Properties of the enzyme peroxidase

Adapted from Cells and Molecules lab

OBJECTIVE:
To perform an assay for a specific enzyme and use it to determine the optimum pH for the enzyme.

BACKGROUND:

Enzymes are protein molecules that function as biological catalysts. Catalysts alter the rate of chemical reactions without undergoing any permanent change themselves. It is estimated that the human body has at least 50,000 enzymes to catalyze the enormous number of biochemical reactions that take place. Enzymes are necessary for most bodily reactions. Some reactions that would otherwise require years for completion can be made to occur almost instantly with the aid of enzymes. For example, the enzyme peroxidase can effect the decomposition of hydrogen peroxide, H_2O_2, so that more than 40,000 molecules are consumed every second. Hydrogen peroxide is formed as a result of several different reactions that routinely take place in all living cells. But it is toxic to cells, so there must be some way of destroying it as soon as it is formed, before any damage is done. That is the job of peroxidase.

$$2H_2O_2 \rightarrow 2H_2O + O_2$$

The oxygen that is released is used just as respiratory oxygen is used. In fact, the peroxidase reaction is an important source of oxygen for routine use in some metabolic pathways. Like the oxygen-carrying protein hemoglobin, peroxidase has a heme group at its active site; an iron atom in the heme is essential for the enzyme to function.

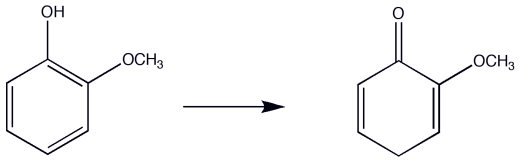
The reaction rate for an enzyme reaction is the speed at which an enzyme converts substrate (reactant) into products. Measuring reaction rate is not hard to do. There are two ways we could measure the rate at which peroxide is broken down into water and oxygen:

- Measure the rate of disappearance of hydrogen peroxide.
- Measure the rate of appearance of oxygen.

The first method turns out to be rather cumbersome; it will be easier to measure the rate of appearance of product than the disappearance of substrate. There are two ways in which oxygen generation could be measured:

- Measure the rate of free oxygen formation, by counting bubbles, or, better still, by measuring the volume of oxygen produced using manometry (similar to what we did last semester in the Gas Laws lab).
- Take advantage of the fact that the oxygen produced can be used to carry out some other chemical reaction. This "secondary" reaction will generate a color that can be monitored using spectroscopy. The color will develop as oxygen becomes available.

The second method is more practical. The reaction that we will be monitoring is the oxidization of the dye guaiacol (this is a common ingredient in cough syrups whose odor you may recognize). The reaction is shown below. When guaiacol is oxidized, it changes color from colorless to brown. The deeper the color, the more molecules of guaiacol that have been oxidized, i.e. the more molecules of peroxide that have been broken down by the enzyme. By taking absorbance readings at frequent intervals we can learn how fast the color is developing.



It is then possible to vary some of the conditions that might affect the enzyme's reaction rate (temperature, pH, presence of inhibitor, etc) and determine their impact.

PROCEDURE:

Extraction of peroxidase:

Some tissues have a higher concentration of peroxidase than others. The best possible sources are horseradish roots and the sap of fig trees. Many other tissues also have high concentrations, turnip roots and potato tubers, for example. We will use both fresh horseradish and turnip roots.

1. Peel the root vegetable and weigh out 1.50 grams. In order to make class data analysis as straightforward as possible, it would be helpful to be fairly precise in this mass.
2. Put the tissue into a clean Waring blender with 75 mL of cold pH 7 buffer. Blend 45 seconds.
3. Pour the homogenate into a clean 100-mL graduated cylinder (WARNING: at all steps in working with enzymes it is essential to have chemically clean glassware, to avoid enzyme inactivation by metal ions. It is also very important not to get traces of the enzyme extract into any stock solution that might contain substrate – or there will be no substrate).
4. Bring the volume up to 100 mL with the pH 7 buffer and mix.
5. Filter through two layers of cheesecloth
6. Store the extract on ice in a clean 125-mL Erlenmeyer flask. This is your turnip extract.

The extract actually contains hundreds of different proteins; one of them is the peroxidase enzyme. The different proteins could be separated from one another but for our purposes it is not necessary. In this crude protein extract, it is not possible to determine the precise concentration of peroxidase present. Instead, we will use relative concentrations based upon the volume of homogenate used.

