Analysis of Polyphenoloxidase Enzyme Activity from Potato Extract
Biochemistry Lab I (CHEM 4401)

Background

Enzymes are protein molecules (primarily) that serve as biological catalysts. They are responsible for the synthesis and degradation of lipids, amino acids, carbohydrates, proteins, vitamins, steroids, hormones, neurotransmitters, nucleic acids, polysaccharides and all their metabolic intermediates. Enzymes are able to perform their functions by binding to reactants in a very specific manner, straining them to increase their reactivity and providing the chemical environment necessary to allow the reaction to proceed quickly and efficiently. The rate at which enzymes can catalyze particular reactions can be truly astonishing. For example, the *catalase* enzyme, which catalyzes the conversion of H_2O_2 (hydrogen peroxide) to H_2O and O_2, can perform this reaction at a rate of 40,000,000 molecules of H_2O_2 *per second*!

In order to understand these biological transformations and how they are catalyzed we need to purify enzymes and study their activity. Enzyme activity concerns factors such as how fast the reaction is catalyzed, how strongly it binds its substrate, sensitivity of the catalysis to changes in pH, substrate or cofactor concentration, temperature or other variables. Today we will be performing a very crude isolation of the enzyme *polyphenoloxidase* from potato. This enzyme catalyzes the hydroxylation of phenolic compounds such as intermediates in amino acid synthesis or degradation pathways. It also catalyzes the oxidation of diphenol compounds, such as those that lead to the production of various melanin pigments.

We will use 3,4-dihydroxyphenyalanine (DOPA) as the substrate for our reaction. Polyphenoloxidase will oxidize DOPA to dopachrome (figure 1), an orange -colored O-quinone that absorbs light at 475 nm (λ_{max}). We will follow the production of dopachrome spectrophotometrically using several different initial concentrations of DOPA. Data from these reactions will be used to produce graphs (Michaelis-Menten, Lineweaver-Burke) in our next laboratory section. These graphs, in turn, will be used to estimate kinetic data on our enzyme, such as the maximum velocity at which it can catalyze the reaction (V_{max}) and the affinity it has for the DOPA substrate (K_m). We will also examine the effect of pH and temperature on enzymatic activity.

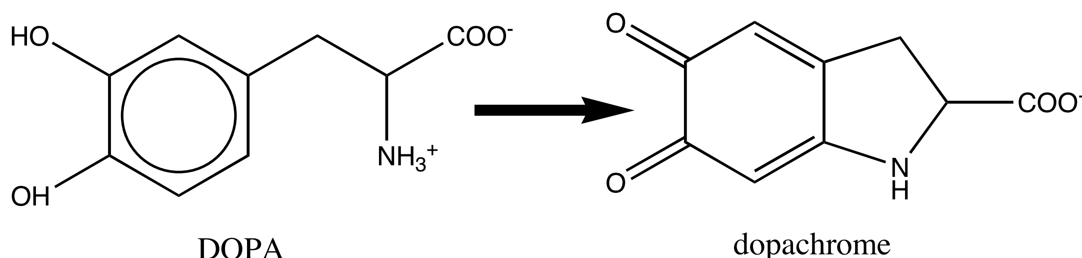

Figure 1. Conversion of DOPA to dopachrome

Prep Sheet

Materials and Reagents	per Group	per Lab
Potatoes	10 g	1-2 potatoes
Potato peelers		1-2
Mortars & Pestles	1	10
Cheesecloth	4 in^2	40 in^2
15 ml centrifuge tubes	2	24
15 ml snap-cap tubes	1	12
aluminum foil	6 in^2	60 in^2
13 x 100 mm test tubes	7	100
razor blades	1	10
DOPA (L-β-3,4-dihydroxyphenylalanine)(20 mM)	10 ml	150 ml
Phosphate buffer (0.1M, pH 6.8, Prep)	10 ml	100 ml
Phosphate buffer (0.1M, pH 6.8, Sp. Activity)	13 ml	200 ml
Phosphate buffer (0.1M, pH 6.8, 0ºC)	3.2 ml	50 ml
Acetate buffer (0.1M, pH 4.0)	5 ml	100 ml
Borate buffer (0.1M, pH 10)	5 ml	100 ml

Reagent Prep (**Instructor**)

DOPA (20 mM):

0.6 g DOPA/150 ml pH 6.8 phosphate buffer. Make fresh for each lab. Store in amber bottle. Solution will settle so it may require a stir bar and possibly low heat to dissolve entirely. Have students mix well prior to addition to assay tubes.

