Lesson plan

Activity of Acid Phosphatase Extracted from Wheat Germ

NanoDrop One/One^{C} Spectrophotometer

Introduction

Enzymes

Essential to living organisms, enzymes are proteins that catalyze chemical reactions required to sustain life. The active site of an enzyme binds with a specific substrate, helping to transform the substrate into a new product with great efficiency (Figure 1).[1,2] Most enzymes are classified by the suffix "-ase" and the substrate they act upon. For example, the enzyme phosphatase is involved in dephosphorylation, or the removal of a phosphate group.[1] Acid phosphatases are widespread enzymes found in plants and mammals that serve to dephosphorylate a substrate to form free phosphate and alcohol products.[3]

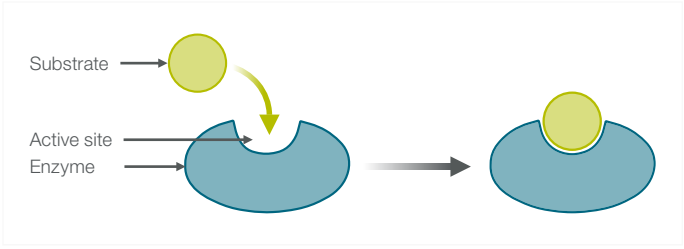


Figure 1. Enzyme binding with a substrate at the enzyme's active site. Image created with BioRender.com.

An enzyme deficiency or surplus can carry clinical significance in the form of diabetes, neurological disorders, or other maladies. This illustrates the importance of classifying enzymes and their activity.[1,4] Enzyme activity describes the amount of product made per unit time, while the specific activity describes the activity per milligram of protein. Specific activity provides a purity assessment of the enzyme because specific activity increases as the enzyme purity increases.[2]

During protein extraction and purification, the plant or mammalian cells are lysed, releasing all the cellular contents including proteins, nucleic acids, carbohydrates, and lipids. The next step in protein purification is called "salting out", which uses salt such as ammonium sulfate to reduce the solubility of protein. This decrease in solubility can also be achieved by using high volumes of organic solvents such as methanol or ethanol. As the protein solubility is reduced either with salts or solvents, the proteins precipitate out of the solution and are pelleted via centrifugation.[5] Confirming an enzyme's purity through specific activity is a key component of protein purification before continuing with additional enzyme studies.

UV-Visible Spectrophotometry

A simple technique to measure enzyme activity is ultraviolet-visible (UV-Vis) spectrophotometry. A spectrophotometer measures the amount of light absorbed by a sample. To measure absorbance, a beam of light passes through the sample to a detector and as the sample absorbs more light, this equates to more molecules in the sample. Thus, absorbance is directly proportional to concentration. To relate absorbance and concentration, Beer's Law describes the following relationship:

$$A = \varepsilon bc \quad or \quad c = \frac{A}{\varepsilon b}$$

Equation 1.

Through rearranging Beer's Law, concentration (c) is determined by dividing the absorbance (A) by the pathlength (b) and the sample-specific extinction coefficient (ε). The extinction coefficient is a measure of how strongly a sample absorbs light and the pathlength is the length the light travels through the sample. Traditional spectrophotometers typically use a 1.0 cm pathlength cuvette but the Thermo Scientific™ NanoDrop™ One/One^C Microvolume UV-Vis Spectrophotometer uses a range of pathlengths to accommodate higher sample concentrations without the need for dilutions.

Isolating one enzyme of interest requires high-end laboratory equipment, but a substrate called a chromogen can help quantify the activity of an enzyme of interest in a mixture of different proteins. The product of an enzyme acting upon a chromogen produces a color whose absorbance can be determined with visible spectrophotometry.[2] Acid phosphatase acts upon the chromogen p-nitrophenyl phosphate (PNPP) via hydrolysis to produce yellow p-nitrophenol (PNP), which can be measured at 405 nm using the extinction coefficient 18,300 M^{-1} cm^{-1} (Figure 2).[6,7]

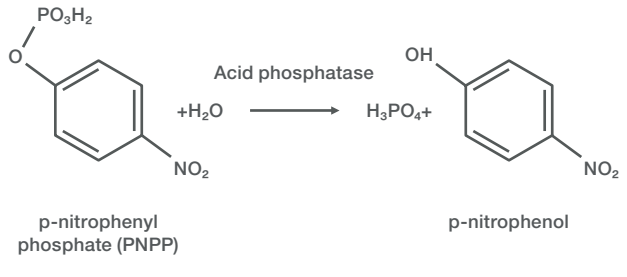


Figure 2. Chemical reaction of p-nitrophenyl phosphate as the chromogen and acid phosphatase as the catalyst to yield p-nitrophenol and inorganic phosphate. Image created with BioRender.com.

