Isolation and Characterization of Wheat Germ Acid Phosphatase

Pilot Project

In order to insure that you can perform the assays for enzyme activity and total protein content correctly you will perform a pilot project before initiating the full-scale experiment. In this project you will purify Fraction I (see below) but utilize only a tenths (1/10) of the listed amounts of materials and reagents. This will provide enough material to perform both an enzyme assay and a total protein assay. You will use the results of these assays to calculate the specific activity of the enzyme. (Specific activity is defined as total activity divided by mg total protein.

Isolation of Fraction I

Add 50g of wheat germ to 200mL of cold deionized water. Stir this gently until a uniform mixture is obtained. Let this mixture stand at room temperature for 30 minutes with occasional stirring (note that over-stirring can result in significant loss of acid phosphatase). Decant the suspension into a 250mL centrifuge bottle (use a 50mL bottle instead for the pilot project) and centrifuge for 10 minutes at 8000 rpm, making sure that the slot opposite your bottle in the centrifuge bucket contains an identical bottle filled to the same level as yours. This will require you to coordinate your efforts with other groups. Carefully decant off the supernatant, measure its volume, and discard the pellet. This solution is regarded as Fraction I and represents the crude isolate. Remove 3mL for analysis. **Do not stop here unless you are performing the Pilot Project.** Continue with the preparation of Fraction II at least to the point of addition of the first aliquot of saturated ammonium sulfate. You can perform the enzyme activity and total protein assays while you continue or do these at the beginning of the next laboratory period. If you choose to analyze the fraction at a later time, store the 3mL aliquot frozen in a clearly labeled microcentrifuge tube. Please be sure that you use no more than 1mL for performing the assays since 2mL will be required for the characterization of enzyme activity that you will perform later in the semester.

Isolation of Fraction II

Add 2.0mL of 1M $MnCl_2$ for each 100mL of Fraction I isolated slowly, while stirring. Centrifuge the resulting suspension at 8000 rpm for 10 minutes. Decant off the supernatant, measure its volume and transfer it to a 600mL beaker immersed in an ice bath. Discard the pellet. Set the ice bath/beaker combination on a magnetic stirrer, add a stir bar to the beaker and begin to gently stir the solution. Add 54mL of cold, saturated ammonium sulfate (pH 5.5) for each 100mL of the supernatant while stirring. **You must get at least to this point on the first day.** After 10 to 15 minutes of stirring (following the ammonium sulfate addition), centrifuge the suspension at 8000 rpm for 10 minutes. Decant the supernatant into an appropriate sized beaker and discard the pellet.

Add 79mL of saturated ammonium sulfate to this supernatant for each 100mL of volume obtained after the addition of $MnCl_2$ with constant stirring. Place the beaker containing this mixture into a water bath adjusted to 65-70^{O}C. Maintain slow and continuous stirring while monitoring the temperature of the mixture. When the mixture temperature

reaches 60 ^{O}C, note the time and keep the mixture at this temperature for two minutes. Transfer the beaker from the water bath to an ice bath and stir the mixture slowly until the temperature reaches 5 ^{O}C. Centrifuge the resulting suspension at 8000 rpm for 30 minutes. Save both the supernatant and the pellet. Although most of the enzyme should be associated with the pellet, assay the supernatant for residual activity to make sure of this. If there is substantial activity in the supernatant you will have to add more saturated ammonium sulfate to it to precipitate the enzyme. See the instructor to develop a protocol to address this problem if it comes up.

Resuspend the pellet in a volume of cold deionized water equal to one-third of that obtained after the addition of $MnCl_2$. Centrifuge this suspension at 8000 rpm for 30 minutes. Decant the supernatant, measure its volume and discard the pellet. The supernatant constitutes Fraction II and represents acid phosphatase purified by salt fractionation. It may be stored frozen.

Isolation of Fraction III

Prior to beginning this portion of the protocol, the total protein concentration of Fraction II must be determined. Ideally, at this stage of the isolation procedure the protein concentration is too high to proceed with the next step. Adjust the protein concentration of Fraction II to 4.5 ± 0.5 mg/mL with cold deionized water. Add 0.11mL of 0.2M EDTA (disodium salt; not pH adjusted) and 0.05mL of saturated ammonium sulfate for each mL of the solution obtained after adjusting the protein concentration. (For example, if the total adjusted volume added up to 100mL, 11mL of 0.2M EDTA and 5 mL of saturated ammonium sulfate would be added.) Place the resulting solution into an ice bath and stir slowly while adding 1.75mL of **very cold** methanol for each mL of the solution. The addition should be carried out over a time interval of five to ten minutes from a container of methanol chilled to – 20^{O}C. The temperature of the methanol and the slow rate of addition are intended to minimize the denaturation of proteins.

