

CSUDH Biochemistry II Laboratory – CHE 453

Lab 1A: The Extraction and Purification of Wheat Germ Acid Phosphatase

INTRODUCTION

Protein Isolation

Proteins play such a variety of important roles in the living cell that it should be no surprise that an enormous amount of time and effort has been spent in elucidating the structure and function of these versatile macromolecules. An absolute prerequisite to meaningful progress in the study of any protein is an ample supply of highly purified material. Unfortunately, few if any proteins exist in nature at the desired concentration or at the degree of purity required for such detailed analysis. Consequently, it has been necessary that laboratory procedures be developed for the extraction, concentration, and purification of cellular proteins.

The development of these procedures, which has taken a considerable period of time, has been hampered by the fact that the concentration of any given protein in a cell is usually extremely low (less than 0.01% of the total mass of the cell) and further complicated by the presence of many other macromolecules (nucleic acids, carbohydrates, etc.) which must be eliminated. The task of a biochemist who chooses to develop an isolation technique for large amounts of purified protein is a difficult one and would typically include the following considerations:

The development or selection of a simple assay procedure that specifically demonstrates the presence and concentration of the protein in question.

The choice of material from which the protein can be isolated. The overriding factors in this choice typically are the absolute concentration of the protein in a given natural source and the cost and availability of the material.

The selection of a cell-disruption technique appropriate to the cell or tissue type being used. Some materials such as plant cells, for example, require harsher techniques than others and run the risk of physical disruption of the protein.

The development of extraction conditions that maintain a pH, temperature, and ionic environment conducive to maintaining the native configuration of the protein. Extremes of these conditions increase the likelihood of inactivating the protein by denaturation.

The selection of a series of precipitation and centrifugation procedures that maximize the concentration of the chosen protein and minimize that of the other contaminating molecules.

Biochemists have, through a process of trial and error, addressed these considerations and have developed an impressive array of techniques for the extraction and purification of proteins. A point that should be emphasized is that there is no single, foolproof protein isolation technique uniformly applicable to all situations. A biochemist with the task of isolating a protein that has not been purified previously develops an isolation scheme by first trying several of the most reliable techniques for that general class of proteins. After each step in the scheme, the effectiveness of the technique is evaluated and subsequent isolation schemes modified accordingly. Thus, any given isolation procedure has in all probability arisen through the application of well-established techniques tested by trial and error.

The Enzyme

The protein to be extracted and purified in the following exercise is an enzyme called wheat germ acid phosphatase. The systematic name of acid phosphatase is orthophosphoric-monoester phosphohydrolase (acid optimum). The enzyme catalyzes the release of inorganic phosphate (P_i) from such diverse substances as ARP, glucose-6-phosphate, and glycerol-2,3-diphosphate at pH values ranging from 4 to 6 (Figure 1).

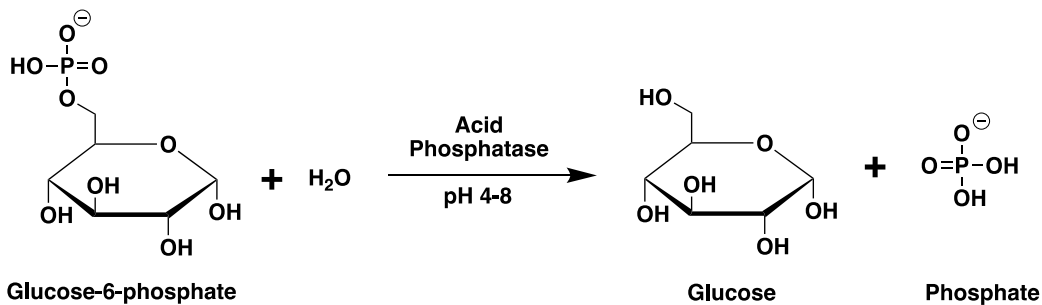


Figure 1. A typical acid phosphatase reaction, in which glucose-6-phosphate is degraded to glucose and inorganic phosphate.

