

## Mushroom Tyrosinase Experiment

Based upon the procedure outlined in:

Rodriquez, M. O. and Flurkey, W. H. A Biochemistry Project to Study Mushroom Tyrosinase  
*J. Chem. Ed.* **1992**, 69(9) 767-769.

**NOTE:** The kinetics part of this experiment (Activity B) requires that you are ultra-prepared for lab. If you are not prepared, you will probably not have ANY data at 5:30 pm. Please come see me before your lab day if you have questions.

For the next few weeks, you will be isolating and examining the enzyme mushroom tyrosinase. Tyrosinases in general are important in synthesis of pigments (i.e. hair color, skin color etc.). Tyrosinase converts tyrosine to L-dihydroxyphenylalanine (L-DOPA) and finally dopaquinone (Figure 1). Dopaquinone then polymerizes and forms biological pigments, such as hair color.

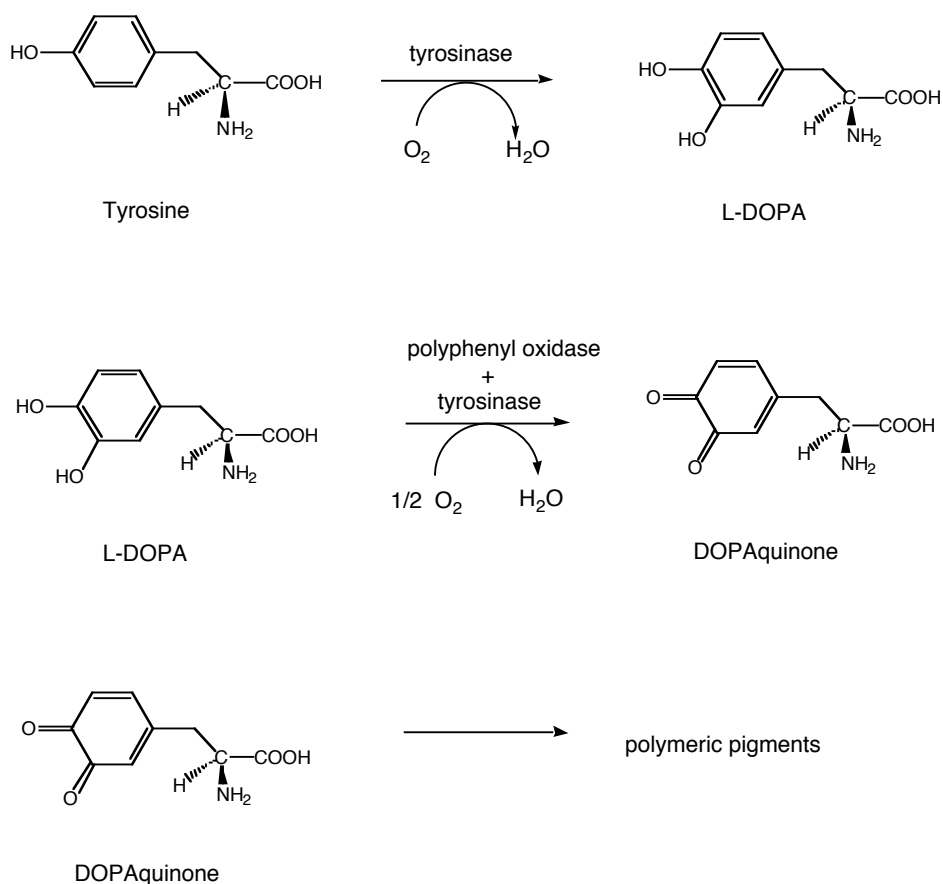


Figure 1. Tyrosinase conversion of tyrosine to biological pigments.

