Enzyme Kinetics: Properties of β-Galactosidase

Preparation for Laboratory: Web Tutorial 4, Beta Galactosidase - submit answers to questions
Additonal background: Freeman, "Proteins" pp 51-54 and Box 3.3 pp56-57,

This week, you will begin your investigation of the activity of an enzyme, β-galactosidase, by using spectroscopy. The power of the techniques that you will use will become obvious when you calculate how many reactions a **single molecule** of β-galactosidase catalyzes in a minute. At the end of the lab, you and your lab partners will design an experiment to be done next week on β-galactosidase, based on what you have learned this week. The same general techniques that you use this week will be used for the next two weeks, so what you learn in lab this week will help your future comprehension.

I. INTRODUCTION

Enzymes:

Chemical reactions are at the heart of all biological processes. The body must regulate precisely all the chemical reactions going on in order to maintain life. Much of this regulation is done by changing the activity of enzymes, which are biological catalysts.

A catalyst is any substance that **speeds** the rate of a chemical reaction. Many biological reactions will progress in the absence of a catalyst, but their speed will be too slow to maintain life. Let's take a look at a hypothetical reaction in which

$$A + B <=> C + D$$

The reaction will eventually reach an equilibrium, that can be described by an equilibrium constant, K_{eq}. For the above reaction,

$$K_{eq} = \frac{[C]\,[D]}{[A]\,[B]}$$

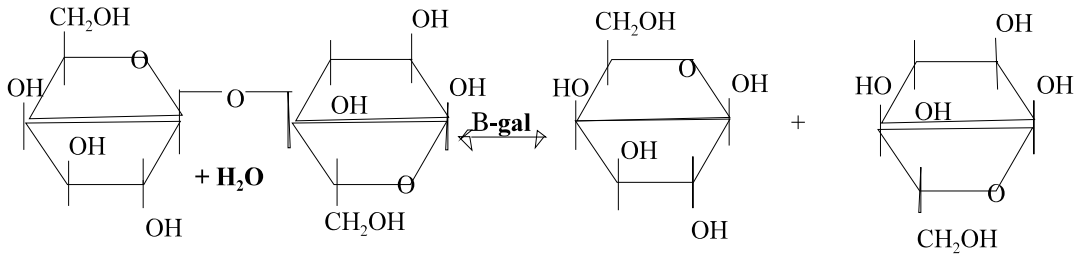
whereby [A], [B], [C] and [D] are the concentrations of A, B, C and D at equilibrium.

This hypothetical reaction may take hours or even days or years to reach equilibrium, but the presence of a catalyst can speed the reaction by many orders of magnitude such that equilibrium is reached in much less time. However, the presence of a catalyst **does not** change the K_{eq} of a chemical reaction, only the speed at which equilibrium is reached.

Today, you will be looking at the characteristics of a biological catalyst, or enzyme. Enzymes are usually proteins, although some RNAs are now known also to act as catalysts in biological reactions. You will investigate the functional properties of the enzyme β-galactosidase and calculate its reaction rate. Next week, you will perform experiments of your own design on the temperature and pH sensitivity of this enzyme. Both weeks, you will be using β-galactosidase from the gut bacteria *Escherichia coli.*

The Reaction:

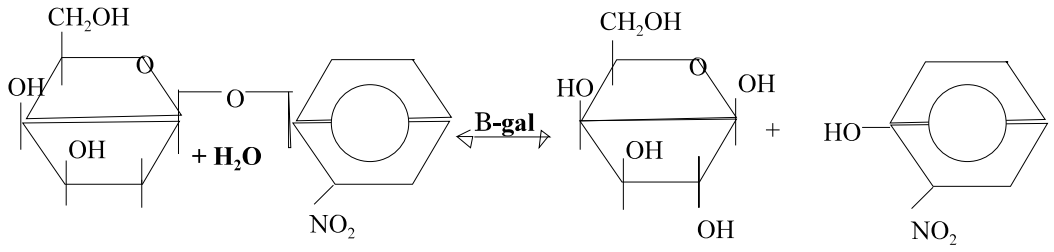
What is the function of β-galactosidase? β-galactosidase catalyzes the breakdown of the substrate lactose, a disaccharide sugar found in milk into two monosaccharide sugars, galactose and glucose. The oxygen bridge connecting the two sides of the lactose molecule is cleaved through the addition of a water molecule. The addition of the water molecule is known as **hydrolysis**.



