BCH377H Module 1
Trypsin Enzyme Kinetics

Enzymes are the “magic bullets” that make life possible. They are the catalysts that speed up reactions that would otherwise be far too slow to sustain life. Many of the important concepts in biochemistry are related to understanding the properties of enzymes. This includes mechanisms of catalysis, enzyme kinetics, and the regulation of enzyme activity.

The purpose of this exercise is to illustrate some of the concepts you learned in your lecture course. You will see what an enzyme reaction looks like by generating a progress curve. You will confirm the effect of enzyme concentration on the rate and extent of the reaction and you will look at the effect of pH on enzyme activity. Finally, you will determine $K_m$ and $k_{cat}$ by putting into practice the theory of Michaelis-Menten kinetics you studied last year.

We will use trypsin as our model enzyme. Trypsin is a protease that cleaves polypeptides on the C-terminal side of lysine and arginine residues (unless the next residue is proline).

\[
\begin{align*}
\text{Trypsin} & \quad \text{H}_3\text{N}-\text{Gly}-\text{Arg}-\text{Ala}-\text{Ser}-\text{Phe}-\text{Gly}-\text{Asn}-\text{Lys}-\text{Trp}-\text{Glu}-\text{Val}-\text{COO}^- \\
& \quad \downarrow \\
& \quad \text{H}_3\text{N}-\text{Gly}-\text{Arg}-\text{COO}^- + \text{H}_3\text{N}-\text{Ala}-\text{Ser}-\text{Phe}-\text{Gly}-\text{Asn}-\text{Lys}-\text{COO}^- + \text{H}_3\text{N}-\text{Trp}-\text{Glu}-\text{Val}-\text{COO}^-
\end{align*}
\]


The *in vivo* reaction is complicated because the substrates are large polypeptide chains and not all potential cleavage sites are equally accessible—especially in native (folded) proteins. However, trypsin will also cleave short peptides and many substrates that don’t look anything like a peptide but have a peptide-like bond. We will use one of these artificial substrates in the enzyme assays.

Recall that the mechanism of trypsin and the other serine proteases involves a catalytic triad of aspartate, histidine, and serine residues. The reaction involves *hydrolysis* of the peptide bond. The mechanism of catalysis requires consumption of a water molecule, making it a bisubstrate reaction. But since water is at such a high concentration in aqueous solutions, its concentration can be considered to be constant and in excess. Thus, serine protease activity can be measured via single substrate Michaelis-Menten kinetics.

**Bovine pancreatic trypsin**

In this module you will be using trypsin obtained from bovine pancreas to carry out *in vitro* enzyme assays. This enzyme has a molecular weight of 23,800 ($M_r = 23,800$). Studies on bovine pancreatic trypsin have shown that it is most stable at pH $\sim 2.3$ but it is inactive at this pH. The optimal pH for activity is between 7 and 9 and its stability is increased in the presence of Ca$^{2+}$ ions. Therefore a higher rate of overall catalysis is found in most assay systems when Ca$^{2+}$ is added to the reaction mixture.
We will use the artificial substrate benzoyl-DL-arginine p-nitroanilide. It allows both continuous assay (direct monitoring of the product liberated, using the spectrophotometer) or discontinuous assay (indirect, determination of the product liberated after a fixed interval of time).

**References**


**Outline of the Schedule**

**Tuesday, Sept. 9 Trypsin Day One**

The first day is about buffers. You will prepare buffers needed to study the activity of the enzyme trypsin at different pH’s. We’ll take advantage of this opportunity to learn a little bit about how to make buffers.

**Tuesday, Sept. 16 Trypsin Day Two**

On the second day you will create and examine a standard enzyme assay (basic reaction progress curve). You will also look at the effect of enzyme concentration on activity.

**Tuesday, Sept. 23 Trypsin Day Three**

You will use the different pH buffers prepared by the class on Day 1 to study the effect of pH on the activity of trypsin. You will carry out Michaelis-Menten (M-M) analyses of trypsin in the presence or absence of the inhibitor benzamidine in order to analyze various kinetic properties of the enzyme ($K_m$, $V_{max}$, $k_{cat}$) and the effect of this inhibitor (including $K_i$).

**Thursday, Oct. 2**: Hand in Lab Report #1: Trypsin
Trypsin: Day One

Your goal for Day 1 is to make up buffer solutions that will be used on Day 3 to measure the effect of pH on the activity of trypsin. At the end of the day you should have two 250 mL bottles of buffer solution that are clearly labeled with your name(s) and the exact pH. One them will be a Tris buffer solution at pH 9.5, or 8.5, or 7.5, or 6.5. (The pH will be assigned to each group at the beginning of the lab.) The other buffer will be an acetate buffer at a pH ranging from 3.5 to 5.5.

The acetate buffers at pH 3.5, 4.5, and 5.5 will be used by the rest of the class on Day 3. The final concentrations of buffer will be 0.14 M and each one will contain 0.025M CaCl₂. (Recall that calcium is required in the trypsin enzyme assays in order to stabilize the enzyme.)

