

Enzyme Kinetics: Properties of β -Galactosidase

Preparation for Laboratory: Read the introduction to this laboratory before doing the **Web Tutorial - Beta Galactosidase**. Additional background: Freeman, skim pp 43-58 for a review of protein structure.

I. INTRODUCTION

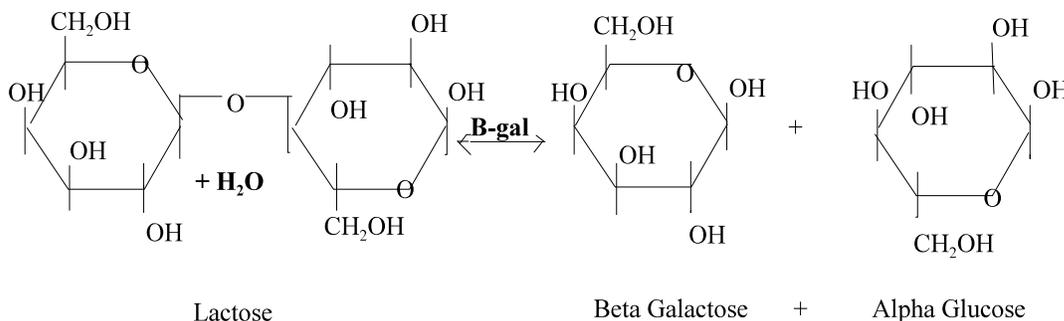
Enzymes: Read Freeman - enzyme action 55-65 before proceeding.

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.

For the next two weeks we 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, determine the reaction rate, and design and conduct an experiment on the potential interaction between pH and temperature on the sensitivity of this enzyme. You will be using β -galactosidase isolated from the gut 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 the enzyme will have the same efficiency over a range of temperatures and pHs?

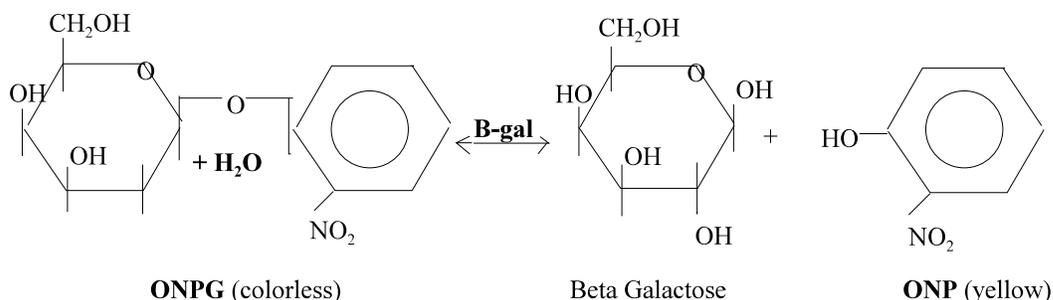
The Reaction:

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**.



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 glucose when lactose is hydrolyzed.



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. Optical density (OD) is a measure of the amount of light absorbed by a solution. O-nitrophenol, the yellow product of the breakdown of ONPG, absorbs light maximally at 420nm. Thus OD 420 in this experiment is a measure of the amount of O-nitrophenol present in the sample and can be used to calculate β -galactosidase activity. The more yellow the solution, the more ONPG has been degraded, and higher the absorbance. In your assays today, you will be using Na₂CO₃ 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?)

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? How would the O.D. of the solution change over time? **Write your hypotheses and sketch a simple plot of O.D. vs. time to show what you think will happen. Briefly justify your answer.**

The tutorial will provide background that will allow the class to design an experiment to investigate the effect of pH and temperature on the activity of β -galactosidase, 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.

As part of the tutorial you will create a standard curve that describes the relationship between absorbance and the concentration of ONP. Record the equations for the relationship below.

Standard Curve Regression Equation: $\text{Conc} = \underline{\hspace{2cm}} + \underline{\hspace{2cm}} * \text{absorbance}$

Data from 2008 is provided in the tutorial so you can also graph the relationship between time and the activity of β -galactosidase at room temperature as measured by the concentration of ONP in the reacting mixture.

