Week 3 - Phosphatase Data Analysis with Microsoft Excel

Week 3 Learning Goals:

- To understand the effect of enzyme and substrate concentration on reaction rate
- To understand the concepts of Vmax, Km and specific activity
- To learn to use conversion factors in calculations
- To learn how to use Microsoft Excel for data manipulation and graphing

Background

Last week, we measured the amount of product formed using the absorbance at 405 nm. A better measure is the actual amount of product in moles, or concentration of product in units of molarity. The absorbance can be converted to concentration using the **Molar Extinction Coefficient (ε), which describes the relationship between absorbance and concentration of a molecule.**

For the nitrophenolate ion, **$\varepsilon = 1.88 \times 10^4$ AU/M**)

The value above means that a 1M nitrophenolate solution would have an absorbance of 18,800. This value is far beyond the range of spectrophotometers, but still can be used to determine the concentration of dilute solutions. For example, a 1 mM solution would have an absorbance of 18.8 (still out of range) and a 1 μM solution would have an absorbance of 0.0188 (within the functional range of spectrophotometers).

To learn how to use the molar extinction coefficient in a real experiment, consider the following example.

If a 15 minute stop tube has an absorbance 0.188, the concentration of NP in the stop tube would be 10 μM.

Because the NP in the stop tube came from the 0.5 mL of reaction mixture that was added to 2 mL of 0.1 M NaOH (a 5-fold dilution), the concentration in the reaction tube would be 5 times higher, or 50μM.

To determine the total amount of product formed, one must take the volume of the reaction mixture (2.5 mL or .0025L) into consideration. Recall that molarity corresponds to moles/L. Therefore
50μmol/L x 0.0025L = 1.25 μmol NP formed

One unit of enzyme is defined as the amount of enzyme that releases 1 μmole of phosphate (or nitrophenol) per hour under standard assay conditions.
Because the 1.25 μmol of NP was produced over 15 min, we must multiply by 4 to determine the amount that would be produced per hour.
1.25 μmol/15 min x 60min/hr = 5 μmol/hr = 5 units of enzyme

The **Specific Activity** of an enzyme preparation is expressed as the **umol of product formed per hour, per mg of protein.**

The first two terms, umol product/hour, indicate the rate or velocity of product formation. The final term, mg protein, refers to the total amount of all proteins in the mixture—not just the acid phosphatase protein, but ALL proteins. Our crude wheat germ extract contains other proteins in addition to the acid phosphatase enzymes, but these other proteins do not catalyze the production of nitrophenol. The other, non-acid phosphatase proteins can be thought of as "bystander proteins."

Comparing the specific activities of two enzyme preparations provides information about the relative purity of the enzyme preparation. The preparation with the higher specific activity is purer, with fewer contaminating or bystander proteins.

For example, if the protein in sample A contains 1% acid phosphatase and 99% other proteins, and the protein in sample B contains 20% acid phosphatase and 80% other proteins, sample B would be more pure and have a higher specific activity.

Consider the following macro-scale example:

> You must choose a team to make birdhouses.
> Team A has one worker who can make 10 birdhouses per hour, and nine other managers and marketing people who don't know how to make birdhouses.
> Team B has 8 workers that can make 10 birdhouses per hour *each*, and one manager and one marketer that don't know how to make birdhouses.
>
> Which team is more efficient at making birdhouses? This is the same as asking which team has higher specific activity for producing birdhouses.
>
> Specific activity = product made per hour per person
> Team A specific activity = 10 houses/hour/10 people
> = 1 house/hour/person
> Team B specific activity = 80 houses/hour/10 people
> = 8 houses/hour/person
>
> Because Team B has fewer bystanders and more workers that can do your desired job, you would want to hire Team B—no matter how great the glossy brochures for Team A looked!

Now let's return to our experiment with acid phosphatase enzyme:

If we were to compare the crude wheat germ protein extract, which contains thousands of different proteins, the vast majority of which do not produce nitrophenol, to a purified

preparation in which perhaps 20% of the proteins do produce nitrophenol, we might expect the following results.