Acid phosphatase is found widely distributed among plants, animals, and microorganisms, although its precise biochemical role remains unclear. Wheat germ has been selected as a source for the enzyme because it is inexpensive and readily available and contains high levels of the enzyme.

Extraction Procedures

The procedures detailed below for the extraction of wheat germ acid phosphatase are representative of a typical protein isolation scheme. The initial water extraction of the enzyme (along with a number of other water-soluble proteins, carbohydrates, etc.) is accomplished by soaking the wheat germ in cold distilled water. The insoluble material is removed by a brief centrifugation which yields a supernatant containing the enzyme and a variety of other soluble contaminants. These contaminants (protein and otherwise) are in turn removed by a series of selective precipitation and centrifugation steps to separate the soluble (supernatant) from the insoluble (pellet) fraction.

Salt fractionation, or salting out, of proteins is often used at the beginning of a protein isolation scheme because it is a convenient method for separating large groups of proteins in one or two steps. The solubility of proteins in aqueous solution depends on the number of hydrophilic amino acids in the protein and on the ionic strength of the solution. When the ionic strength is gradually increased by the addition of manganese chloride or ammonium sulfate, fewer and fewer water molecules are available to solubilize the proteins and eventually the proteins begin to come out of solution (salt out). Thus, by carefully controlling the salt concentration, one can selectively precipitate whole groups of proteins while leaving others in solution. Previous experience has demonstrated that acid phosphatase is soluble in a 35% ammonium sulfate solution and insoluble in 57% ammonium sulfate.

The subsequent heat treatment and methanol fractionation operate in much the same way, precipitating some proteins and leaving the other in solution. Since this procedure, like all others, is not foolproof, it is highly visible to verify the presence of the enzyme in each action before moving to the next step.

The completing of this purification scheme is accomplished by dialysis, a technique designed to remove small-molecular-weight contaminants. This enzyme extract is enclosed within a dialysis sac (a semipermeable membrane) and suspended in a large volume dilute salt. As the solution is gently stirred, the small molecules pass out through the pores, leaving the large-molecular-weight enzyme inside the sac. At equilibrium, the majority of the small molecules are removed in this fashion.

Assay Procedures

The method of choice for protein measurement during enzyme purification is the Biuret reaction. The Biuret reagent contains copper ions in alkaline solution, and these ions form colored complexes with the peptide linkages of protein. The concentration of protein is then determined colorimetrically. This method is advantageous because it is quick and easy and because the high concentrations at ammonium sulfate typical of early fractionation steps do not interfere with the color reaction.

The assay for acid phosphatase activity depends upon the fact that the enzyme catalyzes the hydrolytic lease of para-nitrophenol and inorganic phosphate from the artificial substrate p-nitrophenyl phosphate (Figure 2). Under alkaline conditions one of the reaction products (p-nitrophenol absorbs light strongly at 405 nm, and its concentration can be determined colorimetrically. The enzyme assay calls for combining the enzyme and substrate for a brief period of time, stopping the reaction, and determining the amount of substrate produced per unit time.

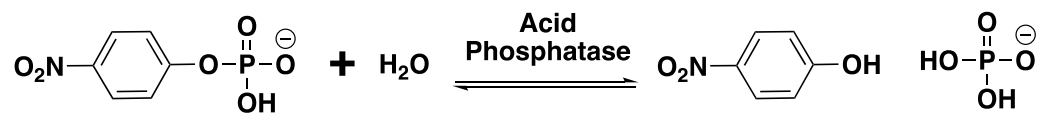


Figure 2. The hydrolysis of p-nitrophenyl phosphate by the enzyme acid phosphatase

Procedure

You have read all the preliminary material before coming to the laboratory and that all of the necessary solutions and equipment are readily available. A brief synopsis of what you should be able to complete in each day follows:

Part I. Extraction of acid phosphatase through supernatant V (SV). This fraction is stable and may be stored frozen for several weeks with little or no loss in enzyme activity.

Part II. Protein and phosphatase assays on all samples (SI, SII, etc.) to assess the effectiveness of the enzyme isolation procedure.

