

Enzyme Analysis: Experiments with Tyrosinase

Enzymes are molecules that regulate the chemical reactions that occur in all living organisms. Almost all enzymes are globular proteins that act as *catalysts*, substances that speed up chemical reactions. Enzymes catalyze reactions by reducing the activation energy for a specific reaction to occur and yet are neither destroyed nor altered during this process. At the molecular level, enzymes catalyze these reactions by briefly binding to the substrate or reactants to form an enzyme-substrate complex. The reaction takes place while the substrate is bound to the enzyme, converting the substrate to the new product. The new product is then released from the enzyme substrate complex and the enzyme is then free to bind with more substrate.



Based on this model, the rate at which the product can be produced depends on the amount of enzyme and substrate that are present during the reaction. If there is excess substrate and a small amount of enzyme in solution, the reaction rate, or velocity of the reaction will increase with the amount of enzyme in the solution. In this case, all of the enzyme molecules are busy catalyzing reactions even though there is still plenty of substrate that can be turned into product. The velocity of the reaction can only increase if the concentration of enzyme is increased. Put another way, the velocity of the reaction should increase in direct proportion to the concentration of enzyme in solution.

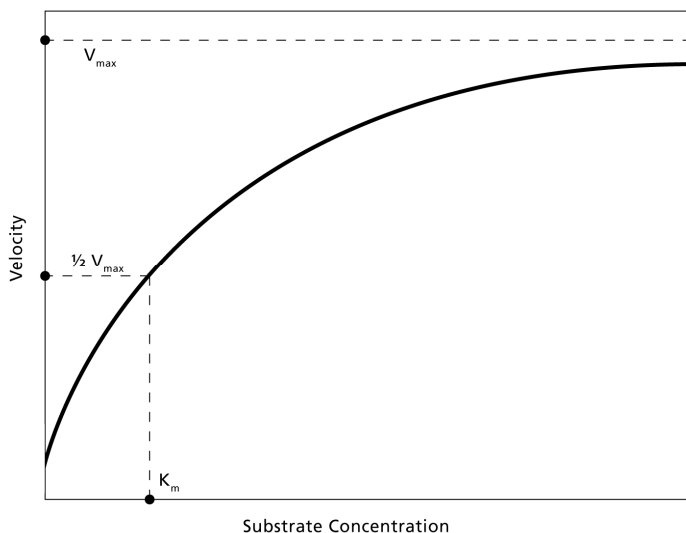


Figure 1

In a case where the enzyme concentration is kept constant and the concentration of substrate is increased, the velocity of the reaction will increase rapidly until $\frac{1}{2}$ of the enzyme becomes saturated with substrate. At this point, the velocity of the reaction will not increase as rapidly. Eventually, the velocity of reaction will approach a constant rate even when the substrate concentration is increased. When the reaction rate ceases to increase, the maximum velocity or V_{\max} for the reaction has been reached. A diagram of this case is presented in Figure 1. Fifty percent or half of the maximum velocity is referred to as $\frac{1}{2} V_{\max}$. The substrate concentration that coincides with $\frac{1}{2} V_{\max}$ is called the K_m or Michaelis-Menten constant. At this concentration, $\frac{1}{2}$ of the enzyme molecules in solution are bound to the substrate. Biochemists use these parameters to characterize enzymes and how enzymes react to different substrates. For example, the K_m is inversely related to enzyme binding affinity for a given substrate. In other words, the smaller the K_m , the greater the binding affinity an enzyme has a substrate.

This exercise is designed to introduce you to the quantitative analysis of enzymes using the enzyme tyrosinase. Tyrosinase is an enzyme that is involved in melanin synthesis, which gives skin its color. Tyrosinase is also involved in the browning of fruits, tubers, and fungi that have been damaged. As shown in Figure 1, in the presence of oxygen (O_2), tyrosinase (E) catalyzes the hydroxylation of tyrosine into the compound 3,4-dihydroxyphenylalanine or DOPA for short. Tyrosinase then catalyzes DOPA into dopaquinone which spontaneously converts into dopachrome. Dopachrome will eventually be turned into melanin. Dopachrome is a colored compound with a peak absorbance at 475 nm that can be monitored using a Spectrometer or Colorimeter.