Experimental

Purpose

The purpose of this experiment is to determine the enzyme activity and specific activity of acid phosphatase extracted from wheat germ. There are four parts to this experiment:

- Part A: Extraction of acid phosphatase from wheat germ (adapted from Joyce & Grisolia, 1960)
- Part B: Determination of protein concentration with the BCA assay
- Part C: Completion of the phosphatase assay with p-nitrophenyl phosphate (adapted from Sigma-Aldrich, doc. no. SSPNPP02)
- Part D: Calculation of enzyme activity and specific activity

Materials

- Thermo Scientific™ NanoDrop™ One/One^C Spectrophotometer
- Deionized (DI) water
- Acid phosphatase control (Sigma-Aldrich, P3627)
- Store-bought wheat germ
- 1.0 M Magnesium chloride ($MgCl_2$)
- 4.10 M Ammonium sulfate (saturated) $(NH_4)_2SO_4$
- Thermo Scientific™ Pierce™ BCA Protein Assay Kit (Thermo Scientific, 23225)
- p-nitrophenyl phosphate (PNPP) (Sigma-Aldrich, 20-106)
- 0.1 M Sodium hydroxide (NaOH)
- 0.09 M Citrate buffer solution, pH 4.8 at 37°C (Sigma-Aldrich, C2488)

Before beginning: write your experimental hypothesis in #1 of the Lab Report.

Part A: Extracting acid phosphatase from wheat germ

A1. Heat water bath to 70°C.

A2. Weigh out 5.0 grams of wheat germ and mix with 20 mL DI water in a 50 mL conical tube. Allow to sit for 30 minutes and stir occasionally to lyse the cells and release the cellular contents.

A3. Centrifuge at 5000 x g for 5 minutes, making sure the centrifuge is properly balanced. You may need to place a counterweight directly opposite the sample tube in the centrifuge.

A4. Decant and save the supernatant by tilting the conical tube into a clean 50 mL conical tube and discard the pellet. Reserve 200 µL of the supernatant for downstream testing. Label as **Fraction 1**.

A5. Add 500 µL of 1.0 M $MgCl_2$ and stir to mix.

A6. Centrifuge 5000 x g for 5 minutes. Decant and save the supernatant then discard the pellet. Reserve 200 µL of supernatant then determine supernatant volume. Label as **Fraction 2**.

   a. Supernatant volume: _____ µL/mL

A7. Add 0.54 volume saturated $(NH_4)_2SO_4$ and slowly stir to mix, avoiding bubbles and foaming as this denatures the enzyme.

   a. Volume of saturated $(NH_4)_2SO_4$ to add: _____ µL/mL

   b. For example: if the supernatant volume in 6a is 5.0 mL, add 2.7 mL of saturated $(NH_4)_2SO_4$ (5.0 mL x 0.54 volume = 2.7 mL).

A8. Centrifuge 5000 x g for 5 minutes. Decant and save the supernatant then discard the pellet. Reserve 200 µL of supernatant then determine supernatant volume. Label as **Fraction 3**.

   a. Supernatant volume: _____ µL/mL

A9. Slowly add 0.51 volume saturated $(NH_4)_2SO_4$ and place the sample tube in a water bath until sample temperature reaches 60°C and hold for 2 minutes.

   a. Volume of saturated $(NH_4)_2SO_4$ to add: _____ µL/mL

A10. Immediately after 2 minutes, place the sample tube in an ice bath until sample temperature reaches 8°C.

A11. Centrifuge 5000 x g for 10 minutes. Discard the supernatant. The enzyme will be present in the insoluble pellet.

A12. Resuspend pellet in 5.0 mL DI water and mix until completely dissolved, then reserve 200 µL. Label as **Fraction 4**.

A13. If Part B is not performed on the same day, all fractions can be stored in a freezer.

A14. If using an acid phosphatase control, prepare a 1.0 mg/mL sample.

Part B: Protein concentration with BCA assay

B1. The microplate protocol can be performed per the Pierce BCA protein assay instructions.

B2. Prepare a BSA standard curve using the serial dilutions provided in Table 1. Use clean pipette tips between each dilution.

Standard #	Volume of Water (µL)	Volume and source of BSA (µL)	Final BSA concentration (mg/mL)
Reference	300	0	0
1	0	300 of stock	2.0
2	150	150 of stock	1.0
3	150	150 of Standard 2	0.5
4	150	150 of Standard 3	0.25
5	150	150 of Standard 4	0.125
6	150	150 of Standard 5	0.0625

Table 1. BSA standard curve 1:1 dilution preparation to provide a working range of 0.0625 mg/mL – 2 mg/mL.