Centrifuge the resulting mixture at 8000 rpm for 30 minutes. Discard the supernatant. Suspend the pellet in 10mL of cold deionized water with the aid of a glass stirring rod and centrifuge the resulting mixture at 8000 rpm for an additional 30 minutes. Remove and save the supernatant, resuspend the pellet in an additional 10mL of cold deionized water and repeat the centrifugation (8000 rpm for 30 minutes). Combine the supernatant obtained from this step with the one from the previous centrifugation and discard the pellet.

At this point the combined supernatants contain unknown amounts of ammonium sulfate and manganese chloride that must be removed through dialysis. Prepare the dialysis tubing (see below) and dialyze the supernatant against 500mL of 5mM EDTA (pH 5.7) at 4 ^{O}C with constant slow stirring overnight. Remove the solution from the bag, measure the total volume, remove and save 3mL for analysis and store the remainder in the freezer. This solution is designated as Fraction III (that purified by methanol fractionation).

Dialysis of Protein-containing Solutions

This protocol is designed to establish the small solute concentrations in a solution of macromolecules. The walls of the dialysis tubing are permeable to the former but impermeable to the latter. The length of tubing required must be enough to contain the solution being dialyzed, allow sealing of both ends and trap an air pocket that keeps the dialysis bag floating in the dialysis solution. The conversion factor between volume and cm of length is usually given on the side of the box containing the dry tubing. Measure the volume of the solution to be dialyzed, evaluate the length of tubing required to hold this volume and add 10 cm to this length to allow for sealing the tubing and trapping the air pocket. (It is better to overestimate than underestimate the length required!) Cut the appropriate length of tubing and soak it in a beaker of deionized water for about ten minutes. This hydrates the tubing that has been previously coated with glycerol. Open the entire length of tubing by rubbing the tubing between thumb and forefinger. Seal the bottom of the bag by tying a knot at one end. Check to make sure that there are no leaks by filling the knotted tubing with deionized water, sealing the top with a thumb and forefinger and gently squeezing the bag between remaining fingers and palm.

To fill the bag, hold the top end open with one hand and carefully transfer the solution into the bag with a pipette, being careful not to damage the bag with the pipette tip. After filling, manipulate the top of the bag with the fingers to open it as wide as possible, the fold over about 1cm of the top, capturing some air inside. Clip the top closed with a dialysis clip and place the sealed bag in the dialysis buffer immediately. (The dialysis bag should never be allowed to become dry!)

Dialysis of protein containing solutions should always be done in the cold to prevent denaturation and minimize the growth of contaminating bacteria and mold. Dialysis buffers should always be stirred during dialysis to insure that equilibrium (in terms of the concentrations of diffusible species) is established between the inside and outside of the bag as efficiently as possible. As a general rule of thumb, equilibration is more than 90% complete in two to three hours if dialysis is carried out at 4 ^{O}C ith constant slow stirring.

Concentration of the Enzyme

The enzyme will be concentrated by precipitation with ammonium sulfate followed by dissolution in a smaller volume of buffer. Add 4g of solid ammonium sulfate for each 10mL of dialysate (Fraction III) while slowly stirring the solution/suspension. Continue stirring for ten to fifteen minutes and then centrifuge the mixture at 8000 rpm for 30 minutes. Decant off the supernatant and dissolve the pellet in 2mL of pH 5.8 0.2M ammonium sulfate containing 1mM EDTA. Remove any insoluble material by centrifugation at 8000 rpm for an additional 30 minutes. **Note:** Check the supernatant obtained from the ammonium sulfate precipitation for residual acid phosphatase activity. There should be very little (if any) present. See the instructor if significant activity remained in the supernatant.

Enzymology

Each group will receive a 0.500mL sample of wheat germ acid phosphatase dissolved in pH 5.8 0.2M ammonium sulfate containing 1mM EDTA. (The concentration of the enzyme is 53mg/3.0mL.) This solution will be used as the source of the enzyme in performing the kinetic experiments that comprise this portion of the characterization of the enzyme. Assume that the activity of the sample is equivalent to that listed for Fraction IV on the first page of the "Phosphatase Assay" in order to calculate the volume that should be used for each sample assuming that an absorbance change of 0.2 AU/min is desired. (A similar calculation was carried out to evaluate the volume of each of the fractions to be used in evaluating enzyme activity.) After carrying out this calculation, check with the instructor to make sure that it is correct. The assays will be done the same way that all of the other activity measurements, i.e. in single cell kinetics mode. It is essential not to waste enzyme once you begin!

Measurement of K_m and k_{cat}

In this portion of the experiment initial rates will be measured for a series of different starting concentrations of p-nitrophenyl phosphate (pNPP). (Recall that the samples have to be kept on ice and that a new assay mixture has to be made each day.)

Prepare a dilution series of pNPP from a concentrated stock solution such that after the addition of the enzyme (see above) the total volume of the solution is 3.00mL and the initial pNPP concentrations are 0.10, 0.25, 0.50, 0.75, 1.00, 2.50 and 5.00 mM. Evaluate samples in order of increasing substrate concentration. Mixing is accomplished by drawing the solution in and out of a Pasteur pipette equipped with a piece of Tygon tubing on the end to protect the cuvette. Rescale the data so that each run can be seen on the same page and print out the results. Repeat this for the second set of kinetics measurements.