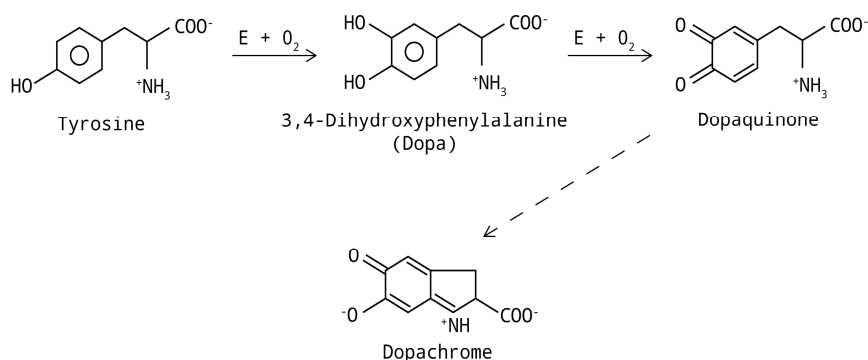


Figure 2

The first step in this series of reactions is the rate-limiting step for the entire process. This means that tyrosinase is much better at converting DOPA to dopaquinone than it is at converting tyrosine to DOPA. Knowing which step is the slowest in a complex biochemical process can be medically important. People with Parkinsons' disease have low levels of dopamine in their brain. Dopamine is synthesized from tyrosine; the first step involves converting tyrosine to DOPA, which is also the rate-limiting step for the entire process. As a result, L-DOPA is given to people with Parkinsons' disease to increase their natural production of dopamine. This in turn alleviates the symptoms of the disease.

OBJECTIVES

In this experiment, you will

- Observe and compare the reaction rate of two substrates, tyrosine and DL-DOPA.
- Determine the effect of increasing enzyme concentration on the reaction rate of an enzyme at a given substrate concentration.
- Determine the effect of increasing substrate concentration on the reaction rate of an enzyme at a given enzyme concentration.
- Estimate the parameters, V_{max} , $\frac{1}{2} V_{max}$, and K_m for your enzyme extract.

MATERIALS

Computer	0.1M Phosphate Buffer, pH 6.8
Vernier computer interface*	20 mM DL-DOPA solution
Logger <i>Pro</i>	1 mM DL-DOPA solution
Colorimeter or Spectrometer	1 mM tyrosine solution
12 plastic cuvettes with caps	15 mL centrifuge tubes
20-200 μ L micropipette**	4 mL enzyme extract (on ice)
100-1000 μ L micropipette**	200 μ L micropipette tips (1 box)
	1000 μ L micropipette tips (1 box)

*No interface is required if using spectrometer


** Appropriate graduated transfer pipettes (1 and 5 mL) may be substituted


PROCEDURE

Both Colorimeter and Spectrometer Users


1. Obtain and wear goggles.
2. Use a 15 mL centrifuge tube to obtain 10 mL of 0.1M Phosphate Buffer, pH 6.8. Use another 15 mL centrifuge tube to obtain 10 mL of 20 mM DL-DOPA solution.
3. Prepare a *blank* by filling a cuvette with 2 mL of buffer. To correctly use cuvettes, remember:
 - Wipe the outside of each cuvette with a lint-free tissue.
 - Handle cuvettes only by the top edge of the ribbed sides.
 - Dislodge any bubbles by gently tapping the cuvette on a hard surface.
 - Always position the cuvette so the light passes through the clear sides.

Spectrometer Users Only (Colorimeter users proceed to the Colorimeter section)

4. Use a USB cable to connect the Spectrometer to your computer. Choose New from the File menu.
5. To calibrate the Spectrometer, place the blank cuvette into the cuvette slot of the Spectrometer, choose Calibrate ► Spectrometer from the Experiment menu. The calibration dialog box will display the message: "Waiting 60 seconds for lamp to warm up." After 60 seconds, the message will change to "Warmup complete." Click .
6. Determine the optimum wavelength for examining the absorbance of dopachrome and set up the mode of data collection.
 - a. Empty the blank cuvette. Fill the cuvette with 2 mL of the 20 mM DL-DOPA solution. Add 100 μ L of enzyme extract into the cuvette. Cap the cuvette and gently invert the cuvette twice. Let the cuvette sit for 2 minutes, and then place the cuvette into the spectrometer.
 - b. Click . A full spectrum graph of the solution will be displayed. Note that one area of the graph contains a peak absorbance. Click to complete the analysis.
 - c. To save your graph of absorbance vs. wavelength, select Store Latest Run from the Experiment menu.
 - d. To set up the data collection mode and select a wavelength for analysis, click on the Configure Spectrometer Data Collection icon, , on the toolbar.