Enzyme activity : $\text{Conc (ONP)} = \underline{\hspace{2cm}} + \underline{\hspace{2cm}} * \text{time}$

Make sure you understand the relationships shown by both graphs. Come to class prepared to design and conduct an experiment on the potential interaction of pH and temperature on the activity of β -galactosidase.

Now proceed to the tutorial.

II. Experimental Design:

When designing experiments to explore the potential interaction between two variables it is important to make sure that a complete design is used. This means that all combinations of the two variables of interest are paired. For this experiment we will use two variables temperature and pH in a 2x2, 2x3, 3x3 or 2x4 design. You have the following choice of pHs: 6, 7, 7.5, 8) and temperatures (0°, ~21°, 37°, 55° C). As a class you will discuss the design and fill in the table below to make sure the design is complete.

Table 1. Experimental design to determine if pH and temperature have an interactive effect on β -galactosidase activity.

	Temperature			
pH				

Setting up the reactions:

Each table will be given a tube of β -galactosidase solution with a concentration of 1.4×10^{12} molecules/ml (0.5 units activity/ml). You will work in pairs.

Room temperature _____ Duration of reaction: _____

- Label the number of test tubes required for your experiment with the pH and temperature
- Add the appropriate buffer and 2.5 mM ONPG
- Equilibrate to the selected temperature for _____ minutes
- Start the reaction by adding β -galactosidase and record the time each test tube is started
- Keep a record of when each reacting mixture should be stopped by adding Na_2CO_3
- Stop the reaction
- Read the absorbance at 420nm using the appropriate pH blank

Start with an 8:1:1 ratio of buffer:ONPG:enzyme. **You will want the final volume of the mixture to be 2ml (2000 μl).** Do your calculations in microliters (μl) and put them in Table 1.

Table 1. Standard quantities of reagents for preparing testtubes to determine β -galactosidase activity.

Buffer	ONPG	Enzyme	Total Volume
μl	μl	μl	μl

A 1:1 ratio of 0.5M Na_2CO_3 : reacting enzyme mixture is used to stop the reaction.

Amount of Na_2CO_3 needed to stop each reaction _____ μl or _____ ml

A blank is used to calibrate the spectrophotometer for O.D. 420nm readings. The blank contains everything except the thing that you want to measure and does not contain the enzyme. The enzyme was prepared using distilled water. You will prepare a blank for **each** of the pH buffers in a cuvette. Record volumes in Table 2.

Table 2. Standard quantities for preparing the blank

Buffer	ONPG	Distilled water	Na_2CO_3	Total Volume
μl	μl	μl	μl	2000 μl

Before setting up the reactions check with the instructor to make sure your calculations are correct.

Reaction Rate of β -galactosidase at Different Temperatures and pHs

Record your data in table 3.

Table 3. The effect of temperature and pH on the absorbance of ONPG and β galactosidase mixtures after a _____ minute incubation. Fill in the pH and Temperatures used. Create a combined code column that will allow you to sort the data. (Eg. 0pH6, 0pH7 etc.)

Absorbance 420nm	pH	Temperature	Combined

A. Calculation of the rate of the reaction using data from the tutorial

As part of the tutorial you graphed the relationship between ONP concentration and time using data from 2008. The slope ($\Delta\text{conc}/\Delta\text{time}$) of linear portion of the plot indirectly represents the maximum rate of ONPG hydrolysis as a function of time. For every molecule of ONPG hydrolyzed one molecule of ONP is produced. Your plot shows the effect of reaction time on the concentration of ONP in the reacting mixture. The rate of the reaction is the change in concentration / change in time ($\Delta\text{conc}/\Delta\text{time}$) and is expressed in **mM ONP/min.**

Since the reaction was linear, the slope of the regression curve represents the rate of the reaction in mM/min.

The room temperature reaction rate from the tutorial was _____ **mM/min**

Using the slope of the regression curve 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.

