

Protocol:

1. Label 7 plastic cuvettes 1 through 7
2. Cut out 7 small pieces of parafilm; you will use the parafilm to cover the cuvettes so you can thoroughly mix the contents by inversion.
3. Press the Kinetics button on your spectrophotometer.
4. Type in the wavelength (405 nm) and press enter.
5. Type in the duration (200 seconds) and press enter.
6. Type in the interval (20 seconds) and press enter.
7. Select no (press enter when it says NO) for subtraction of background.
8. Prepare your first cuvette according to the table above. Add the reagents one a time, from left to right. DO NOT ADD TRYPSIN until you are totally ready to collect data (spec is ready, etc.).
9. When ready, add the trypsin to the first cuvette. Mix the contents thoroughly by inverting the parafilmed cuvette 5-6 times. Check that there are no bubbles in the light path. (If there are, tap the cuvette to dislodge them.)
10. Place the cuvette in the spec and press Read Blank.
11. As soon as the blank has been read, press the right arrow button to continue, then press Read Sample. The spec will read the absorbance every 20 seconds for 4 minutes.
12. When cuvette 1 is complete, print the report. Then set up and collect data for each remaining cuvette as in steps 8-11, except that *you do not need to reblank the spec with each new cuvette*.

Data Analysis

1. You should now have a table of data reporting absorbance at 405 nm over time for each the 7 cuvettes you utilized in this experiment.
2. Determine the concentration of trypsin in each cuvette, in mg/mL.
3. Enter your data into Excel. The first column will be time in seconds (the spec takes a reading every 20 seconds, so it's 0, 20, 40, etc.). In the seven subsequent columns, you'll enter the absorbance values for each of your seven cuvettes. Make the column headings the concentration of trypsin in each cuvette.

	A	B	C
1	Seconds	0 mg/ml	0.02 mg/ml
2	0		
3	20		
4	40		
5	60		

4. Highlight the entire table, go to the Insert tab, and click on the scatter plot with no lines.
5. You should get a graph. Click on Chart Layout 1 so you can enter a meaningful title and axis labels.
6. Each of your columns (each concentration of trypsin) should be pictured on the graph. For each one, right-click on a data point. Select Add Trendline. Choose a Linear Trendline, and check the boxes to display the equation and R-squared values.
7. Neatly arrange the equations and R-squared values on the graph, with highest [E] at the top, down to lowest [E] at the bottom. (You may need to make your graph bigger to do this.)
8. The equations are in the format $y = mx + b$, where m is the slope of the line. The slope of the line is the rate of change of the concentration (in Abs / second). Because the change in absorbance tells us the change in the amount of colored product, this value is also a measure of the rate of trypsin's activity.
9. In a new Excel sheet (see the tabs at the bottom), enter your data for concentration of trypsin in each cuvette in one column, and then reaction rate (Abs / second) in the next column.
10. Make a scatter plot of this data, label it well, and determine the equation (and goodness of fit, from the R-squared value) of the trendline. Print this graph for your notebook as well.
11. Questions to consider and discuss with your instructor:
 - a. What was the reaction rate in cuvette #1? Was it what you expected? (If not, what might have happened?) What are we controlling for with this reaction?
 - b. How good are your line fits for the Abs vs. time graph? Do you have deviations from linearity anywhere? If so, what might explain your results?
 - c. What relationship do you see in the velocity vs. [trypsin] graph? Is it what you expected? If not, what might explain your results?

Day 2: Measure reaction velocity as a function of substrate concentration with and without soybean trypsin inhibitor, and determine V_{\max} and turnover number (K_{cat}) in each case.

Solutions required:

- Tris buffer: 50 mM Tris, 1 mM CaCl_2 , pH 8.2. (Calcium helps stabilize the trypsin.)
- Stock trypsin: 2 mg/mL in 1 mM HCl. (At acidic pH, the activity of trypsin is inhibited, thus inhibiting the proteolysis of the trypsin itself.)
- Chromogenic substrate: BAPNA, 2.5 mM in Tris buffer. Check that the substrate does not have a yellow precipitate; such a solution will not work.
- Soybean trypsin inhibitor: 0.1 mg/mL in Tris buffer.

Protocol, V vs. $[S]$, no inhibitor:

1. Cut out 7 small pieces of parafilm; you will use the parafilm to cover the cuvettes so you can thoroughly mix the contents by inversion.
2. Press the Kinetics button.
3. Type in the wavelength (405 nm) and press enter.
4. Type in the duration (200 seconds) and press enter.
5. Type in the interval (20 seconds) and press enter.
6. Select no (press enter when it says NO) for subtraction of background.
7. Prepare your first cuvette according to the table below. DO NOT ADD TRYPSIN until you are totally ready to collect data (spec is ready, etc.).
8. When ready, add the trypsin to the first cuvette. Mix the contents thoroughly by inverting the parafilm cuvette 5-6 times. Check that there are no bubbles in the light path. (If there are, tap the cuvette to dislodge them.)
9. Place the cuvette in the spec and press Read Blank.
10. As soon as the blank has been read, press the right arrow button to continue, then press Read Sample. The spec will read the absorbance every 20 seconds for 4 minutes.
11. When cuvette 1 is complete, print the report. Then set up and collect data for each remaining cuvette as in steps 7-10, except that *you do not need to reblank the spec with each new cuvette*.