Crude extract with 10 units of activity in 0.5 mL of 0.1XE (1.2 mg/mL)
1.2 mg protein/mL x 0.5 mL = 0.6 mg protein
Specific activity = 10 units/0.6 mg protein = 16.6 units/mg

Purified extract with 5 units of activity in 0.5 mL of 0.1XP (0.05 mg/mL)
0.05 mg protein/mL x 0.5 mL = 0.025 mg protein
Specific activity = 5 units/0.025 mg protein = 200 units/mg

Note that the total activity (10 units) of the crude extract is higher than the purified extract (5 units), yet when the activity is normalized to the amount of protein, the **specific activity of the purified preparation is much higher than that of the crude preparation** because there is a lower percentage of contaminating, bystander "non-acid phosphatase" proteins to increase the denominator of our ratio.

In the purified preparation, a higher percentage of the proteins are acid phosphatase enzyme molecules that are working on the reaction being studied. In the crude extract, many of the proteins are not working on this reaction. **Therefore, the purified preparation has a higher specific activity than the crude extract.**

Data Analysis using Microsoft Excel:

A. Time course

Set up your data in an Excel spreadsheet:

1. Open a new excel document. Double click on the tab at the bottom of the worksheet and change the name to "time course." Change the name of the other worksheets to "substrate varied," and "Standard Curve." Click on the Time Course Tab.

2. On the time course worksheet, click and drag to select cells B1 – E1, right click on the selected cells, choose format cells, click the alignment tab, then click the Merge cells box and OK. Enter A405 into the merged cells.

3. Click the upper left box to select the entire worksheet, then click the center alignment button.

4. Enter "time (min)" into cell A2, ".1xP" into B2, ".01xP" into C2, etc.
   Note: Each line you want to produce in a graph should get its own data column in your spreadsheet.

5. Enter your data in rows 3-7. Save the file on your network space (H drive) as phosphatase.xlsx.

Convert your A_{405} readings to micromoles of product made:

6. Click and drag to select cells F1 – I1, right click on the selected cells, choose format cells, click the alignment tab, then click the Merge cells box and OK. Enter "NP (umol) into the merged cells.

7. Click and drag to select cells B2 – E2, right click on the selected cells, choose copy, right click in cell F2, choose paste.

8. To calculate NP in μmol, use the conversion factor explained below:
   NP in umol = (Absorbance reading) (1 μmol NP/L 0.0188 AU) * 5 (dilution factor into stop tube) * .00375 L (volume of reaction tube)

   the molar extinction coefficient for your nitrophenol (NP) product is:
   1.88×10^4 Absorbance units/1 Molar solution of NP
   So for every umol NP/L, the absorbance is 0.0188 AU

   You diluted 0.75 ml of your reaction into a stop tube with 3ml of NaOH, OR
   You diluted 0.5 ml of your reaction into a stop tube with 2ml of NaOH,
   These correspond to a 5-fold dilution, and to correct for this dilution you must multiply your measurements by a factor of 5.

   Interestingly, in this case, the conversion factor = 0.997 or when rounded, 1.0.

9. In cell F3, enter "=b3*1". To copy this formula into other cells in column F, select cell F3 by right clicking on it, then choose copy. Click and drag to select the other cells in column F, then choose paste from the edit menu or after right clicking on the selected cells. To copy the formula into the other cells, click and drag to select cells G3 through I7, then choose paste.

   Notice that Excel edits the cell numbers for you as you paste the formula into new cells.

