Chem 460 Laboratory—Fall 2008
Experiment 6: Kinetics of Lactase

Before Lab

- Read section 12-1B "Enzyme Kinetics" and section 12-1C "Analysis of Kinetic Data" in Voet, Voet, & Pratt
- For Week 2 of this experiment read section 12-2 "Enzyme Inhibition"
- Review how to do the BCA protein assay
- In your notebook. Write the reaction for the hydrolysis of ONPG by lactase.

Introduction

In this two week lab you will be studying the kinetics of the enzyme lactase, a.k.a β-galactosidase. This enzyme catalyzes the hydrolysis of lactose to galactose and glucose. Lactase is the active ingredient in several products sold to treat lactose intolerance. Since the hydrolysis of lactose doesn't produce any significant change in UV/Visible absorbance, it is more convenient to use the synthetic substrate *o*-nitrophenyl-beta-galactoside (ONPG), which is hydrolyzed to galactose and *o*-nitrophenol, which you may recall produces a yellow color in its ionic form. Your job is to accurately determine kinetic parameters (specifically K_M, V_{max}, k_{cat} and k_{cat}/K_M) for this substrate. In addition, you will investigate potential inhibitors of this reaction and accurately determine K_i for the most effective inhibitor.

Earlier in this course you carried out kinetic assays on the enzymes fumarase and lysozyme. Using the expertise you gained in these previous labs, you will design and carry out assays to determine various kinetic parameters for the enzyme lactase. Note that the instructions below only provide guidelines for how to do this. It is up to you to put the pieces together and to understand how to obtain the data you need for your final plot and table. Also note that you will spend the first week of this lab becoming familiar with the enzymatic assay, determining protein concentration and figuring out how to do the calculations for K_M, V_{max}, k_{cat} and k_{cat}/K_M. The second week will be spent determining which sugar (glucose, galactose or sucrose) is the most potent inhibitor of lactase and then carrying out enzymatic assays, in duplicate, in the presence or absence of this inhibitor. <u>It is the data from the second week that you will use to create the plot and table you will be turning in for this lab.</u>

Week 1—Laying the groundwork for Week 2

The following points serve as guidelines for how to set up the enzymatic assay to determine K_M, V_{max}, k_{cat} and k_{cat}/K_M for lactase:

- Keep all solutions at 25°C and conduct assays at this temperature.
- Make a 20.00 mM ONPG stock solution using a 50.00 mL volumetric flask (note that ONPG takes a little while to dissolve).
- Follow ONPG hydrolysis by monitoring the absorbance change over time at 420 nm.

- Use a final concentration of 50 mM potassium phosphate buffer pH 7.4 in each assay.
- Prepare your enzyme stock from a commercial lactase product by taking 1 pill and grinding it in a mortar and pestle. Add 2 mL of cold phosphate buffer and grind some more. (Most of the pill will not dissolve.) Place as much of the liquid as possible in an eppendorf tube. Remove any remaining solid by centrifuging the tube and placing the supernatant in another eppendorf. Store it on ice. To prevent the cold enzyme from significantly changing the temperature of the solution, the stock enzyme solution should be concentrated enough that only a small amount (10-20 μL for a 1 ml assay) needs to be added.
- As your textbook states, it is best to determine initial rates (v_0) at substrate concentrations from 5 to 0.5 K_M (remember K_M is the concentration of substrate when the enzyme is functioning at $\frac{1}{2}V_{max}$). Since you do not know K_M when you start, you have to calculate a preliminary K_M, and then adjust the concentrations for subsequent assays. Start by assuming that K_M is 1 mM and use 5 different concentrations above and below this value.
- Determine how much enzyme is needed for ~10% of the ONPG to react over a 1 minute period. This ensures we are determining the initial rate of the reaction.
- To determine V_{max}, we need to convert the rates given by the spectrophotometer (in AU/s) to mM/s. To do this we need to use Beer's law. However, we need to determine the absorptivity coefficient (ε) for ONP at pH 7.4. The most convenient way to do this is to treat a known concentration of ONPG with enzyme and let the reaction go to completion. Try starting a concentration of 0.1 mM ONPG and enough enzyme (say 10 μL of the stock enzyme) such that the reaction comes to completion in a reasonable amount of time. Be sure that you have blanked the solution before you add the enzyme. Do at least two runs and check that the total absorbance changes agree within 5%.
- k_{cat} is the 'turnover number' and is calculated by dividing V_{max} by the molar concentration of enzyme, resulting in units of s^{-1}. Use the BCA assay to determine the concentration of protein in your enzyme stock solution. (We will assume that the only protein present is the lactase.) Since you don't know the concentration of your stock, you will probably want to do a couple different dilutions so that at least one of them falls on your standard curve. Once you know the concentration in mg/mL you will need to obtain the molecular weight of lactase to convert to concentration in molarity. Keep in mind you are trying to get the molar concentration of enzyme <u>in your kinetic assays</u>, so account for that dilution accordingly.
- To obtain good kinetic data you need to be as careful as possible to do each assay under the same conditions. Triplicate runs are usually considered to be the minimum needed for high quality work, but we will see how many time permit.