During the first week, you will isolate tyrosinase from portabella mushrooms. There are many different types of tissue in a mushroom (see *Rodriquez and Flurkey*). Each section of the mushroom has a characteristic tyrosinase enzymatic activity. Each group will choose a different portion of the mushroom from which to isolate tyrosinase (Activity A). The second and third weeks of this experiment involve characterizing tyrosinase kinetics (Activity B) and quantifying the concentration of enzyme isolated (Activity C). In the second and third weeks, we will divide the class into two parts and switch off between Activities B and C. In Activity B, you will determine Michaelis-Menten kinetic parameters ( $k_{cat}$ ,  $K_m$ ) for tyrosinase by itself, in the presence of an inhibitor (sodium benzoate) and an activator (sodium dodecylsulfate, SDS). In activity C, you will determine the concentration of protein in your crude extract by the Lowry assay (see below at Activity C for more details).

### **General guidelines for making buffers**

In biochemistry, we use buffers solutions to run our reactions because they stay around a particular pH of solution and they mimic the native environment. When you make a buffer you need an acid and a conjugate base. You can use the Henderson-Hasselbach equation to calculate relative quantities but the easiest way to make buffers is to make a solution of acid and another solution of base. Then mix them together until you get the right pH. Remember that because the counterions are the same, 1 M acid + 1 M base is 1 M buffer no matter the proportions you use.

### Activity A (1<sup>st</sup> week)

Tasks:

- prepare solutions to use in the isolation of tyrosinase (Activity A) and enzymatic reactions (Activity B)
  - 25 mM phosphate, 1 mM ascorbate buffer, pH 6.0
  - 100 mM phosphate buffer, pH 6.0
  - 100 mM phosphate buffer with 1 mM sodium benzoate, pH 6.0
  - 100 mM phosphate buffer with 0.1% SDS, pH 6.0 (Dr. Delaware's class only)
- isolate tyrosinase from one section of a portabella mushroom

### **Before you come to lab:**

- Obtain a diagram of the mushroom from *Rodriquez et al.*
- Figure out how to make buffer solutions.
- Calculate appropriate masses needed to make solutions.

### **Supplies/Equipment Provided**

pH meters  
pH standards (4, 7, and 10)  
KH<sub>2</sub>PO<sub>4</sub> solid  
K<sub>2</sub>HPO<sub>4</sub> solid

Ascorbic acid ( $C_6H_8O_6$ )  
sodium benzoate solid ( $C_7H_5O_2Na$ )  
Sodium lauryl sulfate (SDS,  $C_{12}H_{25}NaO_4S$ )  
deionized distilled water (DDI  $H_2O$ )  
portabella mushroom from Hy-Vee  
biohomogenizer  
volumetric flasks (10, 15, 50, 100 mL)

### *Some issues when making buffers*

In biochemistry, we also commonly have multiple chemicals in one solution. We might have 1 M buffer but it also might have 1 M something else in solution. These ingredients all go in the same flask. You can make solutions two ways. You can put the “something else” in both the acid and the base solutions or you can make a third solution in which you put the “something else” in the flask and dilute to the mark with your acid/base mixture. If you do the latter, you need to be careful that the “something else” doesn’t affect the pH of the solution (i.e. it isn’t an acid or base itself). If it will affect the pH, the first option is better.

### *Preparation of solutions*

You will need to prepare 25 mL of 25 mM phosphate, 1 mM ascorbate buffer, pH 6.0 for homogenizing your mushroom. You will also need to prepare three buffers for enzymatic reactions. You will use about 50 mL of 100 mM phosphate buffer, pH 6.0 for your enzymatic reactions with just tyrosinase and 50 mL of 1 mM sodium benzoate in 100 mM phosphate buffer, pH 6.0 for reactions with the inhibitor, sodium benzoate. Please note that these volumes are approximate and you may consider planning Activity B before you decide on final volumes.

### *Isolation of tyrosinase*

Choose a section of portabella mushroom from which to isolate tyrosinase. If you choose part B<sub>1</sub>, notice that the gills (G) should *not* be included in your sample. Also notice that all cap parts B should have D removed and similarly in the stalk region, part S should be removed before homogenation.