Part III. Final purification of the enzyme starting with SV and continuing through the dialysis step.

Unless otherwise indicated, all procedures should be carried out at 0°C to 4°C to minimize heat denaturation and protease damage to the enzyme. The flow sheet shown in Figure 3 illustrates in an abbreviated fashion all the steps in the procedure and may be used as a checklist throughout the process.

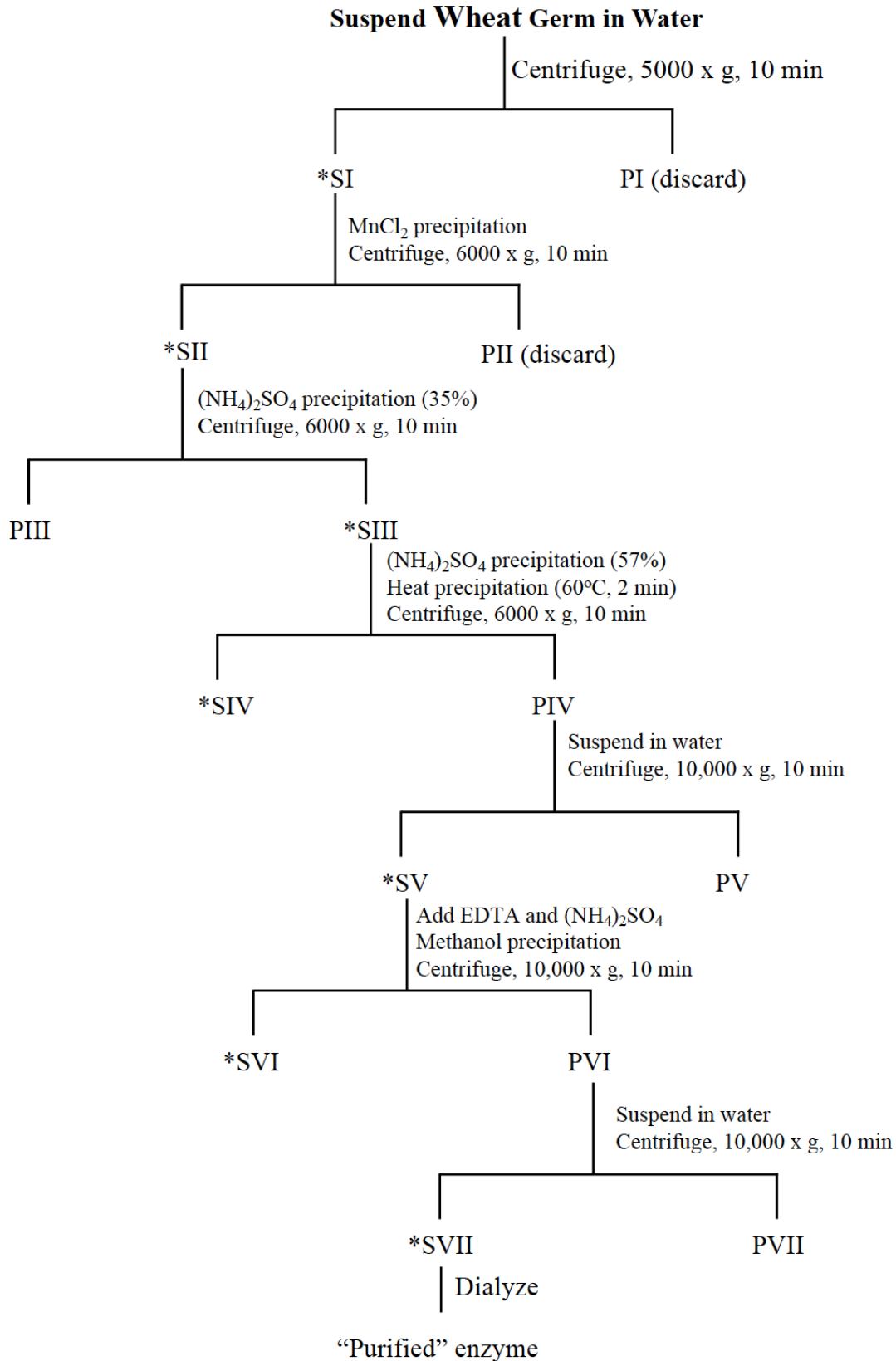







Figure 3. A flow sheet illustrating the entire isolation procedure for wheat germ acid phosphatase. Asterisks indicate fractions for which a small amount should be set aside for later analysis. P indicated a pellet, and S indicates a supernatant.

