ENZYME ACTIVITY

Readings: Review pp. 51-58, and 128-139 in your text (POHS, 5th ed.).

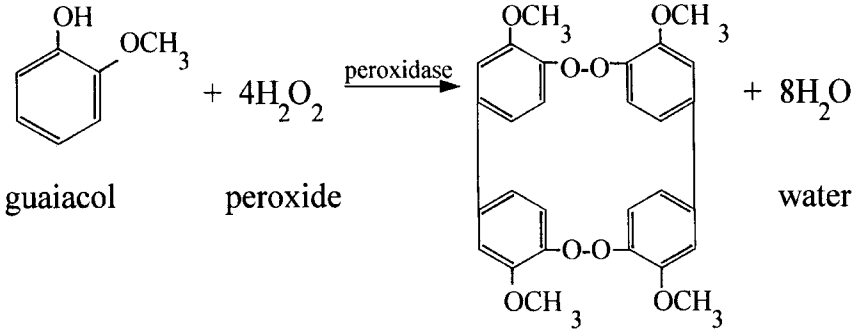
Introduction

Enzymes are biological catalysts; that is, enzymes are able to mediate the conversion of substrate into product(s) without being changed or destroyed in the conversion process. Almost all enzymes are proteins. The only known exceptions are ribozymes, which are composed of RNA. Individual enzymes have unique primary structures – the linear sequence of amino acids of which they are made. As a consequence of hydrogen bonding and R group interactions, the primary structure of the protein will fold and twist upon itself to form characteristic secondary and tertiary structures. The tertiary structure of an enzyme is crucial to its role as a catalyst because the maintenance of a three-dimensional "active site" in which the enzyme contacts the substrate is required for an enzyme to remain biologically active. In some instances, quaternary structure or the interaction of two polypeptides (either identical or different) is involved in the formation of an enzyme's active site.

Several factors can influence enzyme activity. Some of these factors are hydrogen ion concentration (pH), the concentration of various metal or other cations, and temperature. These factors influence enzyme activity by altering the three-dimensional shape of the enzyme thereby changing the three-dimensional shape of the active site, which is frequently a pocket, cleft, or groove. Inhibitors (specific or nonspecific) are able to interfere with an enzyme's activity by binding directly to the active site or to a different part of the enzyme causing indirect deformation of the active site. An enzyme's activity can also be affected if the gene that encodes the enzyme is mutated such that the enzyme's amino acid sequence is changed or truncated.

LAB GOALS

In this exercise, you will **examine** and **quantify** the activity of horseradish peroxidase. This enzyme catalyzes the conversion of guaiacol (a colorless liquid substrate) and peroxide into tetraguaiacol (a brownish product) and water. The reaction is illustrated in the following figure:



tetraguaiacol

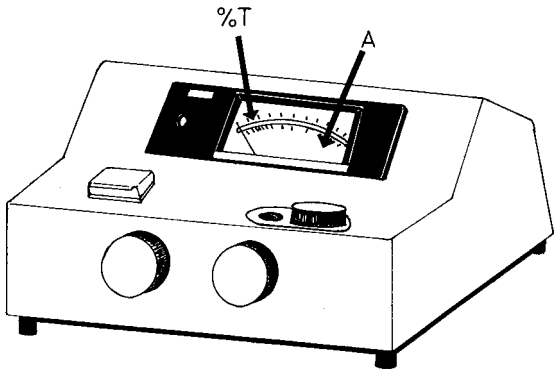
Because one of the products of the reaction is colored and absorbs visible light, you can monitor the peroxidase's activity by using a colorimeter. To use the "Spec 20" (see Appendix A for more information about the "Spec 20"):

(1) Plug in the "Spec 20" and set the wavelength to 470 nm. Let it warm up for 15-20 minutes before use.

(2) With the portal empty and the lid closed, turn the lower left knob until the needle rests on on ∞ on the absorbance scale (0% transmittance). This sets the dark current for the "Spec 20" at this wavelength.

(3) Place a clean colorimeter tube containing the blank solution in the portal and close the lid. Make sure the white line on the tube is aligned with the line on the portal, i.e., in the correct orientation. Adjust the lower right knob until the needle rests on 0 absorbance (100% transmittance). This "zero" adjustment compensates for any absorbance inherent in the blank solution.

