would have preferred a diverse set of individual lab experiments that did not span multiple weeks." 22% disagreed with this statement and the remaining 24% were "neutral." There are a number of reasons students might prefer a traditional laboratory sequence of isolated experiments; in this case the low popularity of the linked series is probably due to a perception of increased workload due to the cumulative nature of the experiments. Students must complete assignments in a timely fashion and be proactive about seeking help when an assignment or procedure is confusing, or their performance in subsequent labs suffers. Some students simultaneously express a desire for fewer assignments and appreciation for the regular feedback possible with frequent assignments, indicating that students do not have a simple opinion on this matter. As this is the first project-based lab that many of our students have encountered, there is a learning curve as they become adjusted to the expectations for their performance. Moreover, the survey was conducted

at the end of the series, while the students were completing their final manuscript, and so they may have been expressing end-of-semester stress as much as evaluating the series of experiments.

Although students did not report a significant appreciation for the series, the majority of students did report a moderate to great gain in their understanding of a number of conceptual details as well as technical lab skills (Table II), indicating that the experimental series did have the desired effect on student learning. Student comments also supported the value of the project: "I was able to learn many aspects of biochemistry that I was unfamiliar with and my problem-solving skills greatly improved because of how challenging this lab was. This lab required you to be engaged in the experimental aspect of biochemistry which caused me to be more intrigued by the subject." Another student commented, "After taking this course, I have gained a considerable amount of knowledge of biochemistry. I have also greatly improved my problem-solving skills and become more of a critical thinker." Even students who were not pleased with the course recognized the amount learned: "It [lab] helped with problem solving and research skills; and, with computer skills. I have no clue what my attitude [is] toward biochem. It is a love hate relationship."

To assess progress in student understanding of relevant concepts during the course, we make use of a pre-test/post-test system. Students are given the pre-test on the first day of the semester, and similar questions are asked as part of the final exam (pre-tests are not returned to the students). Several of the questions directly relate to basic skills required to work with the data obtained during this experimental series (Table III). Questions are open-ended, and students were judged as understanding the concept if their final answer contained no more than one minor error unrelated to the overall concept (e.g. math error due to mistyping numbers in a calculator).

This 6-week experimental series has an advantage over many of the current undergraduate laboratory experiments in the literature because it is designed to work with large groups of students in a short time period. Very little specialized equipment is required, although the use of the Bio-Tek plate reader does allow for students to take triplicate measurements of all absorbance readings and perform kinetic assays. The modular nature of the project is also advantageous; for example, the series could begin in week 3 with instructor prepared samples or weeks 1 and 2 could be performed as an isolated series to demonstrate differences in protein solubility and activity.

The majority of students were able to successfully complete the series, with less than 10% having inactive or missing enzyme by the end of the series. With up to 24 students per section, it is impossible for the instructor to continuously monitor all groups and prevent mistakes in the procedure; the overall success of the series despite these challenges demonstrates the robust nature of these experiments. Requiring students to develop their own protocols for several experiments fosters critical thinking skills and forces students to think more about why they are performing a particular step rather than having them simply follow a set of instructions. We hope that the details of this experimental series will be of benefit to other universities that struggle to balance the desire to offer a project-based experience to their students with the demands of a large student population and limited resources.

Acknowledgments

This work was supported by the Louisiana Board of Regents [LEQSF (2011-12)ENH-UG-24]. Th authors thank Xavier Chemistry department chairs, Cheryl K. Stevens and Maryam Foroozesh, for their support of this project.

References

[1] Hurst, M. O., Keenan, M. V., and Son, C. C. (1992) Purification of lysozyme by linear salt gradient and SDS gel electrophoresis. J. Chem. Educ. 69:850–851; doi:10.1021/ed069p850.

[2] Brockman, M., Ordman, A. B., and Campbell, A. M. (1996) Research in undergraduate instruction: A biotech lab project for recombinant DNA protein expression in bacteria. J. Chem. Educ. 73:542–543; doi:10.1021/ed073p542.

[3] Deal, S. T., and Hurst, M. O. (1997) Utilizing isolation, purification, and characterization of enzymes as project-oriented labs for undergraduate biochemistry. J. Chem. Educ. 74:241–242; doi:10.1021/ed074p241.

[4] Craig, P. A. (1999) A project-oriented biochemistry laboratory course. J. Chem. Educ. 76:1130–1135; doi:10.1021/ed076p1130.

[5] Hunnes, C. H. (2003) Isolation, purification, and characterization of bovine heart and yeast cytochromes c: An integrated biochemistry laboratory experience. Biochem. Mol. Biol. Educ. 31:242–248; doi:10.1002/bmb.2003.494031040248.

[6] MacDonald, G. (2008) Teaching Protein Purification and Characterization Techniques. A Student-Initiated, Project-Oriented Biochemistry Laboratory Course. J. Chem. Educ. 85:1250–1252; doi:10.1021/ed085p1250.

[7] Timerman, A. P., Fenrick, A. M., and Zamis, T. M. (2009) The Isolation of Invertase from Baker's Yeast: A Four-Part Exercise in Protein Purification and Characterization. J. Chem. Educ. 86:379–381; doi:10.1021/ed086p379.

[8] Jewett, K., and Sandwick, R. K. (2011) Ribose 5-Phosphate Isomerase Investigations for the Undergraduate Biochemistry Laboratory. J. Chem. Educ. 88:1170–1174; doi:10.1021/ed100670a.

[9] Melius, P. (1971) Isolation of yeast invertase by Sephadex gel chromatography. A biochemistry laboratory experiment. J. Chem. Educ. 48:765–766; doi:10.1021/ed048p765.

