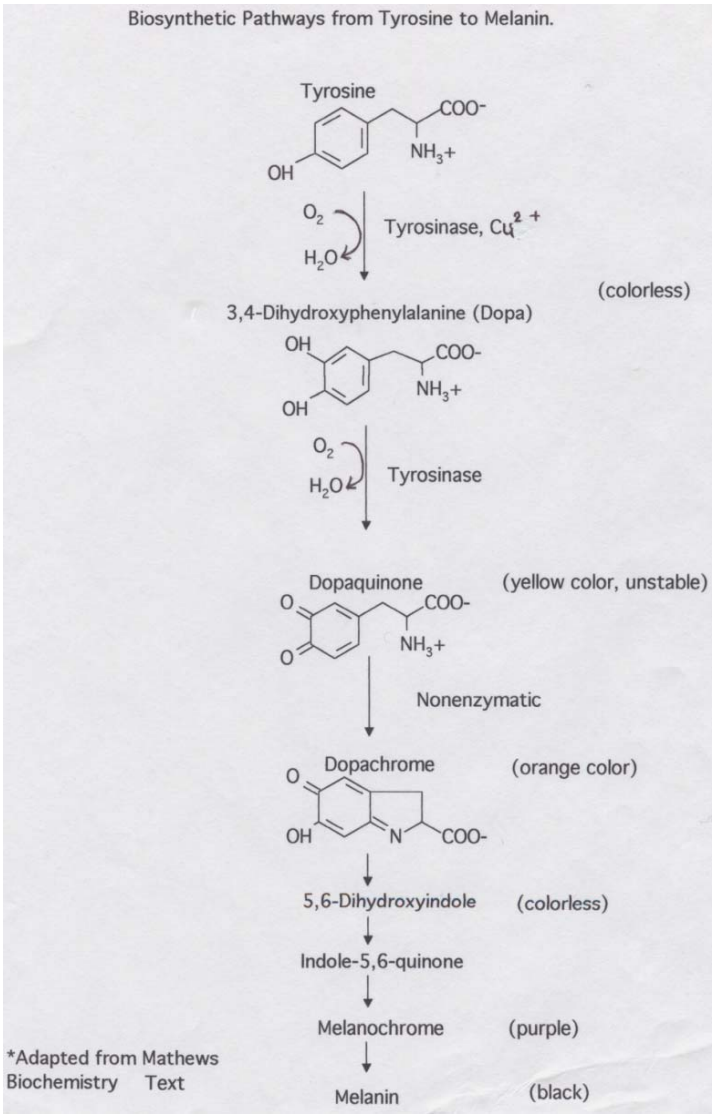
SPECTROPHOTOMETRIC DETERMINATION OF ENZYME ACTIVITY

In this laboratory, we will study the catalyzed oxidation of "dopa" to "dopaquinone." Because hundreds of reactions are simultaneously carried out in the living cell, it is difficult to study a single reaction in an intact cell. However, it is possible to extract enzymes from cells and thus study enzyme–controlled reactions in a test tube. The extraction procedure we will use involves fractionating (separating) the plant proteins with a neutral salt, ammonium sulfate, to isolate the enzyme **tyrosinase**.

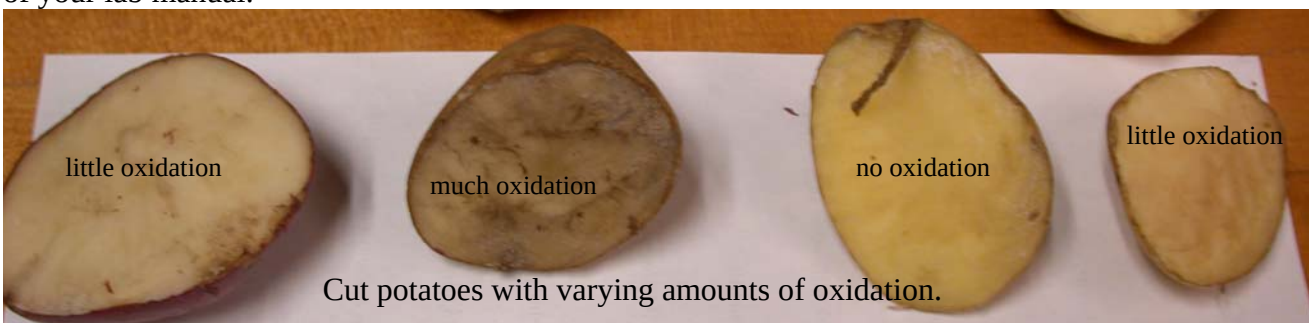


The dynamics of enzyme–catalyzed reactions can best be understood if one can quantify the extent of reaction at a given time. This means that one can know how much substrate has been converted to product at a certain point during the reaction. A common way of reporting the extent of reaction is to define how many moles of substrate or product are present. Recall that a mole is the gram molecular mass of a substance. Because reaction mixtures typically are very dilute, one commonly uses millimoles, mM (10^{-3} moles), or micromoles, μM (10^{-6} moles), as units of amount. A molar solution (1M) contains one mole of substance per liter of solution.