(4) Place a clean colorimeter tube containing a well mixed reaction solution in the portal and close the lid. Make sure the outside of the tube is clean and dry, and that the tube was inserted in the correct orientation. This absorbance reading is due to the reaction between the enzyme and substrate.



"SPEC 20"

In order to **quantify** enzyme activity, you will calculate the **turnover number** which is the number of substrate molecules converted to product molecules per enzyme molecule per unit time. To calculate the turnover number, you need to know two things: How quickly the substrate is converted to product (the rate of the reaction), and how much enzyme is responsible for the rate you observe (enzyme concentration).

$$\text{Turnover number} = \frac{\text{Rate of the reaction}}{\text{Enzyme concentration}}$$

The following paragraphs explain how to use the data you will obtain with the "Spec 20" to quantify the activity of horseradish peroxidase.

Step 1. *Determining the rate of the reaction*. The reaction rate is determined by plotting Absorbance vs. Time. The slope of the line is the rate of the reaction in Absorbance units/second. Reaction rates are typically expressed in terms of concentration (moles/liter/second). To convert Absorbance units/second to moles/liter/second, the slope of the line (A/sec) is divided by the molar extinction coefficient of the product, tetraguaiacol. The extinction coefficient is a constant that allows us to convert A units into concentration units (moles/liter). The molar extinction coefficient of tetraguaiacol is 26,600 at 470 nm. You now have a value for the rate of the reaction in terms of moles/liter/second. This is the rate you will use to calculate the turnover number.

Step 2. *Determining the enzyme concentration*. The enzyme concentration in moles/liter is calculated by multiplying the concentration of the stock enzyme solution (g/L) by the inverse of the molecular weight of the enzyme. The molecular weight of horseradish peroxidase is 40,000 g/Mole.

$$\mathbf{X}\ \frac{\text{g}}{\text{L}} \times \frac{1\ \text{mole}}{40{,}000\text{g}} = \mathbf{Y}\ \frac{\text{mole}}{\text{L}}$$

This is the molarity of the stock solution of enzyme, but it is not the concentration of diluted enzyme in your tube. You will dilute the enzyme by adding 0.1 ml of enzyme to 5.0 ml substrate. To account for this dilution, multiply the molarity of the stock solution by the dilution factor (volume added/ total volume).

$$\mathbf{Y}\ \frac{\text{mole}}{\text{L}} \times \frac{0.1}{5.1} = \mathbf{Z}\ \frac{\text{mole}}{\text{L}}$$

Z is the value you will use to calculate the turnover number.

Step 3. *Determining the turnover number*. Divide the rate of the reaction (Step 1) by the concentration of enzyme (Step 2) to determine the turnover number of the enzyme. Note that the "official" units will be "per second". For the purposes of this lab, however, you will need to write out the **full units** (molecules substrate converted to product per molecule enzyme per second).

EXPERIMENTAL PROTOCOLS. Work in pairs.
Your instructor will outline the specific variables your laboratory section will test, and direct your attention to **Protocol 1, 2**, or **3** below.

Protocol 1. The effects of variations in substrate and enzyme concentration.
Reagents:
(1) Stock solution of substrate: 5 mM H_2O_2 + 25 mM guaiacol in 100 mM Tris acetate at pH 6.5.This reagent is light sensitive and volatile, and is in the fume hood.
(2) Horseradish peroxidase: _____mg/ml and _____mg/ml in distilled H_2O; stored in ice.

Procedure:
Part A.
(1) Set the "Spec 20" to 470 nm and warm up 15 minutes. Zero the "Spec 20".
(2) Pipet 5.0 ml of guaiacol solution into a colorimeter tube.
(3) Zero the "Spec 20" with the substrate blank.
(4) Add 0.1 ml enzyme (use the least concentrated solution) to the substrate, cover the tube with a parafilm square and invert the tube to mix. Quickly place the tube into the "Spec 20".
(5) Read and note the change in Absorbance at 15 second intervals. You will need at least 5 readings.
(6) Graph your data. Plot Absorbance values on the Y axis vs. Time on the X axis.

Part B.
(1) Prepare 11 ml of substrate solution that has been diluted by a factor of 100 in 100 mM Tris acetate, pH 6.5. Remember to include this calculation in your write up.

