BCH3033 ENZYMES, Part II

Purpose: To study the effect of pH and inhibitors on the rate of a peroxidase reaction.

Effect of Inhibitors and Activators

Toxins and inhibitors affect everyone's daily life, and have since organisms first began using chemical warfare to gain evolutionary advantage over their competitors. Those that inhibit enzymes can be classed as either irreversible, in which case active enzyme cannot be recovered once it has been inhibited, or reversible, where the inhibitor can be physically removed leaving the enzyme active again. This class can be further subdivided into competitive inhibitors, in which the inhibitor binds to the active site of the enzyme, blocking the substrate's access, or noncompetitive inhibitors, in which the inhibitor binds elsewhere on the enzyme, changing its shape and, thus, its activity. If the substrate concentration is increased relative to inhibitor, a competitive inhibition will be overcome, while a noncompetitive will not.

In this lab, you will be given different inhibitors (I) of peroxidase and asked to determine i) the level of inhibitor (I) necessary to effectively inhibit peroxidase activity, and ii) the type of inhibition characteristic of specific inhibitors.

Inhibitors of peroxidase: p-aminobenzoic acid, sodium azide (NaN_3), cyanide, cyclopropanone, L-cystine, dichromate, ethylenethiourea, hydroxylamine, sulfide, sulfite, vanadate and a number of divalent anions of Cd, Co, Cu, Fe, Mn, Ni and Pb (Horseradish; see http://www.sigma.sial.com/sigma/proddata/p8000.htm).

Protocol

1. Before you can start this week's lab you need two pieces of information from the first week of lab:
    a. the enzyme [E] concentration (i.e., dilution) that gave you a good rate of product formation (i.e., color); and
    b. the Km value for peroxidase (remember, this is the magical concentration of substrate [S] that is necessary for the reaction to proceed at ½ of the maximum velocity of the reaction (1/2 Vmax). Why would this be a good [S] to work with?
2. Confirm that the enzyme activity is similar to week 1 – since the enzyme is extracted fresh each week, there may be some variation in activity. Thus to start you need to grind up 40 grams of horseradish or turnip root just like last week. Do a quick check on enzyme activity. If the activity is noticeably different, you may need to adjust your dilution or amount to get an appropriate level of enzyme activity (Δabs of 0.4 to 0.7 over 5 minutes; discuss this with your lab instructor). Once you know this, the Km does not change. Now you can begin to study how other molecules (specific inhibitors) interact with the peroxidase enzyme.
    a. Prepare a "control" set of reaction tubes exactly as you did during week 1 of this lab (see Table in **III. Change in Enzyme Kinetics with Substrate Concentration).** Label these tubes the "control" set.

3. Last week in part **III. Change in Enzyme Kinetics with Substrate Concentration,** you varied one substrate, H_2O_2 , but kept [E] constant. You will repeat this procedure in this week's lab, but you will add a constant level of inhibitor [I] to one reaction set to see how the velocity of the reaction is impacted. Do this as follows:

   a. REPEAT the above experiment with *two sets* of idential reaction tubes. One set of these tubes should contain one of the enzyme inhibitors (10 µL per tube) – either hydroxylamine or sodium sulfite. Label the tubes "experimental" to denote that they contain inhibitors**. Be careful and wear gloves.**

   b. As above, when you are ready to initiate the enzyme reaction in each tube, add the specified amount of enzyme and monitor the reaction (color development) in the spectrophotometer.

4. Once you have collected all of the data, plot the change in Abs/min (y-axis) vs. [Substrate] (x-axis) on the same graph. How has the line changed due to the inhibitor?

5. To determine the type of inhibition characteristic of each inhibitor, you will need to plot the data using the Lineweaver-Burke (double reciprocal) plot. What type of inhibition do your inhibitors cause based on this plot?

The Effect of pH

All enzymes display a characteristic range of pH at which they are most active. This "pH optimum"may be due to several factors involving the structure and ionic state of the enzyme, substrate, or cofactors (see your text). In many cases it reflects the pH of the organelle in which the enzyme is active. What is the pH optimum for peroxidase, and the probable pH inside the peroxisome?

Procedure:

1.Prepare a set of reaction mixtures as in Table 2., but substitute buffers of varying pH in all but
one tube and the blank. Use pH 2, 4, 5, 6, 7, 8, & 10 if possible.
2.Start each reaction with the [Enzyme] that gives change in Abs/5 min = 0.6 units only.
3.Plot V0 vs. pH (x-axis).

At what pH is peroxidase most active? Are you sure?

Did you notice anything unexpected? Reevaluate your conclusions.

The Effect of Temperature

At low initial temperatures, increasing it increases the rates of all reactions, whether catalyzed or not. At higher temperatures, however, proteins denature (as in "to cook"). Since most enzymes

are proteins, temperature changes will produce something like a temperature optimum, although at this point the protein is already being denatured.

Can you plan a properly controlled test of the effects of temperature on the peroxidase assay? Try! .....if there is enough time.