Calculate the initial velocities for each of the measurements in terms of AU/minute. Plot the results in terms of double reciprocal (1/v vs. 1/[S]) format. If any of the data points are a poor fit, repeat the kinetics measurement for the associated value(s) of [S]. Once a reliable set of data has been collected, convert velocities into units of activity and plot the data both in double reciprocal and Hanes-Woolf ([S]/v vs. [S]) formats. Use linear regression to determine slope and intercepts and associated standard deviations for each format and use these to calculate the values and standard deviations of K_m and V_{max}. Determine the concentration of enzyme used in the assays and use this to calculate k_{cat}.

Inhibition by P_i at a Single Inhibitor Concentration

This portion of the experiment is carried out exactly the same way as the above with the exception that inorganic phosphate (P_i) will be present in the assay mixture in addition to substrate. The concentration of P_i is adjusted to be 1.0mM once the addition of the enzyme produces a total volume of 3.00mL. Analyze the results of these experiments in the same way as above, plot the results for both the non-inhibited reactions and the ones carried out in the presence of P_i on the same page to determine the type of inhibition exhibited by P_i, and

use the apparent and true values of K_m and V_{max} as well as the inhibitor concentration to determine the value of $K_i/K_{i'}$.

Dixon Plot Analysis of Inhibition

In this portion of the experiment initial velocities are determined as a function of $[P_i]$ at two different values of [S]. Prepare a series of assay mixtures such that the concentrations of P_i and pNPP will correspond to those given below after addition of the enzyme containing solution:

- $[P_i]$ = 0,0.2, 0.5, 1.0, 2.0 mM
  [pNPP] = 1.0mM in all solutions

- $[P_i]$ = 0,0.2, 0.5, 1.0, 2.0 mM
  [pNPP] = 2.5mM in all solutions

(Note that some of these solutions have already been examined-these measurements need not be repeated.)

Determine the initial velocities in the above kinetics experiments and plot the results in the Dixon format (1/v vs. $[P_i]$; note that two lines are required, one at each value of [S]). Use linear regression to determine slopes, intercepts and associated standard deviations and determine the value of Ki from the value of $[P_i]$ at the intersection point of the two lines.

SDS-PAGE

Fractions I, II and III will be analyzed using SDS-polyacrylamide gel electrophoresis to evaluate their protein compositions. Pre-cast Nu-PAGE gradient gels (4-12% acrylamide) will be used for this purpose. 10 μL of each of the samples as well as molecular weight standards will be loaded into the wells on the pre-cast gels. Two gels will be utilized to analyze the samples from all of the groups; the details of most of the methodology will be discussed in class. The salt concentration of the samples you apply to the gel cannot be higher than 0.2M. Check the concentration of salt in your samples and dilute as necessary to achieve the above concentration.

Once this has been done with each of the samples, their protein concentration has to be adjusted to 20mg/mL. If the initial protein concentration is too high, it can be adjusted by appropriately diluting the sample. If however the protein concentration is too low, the sample has to be concentrated by ultrafiltration. The samples will be transferred into a cell equipped with a membrane filter permeable to molecules with MW less than or equal to 10kD. The cell fits into a centrifuge tube that can be spun in a centrifuge. The centrifugal force will push solvent and small solutes through the filter while the desired macromolecules will be retained in the cell. The length of centrifugation time required to concentrate a sample depends on the original protein concentration-this will determine what volume reduction is required to achieve the target concentration. To evaluate the extent of volume change per unit time, measure the initial volume of sample loaded into the cell,

centrifuge for 10 minutes at 5000 rpm and check the volume of the flow-through. This should provide you enough information to calculate the additional time of centrifugation required.

Note that before applying a sample to the gel it will be mixed with an equal volume of 2X sample application buffer, so actual protein concentration of the sample to be loaded is 10mg/mL.

Staining and Destaining the gel

After electrophoresis, place the gel in 100mL of ultrapure water in a loosely covered container and microwave on High for about 45 seconds (s). Swirl the gel/water combination for one minute and discard the water. Repeat the above procedure two more times. (This means that at this point the gel has been washed three times with 100mL portions of water.) Next, add 20mL of SimplyBlue SafeStain and microwave on High for 15 to 30s until the solution almost boils. (Watch the container and remove it from the microwave as soon as bubbles begin to form.) Gently swirl the gel in the container for five minutes. Pour off the stain, wash the gel with a 100mL aliquot of ultrapure water as above, pour off the water, add a second 100mL portion and place a Kimwipe on top of the gel as it sits in the wash. (This is to assure that excess stain is efficiently removed from the gel.) Continue this process until the appearance of the gel is satisfactory for clearly identifying bands of protein.