Cuvette	Tris buffer (μL)	2.5 mM BAPNA (μL)	2 mg/mL Trypsin (in 1 mM HCl, μL) ADD LAST
1	965	0	35
2	765	200	35
3	665	300	35
4	565	400	35
5	465	500	35
6	365	600	35
7	265	700	35

Protocol, V vs. [S] with inhibitor:

Follow instructions as in the previous section using the table below.

Cuvette	Tris buffer (μL)	2.5 mM BAPNA (μL)	0.1 mg/mL trypsin inhibitor (μL)	2 mg/mL Trypsin (in 1 mM HCl, μL) ADD LAST
1	935	0	30	35
2	735	200	30	35
3	635	300	30	35
4	535	400	30	35
5	435	500	30	35
6	335	600	30	35
7	235	700	30	35

Data Analysis

1. You should now have two tables of data, displaying the absorbance values over time for 7 cuvettes with varied substrate concentrations, with and without inhibitor. For now, start with just your no inhibitor data.
2. Determine the substrate concentration in each cuvette.
3. Open an Excel file. Name the sheet you are working on (it's like a tab at the bottom) something like "no inhibitor raw data", to make sure we keep the data straight. Enter your no inhibitor data, making the column headings reflect the concentration of substrate in each cuvette.

	A	B		C
1	Seconds	0 mM	0.5 mM	0.75 mM
2	0			
3	20			
4	40			
5	60			
6	80			

4. Make a graph of A_{405} over time (you'll have one line for each different substrate concentration).
5. As you did last time, use the graph to determine the reaction rate (or velocity) for each cuvette.
6. In a new Excel sheet (call it "no inhibitor velocities"), enter your data for concentration of substrate in each cuvette in one column, and then reaction velocity (Abs units / second) in the next column.

	A	B
1	[S] (mM)	V (Abs/sec)
2	0	
3	0.5	
4	0.75	

- Make a scatter plot of these data. This is a Michaelis-Menten graph: velocity vs. [S] (on horizontal, X-axis). Does it look the way you expect it to?
- Remember that Excel can't fit a line to this type of equation, so leave it as just unconnected dots. We will have to convert the data so we can make a Lineweaver-Burk plot.
- In your same Excel sheet, create two new column headings in the first row: 1/[S] and 1/V.
- Skip the first data row in your 1/[S] and 1/V columns. (This row corresponds to [S]=0. 1/0 does not exist.)
- In the second row of your 1/[S] column, type "=1/", then click on the corresponding cell in your original [S] column. It should enter the cell number for where you just clicked.

	A	B	C	D	E
1	[S] (mM)	V (Abs/sec)		1/[S] (1/mM)	V (Abs/sec)
2	0	0			
3	0.5	0.01		=1/A3	
4	0.75	0.015			

- Press enter, and Excel will do the math for you. Once that is done, move your cursor to the bottom right corner of the cell where Excel just did the calculation. Your cursor will change to a "+" sign. Drag this + down, and Excel will complete that same operation on all the cells you select.
- Go through the same steps (10-12) to calculate your 1/V values. (If you're unsure that this was done correctly, you can always double check a few values using your calculator.)
- Now, make your Lineweaver-Burk plot: a scatter plot of 1/V vs. 1/[S]. (Make sure that 1/[S] is on the horizontal (X) axis.

15. Label the graph well, and do a linear fit as usual.
16. The equation is in the format $y = mx + b$, where b is the y-intercept.
17. The y-intercept is equal to $1/V_{\max}$. Calculate your V_{\max} (remembering units).
18. Calculate the turnover number (k_{cat}) for trypsin under these conditions, recalling that $k_{\text{cat}} \cdot [E] = V_{\max}$.
19. Open a new sheet (name it "+ inhibitor raw data"), and go through steps 1-18 to also analyze the data obtained in the presence of the trypsin inhibitor.
20. Make two last graphs: Michaelis-Menten and Lineweaver-Burk plots that show the data for both + and – inhibitor. (Do this by compiling the relevant data in a new sheet, highlighting the data you want to include, and then inserting the scatter plot.) These graphs will allow us to directly compare your two sets of results.
21. As part of your data analysis, make sure you submit through Canvas of the following well-labeled graphs in your notebook:
 - a. Abs. vs. time for both sets of experiments (the raw data)
 - b. the combined Michaelis-Menten graph, showing both + and – inhibitor (and identifying which is which)
 - c. the combined Lineweaver-Burk plot, showing both + and – inhibitor (and identifying which is which)
22. Questions to consider and discuss with your instructor:
 - a. What was the reaction rate in cuvette #1 (both sets of data)? Was it what you expected? (If not, what might have happened?) What are we controlling for with this reaction?
 - b. How good are your line fits for the Abs. vs. time graphs? Do you have deviations from linearity anywhere? If so, what might explain your results?
 - c. Do your Michaelis-Menten plots show the expected pattern?
 - d. How do your V_{\max} and turnover number (k_{cat}) values compare with and without the inhibitor? Is this what you expected? What type of inhibitor are they characteristic of?