[10] Munson, L., and Fall, R. R. (1978) Purification and characterisation of Escherichia coli alkaline phosphatase. A biochemical experiment. Biochemical Education 6:53–56; doi:10.1016/0307–4412(78)90059-6.

[11] Howitt, S. M. (2007) Investigation of the properties of wild type and mutant alkaline phosphatase variants: A laboratory project linking genotype and phenotype. Biochem. Mol. Biol. Educ. 35:350–354; doi:10.1002/bmb.91.

[12] Ninfa, A. J., Ballou, D. P., and Benore, M. (2010) Fundamental Laboratory Approaches for Biochemistry and Biotechnology, John Wiley and Sons, Hoboken, New Jersey.

[13] Schurr, A., and Yagil, E. (1971) Regulation and characterization of acid and alkaline phosphatase in yeast. J. Gen. Microbiol. 65:291–303.

[14] Onishi, H. R., Tkacz, J. S., and Lampen, J. O. (1979) Glycoprotein nature of yeast alkaline phosphatase. Formation of active enzyme in the presence of tunicamycin. J. Biol. Chem. 254:11943–11952.

[15] Tuleva, B., Vasileva-Tonkova, E., and Galabova, D. (1998) A specific alkaline phosphatase from Saccharomyces cerevisiae with protein phosphatase activity. FEMS Microbiol. Lett. 161:139–144.

[16] Carlson, M., Osmond, B. C., and Botstein, D. (1981) Genetic evidence for a silent SUC gene in yeast. Genetics 98:41–54.

[17] Carlson. M, Osmond, B. C., and Botstein, D. (1981) SUC genes of yeast: A dispersed gene family. Cold Spring Harb. Symp. Quant. Biol. 45 Pt 2:799–803.

[18] Carlson, M. and Botstein, D. (1983) Organization of the SUC gene family in Saccharomyces. Mol. Cell Biol. 3:351–359.

[19] Carlson, M., Taussig, R., Kustu, S., and Botstein, D. (1983) The secreted form of invertase in Saccharomyces cerevisiae is synthesized from mRNA encoding a signal sequence. Mol. Cell. Biol. 3:439–447.

[20] Williams, R. S., Trumbly, R. J., MacColl, R., Trimble, R. B., and Maley, F. (1985) Comparative properties of amplified external and internal invertase from the yeast SUC2 gene. J. Biol. Chem. 260:13334–13341.

[21] Tarentino, A. L., Plummer, T. H., Jr., and Maley, F. (1974) The release of intact oligosaccharides from specific glycoproteins by endo-beta-N-acetylglucosaminidase H. J. Biol. Chem. 249:818–824.

[22] Chu, F. K., Trimble, R. B., and Maley, F. (1978) The effect of carbohydrate depletion on the properties of yeast external invertase. J. Biol. Chem. 253:8691–8693.

[23] Barbaric, S., Kozulic, B., Ries, B., and Mildner, P. (1984) Physicochemical and kinetic properties of acid phosphatase from Saccharomyces cerevisiae. J. Biol. Chem. 259:878–883.

[24] Barbaric, S., Mrsa, V., Ries, B., and Mildner, P. (1984) Role of the carbohydrate part of yeast acid phosphatase. Arch. Biochem. Biophys. 234:567–575.

[25] Kaneko, Y., Hayashi, N., Toh-e, A., Banno, I., and Oshima, Y. (1987) Structural characteristics of the PHO8 gene encoding repressible alkaline phosphatase in Saccharomyces cerevisiae. Gene 58:137–148.

[26] Kaneko, Y., Toh-e, A., Banno, I., and Oshima, Y. (1989) Molecular characterization of a specific p-nitrophenylphosphatase gene, PHO13, and its mapping by chromosome fragmentation in Saccharomyces cerevisiae. Mol. Gen. Genet. 220:133–139.

[27] Klionsky, D. J. and Emr, S. D. (1989) Membrane protein sorting: Biosynthesis, transport and processing of yeast vacuolar alkaline phosphatase. EMBO J. 8:2241–2250.

[28] Mackintosh, C. Assay and purification of (serine/threonine) phosphatases in Protein Phosphorylation: A Practial Approach. Hardie, D. G., Ed. (1993), IRL Press, New York.

Purification and Characterization of Invertase and Phosphatase from Baker's Yeast

Over the next six weeks, you will be performing a series of experiments designed to introduce you to common techniques used in the purification and characterization of proteins. This handout contains background information you may need during this process. At the end of this experimental series, you will present your findings by writing a manuscript in the style of the journal *Biochemistry*.

Since more than one form of each enzyme exists, it is also necessary for you to determine which form has been purified. Based on our starting material and reaction conditions, you can start to formulate a hypothesis as to which form(s) of invertase and phosphatase are present in your samples. Information from the enzyme activity assays and gel electrophoresis will provide the data necessary to accept or reject your hypothesis. At the end of this series of labs, you should be able to decide which form(s) you have purified and use your data to support your decision.

Enzyme background

You will be purifying the enzymes invertase and phosphatase from Baker's yeast (*S. Cerevisiae*). Both invertase and phosphatase catalyze hydrolysis reactions; therefore they fall into the Class III (Hydrolases) group of enzymes according to the International Union of Biochemistry and Molecular Biology (IUBMB).

Hydrolase reactions usually follow the scheme:

Hydrolases are further classified according to the type of bond broken. Invertase (E3.2.1.26) is a glycosylase while phosphatases (E3.1.3-5) are phosphoric ester hydrolases. Although they both catalyze reactions according

to the general scheme shown above, their substrate specificities are very different. This difference is important because it allows us to test the activity of each enzyme even when they are present in a mixed sample.

Invertase: Invertase primarily catalyzes the breakdown of sucrose by hydrolyzing the glycosidic bond between glucose and fructose. The structure of sucrose is shown in Figure 1.