SAFETY CONSIDERATIONS

Although most of the reagents in this laboratory are being used in dilute concentrations, many can still cause you harm if they come in contact with your skin and/or eyes. Therefore, you **MUST** wear personal protective equipment (PPE) [lab coat, goggles, close toed shoes, and appropriate clothing] in the laboratory at all times.

Substance	GHS Pictogram	Signal Word
1.0M Manganese(II) chloride, $MnCl_2$		Danger Toxic if swallowed. Causes serious eye damage. May cause damage to organs (Brain) through prolonged or repeated exposure. May cause damage to organs (Brain) through prolonged or repeated exposure if inhaled. Toxic to aquatic life with long lasting effects.
Saturated ammonium sulfate, $(NH_4)_2SO_4$	none	none
bovine serum albumin (BSA)	none	none
Biuret reagent	none	none
0.05 M <i>p</i> -nitrophenyl phosphate		Danger Causes skin irritation. Causes serious eye damage. Suspected of causing cancer. May cause damage to organs through prolonged or repeated exposure.
0.5 M Potassium hydroxide, KOH		Danger May be corrosive to metals. Harmful if swallowed. Causes severe skin burns and eye damage. Harmful to aquatic life.
0.2 M EDTA		Warning Causes serious eye irritation.
Methanol		Danger Highly flammable liquid and vapour. Toxic if swallowed, in contact with skin or if inhaled. Causes damage to organs (Eyes).

PART I

1. Water extraction at the enzyme from wheat germ

- a. Transfer 25 g of wheat germ to a 125-ml beaker surrounded by crushed ice in an ice bucket.
- b. Add 100 ml of cold distilled water, mix, and allow to stand for 30 minutes with occasional stirring. This procedure will dissolve the water-soluble proteins, including the acid phosphatase.
- c. Centrifuge the mixture at 6000 x g for 10 minutes to separate the heavier cellular material from the soluble proteins.

NOTE: Operation of the centrifuge according to proper guidelines is absolutely essential for safety in the laboratory and for reliable results.

- 1. Be sure that the centrifuge tubes and inserts you are using are those designed for your particular centrifuge and rotor.**
- 2. Do not fill the centrifuge tubes to more than two thirds of their total capacity.**
- 3. Be certain that, as you place filled tubes in the rotor, there is a balancing tube of equal weight on the opposite side of the rotor.**
- 4. Consult your laboratory instructor for specific instructions regarding the operation of the centrifuge.**

- d. When the centrifugation is complete, remove the tubes and carefully decant the supernatant (SI) into a chilled graduated cylinder, recording its total volume. The pellet (PI) at the bottom of the tube may be discarded.
- e. The water extract (SI) contains the enzyme as well as a number of other soluble molecules. Set aside a small volume (0.5 ml) of SI for subsequent protein and enzyme analysis.

2. Salt fractionation of the enzyme with manganese chloride

- a. Transfer SI to a large beaker in an ice bath on top of a stirring motor. Place a magnetic stirring bar in the solution and turn the motor on low speed to stir the mixture gently.
- b. Add 1.0 M MnCl_2 (1.0 ml for every 50 ml of SI) to the mixture. The MnCl_2 solution should be added slowly and with gentle mixing.
- c. Centrifuge at 6000 x g for 10 minutes, reserving the enzyme-containing supernatant (SII) and discarding the pellet (PII).
- d. Determine the total volume of SII and set aside a small amount (0.5 ml) for later analysis.