B3. Prepare the BCA working reagent as follows where the control is included as an "unknown":

   a. (7 standards + 5 unknowns) x (2 replicates) x (200 µL) = 4.8 mL working reagent required

   b. Mix 50 parts BCA Reagent A with 1 part BCA Reagent B: 5.0 mL Reagent A with 100 µL Reagent B.

   c. Note: the working reagent will appear light green.

B4. Mix 25 µL of each standard and unknown with 200 µL working reagent. Incubate at 37°C for 30 minutes.

   a. Note: the solution will turn purple as the colorimetric reaction takes place.

B5. On the NanoDrop One instrument, select the tab **Proteins**, open the **Protein BCA** application, and enter the following in the Setup:

   a. Curve type: Linear

   b. Replicates: 2

   c. Standard Amount: 6

   d. Enter the BSA concentrations listed in Table 1. The Reference is already included in the software. Press "Done" when finished.

   e. Clean the pedestals by wiping with a lint-free lab wipe. Make a Blank measurement with 2.0 µL of DI water.

   f. Follow the on-screen prompts for measuring the standards in 2.0 µL volumes, making sure to clean the pedestals with a lint-free lab wipe before each new measurement. Once the standards are complete, select **Run Samples** to measure the unknowns in 2.0 µL volumes.

   g. Record the reported concentrations for the unknowns in Data Table 1 along with the R^2 of the BSA standard curve.

Part C: Single-point phosphatase assay and absorbance measurements

C1. Label six 1.5 mL microcentrifuge tubes as **F1**, **F2**, **F3**, **F4**, **C**, and **B**, where F1-4 correspond to the four fractions from part A, C corresponds to the control (if using), and B corresponds to the blank.

C2. Add 125 µL of 0.09 M citrate buffer and 125 µL of PNPP to all tubes.

C3. Mix each tube and equilibrate to 37°C for 5 minutes in a water bath or heat block.

C4. Add 25 µL distilled water to tube **B** and place back in the 37°C bath or block.

C5. Add 25 µL of the acid phosphatase control to tube **C** (if using), immediately mix by inversion, place back in the 37°C bath or block, and start a timer for 5 minutes.

C6. Working quickly, add 25 µL of Fraction 1 to tube **F1** and continue with fractions 2-4, mixing by inversion and then placing the tubes in the 37°C bath or block.

C7. After exactly 5 minutes, add 1.0 mL 0.1 M NaOH to all tubes in the same order the enzyme solutions were added (e.g., B, C, F1, F2, F3, F4). Adding NaOH stops the enzymatic reaction from continuing.

C8. From the NanoDrop One instrument home screen, select the tab **Custom** and select the application **UV-Vis**.

C9. Enter 750 nm for the **Baseline Correction**, enter 405 nm for the **Analytical Wavelength**, and add 405 nm as a **Monitored Wavelength**.

   a. The baseline correction anchors the spectrum to zero at 750 nm and is used to correct for a baseline offset due to the presence of light-scattering particles.

   b. The analytical wavelength represents the PNP peak at 405 nm and instructs the NanoDrop software to use the optimal pedestal pathlength depending on the absorbance intensity. Regardless of the pathlength used, all displayed absorbance measurements are normalized to a 1.0 cm pathlength for application to Beer's Law.

C10. Follow the on-screen prompts for measuring the blank (tube B) and the samples in 2.0 µL volumes on the microvolume pedestal, making sure to clean the pedestals with a lint-free lab wipe before each new measurement.

   a. *Note: the best practice after making a Blank measurement is to measure the blank solution as a sample. For the NanoDrop One instruments, this measurement should be within ± 0.04 absorbance units at the analysis wavelength (405 nm). If the measurement is outside of this range, measure a new Blank.*

C11. Record the absorbance results in Data Table 2.

Part D: Calculate enzyme activity and specific activity

D1. Calculate the concentration of PNP in µmol/mL using Beer's Law, where pathlength (l) = 1.0 cm, extinction coefficient (ε) = 18,300 M^{-1} cm^{-1}, and use the reported absorbance in Data Table 2.

$$\text{PNP concentration}\left(\frac{\mu mol}{mL}\right) = \frac{A}{\varepsilon * l} * 1.0\varepsilon 3\left(\text{conversion from } \frac{mol}{L} \text{ to } \frac{\mu mol}{mL}\right)$$

   a. Record the concentration in Data Table 3.

D2. Calculate the enzyme activity in µmol/min using the total reaction time (5 minutes) and the total assay volume (1.275 mL).

$$\text{Enzyme Activity}\left(\frac{\mu mol}{min}\right) = \left(\frac{\text{PNP}\left(\frac{\mu mol}{mL}\right)}{\text{Reaction time (min)}}\right) * \left(\text{total assay volume (mL)}\right)$$

   a. Record the enzyme activity in Data Table 3.