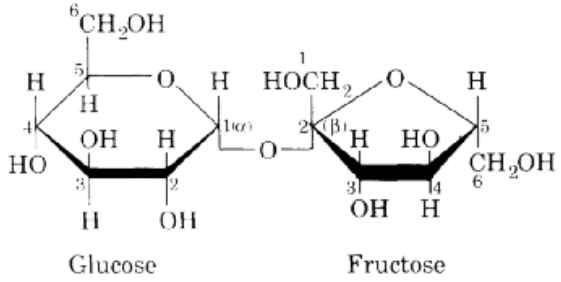

Figure 1. The strucutre of sucrose. Glucose and fructose are shown joined by an α(1→2) glycosidic bond with the individual carbon atoms in each monosaccharide

Yeast can contain up to seven sucrose fermentation (SUC) genes that code for invertase; however, all loci code for nearly identical forms of the protein [1-3]. The distribution of these genes varies among yeast strains, with SUC2 being the most commonly identified and therefore the most studied of the SUC genes [2]. Transcription of the SUC2 gene results in two mRNAs that differ by 100 bases at their 5' end. The longer mRNA is translated to produce a form of invertase that is secreted from the cell into the periplasm [4] [5]. The extracellular form of invertase is also glycosylated; it contains an average of nine oligosaccharide chains with each chain containing between 26 and 54 residues [6, 7]. Glycosylation increases the molecular mass of the enzyme from 60,000 Da to 125,000 Da per subunit, with the active form usually found in a dimeric state [5, 7]. Translation of the smaller mRNA results in invertase that remains in the cell and is relatively free of carbohydrates giving it a molecular mass similar to that of the extracellular form prior to glycosylation [5, 7]. Both forms of the enzyme have similar kinetic properties and operate in the same pH range (4-6), although the glycosylation appears to give the extracellular form more stability and therefore more activity at low pH [7].

Phosphatase: Phosphatases are enzymes that catalyze the removal of a phosphate group from a substrate. There are many different subtypes of phosphatases, and they are grouped according to the chemical nature of the substrate or the type of hydrolytic reaction catalyzed. Phosphomonoesterases (EC3.1.3) are a group of

phosphatases that catalyze the hydrolysis of a single phosphate ester. While some phosphomonoesterases are highly specific, others act on a broad range of substrates. The phosphomonoesterases that can act on a broad range of substrates are classified according to the pH at which they show optimum activity, and are named either acid or alkaline phosphatases.

Yeast have several genes that encode nonspecific phosphomonoesterases as well as several other related genes involved in phosphate metabolism (PHO). Three genes, PHO3, PHO5, and PHO11, encode acid phosphatases that range from 61,500 – 73,000 Da [8]. These acid phosphatases are secreted from the cell, glycosylated, and are found in large oligomeric complexes containing roughly 50% oligosaccharide residues and 50% protein, giving them a molecular mass of 200,000 Da or greater [9]. They are active in a small pH range (3-5) and denature easily at pH values outside of that range [9]. Two genes, PHO8 and PHO13, encode alkaline phosphatases [10, 11]. Unlike the interchangeable acid phosphatases of yeast, the enzymes encoded by PHO8 and PHO13 do not have the same structure or substrate specificity. PHO8 encodes a dimeric protein of 130,000 Da containing a small amount of glycosylation, as each subunit is predicted to have a molecular mass of 63,000 without oligosaccharides [12, 13]. This phosphatase is located in the vacuole of the cell [14] and seems to have no preference for any particular monophosphate esters [13]. By contrast, the protein encoded by PHO13 is monomeric with a molecular mass of 60,000 Da and has a strong substrate preference for para-nitrophenol phosphate along with a few phosphorylated proteins [13]. The cellular location of this phosphatase is unspecified. Both alkaline phosphatases have a similar pH range (8-10) for activity [13].

Enzyme activity

As you proceed with the purification, you must monitor the activity of both invertase and phosphatase to ensure that the purification is proceeding successfully. The activity will be tested through the use of a specific assay or test for each enzyme. In both assays, you will be detecting the product of the enzymatic reaction. You should be familiar with the reactions described below and be able to understand and explain the results of each assay.

Assay for invertase activity: The main difference between the substrate and products of the invertase-catalyzed reaction is that a nonreducing sugar has been converted to a reducing sugar. A reducing sugar cannot be differentiated from a nonreducing sugar by the naked eye; however, reducing sugars have the ability to reduce other compounds. This assay proceeds in three steps: Step I: Sucrose is mixed with a protein sample. If active invertase is present, sucrose will be hydrolyzed to yield equal amounts of glucose and fructose. Step 2: The sample is heated to encourage the isomerization of fructose to glucose. Step 3: DNS (3,5-dinitrosalicylate, Figure 2) is added to the mixture. Glucose reduces DNS to 3-amino-5-nitrosalicylate and in turn is oxidized to gluconate. Steps 2 and 3 are often combined when performing this experiment in the laboratory.

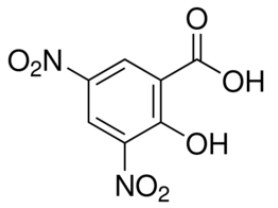

Figure 2. Structure of DNS. Here DNS is shown in the acid form.

Because you are not directly detecting the amount of glucose present, this is known as a coupled assay. Here the production of glucose is coupled to the reduction of DNS. 3-amino-5-nitrosalicylate can be distinguished from DNS by a difference in their color. This difference can be detected by reading the absorbance of the sample at 540 nm, which is the absorbance maximum for 3-amino-5-nitrosalicylate. This is a stopped assay, because the heat in step 2 will denature the enzyme and prevent any further activity.

