Laboratory 5: Properties of Enzymes

Technical Objectives

1. Accurately measure and transfer solutions with pipettes
2. Use a Spectrophotometer to study enzyme action.
3. Properly graph a set of data.

Knowledge Objectives

1. Know what an enzyme is and how it functions
2. Know why peroxidase is an important enzyme for all oxygen-using cells.
3. Know how various environmental factors and enzyme inhibitors can affect an enzyme's activity.
4. Be able to interpret data to determine the effect of different conditions on enzyme activity.
5. Understand what a reaction rate is, be able to determine or calculate a reaction rate, and understand what a reaction rate means in this experiment.
6. Key terms: catalyst, enzyme, product, activation energy, active site, enzyme-substrate complex, denaturation.

Overview

In this exercise you will observe the action of peroxidase, an enzyme commonly found in turnips, potatoes, and horseradish. You will use pipettes to set up the enzyme reaction and use the Spectronic 20 spectrophotometer to measure the enzyme’s activity.

Introduction

All chemical reactions require an input of energy in order to "get started". This is known as activation energy. A catalyst is a molecule that speeds up a chemical reaction by lowering the activation energy required to start the reaction. Figure 1 shows the energy required for a biochemical reaction to proceed. Notice that the energy required to start this reaction is much lower in the presence of an enzyme than without an enzyme. Enzymes are biological catalysts that carry out thousands of chemical reactions, which occur in living cells. Enzymes are large protein molecules with specific three-dimensional structures. Each enzyme catalyzes a specific reaction, or specific type of reaction, that involves specific substrates.
In an enzyme-catalyzed reaction, the **substrate** (the substance to be acted upon) binds to the **active site** of the enzyme. The active site has a specific three-dimensional shape that corresponds to the appropriate substrate. This assures that only the substrate can bind to the enzyme and prevents the thousands of other compounds present in the cell from binding to the enzyme and interfering with the reaction. Once the substrate binds to the enzyme it is called an **enzyme-substrate complex**. This complex goes through the biochemical reactions and the **product(s)** of the reaction is released into solution. The enzyme molecule, like all catalysts, is not used up in the reaction. It is released to bind to another substrate molecule and is recycled over and over in the cell. Figure 2 shows how enzymes and substrates bind before the reaction occurs and how the enzyme is released unchanged after the reaction.
Any substance that blocks or changes the shape of the active site will interfere with the activity and efficiency of the enzyme. **Inhibitors** are molecules that are similar in shape to the substrate. These molecules bind to and block the active site. **Denaturation** is the irreversible change in an enzyme's, or any protein's, three-dimensional structure, which leads to a loss of its catalytic abilities. Because an enzyme's shape is critical to its proper functioning, the cell must maintain optimal or near-optimal conditions to preserve the enzyme's shape. If an enzyme's three-dimensional structure changes, the enzyme is unable to act as a catalyst. Enzymes function in different cells and in different organelles within cells. Consequently, different enzymes are most efficient under different conditions. Salt concentrations, pH, and temperature are several factors that affect an enzyme's shape and are important in determining the most efficient enzyme activity.

In this experiment we will study the enzyme **peroxidase** from turnips. Peroxidases are found in plant and animal cells and function to convert toxic hydrogen peroxide ($H_2O_2$) into water and oxygen. The basic equation for a peroxidase reaction is:

$$2 \; H_2O_2 \xrightarrow{\text{peroxidase}} 2 \; H_2O + O_2$$

All cells that use molecular oxygen in metabolism (respiration) will produce small amounts of hydrogen peroxide. Hydrogen peroxide is a free radical, which is a molecule that is extremely reactive and can cause cellular damage. It is critical that any hydrogen peroxide molecules be quickly removed before they can damage the cell. (Remember, hydrogen peroxide is often used to sterilize a wound - to kill bacteria that could lead to an infection).

The oxygen produced from the breakdown of hydrogen peroxide then reacts with organic compounds to form secondary products in the cell. The basic equation for this reaction is:

$$H-R-O-H + O_2 \xrightarrow{} R=O + H_2O$$

R is used to symbolize an organic compound.

Thus the overall equation for the process we will study in lab today is:

$$H-R-O-H + H_2O_2 \xrightarrow{\text{peroxidase}} R=O + 2 \; H_2O$$

In this experiment we will use the dye guaiacol (i.e. The organic compound “R” in the above reaction) to determine the rate of the reaction. When guaiacol is in its normal state it is colorless; when it is oxidized, after having gone through the above reaction, it is brown. The dye does not interact with or affect the enzyme, but is oxidized by the oxygen. The color change, which indicates that the reaction is occurring, allows us to monitor the rate of the reaction under different conditions. We will use a spectrophotometer to measure the color change.
**Procedure**

**A. Spectronic 20 Spectrophotometer**

A colored solution appears colored because some of the light that enters the solution is absorbed by the colored substance (pigments) while a clear solution allows almost all the light to pass through. A **spectrophotometer** is an instrument that allows us to measure the amount of light that is transmitted through a specimen and the amount of light that is absorbed by a specimen. The darker the solution, the greater the absorbance.

The spectrophotometer has a light source that shines light through a diffraction grating. The diffraction grating separates light into its component colors and the spectrophotometer can be adjusted so that only light of the desired wavelength (color) enters the sample. The spectrophotometer contains an absorbance meter that measures the fraction of light that has been blocked, or absorbed by, the sample. The scale on the spectrophotometer is calibrated in **absorbance**, which runs from 0 to 2; and **percent transmittance**, which runs from 0 to 100.

Follow the steps below whenever you want to read a sample in a Spec 20.