3. Salt fractionation of the enzyme with ammonium sulfate (35% cut)

- a. Transfer SII to a 250-mL beaker situated in a gently stirring ice bath and add 27 ml of cold saturated $(\text{NH}_4)_2\text{SO}_4$ (pH 5.5) for every 100 ml of SII.
- b. The addition of ammonium sulfate should be done slowly to avoid localized denaturation of protein. This can be accomplished best by using a 10-ml pipette to add the salt solution to the slowly stirring supernatant over a 10 to 15-minute period. If there is any sign of foaming, the rate of stirring should be reduced because foaming denatures protein and may render the enzyme inactive.
- c. Centrifuge at 6000 x g for 10 minutes, saving the supernatant (SIII) and discarding the pellet (PIII).
- d. Measure the volume of SIII, which is now 35% with respect to ammonium sulfate and contains the bulk of the acid phosphatase. Set aside a small sample (0.5 mL) of SIII for subsequent analysis.

4. Precipitation of the enzyme by ammonium sulfate (57% cut) and heat treatment (60°C)

- a. For every 50 ml of SIII, add 39.5 ml of cold saturated ammonium sulfate (pH 5.5) in precisely the same fashion as described above.
- b. When all of the ammonium sulfate has been added, transfer the beaker to a hot water bath (65 to 70°C) and

swirl the mixture gently until the temperature reaches 60°C. Maintain the mixture at that temperature for exactly 2 minutes and then immediately return the beaker to the ice bath, cooling the mixture to 5°C as rapidly as possible. The temperature of the mixture should be monitored throughout this procedure with a thermometer, which can also be used (with care) to mix the solution.

- c. Centrifuge at 6000 x g for 10 minutes to collect the enzyme, which should now be in the insoluble portion (PIV).
- d. Retain the pellet (PIV) but do not discard the supernatant (SIV) until the presence of acid phosphatase in PIV has been verified by enzyme analysis. The supernatant should be stored frozen until PIV has been tested for enzyme activity.

5. Water extraction of the enzyme from PIV

- a. Add a small volume (2 to 5 ml) of cold distilled water to each centrifuge tube containing an enzyme pellet (PIV). Resuspend the pellets by scraping and agitating the precipitated material with a blunt glass rod.
- b. When a uniform suspension has been achieved, measure the total volume and add enough cold distilled water to give a final volume equal to one third the volume of the MnCl₂ supernatant (SII).
- c. Centrifuge the suspension at 10,000 x g for 10 minutes, keeping the supernatant (SV) and discarding the pellet (PV).
- d. Measure the volume of SV and save a small amount (0.5 mL) for later analysis.
- e. Transfer SV to a plastic bottle and store it in the freezer. The acid phosphatase may be stored in this condition for several weeks with little decrease in enzyme activity.

6. Place all of the 1.0-mL samples (SI, SII, etc.) you have collected in the freezer.

PART II

It is your task at this point to evaluate the effectiveness of the protein isolation procedure through an analysis of all the fractions thus far collected (SI, SII, etc). Test each sample for the amount of protein present and the level of enzyme activity. These and other relevant data should be entered in the enzyme purification table (Table 1) ***in your lab notebook***, from which the percent recovery at each step can be determined.

1. Protein concentration- Biuret test

a. Protein standard curve using bovine serum albumin

- 1) Obtain a sample of bovine serum albumin (BSA) having a concentration of 10 mg/mL and prepare a series of 1.0 mL solutions containing a range of protein concentration from 0 to 10 mg/mL.
- 2) To each 1.0 ml sample, add 4.0 ml of Biuret reagent, mix well and allow to stand at room temperature for 30 minutes.
- 3) Determine the absorbance of each sample at 540 nm (A_{540})
- 4) Prepare a standard curve by plotting A_{540} versus protein concentration (mg/mL).