(2) Pipet 5.0 ml of the diluted substrate solution into a colorimeter tube and zero the "Spec 20".
(3) Add 0.1 ml of the more concentrated enzyme prep to the substrate in the tube, cover with a parafilm square and invert to mix. Place the tube quickly into the "Spec 20".
(4) Note changes in Absorbance at 30 second intervals until there is no further change.
(5) Graph your data. Plot Absorbance values on the Y axis vs. Time on the X axis.

Part C.
(1) Dilute the more concentrated enzyme solution with distilled H_2O so that the final enzyme concentration is 0.02 mg/ml.
(2) Re-zero the "Spec 20" as in step 2 above.
(3) Add 0.1 ml of the dilute enzyme to 5.0 ml of the dilute substrate solution you prepared in Part B, Step 1. Cover the colorimeter tube, invert to mix, and quickly place the tube in the "Spec 20".
(4) Note changes in Absorbance at 30 second intervals until there is no further change.
(5) Graph your data. Plot Absorbance values on the Y axis vs. Time on the X axis.

Protocol 2. The effects of pH.
Reagents:
(1) Stock solutions of substrate: 5 mM H_2O_2 + 25 mM guaiacol in 100 mM Tris acetate at pH 4.5, 6.5, and 8.5. These reagents are light sensitive and volatile, and are in the fume hood.
(2) Horseradish peroxidase: 0.01 mg/ml in distilled H_2O, stored on ice.

Procedure:
(1) Zero the "Spec 20" with 5.0 ml of substrate at pH 6.5.
(2) Add 0.1 ml enzyme to this tube, cover the tube with a parafilm square, invert to mix, and quickly place the tube in the "Spec 20". Be sure to carefully dry your tubes before inserting them into the "Spec 20".
(3) Note the change in Absorbance at 15 second intervals (take at least 5 readings).
(4) Repeat steps 1, 2, and 3 with substrate mixtures at pH 4.5 and 8.5.
(5) Graph your data. Plot Absorbance values on the Y axis vs. Time on the X axis.

Protocol 3. The effects of temperature.

Reagents:

(1) Stock solution of substrate: 5 mM H_2O_2 + 25 mM guaiacol in 100 mM Tris acetate at pH 6.5. This reagent is light sensitive and volatile and is stored in the fume hood.

(2) Horseradish peroxidase: 0.01 mg/ml in distilled H_2O, stored on ice.

Procedure:

(1) Zero the "Spec 20" with 5.0 ml substrate solution.

(2) Add 0.1 ml enzyme to the tube, cover the tube with a parafilm square, invert to mix, and quickly place into "Spec 20". Be sure to carefully dry your tubes before inserting them into the "Spec 20".

(3) Note the change in Absorbance every 15 seconds (take at least 7 readings).

(4) Warm a tube containing 0.3 ml enzyme solution in a heat block and/or waterbath for 10 minutes. Heat blocks and/or waterbaths are set at 40°C, 60°C, & 95°C. After warming the enzyme, immediately place the tube on ice.

(5) Zero the "Spec 20" with 5.0 ml substrate solution.

(6) Add 0.1 ml of one heated enzyme solution to the tube containing 5.0 ml substrate solution at room temperature. Cover the tube with a parafilm square, invert to mix., and quickly place into "Spec 20". Note the change in Absorbance every 15 seconds (take at least 7 readings). Be sure to carefully dry your tubes before inserting them into the "Spec 20".

(7) Repeat steps 5 and 6 for the other heated enzyme solutions.

(8) Graph your data. Plot Absorbance values on the Y axis vs. Time on the X axis.

DATA ANALYSIS and WRITE UP

(1) Using the data you have accumulated and plotted, calculate the turnover number for the reactions in Protocol 1, Part A; Protocol 2 at pH 6.5; and Protocol 3 at each temperature. Using your own data and the data from other sections, define the optimum pH for the reaction. Also discuss the effects of elevated temperature on the reaction using data pooled from all sections.

(2) For Protocol 1, Parts B and C, plot your data on the same graph and compare them with these questions in mind:

(a) How does the dilution of substrate affect the rate of the reaction?

(b) How does the dilution of enzyme affect the rate of the reaction? How does dilution of enzyme affect the total amount of product produced?

(c) How would increasing the concentration of enzyme affect the rate of the reaction?