

Purpose: This multi-week lab will demonstrate how to extract and assay the enzyme peroxidase from plants. You will use this knowledge to study the effect of enzyme and substrate concentration, pH, inhibitors and temperature on the rate of a peroxidase catalyzed reaction.

Key Concepts and Terms:

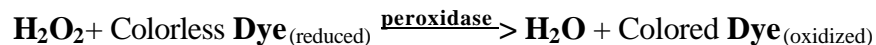
[Enzyme]	inhibitors
[Substrate]	Lysosome
Active site	Peroxisome
Catalyst	V_{\max}
E.C. 1.11.1.7	V_o
Inhibition – reversible/non-reversible	

Background:

Enzymes are usually proteins that act as catalysts in biochemical reactions. Catalysts cannot initiate reactions that would not happen in their absence, but can, and do, radically affect reaction rates with the result that the cell can carry out rapid and complex chemical activities at relatively low temperatures. Most enzymes are highly specific. They tend to accelerate only one or a group of related reactions. The result is that many different enzymes may be present in a cell and may act simultaneously without mutual interferences. Here we demonstrate the characteristics of enzyme catalyzed reactions by examining peroxidase (E.C. 1.11.1.7) from plants. What does the first “1” in EC 1.11.1.7 mean?

Hydrogen peroxide (H_2O_2) is a common end product of oxidative metabolism and, being a strong oxidizing agent, would be toxic if allowed to accumulate. To prevent this, eukaryotic cells have enclosed the enzymes producing peroxides within a membrane-bound organelle, the peroxisome, which is similar in size and appearance to a lysosome. Peroxisomes also contain high concentrations of peroxidase – the enzyme that functions to reduce the peroxide to water, rendering it harmless. A variety of electron donors can be used, including aromatic amines, phenols, and enediols like ascorbic acid.

A dye like o-dianisidine can be used as the electron donor (colorless) to easily detect peroxidase *in vitro* because its oxidized product is highly colored (Extinction coefficient is $11.3 \text{ mM}^{-1}\text{cm}^{-1}$). The rate of appearance of this colored pigment can be measured colorimetrically and is equivalent to the rate of reaction.



When first measuring enzyme activity from a tissue (in this case turnip or horseradish root) one must first “range find” to determine the amount (extent) of enzyme activity in the tissue. So, right after grinding up the root to make a crude enzyme extract, we will dilute the extract (enzyme) and measure the rate of reaction. This will tell us what amount of enzyme to use.

In these assays, the tubes will be made up with buffer and substrates (completely mixed). This tube is used to blank the spectrophotometer BEFORE adding enzyme dilution. Then the tube is removed, the enzyme dilution is added and immediately returned to the cuvette chamber of the spectrophotometer. OD readings are made immediately and then at 30 second intervals to get a rate

of reaction from a plot of the OD (ordinate or y-axis) against time (abscissa or x-axis). At low dilutions (highest amount of enzyme, the rate may go too fast to accurately measure, at high dilutions (lowest amount of enzyme) the rate may be too slow. Thus, the first range finding will allow each group to find a reasonable rate for future experiments: the amount of enzyme that will produce a change in OD of 0.4 to 0.7 in 5 minutes. Then using this amount each time, we can examine the effect of substrate concentration on this rate; that is do the classical Michaelis-Menten experiment.

Materials and Reagents:

Equipment Needed:

- Spectronic 20 spectrophotometer set at 460 nm.
- One box of spec 20 tubes per group.
- P200 and P1000 pipets and tips.
- Vortex mixers – one per group.
- Blender.
- Small Buchner funnel, Whatman #1 filter paper, single hole stopper.
- 1 500 ml side arm flask.
- Timers.
- 13x100 glass test tubes & racks.
- 1 500 ml Beaker for o-dianisidine waste

Reagents Needed:

- Fresh turnip or horseradish root. 40 grams needed per group.
- 1 liter 0.10M phosphate buffer, pH 7.0
- 200 ml each of 0.10M phosphate buffer, pH 2.0, 4.0, 5.0, 6.0, 8.0 and 10.0.
- 200 ml of 8.8mM H₂O₂ substrate one.
- 50 ml 0.5% w/v o-Dianisidine dye (Sigma #D-3127) in methanol, substrate two.
WARNING: O-Dianisidine is a proven carcinogen and toxic. Avoid contact with skin. Dispose of material as directed by TA. MW o-dianisidine (=3,3-dimethoxybenzidine) is 244.3
- Acid-alcohol bath.

Procedures:

I. Extraction of Horseradish Peroxidase

1. Peel, wash and cut ~40g fresh turnip or horseradish root (best to use) into ~1 inch cubes.
2. Homogenize with 100 ml water in a blender.
3. Filter with buchner funnel and Whatman filter paper by vacuum into a sidearm.
4. Keep filtrate cool, on ice during the experiment.