Chop up the mushroom section into small ~0.5 cm cubes. Weigh and record the mass of mushroom to be homogenized. Put some mushroom in a large centrifuge tube. **For everything from here on, the enzyme should be on ICE to preserve the enzymatic activity of tyrosinase.**

Add 1-2 volumes of chilled 25 mM phosphate, 1 mM ascorbate buffer, pH 6.0 (A volume is approximate – i.e. if your mushroom is about 1 mL, 1-2 volumes is 1-2 mL). Use the biohomogenizer to homogenize the sample. Continue adding buffer (up to 10 volumes) until the homogenate is thick like a milk shake but not soupy. Be careful not to add too much buffer, as your tyrosinase will be too dilute to run reactions. However, you also need enough liquid in the sample to isolate the tyrosinase.

Centrifuge the sample at 4 °C to remove excess solids. Your tyrosinase is in the supernatant. You should have greater than 5 mL of crude isolate.

Mix your crude isolate. Save 1 mL of your sample in a small test tube for determination of the protein concentration in Activity C. Save the rest in another test tube for your enzymatic reaction in Activity B. Parafilm both test tubes. Label them and put them in a paper towel-lined beaker in the freezer. Paper towel helps separate glass-glass contacts so that in the freezing process your glass doesn't crack.

### Activity B (2<sup>nd</sup> or 3<sup>rd</sup> week)

Tasks:

- prepare substrate in appropriate buffers
  - 10 mL 10 mM L-DOPA in 100 mM phosphate buffer, pH 6.0
  - 10 mL 10 mM L-DOPA in 1 mM sodium benzoate, 100 mM phosphate buffer, pH 6.0
  - 10 mL 10 mM L-DOPA in 0.1% SDS, 100 mM phosphate buffer, pH 6.0
- determine the linear range of the enzyme
- carry out enzymatic reactions with tyrosinase alone, with an inhibitor and SDS (if Delaware)

### **Before you come to lab:**

- Calculate how to make L-DOPA solutions
- Map out which reactions you will carry out
- Make tables with volumes you may use for each reaction

### **Supplies/Equipment Provided**

DL-DOPA OR L-DOPA MW=197.2 g/mol – Check bottle (assume 50% L-enantiomeric purity if DL-DOPA)

3 mL cuvettes

parafilm

razor blades

### *Preparation of L-DOPA solutions*

L-DOPA is difficult to dissolve. You may consider using a small stir bar (ask for one from your professor if you don't have one).

### *General notes about tyrosinase reactions*

In the absence of polyphenyl oxidase, tyrosinase catalyzes the conversion of L-DOPA, a diphenol, to dopachrome, a diquinone that can be detected by colorometrically at 475 nm.

Each group will carry out three distinct sets of reactions:

- tyrosinase
- tyrosinase and inhibitor
- tyrosinase and SDS (if Delaware)

With each set of reactions, the concentration of the substrate will be varied. In addition, each set needs proper control reactions. These are reactions with no enzyme (enzyme control), with no substrate (substrate control), and with neither substrate nor enzyme (reagent control). Each group should carry out the three sets of reactions with 5 final substrate concentrations ranging from 0.1-5 mM L-DOPA (remember your stock is 10 mM).

Each of your reactions should have a constant total volume that falls in the range of 2-2.5 mL. All of the reactions will be carried out in 3 mL disposable cuvettes. Each cuvette should have a square of parafilm over it with a razor blade slit. Note that your reaction sans enzyme should be kept at room temperature so that your kinetics are carried out at room temperature. Your enzyme should be kept on ice until it is injected into the cuvette. *These reactions are all at constant enzyme concentration.*

Since this is a timed reaction you can only carry out as many reactions at a time as you have pipetmen to simultaneously inject enzyme. Each person will inject the enzyme simultaneously. One person will quickly mix the reactions by holding their fingers over the parafilm and flipping the cuvettes in the cuvette holder twice. The mixer will then quickly put the cuvette holder with reactions into the spectrophotometer and start collecting data. Make sure that the spectrophotometer is set to collect data at 475 nm every 20 seconds for 3 minutes.