Assay for phosphatase activity: Since it would be impractical to use a variety of monophosphate esters as substrates, the molecule para-nitrophenol phosphate is used as a standard substrate to detect phosphatase activity. This is a direct assay; para-nitrophenol phosphate is mixed with a protein sample. If the sample contains active phosphatase, para-nitrophenol is produced. Para-nitrophenol has a strong, yellow color under basic conditions that can be detected by reading the absorbance of the solution at 400 nm. If the assay is not performed under basic conditions, the pH of the solution must be raised prior to reading the absorbance. Unlike the invertase assay, this is not a stopped assay and the enzyme will continue to convert substrate to product until all substrate has been hydrolyzed.

Schedule of activities

Below is a schedule of experiments for the next six labs.

Week	Experiment
1	Ethanol fractionation of yeast cells
2	Specific activity I
3	Anion exchange chromatography
4	Specific activity II
5	SDS-PAGE
6	Kinetics of phosphatase

A note about naming conventions: Gene and protein names are written differently depending on the organism. For yeast, it is standard convention to write the name of the gene in all capital letters followed by a number (if applicable). If you wish to refer to the protein encoded by the gene, only the first letter is capitalized and a p is added after the name to indicate protein. For example, SUC2 is a gene and Suc2p is a protein. You may also refer to the protein by its activity. As it is unlikely that you will know exactly what form of the protein you have purified until the end of the experimental series, it is best to refer to the enzymes by their generic names (i.e., invertase or phosphatase) in your writings unless you are hypothesizing about your results. In your final analysis, you should be able to identify the specific form of invertase and phosphatase and refer to it by the correct protein name.

Ethanol Fractionation of Baker's Yeast

Introduction:

In today's experiment, we will begin the process of purifying the enzymes invertase and phosphatase from yeast. Ethanol fractionation is used here to separate the periplasm from other cellular components as the first step in purification. If the procedure today is done correctly, you will end up with one fraction that contains a higher concentration of enzyme than the others. However, you will not be able to determine the identity of this fraction today, so you must save all of your samples. Next week, you will analyze the enzyme activity in each sample to determine which has the most enzyme activity.

It is critical that you keep a good record of both your activities in lab and the content and identity of all samples. Organization is essential to the success of these experiments. Be sure to label all tubes so that you can uniquely identify your tubes when mixed in a box with other tubes from your class.

Hazards:

Ethanol is flammable.

Preparation of yeast extract:

This part of the experiment has been done for you. However, these details are an important part of the experiment and should be included in your lab notebook and Materials and Methods section of your manuscript.

28.0 g of dry yeast was suspended in 100 mL of 0.100 M sodium bicarbonate solution, loosely capped, and placed at 35˚C overnight. The next day, the suspension was centrifuged at 7,500 rpm for 30 minutes at 4˚C. The supernatant was transferred into a separate container and stored at -20˚C.

Ethanol Fractionation:

1. Obtain a tube of yeast extract from your instructor.

2. Centrifuge the extract for 2 minutes at 7500 rpm to clarify the extract. Transfer the supernatant to a new tube and label it "sample 1" or "CYE". You do not need to save the pellet produced at this stage and can discard it in the trash.

3. Using ice-cold absolute EtOH, mix EtOH and clarified yeast extract together in a new microcentrifuge tube to give 1.4 mL of a solution that is 29% EtOH. This is sample 2.

4. You should have some clarified yeast extract left over. Save this on ice. If you do not have any left over, first check to see that you have completed step 3 correctly. If you believe that you have performed that step correctly, immediately notify your instructor so that he/she may check your sample.

5. Mix sample 2 by inverting (*i.e.*, turn the tube upside down several times). Place the tube on ice for at least 5 minutes. It is fine if the tube remains on ice for more than 5 minutes.

6. Centrifuge at 13,000 rpm for 10 minutes at 4˚C.

7. Transfer the supernatant to a new tube. Try to transfer as much of the supernatant as possible without disturbing the pellet. This is sample 3. Save the pellet (in sample 2) on ice.

8. Combine the supernatant and ice-cold absolute EtOH in a new tube to give 700 µL of a solution that is 40% EtOH.* This is sample 4.

*Remember that your solution is already 29% EtOH. You will need to solve $C_1V_1 + C_2V_2 = C_3V_3$.

9. Repeat steps 5-7 with sample 4. Label the new supernatant tube sample 5.

10. Resuspend the pellets in sample 2 and 4 tubes in 600 µL of 5.0 mM Tris pH 7.4. The pellets may not go into solution easily. You may vortex to resuspend. If a small amount of pellet remains after several attempts to resuspend, you may stop at this point.

11. You should now have five samples in clearly labeled tubes. Give these five samples to your instructor. Be sure that you have a description in your notebook of what is in each tube and exactly how each tube is labeled.

12. Your instructor will place these samples in the -20˚C freezer for storage.

Specific Activity I

Introduction:

Last week you used ethanol to fractionate yeast cells into different components as an initial purification technique. If fractionation was performed correctly, one fraction should have more invertase and phosphatase than the other fractions. It is important to identify the fraction with the highest enzyme concentration so that it can be used as the starting material for the second stage of purification. Since there are also many other proteins in the fractions, the only way to identify the presence of either enzyme in each fraction is by measuring their activity.

As we have already discussed, invertase and phosphatase catalyze similar reactions, but have very different substrate specificities. Therefore, we can perform two different enzyme activity assays on each sample to determine the relative activity of each enzyme in that sample.

You will be expected to perform these activity assays a number of times in the coming weeks. This will be the only time that you will be given a step-by-step protocol for each assay. Be sure to take good notes and annotate your lab notebook so that you understand exactly how to perform these assays and feel confident that you can repeat them without additional instruction.