Lactose Beta Galactose + Alpha Glucose

The Assay:

Since it is difficult to assay for the activity of β-galactosidase when lactose is the substrate, we will use the lactose analog **ONPG** (o-nitrophenyl β-D-galactopyranoside) in our experiments. Note the similarity in the structure of ONPG and lactose. The enzyme does not distinguish between lactose and ONPG, and cleaves the oxygen bridge between the two sides of the molecule, resulting in the products galactose and the o-nitrophenol . Note how this is similar to the production of galactose and glucolse when lactose is hydrolyzed.



ONPG (colorless) Beta Galactose **ONP** (yellow)

The advantage of using ONPG as the substrate is that it is relatively easy to determine the amount of ONPG cleaved by using a spectrometric assay. ONPG is colorless, but the product **ONP** (o-nitrophenol) is yellow, so that as the β-galactosidase continues to work, more and more ONPG is degraded, and the solution turns more and more yellow. By measuring the rate at which the color intensity increases we can calculate the activity of the enzyme. In your assays today, you will be using Na_2CO_3 to stop the enzyme by making the solution too basic for the enzyme to function. (Why might the pH of the solution affect enzyme activity?) You will then use the spectrophotometer to measure the O.D. (optical density) or absorbance of the solution. The more yellow the solution, the more ONPG has been degraded, and higher the absorbance. Using this basic technique, you will do experiments to assay the activity of the enzyme over time.

II. The Questions:

If you added β-galactosidase to a solution of pure ONPG, which direction would the reaction run? Would it catalyze the hydrolysis of ONPG at a constant rate? Or would the reaction rate change over time? Why? **Write down below your hypotheses and your justification.**

How would the O.D. of the solution change over time? **Sketch below a simple plot of O.D. vs. time to show what you think will happen.**

III. The Procedures:

Each table will be given a tube of β-galactosidase solution with a concentration of $1.4x10^{12}$ molecules/ml (0.5 units activity/ml). You will work in pairs. This week you will create a standard curve (part A) and then calculate the maximum speed of the enzyme at room temperature (part B). Make sure you understand what is happening in experiment. Discuss what you think is happening at each step. Once the data are entered, each pair should do **all** the calculations and make a figure. You will then be ready to move on to designing experiments to explore the effect of temperature and pH on the activity of β-galactosidase. These experiments will be conducted next week.

When using Minitab to analyze your data, save the ***data*** on the H drive so they can be used later. **Place the data for each experiment in a different file.** This will allow you to reuse column names. To help you keep track of things use the part of the exercise as the file name (e.g. PartA.mtw). Make sure you clearly identify the type of data in each column.

A. Standard Curve

In order to accurately measure the activity of the enzyme, it will be necessary to construct a standard curve linking absorbance readings to known concentrations of ONP. These concentrations are millimolar (mM). This means that **a liter of 1mM ONP contains 6.023×10^{20} molecules**. The solutions you will use for the standard curve were made by diluting 2.5mM ONP in a pH 7.7 buffer. Although the solutions for creating a standard curve have already been made for you. It is important to understand how this was done. Assume that you will need 10 ml of each concentration to make a standard curve. The stock solution will contain 2.5mM ONP. Record in the table below the dilution factor required to make each concentration, and the amount of stock solution and buffer that will be required. The formulas below will help you with the calculations

Table 1. Preparation of ONP solutions with known concentrations for use in making a standard curve.

Desired Concentration (mM)	Dilution factor[1]	ml 2.5 mM ONP Stock Solution[3]	ml pH 7.7 Buffer[4]
.000	-	-	
.025			
.050			
.075			
.100			
.125			
.150			
.200			
.250			

Below you are walked through the thought processes that allow you to make dilutions of a stock solution.