The pKₐ’s of the buffer compounds to be used in class are:

<table>
<thead>
<tr>
<th>Compound</th>
<th>pKₐ (at 25°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>trishydroxymethylaminomethane (a.k.a. tris or trizma)</td>
<td>8.06</td>
</tr>
<tr>
<td>acetic acid</td>
<td>4.76</td>
</tr>
</tbody>
</table>

Here are the assignments for each group. The buffers that we will use on Day 3 are marked with an asterisk (*).

<table>
<thead>
<tr>
<th>Group</th>
<th>Tris</th>
<th>acetate (titrate)</th>
<th>acetate (mix)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>9.5*</td>
<td>3.5*</td>
<td>3.5</td>
</tr>
<tr>
<td>2</td>
<td>8.5*</td>
<td>3.7</td>
<td>3.7</td>
</tr>
<tr>
<td>3</td>
<td>7.5*</td>
<td>3.9</td>
<td>3.9</td>
</tr>
<tr>
<td>4</td>
<td>6.5*</td>
<td>4.1</td>
<td>4.1</td>
</tr>
<tr>
<td>5</td>
<td>9.5*</td>
<td>4.3</td>
<td>4.3</td>
</tr>
<tr>
<td>6</td>
<td>8.5*</td>
<td>4.5*</td>
<td>4.5</td>
</tr>
<tr>
<td>7</td>
<td>7.5*</td>
<td>4.7</td>
<td>4.7</td>
</tr>
<tr>
<td>8</td>
<td>6.5*</td>
<td>4.9</td>
<td>4.9</td>
</tr>
<tr>
<td>9</td>
<td>9.5*</td>
<td>5.1</td>
<td>5.1</td>
</tr>
<tr>
<td>10</td>
<td>8.5*</td>
<td>5.3</td>
<td>5.3</td>
</tr>
<tr>
<td>11</td>
<td>7.5*</td>
<td>5.5*</td>
<td>5.5</td>
</tr>
</tbody>
</table>
In order to prepare buffers at the desired pH you begin with concentrated stock solutions of 1.4 M Tris, 1.4 M acetic acid, and 1.4 M sodium acetate.

**To prepare buffers between pH 6.5 and pH 9.5:** The Tris stock solution will be titrated with 5 M maleic acid to produce tris-maleic acid buffers at pH’s 9.5, 8.5, 7.5 and 6.5.

**To prepare buffers between pH 3.5 and 5.5:** There are two ways to prepare acetate buffers.

1. You start with a solution of concentrated acetic acid titrate it with NaOH to reach the desired pH. Then you dilute the buffer to the desired concentration. This is analogous to the way you prepare the Tris buffer.

2. You calculate the concentrations of the acid ([\(\text{CH}_3\text{COOH}\)]) and base (\(\text{CH}_3\text{COO}^-\)) components required for a given pH and then combine the appropriate amounts of acetic acid and sodium acetate. In some cases the resulting mixture will have the correct final concentration but in this exercise we will mix concentrated solutions of acid and base then dilute to the final concentration.

You will use both methods in this exercise. The most difficult part of this lab is calculating the correct amounts of acetate and acetic acid solutions to make a specific buffer at the desired pH. You might want to review/learn the equations before the lab day by looking them up online or by consulting your textbook. You are responsible for knowing how to do this.

**Assignment:** You will be given a form that you must complete and hand in at the end of the lab. Show your calculation of the volumes and the final pH you obtained.

**Reagents**

<table>
<thead>
<tr>
<th>Buffer</th>
<th>TRIS buffers: pH 6.5, 7.5, 8.5 &amp; 9.5</th>
<th>ACETATE buffers: pH 3.5, 4.5, &amp; 5.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH Calibration Solutions</td>
<td>pH 7.0 (phosphate buffer) &amp; pH 10.0 (potassium borate buffer)</td>
<td>pH 7.0 (phosphate buffer) &amp; pH 4.0 (potassium hydrogen phthalate)</td>
</tr>
<tr>
<td>Buffer Stock</td>
<td>10X Tris stock solution (1.4 M tris)</td>
<td>10X acetic acid stock solution (1.4 M acetic acid)</td>
</tr>
<tr>
<td></td>
<td>10X sodium acetate stock solution (1.4 M sodium acetate)</td>
<td></td>
</tr>
<tr>
<td>Titrant</td>
<td>5 M maleic acid</td>
<td>1.5 M NaOH</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>2.5 M CaCl₂</td>
<td>2.5 M CaCl₂</td>
</tr>
</tbody>
</table>
NB: pH is affected by temperature. All solutions used today should be at room temperature before measuring the pH.

Lab Report: Why is pH affected by temperature?

CAUTION

Concentrated acids and bases can cause severe irritation to skin, eyes and the respiratory tract. Do not inhale solutions, wear gloves at all times and handle with care! Rinse immediately and thoroughly with water should you get any of these solutions on your skin.

How to prepare your assigned buffer

The class will prepare seven buffers that will be used on Day 3 of the trypsin module. These are the buffers marked with an asterisk in the table above. All groups will prepare two bottles of buffer solution (250 mL) but only some of them will be used later on.

Each group is assigned one Tris buffer (pH 6.5, 7.5, 8.5, and 9.5). In addition, three of the groups will be making one of the required acetate buffers (pH 3.5, 4.5, and 5.5).