Make plots of your data

The following points should help you in preparing your plot(s).

- Determine the molar absorptivity (in units of $M^{-1}cm^{-1}$) for the complete hydrolysis of ONPG under the conditions that you used for the assay.

- Enter your data into an Excel spreadsheet. Using the molar absorptivity you determined above, convert your rates from change in absorbance/second to change in concentration per second (e.g., mM/s). Choose units so as to make your numbers convenient. For example, report your substrate concentrations in mM, not M.
- In your Excel worksheet make a plot of the rate versus substrate concentration. Then use the Michaelis-Menten equation to construct a theoretical fit to your data that you can adjust by changing K_M and V_{max}. Calculate the sum of the least squares differences between your theoretical fit and your data. Minimize this quantity by adjusting K_M and V_{max}. Calculate the R^2 value to determine how well the curve fits your data.
- Replot your data as a double-reciprocal (Lineweaver-Burk) plot with a line through the data based on the K_M and V_{max} values you determined through least squares. To do this, create a line with the x-intercept = $-1/K_M$ and the y-intercept = $1/V_{max}$. (It should look similar to Figure 12-4 in your textbook.)

Week 2—Obtain kinetic data including inhibitors

Determine an approximate K_i for galactose, glucose and sucrose. Using a concentration of substrate that is 0.5 K_M, do some quick assays to determine the approximate concentration of inhibitor that gives 50% inhibition. In consultation with your instructor, pick one of these compounds and do a thorough (i.e duplicate data that agree) kinetic analysis of lactase by measuring the rates at 5 different substrate concentrations with [I] = 0, K_i and $2K_i$.

Analyze your data following the guidelines for Week 1 but with the following additions:

- Add the inhibitor data to your Michaelis-Menten plot. For each of the different data sets (0, K_i and $2K_i$) separately determine K_M^{app} and V_{max}^{app} using least squares analysis. Then display your data in a double reciprocal plot with 3 lines corresponding to the three different values of K_M^{app} and V_{max}^{app} you obtained.
- Determine K_i of the inhibitor. You can do this by solving for α. Alternatively, you can replot your data as K_M^{app}/V_{max} versus inhibitor concentration and do a linear fit extrapolated to the x-axis to determine the K_i. (Look at page 374 in your book to understand why this plot can be used to determine K_i.)

<u>Report</u> (100 pts)—Due @ 8:00 am Wednesday, December 17, 2008

For this experiment you will be turning in the <u>Lineweaver-Burk plot</u> you generated from your Week 2 data and a <u>table of the kinetic parameters</u> you determined for lactase. You will also turn in your lab notebook pages and an Excel file containing your kinetic data and analyses.