The protocols for the phosphatase assay, invertase assay, and A_{280} measurement are described below. *These are three separate experiments and do not need to be performed in the order they are listed.*

Hazards:

DNS is harmful if swallowed or absorbed through the skin.

Phosphatase activity assay:

This assay will test the ability of phosphatase to remove the phosphate group from paranitrophenol phosphate (PNPP), producing paranitrophenol (PNP).

1. Prepare your positive control: In a microcentrifuge tube, dilute 0.500 mM PNP to 0.100 mM in a final volume of 700 µL with 10.0 mM Tris pH 9.0. This solution should have a significant yellow color.

2. Determine the number of samples you need to test for activity. You will also need a negative control, which will increase your number of samples by one (so a total of 6 samples for this lab).

3. To test each sample, you will need PNPP diluted to a final concentration of 1.0 mM. Use 200 µL as your final volume to perform the dilution calculation and determine the volume of 10.0 mM PNPP needed for each sample. Once you calculate the volume of 10.0 mM PNPP needed for one sample, multiply it by the total number of samples. *

4. The PNPP will be diluted with 10.0 mM Tris pH 9.0. Calculate the volume of 10.0 mM Tris pH 9.0 needed for a single sample, then subtract 10 µL to allow for the addition of your sample to the reaction. Once you have calculated this volume, multiply it times the total number of samples.*

*Preparing solutions in bulk rather than mixing each reaction individually saves time and usually results in more precise data. However, we must also account for a small, unavoidable amount of error while pipetting. Before you proceed to step 5, multiply each of the volumes you calculated above by 1.2. This 'extra' 20% will ensure that you actually have enough solution for all of your samples.

5. Today you will test each sample in triplicate to produce more reliable data. Multiply the volumes of PNPP and Tris calculated above by 3.

6. Mix the calculated total volumes of 10.0 mM PNPP and 10.0 mM Tris pH 9.0 together in a single 15 mL tube. This solution should be colorless to very pale yellow.

7. Pipet 190 µL of the PNPP/Tris mixture into wells of a 96 well plate in sets of three (*e.g*, A8,A9, A10).

8. Determine the set of three wells that you wish to use as your negative control. Add 10 µL of 5.0 mM Tris pH 7.4 buffer to each of these wells. Make a note of these wells in your lab notebook.

9. Pipet 200 µL of your positive control into three empty wells.

10. When it is your turn at the plate reader, quickly add 10 µL of each sample to a set of three wells. The reaction will begin immediately, so it is important to get your plate into the BioTek plate reader as quickly as possible after adding the samples.

11. Read the absorbance of the plate at 400 nm. The program you use will read the absorbance of each sample at zero and five minutes. You will need the five-minute absorbance reading for calculation of phosphatase activity.

12. You should check to see if the absorbance of your sample wells has increased from zero to five minutes (i.e., are the absorbances greater at 5 minutes than at 0 minutes). If at least some of your samples do not show a change, then there is no enzyme activity in your sample or you waited too long between adding your samples and reading the plate. You should also note that the absorbance of the positive control should be much different from that of the negative control, and that the controls do *not* show any difference between zero and five minutes. If you observe a lack of change in your samples or a problem with your controls, notify your instructor so that you can plan to redo this experiment appropriately.

13. Average the triplicate absorbances for each sample and control at 5 minutes. Subtract the average absorbance of the negative control from the average absorbance for each sample (including the positive control). Negative values after this subtraction indicate no enzyme activity. If none of your samples have enzyme activity (*i.e.*, they are all negative), then an error has been made during the experiment and you should notify your instructor so that you can redo this experiment.

Invertase activity assay:

To measure the activity of invertase, you will use its natural substrate, sucrose. However, in order to detect the products of the reaction, the hydrolysis of sucrose will be coupled to the reduction of 3,5 dinitrosalicylate (DNS), producing an orange-colored product, 3-amino-5-nitrosalicylate.

1. Prepare your substrate: Dilute 0.100 M sucrose to 0.020 M in a final volume of 1.00 mL using 50.0 mM acetate pH 4.8. Sucrose is hydrolyzed readily even in the absence of enzyme, so a fresh dilution must be prepared each time you perform this assay.

2. Prepare your positive control: 10.0 µL 5.0 mM Tris-Cl pH 7.4 + 100 µL 0.020 M hydrolyzed sucrose in a labeled microcentrifuge tube. Be sure you are using the correct form and concentration of sucrose here.

3. Prepare your negative control: 10.0 µL 5 mM Tris-Cl pH 7.4 + 100 µL 0.020 M sucrose (the substrate prepared in step 1) in a labeled microcentrifuge tube.

4. For each sample to be tested, combine 10.0 µL of that sample with 100 µL 0.020 M sucrose (prepared in step 1) in a labeled microcentrifuge tube. For this lab, you should be testing 5 samples.

5. Allow all samples and controls to sit on the bench for approximately five minutes. During this time, the hydrolysis of sucrose will be catalyzed by any invertase present in your sample; therefore it is important to note if this reaction proceeds for longer than 5.0 minutes so that the actual time can be used in the activity calculation. Be sure that you record the actual time from addition of sample to the sucrose (step 4) to addition of alkaline DNS (step 6) in your lab notebook, as this is the full reaction time.

6. Add 400 µL alkaline DNS to each tube (samples and controls).

7. Heat all tubes at 90˚C for 5 minutes. Heating the samples will both denature the enzyme (stopping the reaction) and facilitate the reduction of DNS by glucose (as described in the handout provided in Week 6). It is not critical that this value be exactly five minutes, and a little longer is fine. You will not use this time value in any calculations.