b. Determination of protein concentration in enzyme fractions

- 1) Set up a series of labeled test tubes containing 0.1 mL samples of the various enzyme fractions you plan to assay (SI, SII, etc.)
- 2) Add 0.9 mL of distilled water to each sample, bringing it to a final volume of 1.0 mL.
- 3) Add 4.0 ml of Biuret reagent to each sample and let stand at room temperature for 30 minutes.
- 4) Determine the absorbance at 540 nm for each sample and, using the protein standard curve, determine the protein level (mg/mL) for each fraction.
- 5) Enter this information plus the total protein per sample information in the appropriate columns of Table 1 ***in your lab notebook*** (not in the printed version of this handout)

c. Notes

- 1) If the absorbance is beyond the sensitivity of the standard curve, it will be necessary to repeat the assay at a more appropriate dilution of the sample.
- 2) As you do the final calculations for protein concentration, be mindful of the dilution factor introduced during the assay. For example, if a 0.1 mL sample has been diluted with 0.9 mL of water prior to the addition of Biuret reagent, the sample has been diluted ten times and the protein concentration must be adjusted accordingly.

Table 1. Enzyme purification table for wheat germ acid phosphatase

Fraction	Vol (mL)	Protein			Enzyme Activity				
		Sample (mL)	Protein (mg/mL)	Total Protein (mg)	Sample (mL)	Activity (units/mL)	Total Enzyme (units)	Specific Activity (units/mg protein)	Percent Recovery
SI									
SII									
SIII									
SIV									
SV									
SVI									
SVII									
Pure Enzyme									

Enzyme activity-acid phosphatase assay

The level of acid phosphatase is determined by an assay which uses the artificial substrate *p*-nitrophenyl phosphate. To assay a single sample, set up two reaction tubes identical with respect to substrate concentration, temperature, etc., and add enzyme to one tube and an equal volume of water to the other. The reaction is terminated after 5 minutes by adding potassium hydroxide to each tube. The KOH stops the reaction by denaturing the enzyme and converts the *p*-nitrophenol to its colored form. The contents of the tubes are compared colorimetrically, and the amount of *p*-nitrophenol produced per minute is used as a measure of enzyme activity.

a. Acid phosphatase assay

- 1) Prepare two labeled test tubes (A and B) each containing the following reaction mixture:
 - 0.5 ml of 1.0 M sodium acetate buffer (pH 5.7)
 - 0.5 ml of 0.1 M MgCl₂
 - 0.5 mL of 0.05 M *p*-nitrophenyl phosphate
 - 3.3 ml of distilled water
- 2) Mix the contents of each tube and place them in a 37°C water bath. Allow the temperature to equilibrate for 5 minutes.
- 3) Add 0.2 mL of distilled water to tube A (blank), mix the contents, and return the tube to the water bath. Add 0.2 ml of the enzyme sample to tube B, start a stop watch, mix and return the tube to the water bath.
- 4) When the reaction has proceeded for 5 minutes, immediately add 2.5 ml of 0.5 M KOH to each reaction tube.
- 5) If the contents of the tubes appear cloudy due to the presence of precipitated protein, centrifuge the samples for 10 minutes at top speed in a desktop clinical centrifuge. Discard the precipitate.

- 6) Using the contents of tube A as a blank, determine the absorbance of tube B at 405nm. If the absorbance is greater than 1.0 the assay must be repeated using a more dilute concentration of the enzyme because such a reading is beyond the sensitivity of the colorimeter.
- 7) Convert the absorbance readings at 405 nm to micromoles of *p*-nitrophenol formed per minute, using the extinction coefficient for *p*-nitrophenol ($18.8 \times 10^3 \text{ liter mole}^{-1}\text{cm}^{-1}$). which assumes a path length of 1.0 cm.

b. Determine the acid phosphatase activity (units/ml) and total phosphatase activity (units) for all samples (SI, SII, etc.) and enter the data in Table 1 ***in your lab notebook***. One unit of acid phosphatase is defined as the amount of enzyme that catalyzes the hydrolysis of 1.0 micromole of *p*-nitrophenyl phosphate per minute at 37°C.

c. Notes:

- 1) The method described above is for a single enzyme sample and should be expanded to test all the fractions (SI, SII, etc.) for phosphatase activity. With proper organization you should be able to set up a series of reaction tubes, start them in sequence (1 minute apart), and stop them in the same sequence 5 minutes later. A single blank tube is sufficient.
- 2) As indicated in the protein determination notes you should keep track of sample dilutions in order to accurately calculate enzyme activity in the samples.