Various ways of determining how much product is present in a mixture have been developed, depending on the chemical nature of the product and the level of sensitivity required. One common method, colorimetry, is used when the material to be quantitated is a colored compound. Colorimetry employs an instrument, either a spectrophotometer or a colorimeter to indicate the concentration of a chemical based on its absorbance of light. Spectrophotometers can be used to determine the amount of a substance in solution by measuring the amount of light transmitted or absorbed as light of a specific wavelength passes through the specimen. For quantitative work, one usually measures light *absorption* because the extent of absorbance (optical density) is directly related to the concentration of absorbing material, that is, if one doubles the concentration of test material the absorbance of the solution will be doubled.

There are other methods of colorimetry which do not require the use of an instrument. In such cases a standardized color coded chart is used to approximate the concentration of a substance. The colors change or increase in intensity based on the substance measured and how it interacts with the particular indicator which changes color with increased concentration. Typical house hold examples are urinalysis test strips that look for glucose, protein or ketone levels. Other common examples are pH or chlorine pool kits, and aquarium water test kits. Many employ impregnated test strips that you dunk into the liquid sample and match up to the closest color code to take a reading or adding an indicator to the liquid sample.

Today you will examine the enzyme activity of tyrosinase by assaying its activity with the substrate DOPA using the spectrophotometer. If you do not remember how to use the spectrophotometer, be sure to review the "Introduction to the Spectrophotometer" lab you have done previously. The question we are asking you to answer is whether or not one type of potato has more enzymatic activity of the enzyme tyrosinase than the typical baking potato, known as Russet potatoes. Be sure to refer to the "Cell Biology Lab Report Guidelines" in the appendix of your lab manual.



Cut potatoes with varying amounts of oxidation.

Preparation of Enzyme Extracts

Each table will process one russet as well as one other type of potato and share their final extracts among the two groups at each table. The two groups need to communicate: one group will process Russett and the other group a different potato: extracts are to be shared (not data).

Hypothesis building: which potato will have more enzyme activity?

Have one member from each group in charge of periodically monitoring how long it takes to see oxidation in a chunk of each of the two types of potato the table is processing and how much oxidation there is by the end of lab on the surface of each, use the image above to gauge. It should correlate with the difference in rates of dopachrome oxidation.

1. Record which potato type you have. Cut a portion of it, about 2 inches wide, peel it and weigh it, you will need to process **30g**. Cut the 30g sample into chunks and place in the blender with **60ml** of phosphate buffer, pH 6.5. Use a graduated cylinder to measure the buffer.

2. Grind the potato and buffer for thirty seconds and pour the frothy mixture, called the homogenate, through several layers of cheesecloth supported by a funnel, into a 250ml flask. **Label it "homogenate" and keep on ice.** Throw out the cheesecloth; rinse the funnel.

3. Transfer from beneath the frothy top layer, 20 ml of the homogenate into a clean beaker, place on ice. Stir while adding 30ml of cold saturated ammonium sulfate, $(NH_4)_2SO_4$, to precipitate some of the proteins. Stir for 1 minute on ice.

4**.** Pour 40ml of the mixture from step 3 into a 50 ml centrifuge tube. **IMPORTANT: as you perform this step, the instructor must check the balancing of all tubes and stand by to start up and run the centrifuge.** Be sure the tube and contents weigh the same as all other tubes to be spun; place them in opposite positions in the centrifuge. Spin the tubes at 1,500 rpm for ten minutes.

5. Notice the pellet (solids at the bottom of the tube) is loose and can easily be disrupted, therefore you must **carefully** decant (pour off) most of the fluid called supernatant into a beaker, try not to disrupt the pellet. If you do, just note it in your notebook. Label the beaker "supernatant" and set it aside. To the pellet in the tube add 30ml of phosphate buffer, pH 6.5. Stir the contents well using a glass rod. Weigh your tube and again, be sure that the tube and contents weigh the same as all other tubes to be spun; then spin them at 1,500 rpm for ten minutes. **Your instructor must stand by as this is done.**

6. When the centrifuge stops, save the liquid from your centrifuge tube; pour it off into a beaker and place this supernatant on ice. This mixture is your **enzyme extract, label the beaker so,** and include in the label which potato it is from (i.e Russet, Main, Yukon Gold, Red Bliss,…) it contains the enzyme tyrosinase. Do not dispose of the enzyme extract until all experimentation is complete. Share your extract (not your data!) with the group at your table so both groups at each table have two different enzyme extracts to test and compare.

7. Be sure to clean as you work: dispose of all **waste:** cheesecloth, potato peels and mash, etc. Rinse the funnel, blender pitcher and cap.

II. Preparation of a Standard Curve.

1. Obtain 50ml of the colorful dark orange 0.015M dopachrome solution. It contains 15 millimoles of dopachrome per liter, which equals 15 micromoles of dopachrome per milliliter.

2. Your group must prepare 3 sets of the tubes # 1-6 as shown in Table 1. You will later calculate the average and standard deviation for each concentration of Dopachrome to make a standard curve with error bars.