Pay close attention to the tips about the appropriate equipment to use provided on the following pages. Note that these are guidelines ONLY—use your best judgement as you see how the titration is progressing and make appropriate adjustments. It’s better to approach the final pH cautiously than to overshoot and have to repeat the whole titration! Use the sample titration curves provided to guide you. Be sure you understand why you are to use these particular reagents and equipment for your assigned buffer BEFORE you begin setting things up!

pH Test Strips

There will be pH test strips available to test the pH of your solution without using a pH meter. The strips are obviously less accurate than a pH meter but they are a “quick and dirty” way of checking that your buffers and solutions have the desired pH.

The proper technique is to withdraw a drop of solution and apply it to the end of the pH test strip. You can only do this with solutions that are at their final concentration because if you remove liquid from a concentrated solution that you will subsequently dilute, then the final concentration will change.
Tris Buffers

Assigned pH: 9.5
- Tip: Use a p200 micropipettor to add maleic acid to 25 mL of your 1.4 M Tris stock solution. You will require less than 1 mL of titrant to reach a pH of 9.5. Begin with 50 µL additions of titrant and reduce to 20 µL when you are within 0.5 units of the desired pH.

Assigned pH: 8.5
- Tip: Use a p200 micropipettor to add maleic acid to 25 mL your tris stock solution. You will require less than 2 mL of titrant to reach a pH of 8.5. Begin with 100 µL additions of titrant and reduce to 20 µL when you are within 0.2 units of the desired pH.

Assigned pH: 7.5
- Tip: Use a p200 micropipettor to add maleic acid to 25 mL your tris stock solution. You will require ~ 3 to 4 mL of titrant to reach a pH of 7.5. Begin with 200 µL additions of titrant and reduce to 50 µL when you are within 0.2 units of the desired pH.

Assigned pH: 6.5
- Tip: Use a p1000 micropipettor to add maleic acid to 25 mL your tris stock solution. You will require ~ 4 to 5 mL of titrant to reach a pH of 6.5. Begin with 500 µL additions of titrant. When you are within 0.2 units of the desired pH switch to a p200 micropipettor and reduce the additions to 50 to 100 µL.

NB. These are just approximations of the volume of acid that needs to be added. The actual volumes depend on the concentrations of the solutions and the exact starting pH.
Acetate Buffers: Titration Method

Assigned pH: 3.5
- Tip: Use a p200 micropipettor to add 1.5 M NaOH to 25 mL of your acetic acid stock solution. You will require ~ 1 to 1.5 mL of titrant to reach a pH of 3.5. Begin with 200 µL additions of titrant and reduce to 50 µL to 100 µL when you are within 0.2 units of the desired pH.
- For pH values 3.7, 3.9, 4.1, and 4.3 you will more NaOH solution. You might want to start with a p1000 micropipettor.

Assigned pH: 4.5
- Tip: Use a burette to add 1.5 M NaOH to 25 mL of your acetic acid stock solution. You will require ~ 7 to 9 mL of titrant to reach a pH of 4.5. Begin with 1 mL additions of titrant and reduce to 200 µL when you are within 0.2 units of the desired pH.
- For pH values 4.7, 4.9, 5.1, and 5.3 you will more NaOH solution.

Assigned pH: 5.5
- Tip: Use a burette to add 1.5 M NaOH to 25 mL of your acetic acid stock solution. You will require ~ 17 to 19 mL of titrant to reach a pH of 5.5. Begin with additions of 1 to 2 mL of titrant and reduce to 200 µL when you are within 0.2 pH units of the desired pH.

Lab Report: You MUST record the exact amount of NaOH that you add to make your buffer. Later on, you will calculate this amount to see if the calculation is accurate. We will collect the data from all of the groups so you can use it to create a graph like the one on the bottom of the page.

Acetate Buffers: Calculate and Mix Method

You will use standard acid dissociation theory to calculate the volumes of 1.4 M acetic acid and 1.4 M sodium acetate that you need to mix in order to get 25 mL of 1.4 M buffer solution at the assigned pH. Record the calculations and the pH of the buffer in your notes and on the page to be handed in at the end of the lab.
Step 1: Calibration of the pH Meter

pH meters are cantankerous and unreliable. Over time, they tend to give false readings unless treated with love and respect. If it has been sitting idle for any length of time, a pH meter must be re-calibrated before it is used. Instructions for calibration of the pH meter will be provided on your bench.

**NB: THE ELECTRODE MUST ALWAYS BE IN A SOLUTION; KEEP IT IN DISTILLED WATER WHEN YOU ARE NOT USING IT.**

1. Fill a small beaker with distilled water. You will keep the electrode in this beaker when you aren’t using it.
2. Use the pH calibration solutions listed in the reagent table above to perform the appropriate two-point calibration that is required for your assigned buffer. 10-15 mL of each calibration solution in small 20 mL beakers is sufficient.
3. Rinse the electrode with distilled water (from a squirt bottle) with a waste beaker placed underneath.
4. Place the pH electrode into the first calibration solution ensuring that the electrode is completely submerged. Follow the instructions for the first calibration step.
5. Rinse the electrode again with distilled water before placing the electrode into the second calibration solution. Follow the instructions to complete the calibration procedure.
6. Rinse the electrode again when calibration is complete and then submerge the electrode into the beaker with distilled water.