PART III

Remove SV from the freezer and thaw it rapidly in lukewarm water. Proceed with the remaining steps in the extraction procedure.

1. Methanol fractionation of the enzyme

- a. Consult Table 1 to determine the protein concentration of SV and if it is higher than 5 mg/mL adjust it down to that level with distilled water. Measure and record the final volume.
- b. For every 0.5 mL of SV, add 0.06 mL of 0.2 M disodium ethylenediaminetetraacetic acid (EDTA)
- c. For every 0.5 mL of SV, add 0.025 mL of saturated ammonium sulfate (pH 5.5). Record the volume of this solution.
- d. Transfer the solution to a stirring ice bath and slowly add 0.875 mL of very cold (-20°C) methanol for each half milliliter (0.5 mL) of enzyme preparation (SV plus additions just made).
- e. Centrifuge at 10,000 x g for 10 minutes, keeping the pellet (PVI) and discarding the supernatant (SVI) after saving 0.5 mL for later analysis.

2. Water extraction of the enzyme from PVI

- a. Suspend the pellet (PVI) in 5 ml of cold distilled water with the aid of a blunt glass rod.
- b. Centrifuge at 10,000 x g for 10 minutes, decant the supernatant (SVII), and save it on ice.
- c. Suspend the pellet (PVII) in 5 ml of cold distilled water and centrifuge at 10,000 x g for 10 minutes. Combine this supernatant with SVII and discard the pellet.
- d. Measure and record the volume of the combined supernatants (SVII). Place about 0.5 mL of SVII in the freezer for later analysis.

3. Dialysis of the enzyme

- a. Obtain a piece of dialysis tubing approximately 20 cm in length (1.3 cm in diameter) and place it in a beaker of distilled water for 5 to 10 minutes. This treatment hydrates the cellulose tubing and makes it flexible.
- b. Rub the tubing between your fingers to separate the side walls and open the lumen. When the dialysis sac is opened fully, tie a double knot in one end.

- c. Using a Pasteur pipette, carefully transfer SVII into the dialysis sac, being careful not to puncture the tubing. Tie two knots in the open end, trapping a small amount of air inside the sac.
 - d. Transfer the filled dialysis sac to a large beaker or flask containing 500 mL of cold 5.0 mM sodium EDTA. Place the container in a cold room or refrigerator at 4°C and mix it gently overnight with a magnetic stirring rod.
 - e. After removing the sac from the dialysis solution, open one end with clean stainless-steel scissors and transfer the contents to a glass test tube.
 - f. This solution is your purified acid phosphatase sample and is used for all subsequent enzyme experiments. Measure the volume of the enzyme experiments. Measure the volume of the sample and store it frozen in a carefully labeled covered test tube.
4. Use the Biuret and acid phosphatase assays to determine protein and enzyme levels in SVI, SVII, and the purified enzyme sample. Enter this data in to Table 1.

Calculations and Questions

1. Enter all remaining information (volumes, etc.) in Table 1.
 - a. Calculate specific activity (units of enzyme per mg of protein) for each fraction.
 - b. Assuming that the total number of enzyme units in SI represents 100 % recovery, calculate the percent recovery for each sample.
 - c. With each subsequent step in the isolation procedure, what happens to the amount of total protein in each fraction? What happens to the specific activity in each fraction? How can you explain these two trends?
 - d. At which point in the isolation procedure (35% ammonium sulfate cut, methanol precipitation, etc.) did you realize the greatest percent recovery of enzyme? If you were planning to improve the isolation procedure to give a higher yield, which step in the procedure would you concentrate on? Explain your answer.
2. Why is it necessary to maintain low temperatures throughout the extraction procedure?
3. The level of acid phosphatase present in the supernatant after the 35% ammonium sulfate cut was higher than that after the 57% ammonium sulfate cut (SIV). How do you explain this?
4. Investigate the literature to determine how it is believed that salts and organic solvents cause the precipitation of proteins.