1. Calculate the dilution factor.

$$\textbf{Dilution factor} = \frac{\textbf{Concentration of Stock Solution}}{\textbf{Concentration of Final Solution}}$$

2. Determine the final volume you will need (in this case 10 ml).

3. Determine how much stock solution it will take to achieve the correct dilution factor.

$$\textbf{Volume of Stock Solution} = \frac{\textbf{Final Volume}}{\textbf{Dilution Factor}}$$

4. Determine how much buffer to add.

$$\textbf{Volume of Buffer} = \textbf{Final Volume} - \textbf{Volume of Stock}$$

To save time the dilutions shown on the previous page have already been made.

In your experiments you will stop the reaction by diluting the reacting solution with an equal amount of Na_2CO_3. Therefore to create a standard curve that allows you to determine the actual concentration of the reacting solution you will need to dilute the ONP standard 1:1 with the stop solution (0.1M Na_2CO_3). This has not been done for you.

The standard curve you create will allow you to determine the concentration of an unknown solution if its absorbance is known. How would your estimate of the concentration of ONP in the experimental solution be affected if you failed to make a 1:1 dilution of the ONP stock with Na_2CO_3 when creating the standard curve?

Follow these steps to create the standard curve.

1. Label the top of a cuvette with the concentration of the appropriate ONP solution.

2. To each of the cuvettes, add 1.5 ml of the appropriate ONP standard.

3. To each of the cuvettes, add 1.5ml of the stop solution (0.1M Na_2CO_3), cover with parafilm and mix the contents by inverting.

4. Carefully clean the sides of the cuvettes just before reading them in the spectrophotometer

5. Set the spectrophotometer to 420nm (the wavelength at which the absorbances will be measured).

6. Calibrate the spectrophotometer with a blank. (0.1M Na_2CO_3 1:1 with pH 7.7 buffer)

How does the blank differ from the other cuvettes? Why calibrate with a blank? Write down your answer below.

7. Read the absorbance for each standard and record below.

Table 2. Absorbance of known concentrations of ONP at 420nm.

ONP (mM)	0	0.025	0.050	0.075	0.100	0.125	0.150	0.200	0.250
Absorbance									

Data Analysis:

By graphing the relationship between a known concentration of ONP and its absorbance you can determine the ONP concentration in an unknown solution if the absorbance is known. Use MINITAB to plot this relationship.

1. Enter the concentration data into **C1** and the absorbance data into **C2**.
2. Before plotting the data you must determine which variable will be on the x-axis and which variable will be on the y-axis. Later in the experiment you will want to determine the concentration from a known absorbance. The independent variable goes on the x axis and the dependent variable goes on the y axis. **What should be on the x-axis?**

To graph the data:

 **Graph** ➛ **Plot** ➛ *(select y variable)* ➛ *(select x variable)* ➛ **Frame** ➛ **Tick** ➛ *(set size to 0.8)* ➛ **Ok** ➛ **Ok** ➛ **Edit** ➛ *(activate both tool bars, add a caption, and adjust font size)*

Note that the relationship between absorbance and concentration is linear. The line can be defined by its intercept on the y axis and its slope. The general form of the equation is shown below.

Concentration = y intercept + slope * absorbance

Minitab can be used to calculate a regression equation that describes this linear relationship. When a regression is done the printout will give the slope and intercept for the line. In addition the regression determines the degree to which the data fit the line by calculating the coefficient of determination (r^2) The coefficient of determination indicates the amount of variance explained by the line. This can range from 0-100% The closer to 100% the better the fit. The analysis of variance test reported with this statistic tests to see if r^2 is significantly different from zero. Determine the regression equation for the line in your plot.

To calculate a regression:

 **Stats** ➛ **Regression** ➛ **(***optional* Fitted Line Plot**) Response** *(conc or C1)* ➛ **Predictor** ➛ *(absorb or C2)* ➛ **Ok**

Scroll backwards in the session window to find the regression equation and write it down so you have it for reference. This equation can be used during the rest of the laboratory to determine the amount of ONP produced under different conditions.