8. Add 400 µL 50.0 mM acetate pH 4.8 to each tube.

9. Pipet into 96-well plate, 200 µL per well. Each sample or control should be placed in the plate in triplicate.

10. Read the absorbance of the plate at 540 nm.

11. You should note that the absorbance of the positive control should be much different from that of the negative control. If you observe a lack of difference with your controls, notify your instructor so that you can redo the experiment with correct controls.

12. Average the triplicate absorbances for each sample and control. Subtract the average absorbance of the negative control from the average absorbance for each sample (including the positive control). Negative values after this subtraction indicate no enzyme activity. If none of your samples have enzyme activity (*i.e.*, they are all negative), then an error has been made during the experiment and you should notify your instructor so that you can redo this experiment.

Protein quantitation:

You will load 2.00 µL of each sample into the Take 3 plate and record the A_{280}. You will also record the A_{280} of 5.0 mM Tris-Cl pH 7.4 (this will serve as your blank). You should measure the A_{280} of each sample at least twice to ensure that your readings are reproducible, and then average the measurements for each. The volume

you are putting on the plate is very small, and it will evaporate if you leave it too long. If you are having difficulty loading the plate quickly enough, you may need to load just a few samples, take a reading, and repeat. The A_{280} will allow you to estimate the amount of total protein in each sample. Use the guide at the end of this protocol to complete the protein analysis.

Data analysis: calculating molar absorptivity

To calculate the molar absorptivity, subtract the average absorbance of your negative control from the average absorbance of your positive control, and combine with the concentration of the positive control and the path length. Use the path length you calculated previously. This is the same Beer's Law approach as you have used several times:

$$\frac{\text{(Ave. ABS positive control - Ave. ABS of negative control)}}{\text{Concentration of positive control (M) * path length (cm)}} = M^{-1}\ cm^{-1}$$

For PNP, use the final concentration of PNP in a single well for the calculation. Your value should ideally fall in the range 1700 - 20000 $M^{-1}\ cm^{-1}$. If it does not, please recheck your calculation. Contact your instructor if you still obtain a number more than 2500 $M^{-1}\ cm^{-1}$ outside of that range.

Next find the molar absorptivity of 3-amino-5-nitrosalicylate using the same approach. Be sure you are using the final concentration in a single well. *Remember that there are several dilutions that occur between preparation of the hydrolyzed sucrose and the actual addition of the sample to the 96 well plate, and you must correct the concentration used based on the changes in volume.* Your value should ideally fall in the range 1500 - 2000 $M^{-1}\ cm^{-1}$. If it does not, please recheck your calculation. Contact your instructor if you still obtain a number more than 200 $M^{-1}\ cm^{-1}$ outside of that range.

Data analysis: determining the specific activity

In general terms, the specific activity is defined as the activity of the enzyme per mg of total protein. If a purification strategy is successful, the specific activity should increase at each stage of purification You will need to determine the specific activity for each of your samples to determine what effect ethanol fractionation has had on the purification of invertase and phosphatase. You will need this information to decide how to proceed in next week's lab.

To determine specific activity, you will first need to calculate the activity (Units, or μmol min^{-1}) in each sample. This is done separately for phosphatase and invertase, as each reaction product absorbs at a different wavelength and has a different molar absorptivity. Note that it is possible that you will generate a negative number after subtracting the absorbance of the negative control from one or more of your samples. In this case, you should mark this sample as "no detectable activity" and should not use this negative number in the activity calculation described below. When reporting the activity in a table, list it as "no detectable activity" or zero, not as a negative number.

1. **Calculate Units (μmol/min)**

a. Phosphatase: Determine the Units (activity) for a single sample using the equation:

$$\frac{(\text{Ave. ABS} - \text{Ave. ABS of negative control}) * \text{volume of 1 well (L)}}{\text{Molar absorptivity } (M^{-1}cm^{-1}) * \text{path length (cm)} * \text{time (minutes)}} = \text{mol } min^{-1}$$

This number should then be converted to μmol min^{-1} to obtain activity (Units). This calculation should be performed for each sample that potentially contains enzyme (*i.e.*, has detectable activity as indicated by an absorbance greater than the negative control). The time used in this calculation is the length of

time the reaction proceeded before making a measurement. For this week, that value is 5.0 minutes because the plate reader took a reading at zero and five minutes.

b. Invertase: Determine the Units (activity) as described above, using the absorbances and molar absorptivity from your invertase assay. Use the actual time your reactions proceeded between adding enzyme and adding DNS.

At this stage, you have calculated the activity of phosphatase and/or invertase in each sample. However, there may be different amounts of enzyme in these samples so we are not able to directly compare activity. Next, you will use the A_{280} to calculate the amount of enzyme in each sample, and use this value to calculate a specific activity that corrects for differences in amount of enzyme.

2. **Calculate the total amount of protein in each sample (mg)**
    a. Protein mg/mL: Subtract the A_{280} of your blank from the average A_{280} of your sample, then convert the resulting A_{280} to mg/mL assuming that a protein sample with an A_{280} = 1.0 has a concentration of 1.0 mg/mL.

    b. Determine the total volume of protein used in each sample (the amount added to each well). For this week, that amount is 10.0 µL.

    c. Sample protein mg: Multiply your mg/mL concentration from (a) by the protein volume of each sample from (b), being sure to convert appropriately to be left with mg. This should result in a calculation that looks like this:

(Ave. A_{280} - A_{280} of blank) * (1 mg/mL) * sample volume (mL) = mg

3. **Calculate specific activity (µmol/min/mg protein):**

   Divide the µmol/min of each sample from (1) by the sample protein mg from (2). This number is the specific activity of the sample and should be reported with units that produce a whole number.

