Exploring Enzyme Function With the Lactase Enzyme

OBJECTIVES:

In this laboratory exploration, you will
- To continue to practice to apply hypothesis testing.
- To continue to practice experimental design.
- To gain a better understanding of enzymes and some conditions (temperature, pH, and enzyme and substrate concentration) that affect enzyme activity and the rate of an enzyme-catalyzed reaction.
- To learn the concepts behind the workings of the spectrophotometer.
- To learn to use the spectrophotometer.
- To understand these terms: enzyme, enzyme activity, active site, substrate, enzyme-substrate complex, product, denature, variable, control.

INTRODUCTION

In this week’s lab we’ll be exploring enzyme function using a familiar enzyme, lactase. You may be most familiar with lactase for its role in lactose intolerance in humans. Lactose is a disaccharide found in milk and many other dairy products. Almost all of us produce the enzyme, lactase, required to break down this sugar as infants. When broken down, these sugars can then be used to produce energy in cellular respiration.

Most of the world’s population, however, stops making lactase during their first few years of life. If you no longer produce functional lactase, you cannot break down lactose and typically experience abdominal bloating and cramps after consuming milk and other dairy products; this is commonly called lactose intolerance. A small fraction of the world’s population, however, has inherited a different promoter for this lactase gene (more on promoters in a few weeks) allowing them to make functional lactase throughout their lives. We hypothesize that this occurred as a result of natural selection in populations that farmed cattle and thus drank milk throughout their lives.

As you may know, individuals with lactose intolerance often take over-the-counter pills (like Lactaid) to help them properly digest milk products. These pills are really just lactase enzymes! In our work today, we’ll investigate the activity of several of these commercial preparations of lactase.

To do this, we’ll need a method to quantify the activity of the lactase tablets. We’ll use an analog of lactose known as ONPG (Ortho-nitro-phenyl galactoside). ONPG is similar enough to the size and shape of the lactase molecule to bind at the active site of the lactase enzyme. The ONPG molecule, shown at right, contains a galactose sugar covalently bound to the molecule ONP. The lactase enzyme catalyzes the breaking of this bond, releasing ONP and galactose as products. Conveniently, ONP is bright yellow once it has been removed from the ONPG molecule. Thus we can monitor the change in color in our experiment to measure lactase activity. Specifically, we’ll use a spectrophotometer to measure the absorbance of light in our reactions, and thus the activity of our enzyme.

SUPPLIES: Your group will need the following supplies:
- One lactase tablet
- 20 mM o-nitrophenyl-β-D galactoside (1.5 mL ONPG)
- 0.1 M PO₄ buffer, pH 7.0 (6 mL)
- 0.01 M PO₄ buffer, pH 7.0 (120 mL)
- 4% K₂CO₃ (12 mL)
- Plastic pipettes
• micropipette kit (Your instructor will demonstrate proper technique for the accurate use of micropipettes. Be sure to take good notes in your lab notebook!)
• 37 °C water bath
• 150 ml beaker
• 100 ml grad cylinder
• 10 ml grad cylinder
• Parafilm
• Test tubes
• Pen
• Ice
• Laptop, spectrophotometer, cuvettes, and rack (again, take good notes on the proper use of this instrument!)
• Mortar and Pestle

PROCEDURE:
1. a: Record the brand of lactase, labeled number of units of lactase/tablet and the expiration date.
   b: Weigh one lactase tablet, note whether 9,000 FCC or 3,000 FCC units/tablet.
   c: Grind in a mortar and pestle until finely ground.
   d: Suspend/dissolve to 100 units/mL: Grind a tablet in about 5 mL of chilled 0.01 M PO4 buffer, pH 7.
      (Solution will be cloudy because of undissolved binder.)
      For 9,000 unit tabs, bring up in 90 mL with same buffer, including mortar and pestle rinses.
      For 3,000 unit tab, bring up in 30 mL with same buffer, including mortar and pestle rinses.
   e: Dilute 1:30: Add 0.2 mL of enzyme suspension into 5.8 mL 0.01 M PO4 in a 16 x 150 mm tube.