Step 2: Preparation of your assigned buffers

1. Using a graduated glass pipette, accurately dispense 25 mL of the appropriate 10X stock solution into a 100 mL beaker.

2. Place a small magnetic stir bar (a.k.a. a flea) into the beaker and place the beaker on a magnetic stirrer. Place the pH electrode into the solution in the beaker ensuring that the electrode is completely submerged. Turn on the stirrer and adjust to a medium stirring speed. **Be sure that the flea is not hitting the pH electrode!** Stir constantly (but gently) throughout the titration procedure.

3. Titrate using either a micropipettor or a burette.

   **If using a micropipettor:** Obtain a small aliquot of the appropriate titrant solution in a small beaker.

   **If using a burette:** Place a 25 mL burette vertically in a clamp. Make sure that the tap (stopcock) of the burette is secure and in the closed (horizontal) position. **OVER A SINK, using a funnel,** slowly fill the burette with the appropriate titrant solution (solution can spill
as you pour so be careful). Raise the funnel up slightly so that the liquid flows freely. Adjust the level of the solution in the burette so that it is at the 0 mL mark at the top of the burette by opening the tap (turning the stopcock) and allowing solution to flow out. Carefully clamp your burette onto a retort stand to titrate your samples. Be sure to flush the sink with water to remove any waste solution when you are done.

4. Make a series of additions of the titrant from the burette/micropipettor into the beaker with the buffer. Record each addition of titrant (total volume added) and the corresponding pH in your notebook as you go along.

Reminder: Adjust the size of the additions depending on the starting pH of the buffer and the pH that you are assigned. Use the tips provided as a guide; if you are really unsure, it’s best to err on the side of caution and make very small additions until you see how quickly the pH is changing. Remember, if you know you are passing through a buffer region, you may increase to larger additions. Make very small additions as you approach an equivalence region or as you approach your assigned pH. **STOP** once you have reached your assigned pH!

Write down the final pH in your lab notes. Do not worry if you overshoot by a few hundredths of a pH unit of what you were assigned (what is important is that you record the actual pH!)

5. Remember to rinse the pH electrode and place it in the beaker with distilled water when you are done.

6. Repeat steps 1-5 for your second buffer.

**Lab Report:** We will collect and tabulate all of the calculations and final pH values of the acetate buffers made by mixing sodium acetate and acetic acid. You should prepare a graph of these results and present them in your lab report. Discuss why they might differ from the data in tables found on the web.

7. For the third buffer, calculate the volumes of acetate and acetic acid that you need and mix them together into a clean 100 mL beaker, The total volume is 25 mL.

8. Measure and record the pH in your notes and on the page that you will hand in at the end of the lab.
Step 3: Preparing the Final Buffer

1. Transfer your prepared buffer into a 250 mL graduated cylinder.

2. Add 2.5 mL of 2.5 M CaCl₂. When diluted, this will give a final concentration of 0.025 M.

3. Bring the volume up to 250 mL with distilled water.

   Record the pH of this diluted buffer in your lab notes.

4. Transfer the buffer into a 250 mL bottle. Securely fasten a cap. Label your buffer by its name and the final experimental pH obtained; also include your group’s initials. Keep in mind that the class will use some of these buffers for the trypsin assays on Day 3 and they will have to be able to easily identify each buffer! Give the flask to your TA for storage in the fridge.

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Trypsin: Day Two

Today you will begin with Step 1 of the GMO lab. When you have completed this step proceed to today’s Trypsin experiments.

Enzyme Assays

Your goal on Day Two is to examine the reaction catalyzed by trypsin by performing enzyme assays. You will produce a standard progress curve using an automated spectrophotometer and you will explore the effects of enzyme concentration on the progress of the reaction.

Trypsin hydrolyses the synthetic substrate benzoyl-arginine-p-nitroanilide to give benzoyl-arginine and p-nitroaniline in the general assay procedure used in this lab. The product is p-nitroaniline. p-Nitroaniline is a chromophore that has a different absorption spectrum when bound in an amide bond. It is yellow whereas the substrate is colourless.

REACTION OF TRYSIN WITH BENZOYL-ARGININE-P-NITROANILIDE

*Synthesis of the L-isomer is expensive; therefore, the DL-compound will be used.
Note that the substrate is not a peptide and does not contain an arginine or a lysine residue. However, it does have a peptide-like bond and that bond is cleaved efficiently by trypsin.

The formation of product is monitored at a wavelength of 405 nm. At this wavelength there is a significant absorbance (i.e. high $\varepsilon$) of product but neither substrate nor any other component of the reaction absorbs. The molar extinction coefficient ($\varepsilon$) for p-nitroaniline at 405 nm is 10,200 M$^{-1}$ cm$^{-1}$ at pH 8.2 (the spectra of both substrate and product are independent of pH over the range used in these experiments).

**Reagents**

- Standard assay buffer: 0.14 M Tris, pH 7.8, 0.025 M CaCl$_2$

- Substrate: $5 \times 10^{-2}$ M N-$\alpha$-benzoyl-DL-arginine-p-nitroanilide dissolved in dimethylformamide (DMF). **Do NOT put on ice!**

- Standard enzyme: 62.5 $\mu$g/mL Trypsin in 0.002 N HCl.