Regression Equation: Conc = _____ + _____ * absorbance

B. Measuring the Reaction Rate Over Time at Room Temperature

1. Record the room temperature _____°C

2. Label 10 test tubes with times at 1 min. intervals, begin at 1 min. and end at 10 min.

3. Fill each test tube with 2.0 ml of 0.1M Na_2CO_3 - "stop" solution. (The Na_2CO_3 will stop the enzymatic reaction.)

4. Set up a large reaction tube with 20.0 ml of phosphate buffer and 2.5 ml of 2.5mM ONPG.

5. Start the reaction by adding 2.5 ml of enzyme stock solution, and vortex quickly.

6. At 1 min. intervals, remove 2.0 ml of the reaction mixture and add it to the stop solution in the appropriately labeled tube. Vortex the stop solution/enzyme mixture to stop the reaction completely. Think about the timing of the removal of the enzyme mixture from the reaction tube. **Which is more crucial, the time at which you remove the aliquot, or the time you add the aliquot to the stop solution? Why? Write down your answer below.**

7. Read each of the 10 tubes at 420nm against a blank tube consisting of 2.0 ml 0.1M Na_2CO_3, 1.6 ml phosphate buffer, 0.20 ml ONPG and 0.20 ml distilled water.

Table 3. The effect of time on the absorbance (420nm) of a solution of ONPG and β-galactosidase.

Time (min.)	1	2	3	4	5	6	7	8	9	10
Absorbance										
ONP (mM)										

Data Analysis:

Calculate the concentration of ONP in each of the tubes by using the y intercept and slope from the **regression equation determined in <u>part A</u>.**

(calculated ONP conc) = _____*(y intercept)* + _____ *(slope)* * C1 *(abs data from experiment)*

(Instructions for the calculation continue on the following page)

1. Enter the absorbance data into C1 of a Minitab data file.

2. Use the ***Calc Menu*** to calculate the ONP concentration (mM).

Calc ➛Calculator ➛ Store variable in *(Conc or C2)* ➛ **Expression (# + # *C1)**. (*In writing the expression substitute the appropriate numbers from above.)*

Record the mM concentration of ONP in the table above.

3. Graph the change in ONP concentration as a function of time (see part A, page 68). **Hint**: you will need to enter the times into an empty column, **e.g. C3**

Calculate enzyme activity

The slope (Δconc/Δtime) of the initially linear portion of the plot indirectly represents the maximum rate of substrate hydrolysis as a function of time. Your plot shows the effect of time on the concentration of ONP. From your graph determine a time interval where the rate of the reaction is linear then use the data from Table 2 to determine the rate of the reaction during that interval. The rate of the reaction is the change in concentration / change in time (Δconc/Δtime) and is expressed in **mM ONP/min.**

Indicate the time interval where the reaction rate was linear _____.

Calculate the reaction rate during this interval (Δconc/Δtime) **_____ mM/min**

For each molecule of ONP that was produced one molecule of ONPG was hydrolyzed. From the slope it is possible to calculate the number of ONPG molecules hydrolyzed/min/enzyme molecule. To do this it is necessary to understand the relationship between the concentration of a solution and the number of molecules in a given volume. For any given concentration the number of molecules present will be directly related to the volume of the solution.

By definition a 1mM solution = 1 mmol/liter and has 6.023×10^{20} molecules.

The formula given below can be used to calculate the rate of hydrolysis of ONPG in molecules/min.

$$\frac{\textbf{mmol/liter/min} * \textbf{0.001 liter/ml}}{\text{a}} * \frac{\textbf{ml}}{\text{b}} * \frac{\textbf{6.023 x } 10^{20} \textbf{ molecules/mmol}}{\text{c}}$$

To help understand the calculation you can do it in several steps (a-c). **Show your work!**

a) From the above definition note that the rate of the reaction in **mM/min** is the same as **mmol/liter/min.** Use **formula a** to convert **mmol/liter/min** to **mmol/ml/min**.

b) If you know the number of **mmol/ml/min** (a) being produced you can calculate the total number of **mmoles** produced per minute by multiplying by the total volume of material (b) in the **large** reaction tube after the enzyme was added. Use the result from **a * b** to calculate the total number of **mmol/min**.

c) Now that you have determined the total number of **mmol/min** you can convert this to the number of molecules of ONP produced per minute. Use the result from **b * c** to convert mmoles to **molecules/min**.

d) The next question is how many enzyme molecules were present in the reaction tube? Your enzyme stock solution has 1.4 x 10^{12} molecules/ml. **How many ml of enzyme did you add to the reaction tube? _____ How many molecules? _____**

e) Now that you know the number of molecules of ONP produced/min (part c) and the number of enzyme molecules present (part d), determine the number of molecules of ONPG hydrolyzed/min/molecule of enzyme.