Phosphate Buffer (0.1M, pH 6.8):

1 L 0.1M Sodium Phosphate, monobasic (NaH_2PO_4)

1 L 0.1M Sodium Phosphate, dibasic (Na_2HPO_4)

Make up 1L each (0.1M) of the monobasic and dibasic solutions. Make ~ 1L phosphate buffer (pH 6.8) by adding monobasic solution to 750 ml of the dibasic solution while stirring until the appropriate pH is reached. Save 350 ml in a separate bottle in the refrigerator for week. Bury in ice bucket at beginning of each lab to serve as the 0º C buffer in the temperature assay.

Acetate Buffer (0.1M , pH 4.0):

0.5 L 0.1 M solution of Sodium Acetate

0.5 L 0.1 M acetic acid (3 g glacial acetic acid in 500 mL DI H_2O)

Add 0.1 M acetic acid solution to 400 ml 0.1 M sodium acetate until pH reaches 4.0.

(May require addition of conc. HCl to achieve pH = 4.0)

Borate Buffer (pH 10):

3.1 g Boric Acid (H_3BO_3)

3.74 g Potassium Chloride (KCl)

1.76 g NaOH

Dilute to 900 mL with DI H_2O. Adjust pH to 10.0 with HCl or NaOH (0.1M soln.) Bring final volume up to 1 L with DI H_2O.

Experimental Protocol – Use in place of Manual

Preparation of Potato extract (Crude Polyphenoloxidase)

1. Weigh out approximately 10g of diced, peeled potato sample. Record weight and mince with a razor blade on a piece of aluminum foil.
2. Transfer the minced potato to a mortar. Add 10 ml of 0.1 M phosphate buffer (pH 6.8). Grind to a fine slurry using a mortar and pestle.
3. Line a small funnel with 4 in^2 piece of cheesecloth. Transfer slurry onto funnel and strain into a 15 ml, screw-top centrifuge tube. Give to your instructor for centrifugation. Be sure all tube-containing buckets are balanced. Centrifuge for 10 min. at 3500 rpm in a Beckman S4180 rotor.
4. After centrifugation, pour supernatant (liquid phase) into a fresh 15 ml centrifuge tube. Label with initials and laboratory section.
5. Use concentrated extract (supernatant from step 4) for assays in tubes 1-7. Do NOT dilute.

Determination of Enzymatic Activity

1. Turn on spectrophotometer. Set to 475 nm and zero according to instructions.
2. Pipet the proper amount of (1) enzyme extract and (2) phosphate buffer into a clean, 13 x 100 mm test tube (tube No. 1), as indicated in the table below. **Do NOT make up the other samples at this time.**
3. Blank the spectrophotometer with your enzyme/buffer solution (i.e. blank)
4. Add the proper amount of DOPA to your enzyme/buffer solution (each student group should keep 10 ml of DOPA in a foil-covered snap-cap tube at their bench).
5. Shake vigorously (use your gloved thumb to stopper the top of the tube). Wipe down test tube with a tissue and place in the spectrophotometer.
6. Record the absorbance every 20 seconds for three minutes. Take your first reading 20 seconds after you have inserted the sample into the spectrophotometer. Record a reading of "0" for your zero time point ("0 seconds").
7. Repeat steps 2-6 for tubes 2-4.