D3. Calculate the total amount of enzyme in the reaction using the protein concentration from the BCA assay in Data Table 1 and the sample volume added to the kinetics reaction (25 µL or 0.025 mL).

$$\text{Total enzyme (mg)} = \left(\text{protein concentration}\left(\frac{mg}{mL}\right)\right) * \left(\text{sample volume (mL)}\right)$$

   a. Record the total enzyme amount in Data Table 3.

D4. Calculate the specific activity using the values calculated for enzyme activity and total enzyme.

$$\text{Specific activity}\left(\frac{\mu mol}{min \bullet mg}\right) = \frac{\left(\text{Enzyme activity}\left(\frac{\mu mol}{mL}\right)\right)}{\text{Total enzyme (mg)}}$$

   a. Record the specific activity in Data Table 3.

Lab Report

1. Hypothesize how the specific activity changes with each fraction number.

2. Record the R^2 of the BCA protein assay standard curve and the protein concentration in mg/mL reported by the NanoDrop One spectrophotometer.

R^2 of the standard curve: _____

Fraction #	Control	1	2	3	4
Enzyme concentration (mg/mL)					

Data Table 1. BCA protein assay results.

3. Discuss the R^2 of the BSA standard curve. Is this value close to 1.0? If not, what are some steps to take to improve the R^2?

4. Record the PNP absorbance results reported by the NanoDrop One spectrophotometer. The reported absorbances are normalized to a 1.0 cm pathlength.

Sample	Blank	Control	F1	F2	F3	F4
Absorbance at 405 nm						

Data Table 2. UV-Vis absorbance results.

5. What did you observe during the 5 minute kinetics experiment?

6. Why is it important to measure the blank solution as a sample with spectrophotometry?

7. Record the calculated PNP concentration, enzyme activity, total enzyme in the reaction, and specific activity.

Sample	Blank	Control	F1	F2	F3	F4
PNP Concentration (µmol/mL)						
Enzyme Activity (µmol/min)						
Total Enzyme (mg)						
Specific Activity (µmol/ min•mg)						

Data Table 3. PNP concentration, enzyme activity, and specific activity results.

8. Do your specific activity results agree with your experimental hypothesis from #1? Why or why not?

9. How does specific activity change in relation to the fraction number and the total enzyme amount? What does this tell you about enzyme purity?

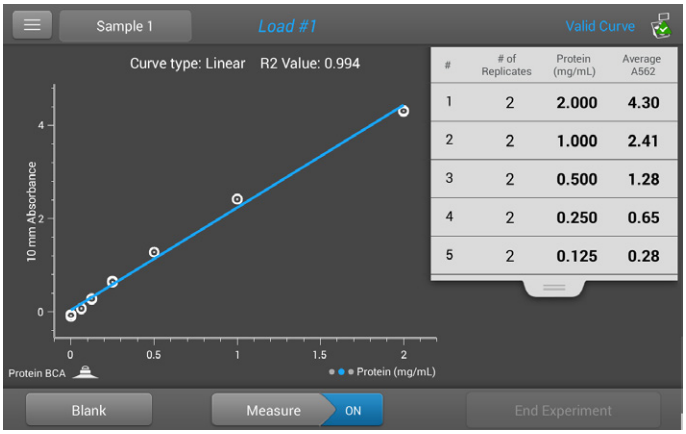
10. What do you predict would happen to specific activity if the reaction temperature was raised to 42°C?

11. Based on the specific activity of Fraction 4, does the enzyme need to be purified further? If so, what should be the next step in the purification procedure?

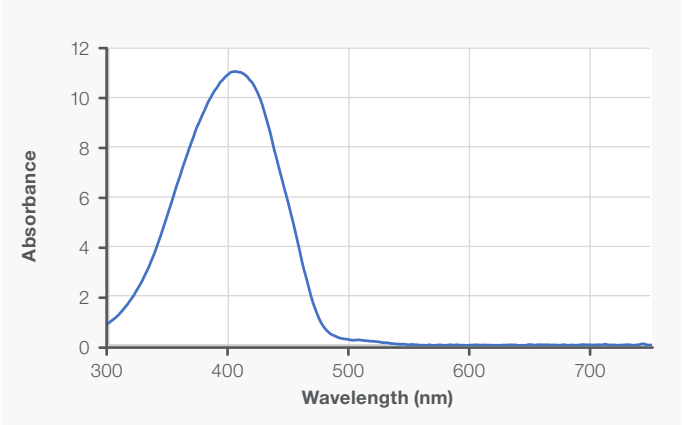
12. Is the enzyme present in Fraction 4 pure acid phosphatase? Why or why not?

Notes for Professors and Teaching Assistants

Expected BCA standard curve results:



Expected PNP absorbance spectrum (405 nm peak):



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