- Enzyme stock (to be used by instructor for Step 2): 12.5 mg/mL Trypsin in 0.002 N HCl

- 0.002N HCl (for instructor to dilute enzyme stock)

- 30% (v/v) acetic acid (to stop the reaction in a fixed-time assay)
A Note on Controls and Blanks

The basic reaction mixture contains standard assay buffer, enzyme and substrate. This assay involves a change in absorbance at 405 nm that is measured over a period of time. In order to show that the change in absorbance is due solely to the action of the enzyme on the substrate, the following four questions must be asked before undertaking a series of experiments. Given time restrictions, you will not carry out all of these controls and blanks yourself but you should note their importance:

The total volume should be kept constant for all measurements.

1. Does the absorbance of the buffer differ from that of water?
2. Does the addition of dimethyl formamide to buffer increase the absorbance? (DMF is the solvent in which the substrate is dissolved.)
3. Does the absorbance of substrate solution added to buffer differ from that of buffer alone? (comparing the same volume of substrate that you would use in the basic assay reaction)
4. Does the addition of enzyme to buffer cause an increase in absorbance in the absence of substrate? (comparing the same volume of enzyme that you would use in the basic assay reaction)

Past experience in carrying out the above controls and blanks indicates that the answer to questions #1, 2 and 4 is NO. Some absorbance may be observed with substrate (question #3) as the substrate can hydrolyze in aqueous solutions over time.

A small amount of absorbance from the substrate solution does not hinder experiments if you are directly measuring initial rates (slopes) (absorbance at the start of the reaction does not matter—what matters is the change in absorbance over a very short period of time). However, if you are carrying out a long assay or a fixed-time (discontinuous) assay in which the substrate would have the time to hydrolyze on its own, you must account for any substrate-only (enzyme-independent) hydrolysis by subtracting the absorbance reading obtained with the appropriate control from the reaction measurements.

(You should also note that if some of the substrate has already hydrolyzed in the stock substrate bottle then the concentration indicated on the substrate bottle will be higher than the true concentration in the solution. This means that the substrate values plotted will be higher than the true values. For simplicity, we will ignore any such problem in our analysis of the data but you should mention this in your lab report if necessary.)

Experiment #1: Basic Reaction Progress Curve

Setting up the Shimadzu spectrophotometer: The Shimadzu instrument that you will use for your spectrophotometric measurements today is a UV-visible dual-beam spectrophotometer. A dual-beam instrument such as this contains a Reference cell to which a Sample cell is always compared as in the diagram on the next page. This allows direct measurement of transmittance, T, by comparing the solvent (blank solution that gives Io (incident light)) vs. the sample I (transmitted light). (Recall that T = I/Io as described in the BCH242 lab manual.)
Set up the Shimadzu spectrophotometer for use in Kinetics Mode. You will use this mode to monitor the progress of the reaction. Kinetics mode monitors change in absorbance over time at a user-specified wavelength.

1. Select Kinetics mode by pressing 4 on the keypad.

2. Press 1 to choose 1 cell kinetics ($1\lambda$).

3. The parameters required for this experiment are shown in the screenshot below. Enter the parameter number to change a particular parameter. Use the keypad to enter the desired number and press ENTER. Change parameter #5 (Time scale) to minutes before you set the other parameters.

Use the GOTO WL button on the keypad to change the wavelength.
Next to each Shimadzu spectrophotometer you should have:
• a pair of cuvettes in a holder
• a beaker to collect liquid waste
• a Pasteur pipette with a plastic tip on the end (to prevent scratches on the cuvettes) and a rubber bulb
• a squirt bottle with distilled water
• a box of KimWipes

Thoroughly rinse the cuvettes with distilled water before beginning any measurements. Remove any residual drops of water in the cuvettes with the Pasteur pipette and dry off the outsides of the cuvette with a KimWipe ensuring there are no spots.

**Blanking the Cuvettes**

4. Before taking any measurements it is necessary to blank the cuvettes with the appropriate blank solution. You will be using 3 mL glass (or quartz) cuvettes in this experiment. The appropriate blank solution for this experiment is the reaction buffer with substrate.

Pipette directly into each cuvette:
1. 2.5 mL of buffer (use a p1000 pipette)
2. 50 µL of substrate solution
Cover with ParaFilm and mix by inversion.

5. Place one cuvette in the Reference cell at the back—this is the Reference Cuvette that will remain with the blank solution throughout your measurements. Place the other cuvette into the Sample cell at the front—this is the Sample Cuvette. Be sure that both cuvettes have the opaque sides facing you so that the clear sides can allow the light beam to pass through from one side to the other, perpendicular to you.

Press the AUTO-ZERO button on the keypad. Wait a few moments until you hear a beep. You should now see the Abs value on the top right side of the screen change to zero.

**Progress curve**

6. Carefully remove the Sample Cuvette from the holder. Initiate the reaction by adding 200 µL of standard enzyme solution (62.5 µg/mL) and mix well, then place immediately back into the Sample cell slot and press the START button on the keypad.

**NB: The buffer and substrate concentrations in the Sample Cuvette will be slightly more dilute than in the Reference Cuvette but this doesn’t matter very much.**
The reaction will be monitored for 20 minutes. Note that once the reaction is started you can leave it running while you do other things in the lab.