- e. Click Abs vs. Time (under the Set Collection Mode). The wavelength of maximum absorbance (λ max) will be selected. Verify that the maximum absorbance is close to 475 nm. Click . Remove the cuvette from the spectrometer and dispose of the solution as directed.
- f. Proceed to Part I.

Colorimeter Users Only

4. Connect the Colorimeter to the computer interface. Open Logger *Pro* and click on the data collection button, . Change the collection length to 200 seconds. Make sure that you are sampling at 2 seconds/sample.
5. Open the Colorimeter lid, insert the *blank*, and close the lid.
6. To calibrate the Colorimeter, press the < or > button on the Colorimeter to select the wavelength of 470 nm (Blue). Press the CAL button until the red LED begins to flash and then release the CAL button. When the LED stops flashing, the calibration is complete. Remove the cuvette from the Colorimeter and proceed to Part I.




Both Colorimeter and Spectrometer Users

Part I. Comparison of tyrosine and DL-DOPA as substrates for tyrosinase




7. Obtain 3 cuvettes. Label the cap of Cuvette 1 with a C. Label the cap of Cuvette 2 with a T. Label the cap of Cuvette 3 with a D.
8. Fill Cuvette 1 with 2 mL of 0.1M phosphate buffer, pH 6.81. Fill Cuvette 2 with 2 mL of 1 mM DL-DOPA solution. Fill Cuvette 3 with 2 mL of 1 mM tyrosinase solution.
9. *Do this quickly!* Add 200 μ L of enzyme extract to Cuvette 1. Cap the cuvette and gently invert the cuvette twice. Place it in the device (close the lid if using a Colorimeter). Click . Absorbance data will be collected for 200 s. Discard the cuvette contents as directed by your instructor at the end of the run.
10. Move your data to a stored run. To do this, choose Store Latest Run from the Experiment menu.
11. Repeat steps 9–10 for Cuvette 2 and Cuvette 3.
12. Using the mouse, select the initial linear region of your data on the graph. This should correspond to the first 60–100 seconds of the record.
13. Click on the Linear Fit button, . Click  and a best-fit linear regression line will be shown for each run selected.
14. Record the value of the slope, m , for each run in Table 1. (The linear regression statistics are displayed in a floating box for each of the data sets.)

Part II. Increasing Enzyme Concentration

15. Create 10 mL of 5 mM DL-DOPA solution. Use the 20 mM DL-DOPA solution as a stock solution and add the appropriate amount of buffer to a 15 mL centrifuge tube. Ask your instructor if you have questions about how to prepare this solution.
16. Obtain 5 cuvettes. Fill each cuvette with 2 mL of 5 mM DL-DOPA solution.
17. Add 200 μ L of enzyme extract to the first cuvette.

18. Cap the cuvette and gently invert the cuvette twice. Place it in the device (close the lid if using a Colorimeter). Click  Collect. Absorbance data will be collected for 200 seconds. Discard the cuvette contents as directed by your instructor at the end of the run.
19. Move your data to a stored run. To do this, choose Store Latest Run from the Experiment menu.
20. Collect data for Cuvettes 2-5:
 - Add 150 μ L of the enzyme extract to Cuvette 2. Repeat Steps 18–19.
 - Add 100 μ L of the enzyme extract to Cuvette 3. Repeat Steps 18–19.
 - Add 50 μ L of the enzyme extract to Cuvette 4. Repeat Steps 18–19.
 - Add 0 μ L of the enzyme extract to Cuvette 5. Repeat Steps 18–19.
21. Use the mouse to select the initial linear region on the graph. This should correspond to the first 60–100 seconds of the record. Click on the Linear Fit button, . Click  and a best-fit linear regression line will be shown for each run selected.
22. Record the value of the slope, m , for each of the 4 runs in Table 2. (The linear regression statistics are displayed in a floating box for each of the data sets.)

