**Becoming Peroxymorons** – CHEM 275 spectroscopy lab

Peroxidases are a class of enzyme that can be found in a wide-variety of organisms. Most organisms contain some form of peroxidase. Humans have several types of peroxidase including the enzyme catalase. Another organism that contains a high concentration of peroxidase is horseradish. Horseradish peroxidase (HRP) is an enzyme commonly used in biological assays such as enzyme-linked immunosorbent assays (ELISA). The utility of HRP derives from its ability to use 2,2,4,4-tetramethylbenzidine (TMB) as a substrate. The reaction of HRP with TMB in the presence of H2O2 produces a molecule with an intense visible absorbance around 650 nm. The intensity of this absorbance can be use to determine the concentration of HRP in a sample.

Peroxidases act as anti-oxidants by catalyzing the decomposition of peroxides. For example, one major role of peroxidases is in the conversion of hydrogen peroxide to water:

peroxidase

H2O2 + ZH2  2 H2O + Z

ZH2 is a generic formula for any molecule that can act as a hydrogen donor. Therefore this redox reaction involves the reduction of hydrogen peroxide and the oxidation of ZH2. TMB is a useful indicator of HRP activity because in its reduced form, the ZH2 form, TMB is colorless. Upon oxidization (ZH2 🡪 Z) TMB turns blue. The reaction progress can be monitored semi-quantitatively by observing the evolution of blue color. In this lab you will quantitatively measure the reaction using a spectrophotometer to measure the absorbance of red light (655 nm). The more the reaction proceeds, the more product Z, is produced. In this reaction, Z is oxidized TMB. The more oxidized TMB is present, the more blue the solution in the tube becomes, and the more it absorbs at 655 nm. In other words, the rate of blue color development and the rate of red color absorbance are both direct measures of the rate of the peroxidase reaction.

In this lab you will use TMB to measure the concentration of peroxidase in various types of food. Your first goal will be to develop a standard assay using the extract of horseradish. You will then develop a method to compare the activity of peroxidase in horseradish to other foods such as green beans, kiwi, carrot, cauliflower, tomato . . . . ?

**Challenge question**

Catalase converts hydrogen peroxide to water and oxygen gas. The electron donor for catalase is a complex and versatile biomolecule known as heme. Your goal is to devise a protocol to measure catalase activity spectrophotometrically. The challenge here is that since catalase uses heme as a hydrogen donor, catalase will not directly react with TMB. However, catalase does react with H2O2. In a solution with both catalase and HRP, these two enzymes will compete for H2O2 and thus the oxidation of TMB can still be influenced by the activity of catalase in the presence of HRP.

**General Procedures**

Peroxidase extraction

* 1.5 g peeled, inner portion of horseradish. The mass used may need to be altered for other vegetables
* 300 mL of ice-cold, distilled H2O
* Mix/blend thoroughly (3-10 min)
* Filter using clean Buchner funnel
* Store at 4oC (stable for days)

Measuring peroxidase activity

* Add 500 L of enzyme extract and 1000 L of cold, distilled H2O to a test tube.
* Add 30 L of 1M citrate buffer (pH 6.0)
* Place a piece of parafilm over the top of the tube. Invert tube several times to mix thoroughly.
* Add 1mL of solution to a cuvette.
* Add 50 L of H2O2. Mix reaction by aspirating with a P1000 micropipettor. Start timing the reaction.
* Collect absorbance readings every 15 seconds for 3 minutes.
* Absorbance should increase by 0.1-0.2 units per minute. If absorbance does not increase at this rate, consult your instructor.
* Plot Absorbance vs. time using excel. If this plot is not linear, consult your instructor.

Solution preparation (prepared by instructor)

* Citrate buffer: Add 125 mL chilled, distilled H2O to a 250 mL Erlenmeyer flask with stirring. Slowly add 26 g citric acid monohydrate and 12 g NaOH pellets. Dissolve with 100 mL distilled water. Adjust pH to 6.0 with HCl or NaOH. Autoclave or filter sterilize. Can be kept at 4 oC for several weeks.
* TMB working solution: Dissolve 50 mg TMB in 2.5 mL of cold, distilled water. Add 835 L of acetic acid. Chill. Mix thoroughly and add 6.65 mL of cold distilled water and mix thoroughly. Keep on ice.
* H2O2 working solution: Add 200 L of 30% H2O2 to 20 mL of cold distilled water. Keep on ice.

**Results**

Plot absorbance vs. time for each assay.

Compare the slopes of you plot for each assay as a measure of relative enzyme activity. Be sure to include the units for your slope.

**Discussion**

Which vegetables had the highest peroxidase activity? Discuss the certainty of your conclusion. Are there any matrix components that may have affected your data? How could/did you control for these factors? Which instrumental/procedural factors may have contributed to error in your data? How could/did you control for these factors?