Lab Module 2 -- ENZYME FUNCTION BIOL116L Spring 2008

(Note to students: this Module covers two weeks, starting the week of Feb. 18)

Introduction

Life depends on many biochemical reactions. Nearly all of these chemical reactions would proceed at a rate that is far too slow to meet the metabolic needs of living systems were it not for the presence of **catalysts**. A catalyst is a substance that increases the rate of a reaction dramatically, without itself being consumed in the process (meaning it can perform its function again and again). In living systems, proteins that act as catalysts are called enzymes. Enzymes allow chemical reactions to proceed at a rate sufficient to sustain life.

Enzymes are, in most cases, proteins with very specific three-dimensional shapes. Each enzyme also has a unique distribution of charge determined by the sequence and arrangement of amino acids, which ultimately are governed by the DNA sequence that encodes the protein.

Each enzyme has a three-dimensional site or pocket on its surface, called the **active site,** which interacts with the charge and shape characteristics of the specific substrate(s) for the enzyme. One or more substrate molecules bind to the active site, fitting into the pocket. The binding of the substrate(s) to the enzyme forms a "strained" complex that lowers the energy of activation for the reaction, and speeds the formation of the product. **(see pp. 150-157 in your textbook for a more detailed explanation)**. At the completion of this process, the enzyme and product dissociate, freeing the enzyme to bind a new molecule(s) of substrate and repeat the catalysis.

enzyme + substrate <==> enzyme-substrate <==> enzyme-product <==> enzyme + product
complex complex

Many factors affect the activity of enzymes, including temperature, pH of the surrounding aqueous environment, and presence of other ions and molecules like cofactors, coenzymes, and inhibitors, to name a few. Because the active site is so critical to the function of the enzyme, it is logical to assume that any factor that modifies the shape or charged character of the enzyme and/or the active site could change the rate of the reaction. While an increase in temperature might be expected to speed up a chemical reaction, excessive temperatures can inactivate (denature) an enzyme by distorting the protein and changing its shape. For some enzymes, a cofactor (usually a metal ion) or coenzyme (often a vitamin) is required for the reaction to proceed. If the metal ion readily gains or loses electrons, it helps catalyze reduction-oxidation reactions (reactions in which electrons are transferred). In some cases, molecules other than the substrate may bind to the active site, making this site unavailable to the substrate and preventing the reaction. Such molecules are called **competitive inhibitors**.

The enzyme that is the subject of this Lab Module is **catecholase**. You have seen the effects of this protein in the discoloration of apples, peaches, or bananas when they are damaged or peeled (or left half-eaten on someone's desk). Also, the enzyme and substrate responsible for production of the dark pigment of human hair and skin are related to the enzyme and substrate you will study today. Both catecholase and its substrate, catechol, are present in undamaged fruit and potatoes, but the chemical reaction does not begin until the enzyme and its substrate are brought together by cell damage in the presence of oxygen, which promotes the exchange of electrons. In this reaction, oxygen "pulls" electrons from catechol, thus oxidizing the catechol molecules and forming a substance called benzoquinone. The benzoquinone molecules then link together to form long branched chains that serve as the backbone of a resulting reddish-brown pigment. The process continues until a complex polymer is formed, similar to the skin pigment melanin. Today you will learn to isolate catecholase and assess its enzymatic activity, and then you will design experiments in order to examine certain factors that affect catecholase activity. Next week, you will perform experiments of your own design, in a self-guided fashion with the lab instructor available to help if needed.

OBJECTIVES FOR THIS 2-WEEK LAB

1. Partially isolate the enzyme catecholase
2. Demonstrate catecholase activity (as measured by rate of product formation)
3. Investigate several factors that affect the function of this protein
4. Write a report describing the results of your investigation in the format of a scientific paper

EXERCISE 1: Isolation of Catecholase and Demonstration of its Activity.

Materials and Methods:

1. Add approximately 50 g of sliced raw potato and 200 ml of water to a blender. Blend the mixture to a fine textured homogenate, and place the mixture in a beaker on ice to cool it and help preserve enzyme activity. Strain the homogenate through two layers of cheesecloth to remove large particles. The liquid filtrate is a crude extract that contains not only the enzyme of interest, catecholase, but also many molecules and other enzymes, a significant amount of cell debris, and a small amount of the substrate catechol found naturally in potatoes. Next, centrifuge a portion of filtrate in two full microcentrifuge tubes, pipet the clear supernatant into a tube on ice, and use this supernatant in your assays for the remainder of the lab period. Also, boil a small portion of this supernatant in a separate tube, place this on ice, and label it as "boiled" for use below (what do you think will be the effect of this treatment on catecholase activity?).

2. Label six test tubes as indicated below and use Table 1 (below) to keep track of the

different experimental conditions. First, add 3 ml of pH 7.0 phosphate buffer to each tube.

3. Add 0.5 ml of catechol to tubes #2, 3, 4, 5, and 6.

4. Add 0.1 ml of cofactor solution (record its identity and concentration) to tube #4.

5. Add 0.1 ml of solution containing the compound phenylthiourea (PTC) to tube #5.

6. Add 0.1 ml of 1% ascorbic acid (Vitamin C) to tube #6. Ascorbic acid binds metal ions, and can also contribute electrons to reverse an oxidation reaction (i.e. it is a **reducing agent**).

7. Add an appropriate volume of ddH_20 to each tube to normalize the volumes to 4.0 mL.

8. To start the reactions, quickly add 0.1 ml of potato extract to tubes #1, 2, 4, 5, and 6. Cap the tubes with Parafilm and gently invert them to mix. Note the precise time at which the extract was added to each tube.