Part III. Increasing Substrate Concentration

23. Obtain 6 cuvettes. Fill Cuvette 1 with 2 mL of 20 mM DL-DOPA solution.
24. Fill Cuvettes 2–6 with 2 mL of 15, 10, 5, 2.5 and 1 mM DL-DOPA respectively. Add the appropriate amount of 20 mM DL-DOPA to the cuvette and then dilute with the proper amount of buffer. Ask your instructor if you have any questions about how to prepare these solutions.
25. *Do this quickly!* Add 100 μ L of enzyme extract to the first cuvette. Cap the cuvette and gently invert the cuvette 2 times. Place it in the device (close the lid if using a Colorimeter), Click  Collect. Absorbance data will be collected for two minutes. Discard the cuvette contents as directed by your instructor at the end of the run.
26. Move your data to a stored run. To do this, choose Store Latest Run from the Experiment menu.
27. Collect data for each cuvette by repeating Steps 25–26.
28. Use the mouse to select the initial linear region of your data on the graph. This should correspond to the first 60–100 seconds of the record. Click on the Linear Fit button, . Click  and a best-fit linear regression line will be shown for each run selected.
29. Record the value of the slope, m , for each of the six substrate concentrations in Table 3. (The linear regression statistics are displayed in a floating box for each of the data sets.)
30. Select Save As from the File Menu. Rename your data set and save it.

DATA

Part I. Comparison of Two Substrates

Table 1	
Substrate	Slope (Δ abs/sec)
Buffer	
1 mM DL-DOPA	
1 mM Tyrosine	
% Difference in Reaction Rate (RateDopa/RateTyrosine *100)	% _____

Part II. Enzyme Concentration

Table 2		
Enzyme (μ L)	Enzyme Conc. (rel.)	Slope (Δ abs/sec)
200	4	
150	3	
100	2	
50	1	
Slope from Linear Fit m = _____		

Part III. Substrate Concentration


Table 3		
Substrate Conc. (mM)	Slope (Δ abs/sec)	
20		
15		
10		
5		
2.5		
1		
Max Rate _____	$\frac{1}{2}$ Max Rate _____	Est. Km _____

PROCESSING THE DATA



Part I. Comparison of two substrates

1. Calculate the percent difference in the rate of dopachrome production for DL-DOPA and tyrosine. Take the slope for DL-DOPA as a substrate and divide it by the slope for tyrosine as a substrate. Multiply this number by 100 to get the percent difference. Write this value in the space provide in Table 1.
2. Create a bar chart of your data. Use the following instructions to do this using *Logger Pro*.
 - a. Open the file labeled EnzymeAnalysis.
 - b. In the Data Set for Part I on Page 1, enter the slope for DL-DOPA and Tyrosine.
 - c. Click the Autoscale button. Change the scale of the graph if necessary.
3. Select Save As from the File Menu. Rename your file and save it.

Part II. Increasing Enzyme Concentration

4. Follow the instructions below to create a graph of your data in *Logger Pro*
 - a. Go to Page 2 of the file labeled EnzymeAnalysis.
 - b. In the Data Set for Part II on Page 2, enter the relative enzyme concentration and the slope you observed for 50, 100, 150 and 200 μL of enzyme extract in the space provided.
 - c. Click the Autoscale button. Change the scale of the graph if necessary.
 - d. Click the Linear Regression button, . Write down the slope value in data Table 2.

Part III. Increasing Substrate Concentration

5. Follow the instructions below to create a graph of your data in *Logger Pro*
 - a. Go to Page 3 of the file labeled EnzymeAnalysis.
 - b. In the Data Set for Part III on Page 3, enter the slope you observed for each substrate concentration.
 - c. Change the scale of the graph if necessary.
 - d. Click the Curve Fit button, .
 - e. Choose Natural Exponent ($y = A \cdot \exp(-Cx) + B$) as your mathematical relationship from the list at the lower left. Click .
 - f. A best-fit curve will be displayed on the graph. The curve should match up well with the points. If the curve has a good fit with the data points, then click .
6. The slope that you observed for 20 mM DL-DOPA will be used as an estimate of V_{\max} . Enter this value into the space provided on Table 3.
7. Multiply the slope you observed at 20 mM DL-DOPA by 0.5. This value will be your estimate of $\frac{1}{2} V_{\max}$. Enter this value in Table 3.
8. Click the Examine button, . Find the point on your graph that corresponds to, or is closest to, $\frac{1}{2} V_{\max}$. Write down the corresponding concentration in mM in the space provided in Table 3. This is your estimate of the K_m of your enzyme extract.
9. Insert Text Annotations that correspond to your estimates of V_{\max} , $\frac{1}{2} V_{\max}$ and K_m .

10. (Optional) Print copies of your graphs. Enter your name(s) and the number of copies of the graphs you want, then click . Save your data.