10. Save your work.

Make a graph from your data:

11. On the **insert** tab, choose **XY scatter** plot with data points connected by smooth lines.

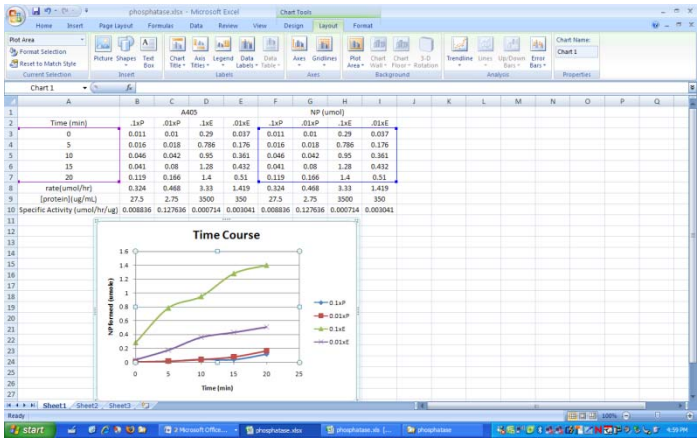
12. Click the select data button, then click the **add** button. Click the worksheet icon on the right side of the X values box, then click and drag to select the **different times**, press enter. Click the worksheet icon on the right side of the Y values box, then click and drag to select the **.1xP NP data points** on the umol side of your data table. Enter ".1xP " in the name box.

13. Click the **add** button and repeat step 12 for the other 3 extracts, naming them appropriately. When finished, Click **OK**.

14. Click the **chart tools** tab, then **layout**, then **chart title**. Enter ”Time Course” in the Chart Title box. Click on the axis titles button and label the horizontal (X) axis as “Time (min.)” and ; label the vertical (Y) axis as “NP formed (umol)” in the Value (Y) axis box.

15. If necessary, click on the graph to select it, then drag the corner handles to position it appropriately.

16. Save your file.



Convert the μmol of NP produced into a reaction rate (product per time):

17. To calculate reaction rate for each extract, the 0 time value must be subtracted from the 20 min value to yield the change over 20 min. This is multiplied by 3 to yield the change per hour.

    Thus, enter the following into cell B8: “=(b7-b3)*3”.

18. Enter “rate (μmol/hr)” in cell A8. Right click on cell B8, choose copy, then click and drag to select cells C8 – I8, right click on the selection and choose paste.

Convert your protein concentrations (μg/ml) to the total amount of μg of enzyme:

19. Enter “[protein] (μg/mL)” in cell A9, and enter the appropriate data into cells B9 – I9. These are the data from your standard curve of protein concentration versus Absorbance, the first graph we made on the first day of this lab exercise. Remember that you used your standard curve to extrapolate the ug/ml of protein in your 0.01XP and 0.01XE extracts. Use these numbers to calculate the μg/ml of protein for each extract (1XP, 1XE, 0.1XP, 0.1XE), and enter the data into cells B9-I9.

20. Enter “Specific Activity (umol/hr/ug)” in cell A10. Enter the following formula into cell B10: “=b8/b9*.75” The .75 is to reflect the fact that we added 0.75 mL of enzyme to the reaction tubes. Copy this formula into cells C10 - I10.

21. Save your work to your network (H: drive) space as phosphatase.xlsx.

B. Analysis of Variable Substrate Concentration Data

Enter your data into the cells on the Substrate Varied worksheet of your Excel document:

1. Enter the following into row 1 for columns A-H :

[NPP] (uM)	0 min	7.5 min	15 min	Vo (umol/hr)	1/[NPP]	1/Vo	Vo/[NPP]

2. Calculate the concentration of NPP in each of the reaction tubes and enter the data into column A.
   You may use the formula $C_1V_1 = C_2V_2$
   C_1 = concentration before dilution
   V_1 = volume before dilution
   C_2 = concentration after dilution
   V_2 = volume after dilution

   Your reaction tube for this part of the experiment had in it 1 ml of citrate buffer, a controlled volume of the 25 mM NPP substrate, water to a total of 2 ml, and 0.5 ml of enzyme added. Total volume of this reaction was 2.5 mL.

   For the reaction tube with 25 uL of 25 mM NPP added into it, the formula looks like this: $C_1V_1 = C_2V_2$
   (25 mM) (25uL) = C_2 (2.5 mL)
   (25 mM) (25 uL) = C_2 (2500 uL)
   0.25 mM = C_2

   So the concentration of NPP in the reaction where you added 25 ul was 250 uM. Enter the concentrations of NPP for each reaction tube in column A.