Assignment

Do all the calculations that are outlined in the text of the lab, fill in the tables, answer the questions in **bold** type**,** and make sure your minitab graphs are properly labeled. Then proceed to planning for next week.

The Experimental Design for Next Week:

Next week, you will be characterizing the temperature and pH sensitivities of β-galactosidase from the bacteria *Escherichia coli*. *E. coli* lives in the gut where the temperature is approximately 37° C and the pH is 7.3-7.7. **Do you think that the** β-galactosidase **will catalyze the reaction at the same speed across a range of temperatures (0°C, 20°C, 37°C, 55°C)? Why or why not? If you think that there will be a difference in speed, at which temperature(s) do you believe that the enzyme will work best. Write down your hypothesis.**

What about pH? You will have buffers with pH's of 6.0; 6.6; 7.2; 7.7; 8.0; 8.3; and 9.0 available for your experiment. At what pH(s) do you think the enzyme will work fastest? Why? Write down your hypothesis.

Would you expect the β-galactosidase from a soil bacterium to have the same pH and temperature sensitivities as β-galactosidase from *E. coli*? Why? Why not?

Now that you have set down your hypotheses, it's time for you and your partner to design experiments to test them. Each pair should design experiments to test the hypotheses about the effects of pH and temperature on the activity of the enzyme. The experiments will be conducted next week.

How to go about designing the experiments? Here are some tips.....

- Start with an 8:1:1 ratio of buffer:ONPG:enzyme solutions. **You will want the final volume of the mixture to be 2ml.**

- For stopping the reaction, use a 1:1 ratio of stop solution (0.1M Na_2CO_3) to the enzyme reaction mixture.

In the table below indicate how much of each solution will be put into each test tube for each treatment.

Table 4. Standard quantities of reagents for determining β-galactosidase activity.

Buffer	ONPG	Enzyme	Total Volume

Amount of NA_2 CO_3 you will add to stop each reaction _____ml

Now, think about how you will be reading the O.D. of the different test solutions. **What will be the composition of your BLANK solution? Consider that the enzyme solution was prepared in distilled water. Record the composition of the BLANK below.**

Design for the temperature experiment

Now, think about the sequence of events by which you will be doing the temperature experiment. What order should the ingredients be mixed in? Should the buffer/ONPG mixture be allowed to equilibrate to the experimental temperature before the enzyme stock is added? If so how long? How long will the reaction be allowed to run before it is stopped? By staggering the start of the different treatments you should be able to run these simultaneously. **In the space below, write down the step by step protocol that you will be following for each of the temperature treatments.**

Design for the pH experiment

Now consider the question of pH. How will the procedure used in the temperature experiment be modified? You have 7 pHs available for testing — do you need to test all of them to evaluate your hypothesis? Determine the pHs that you will use and outline the procedure you will follow. Since this will be done at room temperature you can significantly shorten the incubation time. To avoid confusion you will probably want to run one test tube at a time. When doing this experiment pay careful attention to the color of the solutions at the time the stop solution is added and record the relative color. Just before reading the samples, note any color changes.

Have your instructor check the experimental design before you proceed!

C. Reaction Rate of β-galactosidase at Different Temperatures

Table 5. The effect of temperature on the absorbance of ONPG and β galactosidase mixtures after a _____ minute incubation at different temperatures.

Temp. (°C)	0	Room ____	37	55
Absorbance (420nm)				

Data Analysis:

1. Enter the absorbance into C1 of a new Minitab data file.

2. Use the ***Calc Menu*** and the regression equation from part A last week to calculate the **mM** concentration of ONP. Store the calculation in **C2.** (See page 64)

3. Use ***Calc Menu*** and the formula given below to convert the ONP concentration (mM) to molecules/min at each temperature. Store these data in **C3** and record above. When entering the expression (*the portion of the equation to the right of the = sign*) make sure you include the parentheses.