1. Turn on the Spec 20 with the power switch knob, and allow it to warm up for 15 minutes.
2. Adjust to the desired wavelength using the wavelength control knob.
3. Use the power switch knob to set the meter needle to read **infinity absorbance** (on the left side of the scale). When setting the Spec 20 to infinity, the chamber should be empty and the cover should be closed.
4. Special tubes, called **cuvettes**, are used with the Spec 20. You should always select two of the same type/brand cuvettes. Make sure to hold the cuvettes near the top of the tube to avoid smudging on the sides; fingerprints will interfere with absorbance readings.
5. Fill a cuvette halfway with distilled water (or other solution, when water is not the solvent in your experiment). This will be your blank or zero tube and it serves as the reference tube. Wipe the outside of the cuvette with a Kimwipe to remove any fingerprints or moisture and insert the cuvette into the sample chamber.
6. Adjust the Spec 20 to **zero absorbance** using the right-hand (light control) knob. This removes the absorbance due to the solution in the tube. Read the blank tube and place it in a cuvette rack.
7. Fill the sample cuvette with your sample solution, wipe the outside of the cuvette with Kimwipe, and insert the cuvette into the sample chamber. Read the absorbance directly from the meter.

Some additional guidelines for using the Spec 20:

- Check both the infinity absorbance and zero absorbance occasionally during an experiment.
- Whenever you change the wavelength you need to reset the infinity and zero absorbance.
- Keep the sample chamber cover closed when you are not using the Spec 20, this prevents dirt and dust from getting into the sample chamber and affecting your readings.

**B. Preparation of the turnip extract**

The turnip extract will be prepared before lab using the following procedure:

1. Weigh out 2 g of peeled turnip, from the inner portion of the vegetable.
2. Blend it thoroughly in a blender with 200 ml distilled water for 1 min.

3. Filter the homogenate into a beaker through 1 layer of cheesecloth. Place the filtered homogenate in a tube on ice. This suspension is the turnip extract and contains the enzyme peroxidase. The activity of the turnip extract will vary from experiment to experiment (from lab section to lab section) depending on the size and age of the turnip.

C. Kinetics of the peroxidase reaction

Baseline Experiment - Make sure you understand the procedure before you begin.

1. Label the pipettes with tape so that each one can be reused with the proper solution. This will also reduce the chance of contaminating the different solutions.

2. Obtain two cuvettes and label them B (blank) and R (reaction). In this experiment the control tube will be used as the blank tube described earlier. Make sure to label the cuvettes above the symbol so as not to interfere with the absorbance readings.

3. Obtain three large test tubes and label them 1, 2, 3. Number 1 will contain a control reaction without \( \text{H}_2\text{O}_2 \). The contents of 2 and 3 will be mixed to start the reaction. Set-up the three tubes as described below: notice that tube 1 contains 10 ml and tubes 2 and 3 contain 5 ml each. Tubes 2 and 3 will be combined later and the total volume of ‘reaction’ solution will also be 10 ml.

4. Tube 1 (blank tube; to be used for blanking the spectrophotometer- cuvette B): 0.1 ml
   a. guaiacol, 1.0 ml turnip extract, 8.9 ml \( \text{dH}_2\text{O} \) - mix well.
   Tube 2 (substrate): 0.1 ml guaiacol, 0.2 ml 1.0% \( \text{H}_2\text{O}_2 \), 4.7 ml \( \text{dH}_2\text{O} \) - mix well.
   Tube 3 (enzyme): 1.0 ml turnip extract, 4.0 ml \( \text{dH}_2\text{O} \) - mix well.

5. Set the wavelength of the Spec 20 to **460 nm** and adjust the light control to infinity. Adjust the Spec 20 to zero absorbance using cuvette B, filled with the solution from tube 1.

6. Have cuvette R, Kimwipes, and tubes 2 & 3 ready. When you are completely ready, mix tubes 2 and 3, pour contents back and forth two times, and then pour some of the mixture into cuvette R. Start timing when you first mix the tubes, this is time = 0. You have 20 sec to mix the contents of tubes 2 and 3, pour the contents into the clean cuvette, wipe the cuvette, and take your first reading.

7. Put cuvette R into the Spec 20 and read the absorbance at 20 sec, or as soon after as possible. Read the absorbance every 20 sec for 1 minute and then every 30 sec for 5 -10 minutes. Record the reading in Table 1. If you do not get your first reading at 20 sec make sure to note the actual time that you took the first reading.

Repeat this procedure again 4 times to get an average of the reaction rate.
<table>
<thead>
<tr>
<th>Time (sec)</th>
<th>Run 1</th>
<th>Run 2</th>
<th>Run 3</th>
<th>Run 4</th>
<th>Run 5</th>
<th>Run 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>40</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>60</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>90</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>120</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>150</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>180</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>210</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>240</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>270</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>300</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>330</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>360</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>390</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>420</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>450</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>480</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>510</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>540</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>570</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>600</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
D. Calculating the reaction rate

Graph absorbance versus time. This curve represents the baseline for this reaction under the specific temperature, pH and conditions we have chosen.

To calculate the initial reaction rate (absorbance change/min) for each run use the following procedure:

1. Extrapolate a straight line through the linear portion of the reaction curve. The slope of the linear portion of the curve is a measure of enzymatic activity.

2. Read off the absorbance value where the linear portion of the line ends and note the time in minutes.

3. Divide the absorbance value by the time in minutes to obtain the absorbance change/min; This is the reaction rate.

It is also possible to calculate the reaction rate by mathematically determining the slope of the linear portion of your graph. (Which is basically what was described above).

Determine the reaction rate for each reaction. Label each line on your graph with its calculated reaction rate. Now calculate the mean reaction rate and record this on your graph.