Anion Exchange Chromatography

Introduction:

In today's experiment, we will continue the process of purifying the enzymes invertase and phosphatase from yeast extract. Using anion-exchange chromatography, we will isolate these two enzymes away from other proteins in the sample and perhaps even separate them from each other. At the end of today's experiment, you will have a number of fractions, which you will test next week to determine which contain active invertase and/or phosphatase.

In the process of preparing and running your anion exchange column, you will use several buffers that differ only in salt concentration. Please read the labels carefully to ensure that you are using the correct buffer. You should also be able to explain why we are using different salt concentrations on the column.

Preparing an anion-exchange column:

1. Each group will be provided with a small, plastic column. Your column will be open on one end and contain a small plastic cap on the other. Check the column to ensure that is clean and free of any debris before beginning. Add approximately 4 mL of dH_2O to the column, remove the column cap, and allow the dH_2O to drain out over an empty beaker or the sink. If the flow is very slow (10 seconds or more between drops), ask your instructor for a new frit for the column. Otherwise, let the column drip freely until all dH_2O has passed through. Replace the small plastic cap after all the dH_2O has passed through.

2. Your instructor will have a beaker containing DEAE-Cellulose resin in 5 mM Tris-Cl pH 7.4/5 mM $MgCl_2$. Transfer 2 mL of the slurry to your column. Allow the column to sit undisturbed on your bench for 5-10 minutes. You should notice that during this time the mixture is separating with the resin (white material) settling in the bottom of the column and the buffer (colorless liquid) remaining on the top layer. For the remainder of the lab, you should never let the buffer layer drop below the top of the resin interface.

3. Place the column over a clean 15 mL tube and remove the column cap to being the flow of buffer.

4. When buffer level is about 1 cm (a little less than one finger width) above the top of the resin, add 5 mL 5 mM Tris-Cl pH 7.4/5 mM $MgCl_2$ to the column and allow it to flow through. Do not allow the buffer to drop below the level of the top of the resin.

5. When the buffer level is again 1 cm above the top of the resin, the column is ready for your sample. If you are not ready to add your sample to the column at this point, cap the end of the column so it does not dry out.

Preparing your sample for chromatography:

1. Centrifuge your sample for 1 minute at 12,000 rpm. Remove the supernatant to a clean, labeled microcentrifuge tube. Do not worry if there does not seem to be a pellet at this stage; this step is simply to remove any remaining cellular debris.

2. Add 5 mM Tris-Cl pH 7.4/5 mM MgCl2 to your sample so that the final volume is approximately 1 mL. You may use the markings on the side of the microcentrifuge tube to estimate this volume. If your supernatant is already at the 1 mL mark, then there is no need to add any additional buffer.

3. Remove 20 µL of your sample to a clean, labeled microcentrifuge tube. You will need this sample for further analysis. Label this tube and give to your instructor for storage at -20 ˚C.

Anion-exchange chromatography:

1. You will now add your diluted protein sample to the column. You will need approximately 15 clean microcentrifuge tubes to collect fractions. Depending on how you collect fractions and the exact amount of resin in your column, you may end up with between 13-16 fractions. Notify your instructor if you end with fewer than 10 fractions or more than 18. Work with your partner to exchange tubes and label each as the fractions are collected.

2. Place your uncapped column over a clean microcentrifuge tube and add your protein sample to the resin.

3. Collect the eluant in 500 µL fractions until the level of buffer is again 1 cm above the resin. You may estimate the collected volume using the markings on the microcentrifuge tube.

4. Add 2.5 mL 5.0 mM Tris-Cl pH 7.4/5.0 mM $MgCl_2$/100 mM NaCl to your column. Be sure you are using the correct buffer (with 100 mM NaCl) at this step.

5. Collect the eluant in 400 µL fractions until the level of buffer is again 1 cm above the resin.

6. Add 2.5 mL 5.0 mM Tris-Cl pH 7.4/5.0 mM $MgCl_2$/300 mM NaCl to your column. Be sure you are using the correct buffer (with 300 mM NaCl) at this step.

7. Collect the eluant in 400 µL fractions until the level of buffer is again 1 cm above the resin.

8. Cap the column.

9. After all fractions have been collected, labeled, and the labels recorded in your notebook, give your fraction tubes to your instructor. They will be stored at -20˚C until next week.

Specific Activity II

Introduction:

Last week you collected fractions from anion exchange chromatography. Today you will assay each fraction for invertase activity, phosphatase activity, and total protein (using A_{280}). Use your own protocol to complete each experiment.. At the end of lab today you should have all of the data necessary to calculate the specific activity of both enzymes in each fraction.

Your protocol must contain the following:

1. Specific instructions for the preparation of all solutions (including volumes, times, etc). Complete all necessary calculations as part of your protocol.
2. Detailed instructions for the collection of data and/or operation of any equipment. This level of detail should be comparable to the protocols you have been given in prior weeks.
3. An explanation of the data analysis required to obtain specific activity values written in your own words (*i.e.*, not copied directly from the previous protocol).

SDS-PAGE

Introduction:

In today's experiment, we will use SDS-PAGE to analyze samples collected throughout the purification procedures. You will be able to use the results of the SDS-PAGE gel to determine the sizes of any proteins in your samples and to determine the purity of your invertase and phosphatase preparations.

Hazards:

Acrylamide is a neurotoxin in its powdered from. While the polymerized form is safer to handle, you should wear gloves if it is necessary for you to handle the gel directly. The 2X SDS loading dye contains β-mercaptoethanol, which can be toxic if swallowed or absorbed through the skin.

Pouring an SDS PAGE gel:

A 10% SDS PAGE gel has already been prepared. Your instructor will discuss the procedure for making this type of gel, and this procedure should be recorded in your lab notebook.