7. When complete, print a copy of the curve by pressing the PRINT button on the keypad. (Note: if your curve exceeds the Absorbance range set, you can ZOOM (F1) to adjust the y axis so that the whole curve fits on the screen.) Remember to print a second copy for your lab partner.

Remove the solution from both cuvettes using the Pasteur pipette provided. Rinse the cuvettes thoroughly with distilled water and discard any waste into the waste beaker. Dry off the cuvettes and place them upside down in the holder as you found them.

The basic reaction progress curve is necessary to determine a suitable length of time for a fixed-time (discontinuous) assay method. You will employ such a method for Experiment #2 of today’s experiments. **Examine your progress curve and consult your TA/instructor before you choose the reaction time for Experiment #2.**

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**Lab Report:** What criteria are used to determine the appropriate reaction time? What time did you choose and why did you choose it?

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**Experiment #2: Effect of Enzyme Concentration on the Reaction**

Your instructor will prepare a series of eight different trypsin solutions by serially diluting the 12.5 mg/mL stock solution in 0.002 N HCl to give the following concentrations:

- 0 µg/mL
- 62.5 µg/mL
- 250 µg/mL
- 1000 µg/mL
- 31.25 µg/mL
- 125 µg/mL
- 500 µg/mL
- 2000 µg/mL

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**Progress curve for an enzyme catalyzed reaction.** [P], the concentration of product, increases as the reaction proceeds. The initial velocity of the reaction, \( v_0 \), is the slope of the initial linear portion of the curve. Note that the rate of the reaction doubles when twice as much enzyme (2E, upper curve) is added to an otherwise identical reaction mixture.

Carry out a series of basic reactions with different concentrations of trypsin using a fixed-time (discontinuous) assay method. Stop the reaction by adding acetic acid. Scale down the reaction carried out in the cuvette in Experiment #1 since you will be using a 96-well plate reader to measure absorbance in this assay rather than the Shimadzu. A well in a standard 96-well plate can accommodate a maximum volume of 300 µL (see image on the right).

Each scaled-down reaction mixture will contain:
- 250 µL buffer
- 5 µL substrate  Total Volume = 275 µL
- 20 µL enzyme

The reaction is stopped by adding 25 µL of acetic acid to give a final volume of 300 µL. Remember to account for the final volume when converting your absorbance readings into Molar values (necessary for your lab report). To do so, use Beer’s Law (\( A = ecl \)) to convert absorbance to molarity.

**NB:** Unlike the constant path length of cuvettes (horizontal width) used in a standard spectrophotometer like the Shimadzu, the path length for a plate reader is dependent upon the volume of sample in the well since the beam passes vertically through the sample (see figure below from www.promega.com).

For standard 96-well plates it has been found that an aqueous volume of 200 µL has a path length of 0.56 cm. (There is a linear correlation between volume and path length so path lengths for other volumes can be easily determined.)

**PLEASE READ THROUGH ALL OF THE FOLLOWING INSTRUCTIONS BEFORE YOU BEGIN!**

**REMEMBER TO KEEP ANY ALIQUOTS OF ENZYME THAT YOU TAKE ON ICE AT ALL TIMES!**

**ALL OTHER SOLUTIONS ARE KEPT AT ROOM TEMPERATURE.**
1. It is easier and faster to prepare a buffer-substrate **master mix** rather than pipetting each solution individually into the wells for each assay. To prepare a master mix, multiply the number of reactions you plan to do by the volume of each component to be used in each assay AND allow for extra reactions. **Do not go overboard as the substrate is very expensive and there is only so much available for the class!**

For example, if you are doing 8 reactions, set up enough master mix for 10 reactions as follows:

\[
\begin{align*}
10 \times 250 \, \mu\text{L} & \text{ buffer} = 2.5 \text{ mL buffer} \\
10 \times 5 \, \mu\text{L} & \text{ substrate} = 50 \, \mu\text{L} \text{ substrate}
\end{align*}
\]

Mix these two components together in a small glass vial by gently vortexing. Allow the master mix to come to room temperature. **CAUTION: do not prepare your master mix too far ahead of time as the substrate may hydrolyze in aqueous solutions over time.**

2. When you are ready to carry out your reaction, you will pipette **255 \, \mu\text{L}** of your master mix directly into each of the wells that you will use in the 96-well plate (this is the equivalent of 250 \, \mu\text{L} buffer + 5 \, \mu\text{L} of substrate).

3. To begin your reaction add 20 \, \mu\text{L} of your enzyme solution to the well, and **mix by pipetting up and down 3-4 times**. After the reaction time \(X\) has passed, stop the reaction by the addition of 25 \, \mu\text{L} of 30\% acetic acid (again, pipette up and down to mix).

**NB:** Stagger the addition of enzyme in your wells (e.g. 1st well at time zero, 2nd well - 30 sec. later, 3rd well - 30 sec. after that, etc.) and then after \(X\) minutes has elapsed (from time zero), similarly stagger the addition of acetic acid (e.g. 1st well after \(X\) min. has elapsed from time zero, 2nd well - 30 sec. later, 3rd well - 30 sec. after that, etc.) Once the reaction has been stopped by the addition of acetic acid, you can read the absorbance at your convenience. See diagram below which illustrates how to set up and stagger the reactions.