3. Enter your absorbance data into columns B-D. Save your tile.

Combine Absorbance readings and time points to calculate reaction rates:

4. To calculate rate, the 0 time value must be subtracted from the 15 min value to yield the change in absorbance over 15 min.

   To calculate rate of NP production in μmol/hr, use the conversion factor (1 μmol/L 0.0188 AU) * 5 (dilution factor into stop tube) * .0025 L (vol of reaction tube) * 4 (rate per hour from 15 minute rate)

   Thus, enter the following into cell E2: "=(d2-b2)*2.66"

5. Select cell E2 by clicking on it, choose copy from the edit menu or after right clicking on the selected cell. Click and drag to select the 4 cells below, then choose paste from the edit menu or after right clicking on the selected cells.

Fill in the formulae for the remaining cells:

6. Enter the formula “=1/a2” into cell f2. Copy this formula into the 4 cells below.

7. Enter the formula “=1/e2” into cell g2. Copy this formula into the 4 cells below.

8. Enter the formula “=e2/a2” into cell h2. Copy this formula into the 4 cells below.

Create a graph of your data:

9. On the **insert** tab, choose **XY scatter** plot with data points connected by smooth lines.

10. Click the select data button, then click the **add** button. Click the worksheet icon on the right side of the X values box, then click and drag to select the **[NPP] (uM) data**. Click the worksheet icon on the right side of the Y values box, then click and drag to select the **Vo data**. Enter Vo in the name box, When finished, Click **OK**. Click the **chart tools** tab, then **layout**, then **chart title.** Enter appropriate titles for the chart (Vo versus [NPP]), X and Y axes ([NPP] (uM) and Vo). Click **next**, then **finish.**
Save your work.

Advanced kinetic analysis: Lineweaver-Burke plots:

11. On the **insert** menu, choose **chart**. On the subsequent dialog box, choose **XY scatter** plot with no lines.

12. Click the select data button, then click the **add** button.. Click the worksheet icon on the right side of the X values box, then click and drag to select the **1/[NPP]**. Click the worksheet icon on the right side of the Y values box, then click and drag to select the **1/Vo**. Enter “1/Vo” in the name box, When finished, Click **next**. Enter appropriate titles for the chart, X and Y axes. Click **next**, then **finish.**

13. Right click on the X axis and choose format axis. Click the scale tab, then uncheck the “auto” boxes. Set the minumum, maximum, major and minor unit values to appropriate settings. Click OK

14. Right click on the data points for a plot and choose add trendline. Click the options tab and enter a trendline name, select display equation on chart, and forecast 0.5 or 1 unit such that the trend line extends to the X axis (in negative values). Repeat this for all three plots. Use this graph to then calculate Vmax (1/Y-intercept).

C. Standard Curve Data – Using what you have learned above, create a spreadsheet and plot the relationship between protein concentration and absorbance.

Phosphatase Data Analysis (40 points) – due in lab next week

Print out and submit each of your worksheets and graphs. (8 points each x 3)
Answer the following questions on a separate sheet. (4 pts each x 4)

1. In the time course experiment, how did the **specific activities** of the different enzyme preparations (e.g. 0.1xP and 0.1xE) compare? Are the values similar (within a factor of 2) or different? Is this what you would expect? Explain the reasoning for this.

2. In the time course experiment compare the **specific activities** of the different dilutions of the same enzyme prep (e.g. compare 0.1xP and 0.01xP). Are the values similar (within a factor of 2) or different? Is this what you would expect? Explain the reasoning for this.

3. In the experiment with different substrate concentrations, what trend in reaction rate was observed with the three lowest substrate concentrations? Why does this occur?.

4. In the experiment with different substrate concentrations, what trend in reaction rate was observed with the three highest substrate concentrations? Why does this occur? How does this relate to Vmax?

5. Make sure you identify and try to explain any discrepancies between your data set and the expected results.
   Remember, for lab reports in this course, I grade you on what you learn and understand, so it is still possible to score highly even when your group's data are not perfect. NEVER fudge data or ignore discrepancies—when things aren't working as expected, that is usually when scientists are about to learn something!