2. Prepare reaction mix (Rxn Mix) per assay set:
   5.6 mL 0.1 M PO4 pH 7 buffer
   1.4 mL 20 mM ONPG

3. Set up a series of numbered 13x100 mm test tubes as follows.
   Add water to the tubes first, then RxnMix. *(But not the enzyme yet!!*)

<table>
<thead>
<tr>
<th>tube</th>
<th>mL dH2O</th>
<th>mL Reaction Mix</th>
<th>µL diluted enzyme</th>
<th>A 450</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.00</td>
<td>1.0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>0.975</td>
<td>1.0</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>0.950</td>
<td>1.0</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>0.900</td>
<td>1.0</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>0.800</td>
<td>1.0</td>
<td>200</td>
<td></td>
</tr>
</tbody>
</table>

4. Pre-warm these tubes in a 37 °C water bath for two minutes.
5. At 30 second intervals, add listed µL of enzyme, vortex, start a stopwatch with 1st tube. Place in 37°C water bath.
6. After exactly 15 minutes, add 1.0 mL 4% K2 CO3 down the side of the first tube, mix and remove from hot block. At 30 second intervals, repeat 4% K2 CO3 addition for each of the successive tubes, mix and set aside.
7. Read the absorbency at 450 nm in the spectrophotometer, as described below. Record the results in your notebook.

USING THE VERNIER SPECTROVIS SPECTROPHOTOMETER
1. Turn on computer and attach SpectoVis via the USB cable.
2. In order to begin, click on the Logger Pro 3.7 icon on the desktop.
3. Once the software is up and running it is necessary to calibrate the Spectrophotometer.
   • To calibrate the SpectroVis, choose Calibrate Spectrometer:1 from the Experiment menu.
   • Fill a cuvette about ¾ full with distilled water and place it in the sample chamber of the SpectroVis.
   • Follow the instructions in the dialog box to complete the calibration, and then click OK.
4. Click on the Configure Spectrometer Data Collection button
5. Select Abs vs. Concentration as the collection mode.
6. Change the Column Name to “Sample #”, the Short Name to “sample”, and delete the Units.
7. Select 450 nm by clicking on the box next to the number, then click OK.
8. Insert your sample into the sample holder and click collect. When reading stabilizes, click keep.
9. Enter Sample # and click ok.
10. Repeat steps 8 and 9 until all samples have been read on the spectrometer.
11. Click Stop to end data collection.
12. In order to work with the data it will be necessary to transfer the data to Excel. The easiest way to do this is to cut and paste the data from the table into Excel. Save your Excel file on a USB drive or email the file to yourself to work with later. (Be sure a copy of this data ends up in your lab notebook!!)

OPTIONAL EXTENSION:
If time and supplies permit, your instructor may ask you to design and conduct a brief experiment to test the influence of either pH or temperature on lactase activity. Again, these additional experiments should be carefully recorded in your lab notebook. Be prepared to share the results of your investigation with the class.

PRE-LAB: Answer the following questions on a separate piece of paper before coming to lab this week. Pre-labs are due at the start of your lab class. Late pre-labs will not be accepted. In addition (as always!) prepare your lab notebook by writing in a title, objective, and your methods.

1. We could measure the activity of the lactase enzyme by incubating our enzyme preparations with the sugar lactose. Why is it important and useful to use ONPG instead?
2. In this experiment, we’ll incubate our samples at 37°C. Is this the temperature at which you’d expect lactase to have the greatest activity? Why or why not?

POST-LAB: In this fictional scenario, you’ve been asked to validate the activity of a particular brand of commercially available lactase. Prepare a brief memo to your supervisor that describes the activity of the lactase preparation, along with the methods you used to determine this activity. Your memo should also include a graph of enzyme absorbance versus enzyme units. As with any figure you include in a report, be sure the text of your method specifically describes and explains the results shown in your graph.

LAB QUIZ PREPARATION: For the lab quiz, be able to write clear and effective hypotheses and predictions for these experiments. Be prepared to graph or interpret results similar to those you’ve obtained today.

LAB NOTEBOOK CHECK: Your lab notebook entry this week should contain a title and objective for this lab. Your data/observations section should include your absorbance values in addition to any graphs you prepare in Excel. You should also record the proper technique for the use of the micropipettes and Vernier spectrophotometers.