Table 1:

Tube	Dopachrome (ml)	Phosphate Buffer (ml)	Micromoles/ ml Dopachrome	Absorbance at 475 nm (O D) **set 1**	Absorbance at 475 nm (O D) **set 2**	Absorbance at 475 nm (O D) **set 3**
1	**3.0**	**2.0**	9			
2	**2.5**	**2.5**	7.5			
3	**2.0**	**3.0**	6			
4	**1.5**	**3.5**	4.5			
5	**1.0**	**4.0**	3			
6	**0**	**5.0**	**0**	**0 blank**	**0 blank**	**0 blank**

3. At 475nm, zero the spectrophotometer (spec 20) with the blank which is tube 6, then record in table 1 absorbance readings for all three sets of tubes 1-6.

4. This data will be used for your standard curve, have your instructor check it before you move on.

III. Effects of Enzyme Concentration

Prepare and label the reaction tubes of the table below. Use a 1ml micropipette to add enzyme extract, be sure to pipette correctly!

Table 2:

Reaction Sets	Tube/ Label	Buffer (ml)	Enzyme Extract (ml)	Distilled Water (ml)	*2.0ml DOPA (Keep on ice!)*
Set 1: 0.2ml Russet Potato enzyme extract					
Blank	B1	2.6	0.2	2.0	0
Reaction 1	1-1	2.8	0.2	0	***Add at reaction start***
Reaction 2	1-2	2.8	0.2	0	***Add at reaction start***
Reaction 3	1-3	2.8	0.2	0	***Add at reaction start***
Set 2: 0.2ml Other ( ) Potato enzyme extract					
Blank	B2	2.6	0.2	2.0	0
Reaction 1	2-1	2.8	0.2	0	***Add at reaction start***
Reaction 2	2-2	2.8	0.2	0	***Add at reaction start***
Reaction 3	2-3	2.8	0.2	0	***Add at reaction start***

Testing Reaction Sets

1. Zero the spec 20 machine at 475 and blank it using the appropriate blank for the first reaction set of tubes (1-1, 1-2 & 1-3). Remove the blank.

2. You will stagger the start and measurements of your three reaction tubes by 15 seconds. The reaction starts when you add the DOPA, so add it only to the tubes you are about to measure when you are ready to start:
Start reaction tube 1-1 of the first reaction set by adding 2.0ml of DOPA, cap and invert to mix, wipe the tube and start the timer. Immediately record into the table the absorbance at 475 and repeat this for tube 1-2. It should be 15 seconds after you started tube 1. Repeat this for tube 1-3, which would be 30 seconds after the timer was started for tube 1. Continue to record absorbance values for tubes 1-1, 1-2 and 1-3 every minute for 15 minutes.

3. When you have finished the first set of reaction measurements, repeat the procedure for the second set of enzyme reaction tubes (2-1, 2-2 & 2-3). Remember to blank with the appropriate blank before starting the second set of reactions.

Table 3

Russet Potato	**0.2ml Reaction 1**	**0.2ml Reaction 2**	**0.2ml Reaction 3**	**Average of Reactions**	
Time (minutes)	Absorbance OD_{475}	Absorbance OD_{475}	Absorbance OD_{475}	Average Absorbance OD_{475}	**Average μm Dopa-chrome**
0					
1					
2					
3					
4					
5					
6					
7					
8					
9					
10					
11					
12					
13					
14					
15					

Table 4

Other Potato	**0.2ml Reaction 1**	**0.2ml Reaction 2**	**0.2ml Reaction 3**	**Average of Reactions**	
Time (minutes)	Absorbance OD_{475}	Absorbance OD_{475}	Absorbance OD_{475}	Average Absorbance OD_{475}	**Average μm Dopa-chrome**
0					
1					
2					
3					
4					
5					
6					
7					
8					
9					
10					
11					
12					
13					
14					
15					

Data Analysis

1. Use Excel and create a standard curve from the averages of part II Preparation of a Standard Curve (use excel to first calculate the averages and standard deviation of your data).
    - **a.** Insert a trend-line and display the equation .
    - **b.** Insert vertical error bars using the standard deviation.
    - **c.** Determine the amount of Dopa converted at each time interval and record in Tables 3 and 4 above & in your excel sheet.

2. Use Excel to calculate the average of the absorbance values per minute for each of your two reaction sets. Calculate the standard deviation as well.

3. Determine the amount of dopachrome present per minute based on the average for each set tested, use the equation of the standard curve graph. (See the intro to computers lab or the appendix if you don't remember how this is done.) Remember that the amount of dopachrome present is equal to the amount of DOPA converted (oxidized) by the enzyme.

4. Plot the tyrosinase data (concentration v. time) on Excel. You should distinguish between your two lines: the data obtained with the Russet potato enzyme extract and the data obtained with other type of potato enzyme extract.

5. How would you compare the results from the two kinds of potatoes quantitatively? Look at your data and suggest a measure that would show how much one potato is different from the other. Do you think this corresponds to the difference in tyrosinase enzyme concentration between the two kinds of potatoes? Why or why not?

Be sure to refer to the "Cell Biology Lab Report Guidelines" found in the appendix of your lab manual.