### *Determining the linear range*

Michaelis-Menton kinetics (Chpt 12 Voet, Voet & Pratt) is based upon the assumption that the rate of reaction is proportional to the substrate concentration or in other words, that the enzymatic reactions are carried out in the linear range of the enzyme. You will want all your data to fall in this linear range. You will need to determine the optimum volume of enzyme to use in your reactions. Since, the theoretical maximum velocity of your reactions will occur in the reactions without your inhibitor and your maximum substrate concentration.

Prepare three different reactions, each with a different enzyme volume (e.g. 50, 75, 125  $\mu$ L). Choose the largest volume of enzyme that is less than 5% of your total reaction volume and that also gives linear kinetic data. Also consider how many pipetmen are available to you. *These reactions are all at constant substrate concentration.*

### *Kinetic assays*

Once you have determined the optimum concentration of enzyme to use in your reactions, you can successfully carry out all of your reactions. If you accidentally get some data (i.e. 1 min of your data) that is nonlinear, just don't use it in the calculation of the rate. Calculation details can be found at the end of this handout. The same enzyme concentration should be used in all of the reactions carried out. For more information about competitive, noncompetitive and uncompetitive inhibition mechanisms, see Chpt 13 Garrett and Grisham.

### Activity C (2<sup>nd</sup> or 3<sup>rd</sup> week)

Task:

- prepare 100 mL of 0.85% NaCl solution
- make dilutions of tyrosinase in 0.85% NaCl solution
- determine protein concentration using the Lowry assay

### **Before you come to lab:**

- Look at the Protein Concentration Lab protocol. It's the same thing as this.
- Calculate how to make 100 mL of 0.85% NaCl solution
- Calculate how to make your dilutions (1:4, 1:9, 10 mg/mL to 1 mg/mL etc.)
- Make tables with volumes you may use for each assay

### **Supplies/Equipment Provided**

Sigma Lowry assay kit

Biuret reagent

Folin-Ciocalteu reagent

5 mg/mL or 30 g/dL bovine serum albumin (BSA)

NaCl solid

1.5 mL or 3 mL cuvettes

parafilm

Cary Vis Spectrophotometer

You will need to know the tyrosinase concentration in your reactions to calculate the Michaelis-Menten kinetics. In general, anytime isolation or purification of a protein is carried out, the protein concentration should also be determined. There are many methods for determining protein concentration. The two most inexpensive and reproducible methods for quantifying protein concentration are the Bradford assay and the Lowry assay. Both are chromogenic assays that determine total protein concentration. A standard curve of known concentrations of protein, usually bovine serum albumin (BSA), is constructed. Then the concentration of the unknown sample is determined by comparison to this curve.

Note that this method is nonspecific. There may be many proteins in the solution since the sample has not been purified. Additionally, there are normally many substances in crude samples besides proteins (e.g. nucleic acids, detergents, carbohydrates). These molecules may interact or interfere with the chromogenic reactions.

### *Lowry assay*

In the Lowry assay, protein samples are treated with the Biuret reagent followed by the Folin-Ciocalteu reagent. The Biuret reaction involves the binding of  $\text{Cu}^{2+}$  under alkaline conditions to nitrogens in the peptide bonds of proteins. The Folin-Ciocalteu reagent, which contains phosphomolybdate acids, is reduced by Tyr, Trp and polar amino acids. This process is facilitated by the copper (II) ions from the Biuret reagent. In this reaction, heteropolymolybdenum blue is detected at 750 nm. It should be noted that ammonia and Tris react with copper (II). Additionally, linear BSA standard curves are only obtained at low protein concentration and timing of reagent addition and mixing is critical.