Since we have no idea what the activity of any of the enzymes in the extract is, we have to standardize the extract. What that means is that, before we can do controlled experiments, we have to determine how much peroxidase activity the extract has. In other words, we must find a volume of extract that has the right amount of enzyme to produce a reaction rate with a convenient steady slope over a two-minute period.

Impact of enzyme concentration on reaction rate:

1. Take about 20 mL of the pH 5 buffer solution to your workbench in a small beaker or tube.

2. Set up 4 clean cuvets. Fill the tubes as indicated in Table 1, except for the turnip extract, which must be added LAST, as you start each reaction at time "zero".

Tube 1 is a control; it contains everything but the extract and can be used to "blank" the spectrophotometer.
Tube 2 will test the enzyme extract's activity at a dilution of 1/25.
Tube 3 will test at a dilution of 1/10.
Tube 4 will test at a dilution of 1/5.

Tube	pH 5 buffer	Peroxide solution	Guaiacol solution	Turnip extract	Total volume
1	2.1 mL	0.7 mL	0.7 mL	0	3.5 mL
2	1.9 mL	0.7 mL	0.7 mL	0.2 mL	3.5 mL
3	1.6 mL	0.7 mL	0.7 mL	0.5 mL	3.5 mL
4	1.1 mL	0.7 mL	0.7 mL	1.0 mL	3.5 mL

How to measure activity:

1. To zero the spectrophotometer, open the LoggerPro software. From the 'Experiment' menu, select 'Calibrate – Spectrophotometer'. Wipe the outside of the cuvette filled with the blank solution to remove fingerprints. Place the cuvette into the spectrophotometer with the clear sides oriented in the direction of the light beam. Follow the on-screen instructions to finish the calibration and then click 'OK'.

2. For the samples, absorbance values need to be collected over the course of several minutes. Click on the 'Configure Spectrometer' button (curve with rainbow underneath in the icon bar) and select 'Absorbance vs Time'. Choose the wavelength closest to 500 nm and hit 'OK'. Click on the 'Data Collection' icon (timepiece icon) and set the duration to 120 seconds with a sampling rate of 5 seconds/sample and then click 'Done'.

3. Place the sample in the cuvette, remembering to add enzyme to the sample last. Once the enzyme has been added, the reaction has begun so it is important to work quickly to quickly mix the sample by inverting the sample several times and then place in the spectrophotometer. To begin recording data, click the 'Collect' icon. Be sure you know what you are doing before you start; otherwise you will have to start all over again. Precise timing is essential.

4. Export the data to Excel by saving as a CSV file. Plot the data from each of the three enzyme concentrations on a single graph. You should be able to observe or calculate three slopes: one for your reaction mixture at each concentration. Determine which enzyme concentration gave a steady (constant) slope for the entire two-minute reaction time. This is the "standardized" enzyme concentration that you will use for all further measurements.

4. Calculate the slope for each curve, which corresponds to the rate of the reaction. Input this data into the computer.

The effect of pH on peroxidase activity:

1. Label 4 test tubes: pH 3, pH 5, pH 7, pH 9, and use them to gather stock buffer solution, taking only the quantity you will need (no more than 10 mL of each).

2. Prepare 4 cuvets as in Table 2. Where the table shows a * for the turnip extract, use the "standard" volume of enzyme extract that you determined in the previous section. Where the table shows a * for the buffer volume, add a volume of buffer that will produce a total volume of 5 mL. Time each reaction as before.

Tube	pH 5 Buffer	Peroxide	Guaiacol solution	Turnip extract	Total volume
1	*	0.7 mL	0.7 mL	*	3.5 mL
2	*	0.7 mL	0.7 mL	*	3.5 mL
3	*	0.7 mL	0.7 mL	*	3.5 mL
4	*	0.7 mL	0.7 mL	*	3.5 mL

3. Determine the reaction rate at each pH in the same way you did before. In order to compare class data, the rates obtained should be divided by the amount of tissue present in the mixture. Input this data into the computer.

5. Now plot a graph of reaction rate vs. pH. You should be able to draw a smooth curve through the points that has a maximum, which is at the pH optimum for your preparation of peroxidase.