QUESTIONS

Part I. Comparison of two substrates

1. Was the change in absorbance faster for DL-DOPA or tyrosine? How many times faster was the change in absorbance for DL-DOPA?
2. Does your data indicate that the conversion of tyrosine to DL-DOPA is the rate limiting step in the production of dopachrome? Why?

Part II Increasing Enzyme Concentration


3. How does changing the concentration of enzyme affect the change in absorbance?
4. Is the change in absorbance proportional to the amount of enzyme in solution? Is the trend linear for your data set?
5. Use your results to predict what the rate of dopachrome production would be if you had used 25 μL of enzyme extract. Predict what the rate would be for 400 μL of enzyme extract.

Part III Increasing Substrate Concentration

6. Does your data for this exercise resemble Figure 1? Is 20 mM of DL-DOPA above or below V_{max} for your sample of enzyme?
7. Were you able to estimate $\frac{1}{2} V_{\text{max}}$ and the K_m for your enzyme extract?
8. What would happen to the V_{max} and K_m if you repeated this exercise with twice the concentration of enzyme? What would happen to these parameters if you cut the enzyme concentration in half?

EXTENSIONS

1. Calculate the rate of dopachrome production for each run.
 - Reaction rate = $(\Delta \text{ abs/sec}) / (3700 \text{ M}^{-1} \text{ cm}^{-1}) * 1 \text{ cm}$.
2. Calculate the *specific enzyme activity* of your enzyme extract. *Specific enzyme activity* can be defined as the reaction rate of the extract for a known concentration of substrate divided by the amount of protein found in 1 mL of the extract. The “Got Protein? Kit” from Bio-Rad Laboratories Inc. (Catalog # 166-2900EDU) can be used for this purpose.
3. Compare the substrate catechol to DL-DOPA. Tyrosinase is also known as catechol oxidase. Tyrosinase will oxidize the compound catechol to *ortho-quinone*, which spontaneously converts to a colored product with a peak absorbance at 540 nm. Repeat Part III of this exercise using catechol as a substrate.
4. Compare stereoisomers of tyrosine or DOPA as substrates for this enzyme. Some enzymes are very specific for a given substrate and will only react with the stereoisomer of a given compound. Repeat Part I of this exercise and compare D to L-DOPA.

5. Compare the activity of tyrosinase from different sources. Tyrosinase can be found in bananas, mushrooms, apples, yams and other fruits, tubers, and vegetables. Prepare enzyme extracts from different sources and compare the activity of tyrosinase from each source. Use the “Got Protein? Kit” from Bio-Rad (Catalog # 166-2900EDU) to control for different amounts of protein that may be found in each source. Your instructor can provide you with instructions on how to prepare the enzyme extract.
6. Using the data from Part III of this exercise, construct a Lineweaver–Burke plot and calculate the apparent K_m , V_{max} , and $\frac{1}{2} V_{max}$ of tyrosinase from your extract. A Lineweaver-Burke plot is a double reciprocal plot of the data in Part III of this exercise. To construct a Lineweaver-Burke plot take the inverse of the slope and substrate concentrations in Part III. Plot the inverse of the slope on the Y-axis and the inverse of the substrate concentrations on the X-axis. Your data should now be linear. Perform a linear fit of the data. The Y intercept of this line is equivalent to $1/V_{max}$ and the X intercept is equivalent to $-1/K_m$. You can perform a Lineweaver-Burke plot using Logger-Pro by doing the following.
 - a. Open the file called EnzymeAnalysis.
 - b. Make sure that you have entered data for Part III of this exercise
 - c. Select Page 4 and click the autoscale button. A double reciprocal plot of your data should now be visible.
 - d. Change the scale of the X axis to a negative value so you can see the X intercept.
 - e. Click the Linear Regression button, . Write down the slope and Y intercept. Take the inverse of the Y intercept to get the apparent V_{max} .
 - f. Multiply V_{max} by 0.5 to get $\frac{1}{2} V_{max}$
 - g. Remember that $X_i = -b/m$, where b is the Y intercept and m is the slope. Find the X intercept using this formula. Take the inverse of the X intercept and multiply by -1 to get the apparent K_m .
 - h. Compare the calculated values to your estimated values in Part III of this exercise.
7. Construct a Lineweaver-Burke plot using the reaction rate for dopachrome production. See extension 1.
8. The substrate concentrations in all of your experiments are off by a small amount. This is because you have added a small volume of enzyme extract to each cuvette. This small volume is diluting the initial substrate concentrations. Go through all of these exercises and correct for this source of error.