Prepare samples for SDS-PAGE:

1. As part of your pre-lab, you should have determined the specific activity for each of your samples with detectable invertase and/or phosphatase activity. Use this information to identify the fraction that contains the highest concentration of invertase and the fraction that contains the highest concentration of phosphatase. If two fractions have roughly equal amounts of invertase or phosphatase, you may wish to pool them. You will need these two samples again – please return them to the -20˚C freezer at the end of lab today.

2. Mix 20 µL of 2X SDS loading dye with 20 µL of sample in a new microcentrifuge tube. (Do this for both fractions identified in #1 as well as the sample you saved before loading the column.)

3. Heat the tubes at 90˚C for approximately 5 minutes. Be sure that you have added dye before heating the samples.

Running the SDS PAGE gel:

1. Load the gels into the apparatus. The short plates need to be on the inside.

2. Fill the apparatus with 1 X SDS* running buffer to the top of the tall glass plate, inside and outside.

   *SDS PAGE is a denaturing gel. The proteins must be boiled in the presence of SDS to denature them. The 2X sample buffer contains SDS to denature the protein as well as dye to track the migration of the sample.

3. Load your samples, any order is fine AS LONG AS YOU RECORD THE ORDER IN YOUR NOTEBOOK. Your instructor will load the molecular weight marker (Bio Rad Broad Range Marker) before you load any of your samples. Your instructor will also load a sample of clarified yeast extract on the gel.

   - Loading the sample can be difficult. Place the pipette tip into the buffer and touch the back plate. Slowly raise the tip until you feel the tip move forward to the tall plate. Place the tip over the well and SLOWLY pipette out the sample without injecting any air (do not go all the way to the second stop). The sample should sink to the bottom of the well.

4. Once all the samples and markers are loaded, your instructor will start the electrophoresis. The gel will be run at 150V for approximately 1.5 hours.

5. Your instructor will demonstrate the proper technique for removing the gel from the apparatus and placing it in the staining solution. As time permits, you may be asked to place your gels in the stain. The gels will stain overnight in Gel Code Blue, and then will be placed in water for 2-4 hours. Your instructor will post a picture of the gel on Blackboard.

Data analysis:

1. Using the information provided by your instructor, identify the size of each band in the molecular weight marker lane.
2. Determine the Rf for each band in your molecular weight marker.
3. Graph log MW vs. Rf.
4. Determine the Rf for each band in your purified samples.
5. Use the MW graph (#3) to determine the molecular weight of each band.
6. Compare the molecular weights to the given MW of invertase and phosphatase to determine which bands correspond to the enzymes.

Kinetics of Phosphatase

Introduction:

In today's experiment, you will determine the effect of pH on the activity of your purified phosphatase. From the activity data, you will be able to determine the optimal pH for the activity of your enzyme. You will then determine the velocity of this enzyme starting from several different substrate concentrations at this optimal pH in order to determine the K_M and V_{max} for your purified phosphatase. Use the information below to write your own protocol for this experiment.

Hazards:

Goggles and gloves should be worn at all times when handling 3 M NaOH.

Determining the activity of phosphatase at different pH.

This assay will be performed in a manner similar to the phosphatase assays done previously. However, because several of the buffers will be at a pH lower than the pK_a of PNP, you will need to perform an additional step to ensure that the absorbance of your reaction product can be detected. You should think carefully about the positive and negative controls you will need for this experiment.

Buffer
0.010 M acetate pH 5
0.010 M citrate pH 6
0.010 M phosphate pH 7
0.010 M tris pH 8
0.010 M tris pH 9
0.010 M glycine pH 10

You will be provided with the six buffers in the table above. The actual pH values may be slightly different from those shown in the table. Please record the actual pH of each buffer before you begin the lab.

Set up a reaction with each buffer using the same substrate concentration as used to determine specific activity. Add the substrate/buffer mixture to your 96 well plate, then immediately add enzyme to the appropriate wells. *Be sure that you have enough enzyme to add to all of your samples and to also complete the second experiment described below; you may need to reduce the amount of enzyme added to avoid running out*. Allow the reactions to proceed for approximately five minutes, then add 10.0 µL of 3.0 M NaOH to each well. Read the absorbance of your plate as soon as possible after adding the NaOH.

Once you have collected your absorbance data, you can quickly examine it to determine which pH is optimal for the activity of your phosphatase. This will be the pH you will use to determine the velocity of the enzyme as described below.

Determining the velocity of an enzyme catalyzed reaction:

The velocity of an enzyme-catalyzed reaction is similar to its activity. In past weeks, we have measured activity by calculating the amount of product formed at a single timepoint. In order to calculate velocity, you will observe the change in product concentration over time. The reaction is assembled in the same manner as was done previously; however, the plate reader will take several readings over a period of three minutes.

Using the buffer of optimal pH, set up reactions at several concentrations of substrate. You will need to include a 0 mM substrate reaction as well as 5 additional substrate concentrations with the highest concentration no greater than 1.0 mM. Once the substrate and buffer have been combined, you can proceed the assay in the same manner as was done to determine activity.

*Do you need to add NaOH to your reaction here? What information will help you make that decision?

Once you have collected your absorbance data, you will be able to determine the velocity using a modification of the equation for activity. You can then graph velocity vs. substrate concentration to construct a double-reciprocal plot to calculate values for V_{max} and K_M.

Your protocol must contain the following:

1. Specific instructions for the preparation of all solutions (including volumes, times, etc). Complete all necessary calculations as part of your protocol.

2. Detailed instructions for the collection of data and/or operation of any equipment. This level of detail should be comparable to the protocols you have been given in prior weeks.
3. An explanation of the data analysis required to obtain velocity values and how to use those values to obtain V_{max} and K_M written in your own words..