II. Reaction Time Course for Enzyme Dilutions

- As a result of the action of horseradish peroxidase, the dye o-dianisidine is converted from a colorless form to a colored form which can be measured at 460 nm. We will be measuring the increase in absorbance over time for different concentrations of the extracts.
- First prepare a set of dilutions of your enzyme extract as shown in the table below:

<u>Tube #</u>	<u>Dilution</u>	<u>Vol Enzyme</u>	<u>Vol Water</u>
1	None	1.0 ml extract	----
2	1:2	1.0 ml extract	1.0 ml
3	1:10	200 µl extract	1.8 ml
4	1:100	20 µl extract	1.98 ml

- These dilutions of extracts will be used in the enzyme reaction. Using clean Spec 20 tubes, prepare a set of reaction mixtures as shown below. The enzyme reaction will be run one tube at a time by adding enzyme extract after blanking the Spec 20, vortexing and then placing the tube back into the Spec 20. Therefore do **not** add the extract to your reaction mixtures until it is time to run the reaction. Note that the “Blank reaction” tube contains water rather than hydrogen peroxide. Why do we start with the highest dilution (lowest amount of enzyme)?.

Reaction Mixtures

<u>Rxn #</u>	<u>Vol pH 7 buffer</u>	<u>Vol Dye</u> o-dianisidine	<u>Vol H₂O₂</u>	<u>When ready to Run</u> Reaction, add Vol Extract
1	2.4 ml	50 µl	0.5 ml	50 µl 1:00 dilution (#4)
2	2.4 ml	50 µl	0.5 ml	50 µl 1:10 dilution (#3)
3	2.4 ml	50 µl	0.5 ml	50 µl 1:2 dilution (#2)
4	2.4 ml	50 µl	0.5 ml	50 µl Undiluted (#1)
Blank rxn	2.4 ml	50 µl	0.5 ml water	50 µl 1:10 dilution (#3)

- The enzyme reaction is run in the Spec 20's at 460 nm. Blank using the “Blank rxn” before adding enzyme, then add 50 µl of 1:10 dilution of enzyme into the “Blank rxn” tube, vortex, then place tube in Spec 20. Using a watch or timer measure the OD at 30 second intervals. This reaction should not change much at all.
- Starting with reaction mixture #1, re-blank the Spec 20, then add 50 µl of the extract dilution indicated, vortex and quickly place the tube in the Spec 20. Read the absorbance every 30 seconds until it reaches 1.0 *OR* 8 minutes have passed. Remove the tube from the Spec 20 and set aside in a rack for disposal. **Warning: These tubes contain o-dianisidine.**
- When you have tested all the dilutions of extracts, plot the data in a graph with time on the x axis and absorbance at 460 nm on the y axis. Draw a separate curve for each dilution. What shapes do the curves have and how do they change with dilution?
- Subsequent experiments will be done with one dilution of extract. Examine your dilution curves. The best dilution produces a reaction rate that results in about 0.4-0.7 absorbance units after 5 minutes. This reading should be near the center of the linear portion of the dilution curve. If all went too fast, then further dilutions are needed, your group may need to run additional dilutions. It may also be that you will have to use a volume different from 50 µl, if one dilution went too fast, the next lower one went too slow (for example suppose the 1:10 dilution produced an absorbance change of 0.2 in 5 minutes, and the 1:2 produced an absorbance change of 1.0 in 5 minutes...then you know the best amount of enzyme is between

these two dilutions. You could either do an intermediate dilution and use 50 µl of enzyme in the assay *or* use the 1:2 dilution at something around 20 µl.

Enzyme Kinetics with Substrate Concentration

In this experiment, the enzyme reaction is run with different levels of substrate (H₂O₂) to examine the effect of substrate concentration on enzyme kinetics.

- Prepare 10 ml of the appropriate dilution of your enzyme extract.
- In a clean set of Spec 20 tubes, prepare the following reaction mixtures. **Remember:** Do not add the enzyme extract until you are ready to do the reaction.

Tube	Vol pH 7 buffer	Vol Dye o-dianisidine	Vol Water	Vol H ₂ O ₂	When ready to Run Reaction, add Extract
1	2.4 ml	50 µl	0.48 ml	20 µl	50 µl or lower
2	2.4 ml	50 µl	0.45 ml	50 µl	50 µl or lower
3	2.4 ml	50 µl	0.40 ml	100 µl	50 µl or lower
4	2.4 ml	50 µl	0.30 ml	200 µl	50 µl or lower
5	2.4 ml	50 µl	0.20 ml	300 µl	50 µl or lower
6	2.4 ml	50 µl	0.10 ml	400 µl	50 µl or lower
7	2.4 ml	50 µl	-----	500 µl	50 µl or lower

- Blank the Spec 20 460 nm for each tube as before.
- Set up a watch or timer with 30 second intervals.
- Starting with tube #1, add 50 µl of the extract dilution indicated, vortex and quickly place the tube in the Spec 20. Read the absorbance every 30 seconds for 5 minutes. Remove the tube from the Spec 20 and set aside in a rack for disposal.
- Repeat with each of the tubes at a different substrate concentration.
Warning: These tubes contain o-dianisidine.
- With the data collected, you will determine the relationship between the velocity (rate) of the reaction (v) and the substrate concentration (s) at the different substrate concentrations. To do this you have to convert your ΔOD/min to concentration/minute. For this, the extinction coefficient of the diansidine oxidized product is 11.3 mM⁻¹cm⁻¹. Remember the Beer-Lambert law: OD = E · c · l where E is the extinction coefficient, c is the concentration and “l” is the light path (here 1 cm). So this can be set up neatly to be: c = (ΔOD)/E. Plot the data as both Michaelis-Menten and Lineweaver-Burke graphs. Calculate the Km and Vmax. Refer to Chapter 8 in Lehninger if you have questions about how to do this, then see the instructor.