---

**Diagram:**

- Prepare master mix (buffer + substrate). Allow to come to RT.
- Aliquot master mix into eight wells of a 96-well plate.
- **Stagger initiation** (30 sec. later) initiation
- Initiate rxn in 1st well by addition of 10 µg/mL trypsin, mix, and begin timer.
- X min. elapsed
- **Stagger stop** (30 sec. later) stop
- Stop rxn by addition of acetic acid, mix.
- **Stagger stop** (30 sec. later) stop
- Stop rxn by addition of acetic acid, mix.
![Image](https://via.placeholder.com/150)

**Trypsin: Day Three**

The goal for Day Three is to observe the effect of pH on the activity of trypsin and to determine $K_m$ and $K_{cat}$ for the enzyme-catalyzed reaction. You will also see the effect of an enzyme inhibitor.

You will need to prepare your own diluted enzyme solution using the 12.5 mg/mL trypsin stock and 0.002 N HCl as diluent according to your “optimal” [E] to use for assays on Day 3.

You will be using a fixed-time assay method for today’s experiments as in the linearity experiment conducted last week. Calculate volumes required accordingly.

We will be using the buffers that were made on Day One. Make sure you have the right buffers.

The most difficult part of this lab is Experiment #4 where you have to prepare reaction mixtures using different concentrations of substrate and inhibitor so that the final volume is 275 µL. The second most difficult part is timing all of the reactions so that they all go for the same length of time. Take your time and plan thoroughly before doing anything. Don’t forget to do the pH experiment and the **PCR reactions for the GMO module**.

---

ΔA = $A_x - A_0$

where $A_x$ is Abs of rxn at particular [E]

and $A_0$ is Abs from no enzyme control (1st well)

Initial rate, $v_i = \frac{\Delta A}{\text{time}}$

Plot $v_i$ vs. [E]$_i$

---

**Lab Report:** What concentration did you choose? What factors did you have to take into account?

---

Don’t forget to complete Step 2 of the GMO module before you leave the lab!
Reagents

- Standard assay buffer: 0.14 M Tris, pH 7.8, 0.025 M CaCl₂
- Substrate: 5 x 10⁻² M N-α-benzoyl-DL-arginine-p-nitroanilide in DMF
- N,N Dimethyl Formamide (DMF)
- Enzyme stock solution (dilute as needed): 12.5 mg/mL Trypsin in 0.002 N HCl. STORE ENZYME ON ICE AT ALL TIMES INCLUDING DILUTED SOLUTIONS!
- 0.002 N HCl (to dilute enzyme stock)
- Acetic acid (30% v/v) (to stop the reactions)
- 0.14 M Tris-maleic acid buffers (prepared by class on Day One), pH’s 9.5, 8.5, 7.5 and 6.5, containing 0.025 M CaCl₂
- 0.14 M Sodium acetate buffers (prepared by class on Day One), pH’s 5.5, 4.5 and 3.5, containing 0.025 M CaCl₂
- Benzamidine solutions: various concentrations, dissolved in standard assay buffer

**Experiment #3: Effect of pH on the Activity of Trypsin**

1. Be sure you have prepared your diluted enzyme solution as described above.

2. Equilibrate the buffer and substrate by substituting one of the class’s buffers for the buffer normally used in the standard assay. Do this for each of the buffers prepared by the class, and also for the standard pH 7.8 buffer for comparison. **Prepare a duplicate set of wells – one set will be used as a control series with no enzyme.** Use 250 µL of buffer and 5 µL of substrate for each assay (set up directly in wells—see diagram on page 23).

3. Be sure the mixtures are at room temperature, then start the reaction by adding either 20 µL of diluted enzyme (one set) OR 20 µL of 0.002 N HCl as a control (the other set). Repeat this for each buffer available by staggering initiation and stop the reaction as you did for the enzyme linearity experiment last week. We suggest that you start the corresponding no enzyme reaction for a particular pH immediately following the reaction with enzyme. Work from the highest pH to the lowest pH.

**Treatment of pH Data**

Calculate initial rates as you did last week, accounting for any absorbance seen with the corresponding no-enzyme controls at each pH. Plot a graph showing activity (initial rate) vs. pH. Recall that trypsin is a serine protease that utilizes a catalytic triad to cleave proteins on the C-terminal side of lysine or arginine residues. Consider how the catalytic triad as well as how the overall structure of the enzyme might be affected by exposure to different pHs and how this, in turn, would affect the ability of trypsin to catalyze the reaction.

**Lab Report:** Trypsin is synthesized in the pancreas as trypsinogen. It is secreted into the duodenum (small intestine) where it is activated. Discuss why the enzyme (gene) has evolved to generate the pH activity profile that you observe.
Experiment #4: Michaelis-Menten Kinetics

In this final trypsin experiment you will study the effect of varying substrate concentration on trypsin activity—you will perform Michaelis-Menten analyses of the enzyme. You will also examine the effect of the inhibitor benzamidine on the activity of trypsin using standard Michaelis-Menten analyses. Please review the relevant theory regarding single substrate enzyme kinetics that you have covered in your lecture courses, including equations and graphical methods of analysis (non-linear (v vs. [S]) and Lineweaver-Burk (1/v vs. 1/[S]) transformation). Also review inhibitor kinetics including competitive and (pure) non-competitive inhibitors, their respective equations and graphical methods of analysis.

