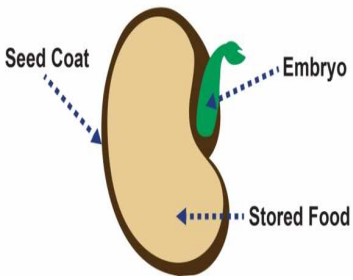
Lab 3 Enzyme Kinetics

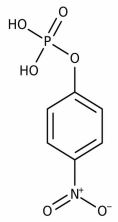
Background

Seeds contain three basic components: the embryo which is also called the germ (and our source of vitamins), the endosperm which is the stored starch or food for the embryo, and third, the tough covering layer or seed coat.



You will be extracting the enzyme acid phosphatase from wheat germ (**wheat germ acid phosphatase**). This enzyme catalyzes the removal of phosphate groups from macromolecules (mostly from proteins).

In order to measure the activity of the enzyme you will be measuring the increase of the product. We will add the synthetic substrate, **nitrophenol phosphate**. This molecule is colorless in alkaline pH. When the phosphate is removed nitrophenol exhibits a yellow color which can be measured in a spectrophotometer.



$$\text{nitrophenol phosphate} \xrightarrow{\text{acid phosphatase}} \text{nitrophenol} + \text{phosphate}$$

(yellow color)

In order to quantitate the amount of nitrophenol produced you will first generate a standard curve using a serial dilution of nitrophenol. Next you will measure enzyme activity from purified enzyme. Finally you will measure the enzyme activity of your isolated wheat germ acid phosphatase.

Spectrophotometry

One of the most important techniques of analytical chemistry used by biologists is spectroscopy. It has had a profound effect in the conversion of biology from a descriptive to the quantitative science it is today.

Theoretical Background

The fact that many molecules of biological importance interact with radiant energy in a predictable fashion is fundamental to spectroscopic measurements. The reflected color of a pigment is qualitative and tells what is happening, but not to what degree (quantitative). A great deal of information can be obtained by quantitating the reflected energy. The human eye is not especially accurate as a quantitative receptor of color, so an instrument known as a colorimeter has been developed. There are three factors which limit the accuracy of the human eye as a colorimeter:

1. The range of radiant energy perceived by the human eye is limited to 380-750 nanometers (the visible range).
2. The human eye cannot discriminate between similar types of light (wave lengths close together).
3. Electrical colorimeters are quantitative (provide a number), while the eye is only semi-quantitative.

Spectrophotometers

The electric photometer apparatus is called a **spectrophotometer**. It has replaced the colorimeter in most laboratories. A spectrophotometer consists of a light source which is focused on a prism to separate the light into its separate bands of radiant energy. The different bands (colors) may be then focused through a narrow slit. The narrower the slit the more precise the measurement since the absorption is then more closely related to a specific wave length. The beam of light then passes through the sample to be measured. The sample is usually dissolved in a suitable solvent and contained in a specially selected tube called a **cuvette**. Most cuvettes have a light path of exactly 1.0 cm.

After the selected beam of light traverses the sample, it emerges as **transmitted light**. The transmitted light is reduced in intensity if the substances in the photocell, including the cuvette, has absorbed some of the transmitted light. If none of the incident light is absorbed, the transmitted light will show the same radiant energy as the original light. The transmitted light then strikes a **photoelectric tube** which generates an electrical current that is proportional to the intensity of the transmitted light. The photoelectric tube is connected to a galvanometer with a graduated scale that permits measuring the intensity of the trans-

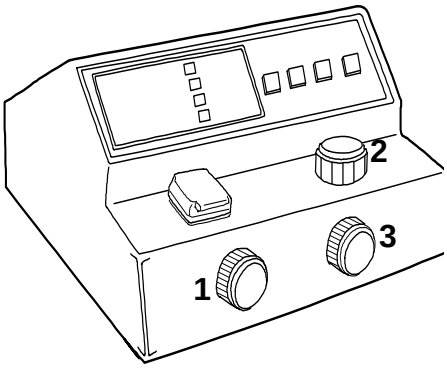
mitted beam. The scale is normally graduated in one of two ways:

1. **%T = percent transmittance** - an arithmetic scale with equidistant units from 0-100%
2. **A = absorbance** - a logarithmicscale with non-equal units from 0.0 - 2.0.