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

To help understand the calculation you can do it in several steps (a-c). The following calculations are based on a reaction containing 20ml of buffer, 2.5ml ONPG and 2.5ml of β galactosidase for a total volume of 25ml. **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 reaction tube after the enzyme was added. The total volume of material used for the example in the tutorial was **25ml**. Multiply 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? The enzyme stock solution had 1.4×10^{13} molecules/ml. **To start the reaction 2.5 ml of β galactosidase were added to the reacting mixture. How many molecules of β galactosidase were added to start the reaction? _____**

- 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. This is the **reaction rate**.

B. Data Analysis: make sure you understand part A before doing this analysis

1. Enter your data and code columns into C1-C4 of the class Minitab worksheet located at p:class\biology\biol110\b-gal.mtw. Do not proceed to analysis until the entire class has added their data.
2. Use the *Calc Menu* and the regression equation for the standard curve to calculate the **mM** concentration of ONP. Store the calculation in **C5**.
3. Use *Calc Menu* and the formula given below to convert the ONP concentration (mM) to molecules/min. Store these data in **C6**. When entering the expression (*the portion of the equation to the right of the = sign*) make sure you include the parentheses.

$$\text{ONP conc (molecules/min)} = (\text{C5}/x) * (.001) * y * 6.023*10^{20}$$

- C5 = **mM** concentration of ONP
- x = time (min) required to reach concentration in C5
- .001 = converts mM to mmol/ml
- y = ml in the reaction flask before adding NA_2CO_3
- 6.023×10^{20} = converts mmol/ml to molecules

4. Determine how many molecules of enzyme were used by multiplying the .2 ml (200 μl) of enzyme added to the flask by 1.4×10^{13} molecules/ml. Record here _____. Now divide the values in **C6** by this value to determine the activity of the enzyme at different temperatures and pHs. Store these data in **C7** (Molecules of ONP/min/molecule of β galactosidase)
5. Do the appropriate statistical test to determine if the two variables had and interactive effect on the rate of the reaction.
6. Visualize the reaction by doing an interaction plot. To decide which variable you want on the x axis and which variable you want to have as the series that make the lines graph the data twice. You will recreate one of these figures in Excel for your final report.
7. Use the **combined** code column and **Store Descriptive Stats** to determine the mean and SEM for each treatment. Copy and paste these data into Excel.
8. Use **Excel** to make a final plot on the effect of temperature and pH on enzyme activity. Instructions for doing this are included in the Appendix of this exercise.

Assignment

The analysis you are doing this week will become a component of the final paper you write on the properties of β -galactosidase. It will be evaluated and returned for inclusion into your final paper.

- Introduction: briefly state your hypotheses concerning pH and temperature,
- Methods: outline the experimental design, refer to manual for methods,
- Results: carefully edit your graph so it can stand alone and include a brief text,
- Discussion: *interpret* your results and indicate whether they support your original hypotheses. Use your text to help explain your results. Keep in mind this would not be done if writing a paper for publication.

Preparation for next week: Web tutorial - Lac Operon

Appendix: Once the data are in Excel you can make a graph without reorganizing the data.

Making the Graph: Your data will probably be organized something like the chart below

Condition	Means	Error
0pH6		
20pH6		
30pH6		
0pH7		
20pH7		
30pH7		

- Insert ► scatter chart with points connected by a straight line
- **RC** inside the box ► **select data**
- **Series name** (Enter either the pH level or temperature that makes up the first line)
- **x-axis** (Enter the numbers that will make up the x-axis with a **comma** between them)
- **y-axis** (Sequentially highlight the cell that corresponds to each data point ► **LC** ► add a comma ► continue to the next data point.
- If you make an error **RC** on the chart ► **select data** ► **edit**

Adding Error Bars

- Highlight the line to which you want to add error bars ► **Layout** ► **Error Bars** ► **More Error Bars** ► Add error bars based on series ► **Select Series** ► **OK** ► **Custom** ► **Specify Value** ► Highlight each positive value for the series with a **comma** between each ► highlight the negative values ► **OK**. The program will automatically add x-error bars. Carefully highlight one of the x error bars ► **Delete**. If you end up deleting your line **Ctrl z** will bring it back. Try deleting the x error bars again.
- Add y-error bars to each series