ONP conc (molecules/min) = (C2/x) * (.001) * y * 6.023*1020**

C2	= **mM** concentration of ONP
x	= time (min) required to reach concentration in C2
.001	= converts mM to mmol/ml
y	= ml in the reaction flask
6.023×10^{20}	= converts mmol/ml to molecules

4. Now determine how many molecules of enzyme were used by multiplying the ml of enzyme added to the flask by 1.4×10^{12}. Record here _____. Now divide the values in **C3** by this value to determine the activity of the enzyme at different temperatures. Store these data in **C4** (Molecules of ONP/min/molecule of β galactosidase)

5. Use **Excel** to graph the relationship between temperature and enzyme activity. Open Excel and enter the temperature data, then paste the activity data from Minitab (**C4** Molecules of ONP/min/molecule of β galactosidase) into Excel and make your graph.

D. The Effect of pH on β-galactosidase Activity

Table 6. The effect of pH on the absorbance of a mixture of ONPG and β galactosidase after minutes.

pH	6.0	6.6	7.2	7.7	8.0	8.3	9.0
Absorbance (420nm)							

Data Analysis:

1. Enter the absorbance into **C1** of a new Minitab data file.

2. Use the ***Calc Menu*** and the regression equation from part A to calculate the **mM** concentration of ONP. **C2**. (See page 64)

3. Use the ***Calc Menu*** and the formula given below to convert the **mM** ONP concentration to **molecules/min** for each pH. Store the results in **C3** and record above. When entering the expression (the portion of the equation to the right of the = sign) make sure you include the parentheses.

ONP conc (molecules/min) = (C2/x) * (.001) * y * 6.023*1020**

C2	= **mM** concentration of ONP
x	= time (min) required to reach concentration in C2
.001	= converts mM to mmol/ml
y	= ml in the reaction flask
6.023×10^{20}	= converts mmol/ml to molecules

4. Now determine how many molecules of enzyme were used by multiplying the ml of enzyme added to the flask by 1.4×10^{12}. Record here _____. Now divide the values in **C3** by this value to determine the activity of the enzyme at different pHs. Store these data in **C4** (Molecules of ONP/min/molecule of β galactosidase)

5. Use **Excel** to graph the relationship between pH and enzyme activity. Open Excel and enter the pH data, then paste the activity data from Minitab (**C4** Molecules of ONP/min/molecule of β galactosidase) into Excel and make your graph.

Assignment

Write a **brief** results and discussion section to summarize your findings on the effects of time, temperature and pH on the activity of β galactosidase. The results section should contain data from both weeks.

- Carefully edit the figures so that they can stand alone.

- The text section should parenthetically refer to the figures you have created. Begin with the number of molecules of ONPG hydrolyzed by each molecule of enzyme each minute. Then consider the effects of **time, temperature and pH** on the activity of the enzyme. In writing this section keep in mind that for each molecule of ONP produced one molecule of ONPG is hydrolyzed. Try to make comparisons between different treatments in terms of molecules of ONPG hydrolyzed/min/molecule of β galactosidase or molecules of ONP produced/min/molecule of β galactosidase (i.e., tell how much faster the reaction progresses at room temperature than at 0^{o}C).

Write a brief discussion section that *interprets* your results and indicates whether they support your original hypotheses. You might try to integrate answers to the following questions when writing this section.

- The slope of your plot of ONP concentration over time should be linear. **What does this mean?**

- As the reaction proceeds, the rate of increase in product diminishes, and the relationship between concentration and time becomes non-linear. **Why might this occur?**

- Does the result of the temperature experiment support your hypothesis? **Why does temperature alter the rate of the reaction?**

- Does the result of the pH experiment support your hypothesis? **Was this what you expected?**

If the results of the pH experiment differed from what you expected, think about each step of the experiment and what type of error might have affected your results. Choose your favorite hypothesis and explain how you would test it.

Excluding figures this assignment should not be more than 2-3 pages.

This experiment adapted from: Straus (1991)
Pew Undergraduate Laboratories in Biology