Biological molecules are usually dissolved in a solvent prior to measurement. Therefore, the solvent itself may absorb light and be a possible source of error. To assure the light absorption of only the solute is determined, a means of subtracting the absorbance of the solvent is necessary. This is done by first using a **blank** (solvent) in the apparatus. The scale is manually adjusted to read 100% T or 0.0 A, after which the sample (unknown plus solvent) is inserted. A reading of less than 100% T or more than 0.0 A is considered to be the result of absorbance of the unknown solute. If other solutes (buffers, salts, etc.) are present in the sample other than the unknown solute, they must be included in the blank.

Using the Spectrophotometer

1. Turn on the instrument by turning knob 1 clockwise. Allow the spec. to warm up for at least five minutes.
2. Make sure the sample compartment is empty the spec is set to Trasmittance mode and adjust to 0 with knob 1.
3. Set the display mode to ABSORBANCE by pressing the MODE button until the appropriate LED is lit.
4. Fill a cuvette with your blank.
5. Wipe the blank cuvette with a kimwipe to remove liquid droplets, dust, and fingerprints.
6. Place the cuvette in the sample compartment and align the mark on the cuvette with the guide mark on the front of the sample compartment. Press the cuvette firmly into the sample compartment and close the lid.
7. Set the desired wavelength with knob 2.
8. Adjust the meter to 0.0A with knob 3 on the far right side of the instrument.



9. Replace the blank cuvette with your unknown cuvette, aligning the guide marks and close the lid.
10. Record the Absorbance from the meter.
    If you have another unknown, place it in the spec and record the new absorbance.
11. When you are finished with all your measurements turn off the spectrophotometer by turning knob 1 counterclockwise until it clicks.

Notes:

- A flashing display indicates that the reading is out of range.
- When changing wavelength it is important to insert the blank and reset the display to 100%T or 0.0A every time.

Generating the nitrophenol standard curve

1. After preparing the spectrophotometer as written above . . .
2. Measure and record the absorbance of the nitrophenol standards provided.
   1. = 0 nmol
   2. = 25 nmol
   3. = 50 nmol
   4. = 100 nmol
   5. = 200 nmol
   6. = 400 nmol
3. Plot the results with the absorbance on the Y axis and the nitrophenol standards on the X axis. You can use computer software (Xact) to determine the regression analysis and the equation for the straight line.

Isolation of wheat germ acid phosphatase

1. Weigh out 0.5 g of wheat germ.
2. Transfer wheat germ to a mortar.
3. Add 5 ml of enzyme extraction buffer.
4. Grind the tissue with a pestle to form a homogenous suspension.
5. Transfer 1.5 ml to a microcentrifuge tube (2 x 750 µl).
6. Centrifuge 1 min. a full speed.
7. Transfer supernatant to a new microcentrifuge tube labeled WGE and place on ice.

Enzyme Reaction

1. Obtain 12 test tubes
2. Label "A1 - A6" and "B1 - B6".
3. Put 1 ml of KOH in each test tube.
4. Obtain 2 25 ml flasks.
5. Label one "A" and the other "B"
6. Add 10 ml of substrate to both A & B flasks.
7. Transfer 1 ml from flask A to tube A1 and transfer 1 ml from flask B to tube B1. These will be used to determine your zero time values.
8. Add 100 µl of pure enzyme (acid phosphatase) to flask A.
9. Add 400 µl of wheat germ enzyme extract to flask B.
10. **Start your clock** and shake both flasks gently.
11. At the correct time point remove 1 ml from each flask and transfer to the appropriate tube.
    - 2.5 min. - tubes A2 & B2
    - 5 min. - tubes A3 & B3
    - 10 min. - tubes A4 & B4
    - 15 min. - tubes A5 & B5
    - 20 min. - tubes A6 & B6

> The KOH in the test tubes will denature the enzyme and stop the reaction. It will also turn the nitrophenol (the product) yellow.

12. Measure the absorbance of each time point in your reactions (tubes A1-A6 and B1-B6) and record the data.
13. Using the equation generated above calculate the amount of nitrophenol generated from each reaction (tubes A and B.
14. Plot your calculations data with the nitrophenol product on the Y axis and the time (minutes) on the X axis.
15. From your graph calculate the initial velocities (V_o) for both reactions.

Reaction A1-A6 (purified enzyme) _____ nmoles nitrophenol / minute

Reaction B1-B6 (extracted enzyme) _____ nmoles nitrophenol / minute