Tube Number	Amount of Extract	Amount of Buffer (pH 6.8)	Amount of DOPA (20 mM
1	0.2 ml	3.4 ml	0.4 ml
2	0.2 ml	3.2 ml	0.6 ml
3	0.2 ml	3.0 ml	0.8 ml
4	0.2 ml	2.8 ml	1.0 ml

Determination of pH and Temperature Inhibition on Enzyme Activity

1. Set spectrophotometer to 475 nm.
2. Pipet the proper amount of (1) enzyme extract and (2) buffer into a clean 13 x 100 mm test tube (No. **5**). Again, **do NOT make up the other samples at this time.**
3. Blank the spectrophotometer with your enzyme/buffer solution (i.e. blank)
4. Add the proper amount of DOPA to your enzyme/buffer solution.
5. Shake vigorously (use your gloved thumb to stopper the top of the tube). Wipe down test tube with a tissue and place in the spectrophotometer.
6. Record the absorbance every 20 seconds for three minutes. Take your first reading 20 seconds after you have inserted the sample into the spectrophotometer. Record a reading of "0" for your zero time point ("0 seconds").
7. Repeat steps 2-6 for tubes 6 & 7.

Tube Number	Amount of Extract	Amount of Buffer	Amount of DOPA (20 mM
5	0.2 ml	3.2 ml (pH 4 acetate buffer)	0.6 ml
6	0.2 ml	3.2 ml (pH 10 Borate buffer)	0.6 ml
7	0.2 ml	3.2 ml (pH 6.8 phosphate buffer, 0 °C)	0.6 ml

Etc.

1. Give enzyme extract from step 7 of *Preparation of Potato extract* to your instructor for storage in freezer (we will use in a subsequent laboratory experiment this semester).
2. Transfer excess DOPA and DOPA-containing enzyme ***assays*** to a specified waste container.
3. Excess ***buffers*** (which don't contain DOPA) can go down the sink (Transfer potato waste to trash)
4. Tranfer used glass test tubes to ***glass waste***. ***Plastic*** test tubes to regular trash.
5. Be sure to clean mortars & pestles. Invert on paper towel to dry.
6. Shut off, cover and put away your spectrophotmeter.
7. Check out with your instructor before leaving.
8. We will go over preparation of the data for your laboratory report next week (supplement to be posted to web site). Be sure to read next week's posted supplement and the *Lab Report Expectations* section in your lab manual *carefully*. If you have a laptop computer, please bring it to lab.

Spectrophotometer Instructions

(Spectronic 20+ spectrophotometer)

1) Turn the spectrophotometer on by turning the "Power Switch/Zero Control" knob (front **left** side of instrument) clockwise. Allow the spectrophotometer to warm up for 10 minutes.
2) Set the filter level to the position appropriate for your desired wavelength (340-599 nm or 600-950 nm).
3) Switch 'mode' to "Transmittance". Adjust the spectrophotometer to 0% T (Transmittance) with the Power Switch/Zero Control knob. Make sure the sample compartment is empty and the cover is closed.
    - The purpose of this step is to zero the detector with the shutter closed and no light hitting the detector.
4) Fill a clean cuvette (test tube) with appropriate volume (about 2/3 of the cuvette) of your blank solution. Wipe the cuvette with a Kimwipe to remove liquid droplets, dusts, and fingerprints.
5) Place the cuvette in the sample compartment. Close the lid.
6) Adjust the spectrophotometer to 100 % T with the Transmittance/Absorbance Control knob (front, **right** side of instrument).
7) Remove the blank cuvette from the sample compartment.
8) Fill a new cuvette (test tube) with appropriate volume (about 2/3 of the cuvette) of test sample. Wipe with a Kimwipe.
9) Insert sample cuvette into the sample compartment and close the lid. Change *mode* to *absorbance*.
10) Read the appropriate value (% Absorbance).
11) When all measurement are completed, turn off the spectrophotometer by turning the Power Switch/Zero Control knob counterclockwise until it clicks. Cover the instrument with a plastic cover.