Attached are the directions from Sigma for carrying out the Lowry assay. You will make a standard curve with various concentrations of the standard BSA. You should add Lowry assay reagents to your standard and your samples *at the same time*. This ensures that your standards are a measure of your experimental error. Since the concentration of protein in your sample is unknown and the absorbance at 750 nm for your sample must fit on the standard curve, you will need to test a few different concentrations of sample. In addition, each concentration of sample should be done in triplicate. Suggested sample concentrations: 1:1, 1:4, 1:9

### *Calculations for Lowry assay*

Plot your standards. Perform a linear fit of the standard data and find the equation of the line. Calculate your unknown protein concentration with this line. Don't forget to take into account your dilution. Calculate a standard deviation in concentration.

### **References**

Lowry, O.H., Rosebrough, N. J., Farr, A.L., and Randall, R.J. (1951) Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**, 265.

Ninfa, A. J. and Ballou, D.P. *Fundamental Laboratory Approaches for Biochemistry and Biotechnology*. 1998 Fitzgerald Science Press, Inc. Bethesda, Maryland.

Peterson, G.L. (1979) Review of the Folin phenol quantitation method of Lowry, Rosebrough, Farr and Randall. *Anal. Biochem.* **100**, 201.

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Sigma Diagnostics. Microprotein detemination: Phenol reagent method for biologic fluids (Procedure No. 690). St. Louis, MO.

### Calculations for kinetics

- 1) You will need to calculate the  $[E_{total}]$ . So you will need to find the MW of tyrosinase or estimate it based upon 110 g/mol per amino acid. For the purposes of this experiment, we will assume your samples only contain tyrosinase.
- 2) Make a spreadsheet of all your data. Subtract from every value in your data set the average of your no enzyme reaction (over all time). Note: Keep each set of reaction calculations separate (two sets: tyrosinase, tyrosinase + inhibitor).
- 3) Make a plot of uM dopachrome synthesized vs. time for each substrate concentration to obtain the rate of reaction for a given substrate concentration. Note:  $\epsilon=3600 \text{ M}^{-1} \text{ cm}^{-1}$ . Also remember Beer's Law:  $A=b\epsilon C$  where A is absorbance, b is the path length (1 cm) and C is concentration.
- 4) Make a Michaelis-Menten plot ( $1/v$  vs.  $1/[S]$ ).
- 5) Determine  $k_{cat}$  and  $K_m$ . For the inhibitor and activator reactions, determine the observed  $k_{cat}$  and  $K_m$  ( $k_{cat,obs}$  and  $K_{m,obs}$ ). For the inhibitor, determine  $\alpha$  and/or  $\alpha'$ . Note:  $k_{cat}$  is in units  $\text{time}^{-1}$
- 6) Calculate  $\Delta\Delta G^\ddagger$ .

$$\Delta\Delta G^\ddagger = \Delta G_{SDS}^\ddagger - \Delta G_{noSDS}^\ddagger$$

$$\Delta\Delta G^\ddagger = RT \ln \left[ \frac{\left( \frac{k_{cat}}{K_m} \right)_{noSDS}}{\left( \frac{k_{cat,obs}}{K_{m,obs}} \right)_{SDS}} \right]$$

$$\Delta\Delta G^\ddagger = \Delta G_{inhibitor}^\ddagger - \Delta G_{no\_inhibitor}^\ddagger$$

$$\Delta\Delta G^\ddagger = RT \ln \left[ \frac{\left( \frac{k_{cat}}{K_m} \right)_{no\_inhibitor}}{\left( \frac{k_{cat,obs}}{K_{m,obs}} \right)_{inhibitor}} \right]$$

- 7) Calculate rate enhancement or fold decrease in activity.

If  $\Delta\Delta G^\ddagger$  is negative, rate enhancement =  $e^{\frac{-\Delta\Delta G^\ddagger}{RT}}$

If  $\Delta\Delta G^\ddagger$  is positive, fold decrease in enzymatic activity =  $e^{\frac{\Delta\Delta G^\ddagger}{RT}}$