9. Add 0.1 ml of the <u>boiled</u> extract to tube #3 and mix. Note the precise time.

10. At several time intervals after adding enzyme (e.g. 0, 5, 10, and 15 min), measure the Absorbance at 520 nm wavelength for each tube, using the spectrophotometer. Be sure to first **zero** the machine using an appropriate blank (which of your tubes is the best "blank"?). The Absorbance should be a number proportional to the amount of pigment or color in the solution, and it allows a scientist to follow quantitatively the rate of the reaction. Product formation must be expressed as a rate (change in Absorbance occurring during a defined time period, which can be expressed as **Δ**Absorbance/minute. You should look for a window of time that shows a reasonably constant rate of product formation in which the reaction rate is linear over the experimental period (example: if the reaction remains linear for 15 minutes, the 10- min reading should be twice the 5-min reading, and the 15-min reading should be three times the 5-min reading).

In the experiment above, you manipulated several factors to determine their effect on the rate of reaction. To determine the effect of each factor, a control tube is needed that is identical (in every way) to the experimental tube *except* for the sole factor being tested. In the table on the next page, indicate the tube that serves as an appropriate control for each experimental tube. Finally, list the sole factor that was tested by the paired tubes (experimental + control) in each case.

Table 1 Experimental versus control tubes and variables tested.

Experimental tube	**Control tube**	**Tested for effects of:**
Tube # 2	Tube # ____	_____
Tube # 3	Tube # ____	_____
Tube # 4	Tube # ____	_____
Tube # 5	Tube # ____	_____
Tube # 6	Tube # ____	_____

EXERCISE 2: Design of your own experiments for next week.

Now that you have experience in this enzyme activity assay, consult with your lab partner(s) and design three separate experiments to determine the effects of various experimental conditions on enzyme activity. **Next week you will perform these three experiments.** On the surface, your goal is very simple. Design each experiment so that you will vary *only one factor* in that experiment. That can be more difficult than it sounds, and care must be taken to establish suitable controls. Remember that for each "experimental" tube, there must also be an appropriate "control" tube. Show your instructor the outline of the experiments you are planning. Lists of acids, bases, cofactors, buffers, inhibitors and other materials you might find useful for your experiments will be on the board. If you cannot find something you need for an experiment you wish to perform, your instructor may be able to help you. Below is a list of some factors you might manipulate, along with some suggestions you might use to examine factors affecting catecholase enzyme activity. You are free to design your own experiments, but have them approved by your instructor before you leave today. The results of next week's experiments will be included in your formal report, *along with* data from the first set of experiments, so make detailed notes on experimental conditions, methods, and results today.

Some suggested variables:

1. Temperature (does catecholase have a temperature optimum for its activity?)
2. pH (does catecholase have a pH optimum for its activity?)

3. Enzyme source (does catecholase from different organisms have different activities?)
4. Substrate concentration (does the enzyme exhibit saturation kinetics?)
5. Different metal ions or cofactors
6. Enzyme concentration (how does enzyme concentration affect activity?)
7. Effect of different inhibitors (is one inhibitor more potent than another?)

Some suggestions and hints:

Effect of temperature

Chemical reaction rates typically increase with increasing temperatures, and enzyme-catalyzed reactions are no different. However, when temperature increases beyond a certain point (the range varies widely- remember an arctic cod functions in temperatures far lower than those for bacteria in hot springs!), the reaction slows, due to partial or complete denaturation of the protein and the loss of its functional three-dimensional shape. In the laboratory, water baths can be set at various temperatures (from 0°C to boiling). Be sure your reaction mixtures come to the desired temperature before adding enzyme, and that the temperature remains fairly constant.

Effect of pH

The pH can vary the shape of a protein, and thus the rate of an enzyme-catalyzed reaction. Different enzymes have different **optimal pH** values at which the rate of reaction is maximal. Buffers of several pH values will be available. Be sure to set up the proper control for each pH value.

Effect of substrate concentration

Chemical reactions typically increase at a rate proportional to the concentrations of the reactants. Enzyme catalyzed reactions generally follow the same rules, but with some important characteristics and variations. As the substrate concentration increases in an enzyme catalyzed reaction, the rate of reaction increases, but at some point, further increases in substrate result in lesser and then no further increases in reaction rate. The enzyme is said to be **saturated** (thus causing the reaction rate to exhibit what are termed "saturation kinetics"). You can conduct this experiment by modifying substrate concentration as the only variable. You must plan this experiment carefully to keep all other variables like volume, enzyme concentration, etc. unaltered to provide appropriate controls.

Enzyme concentration

In another experiment, you might monitor the effects of varying enzyme concentration on reaction rate.

Inhibitors

Enzyme-catalyzed reactions may be inhibited by several different chemical factors. Some compounds are structurally similar to the substrate, and may bind temporarily with the active site but not form product, thereby slowing the rate of the reaction. Two different inhibitors could be compared in terms of their potency (in other words, how small a concentration of inhibitor can be rendered and still be effective). A much more sophisticated analysis would be to determine whether a particular inhibitor is *competitive* or *non-competitive*. When an inhibitor competes with the normal substrate molecule for the enzyme active site (and therefore the reaction rate depends on the concentration of both the substrate and inhibitor), this is called **competitive inhibition** and exhibits a characteristic saturation kinetics curve. Some compounds will bind at a site on the enzyme separate from the active site, and inhibit enzyme activity without changing the initial affinity of the substrate and enzyme for each other. Since such inhibitors do not compete with the substrate molecule for the active site, they should exhibit **noncompetitive inhibition** (or other) kinetics. An analysis of the shape of the curve (after plotting substrate concentration on the X-axis vs. reaction rate on the Y-axis) can yield important information of the nature of the interaction of the enzyme, substrate and inhibitor.