Leonor Michaelis (1875-1949) and Maud Menten (1879-1960) are very famous biochemists. Do you have a photo (e.g. selfie) of you celebrating some personal connection to one of these scientists? If so, we would be happy to post it. Be imaginative and creative (and observant).

PLEASE READ THROUGH ALL OF THE FOLLOWING PROCEDURES BEFORE YOU BEGIN SETTING UP YOUR EXPERIMENT.

You will be running a total of 30 different reactions in the wells of a microtiter plate. There are six series (A, B, C, D, E, and F) each with a different concentration of substrate. Within each series, there are five samples (A1, A2, A3, A4, A5). One of them will have no enzyme (A1). One of them will have only enzyme and substrate (A2). The other three will have enzyme plus substrate plus an inhibitor at three different concentrations,

This experiment is carried out under standard conditions, except that you vary the substrate concentration over approximately a ten-fold range. Set up SIX different master mixes containing different substrate concentrations as shown in the example table below (Set up of Master Mixes).
The smallest volume of substrate to be used is 1 µL and the largest is 10 µL. Recall that the substrate is dissolved in DMF so any changes in substrate volume should be compensated for by adding the appropriate amount of DMF so that the total volume of this solvent remains constant at all times. In the example shown below, the total volume of substrate + DMF is always 10 µL. As always, total volume in the reactions must remain constant.

**NB: When calculating the amount of Master Mix to prepare, you should always allow for extra reactions. Prepare Master Mixes in 2 mL microfuge tubes.**

<table>
<thead>
<tr>
<th>Example: Set up of Master Mixes</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>#txns x 240 µL</td>
<td>#txns x 9 µL</td>
<td>#txns x 1 µL</td>
</tr>
<tr>
<td>B</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>#txns x 240 µL</td>
<td>#txns x 5 µL</td>
<td>#txns x 5 µL</td>
</tr>
<tr>
<td>E</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>#txns x 240 µL</td>
<td>#txns x 0 µL</td>
<td>#txns x 10 µL</td>
</tr>
</tbody>
</table>

**NB: Prolonged exposure to aqueous environment may cause hydrolysis of the substrate. Do NOT prepare your master mixes all at once. Prepare one at a time, complete that series of assay measurements, then prepare the next master mix, and so on. Prepare each master mix in a small test tube when ready and vortex gently to mix.**

You will measure each series of substrate concentration at four different constant concentrations of benzamidine **PLUS** a no-enzyme (Benz0) set of reactions as your controls. Benzamidine solutions have been prepared by dissolving in standard assay buffer. The benzamidine solutions are labelled Benz10, Benz25, and Benz50.

<table>
<thead>
<tr>
<th>Tube Name</th>
<th>Concentration of Benzamidine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benz0, a.k.a. Standard buffer</td>
<td>0 M</td>
</tr>
<tr>
<td>Benz10</td>
<td>10 x 10^{-4} M</td>
</tr>
<tr>
<td>Benz25</td>
<td>25 x 10^{-4} M</td>
</tr>
<tr>
<td>Benz50</td>
<td>50 x 10^{-4} M</td>
</tr>
</tbody>
</table>

Each group should take a **60 µL aliquot** of each concentration of benzamidine.

The table provided below gives an example of how to set up the reactions required for this experiment. You will need to prepare a similar table containing your six different substrate concentrations.
The set of reactions named Benz0 contain no inhibitor (benzamidine) and will be used to determine $K_m$, $V_{\text{max}}$, and $k_{\text{cat}}$ for trypsin (i.e. the standard M-M analyses in the absence of any inhibitor). A duplicate set of Benz0 reactions should be run without added enzyme (your no-enzyme controls).

Each well will contain:

- 240 µL of buffer
- 10 µL of substrate at various concentrations
- 5 µL of inhibitor solution ranging from 0 to 0.005 M
- 20 µL of enzyme

for a total volume of 275 µL.

**NB:** In choosing the volumes of substrate to use you must keep in mind that we would like to have an even spread of points on the reciprocal $1/v$ vs. $1/\left[S\right]$ (Lineweaver-Burk) plot, rather than having the points evenly distributed on the $v$ vs. $\left[S\right]$ (non-linear) plot. Work backwards to determine suitable volumes of substrate to use in your assays!

The table on the next page provides an example of how to set up your reactions in your wells. The diagram on the following page suggests a plate layout for your reactions for both experiments done today. Your solutions should be at room temperature before you begin any assays (except the enzyme which is always kept on ice). Once a series of reactions are set up, initiate by the addition of 20 µL of diluted enzyme (staggered). After Time X has elapsed, similarly stop reactions by staggering addition 25 µL of 30% acetic acid. (The final volume in the well will be 300 µL.)

<table>
<thead>
<tr>
<th></th>
<th>Benz0</th>
<th>Benz10</th>
<th>Benz25</th>
<th>Benz50</th>
<th>Benz0 (ctrl)*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Series A</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A0: 250 µL series A master mix + 5 µL Benz0 solution</td>
<td>A0: 250 µL series A master mix + 5 µL Benz10 solution</td>
<td>A25: 250 µL series A master mix + 5 µL Benz25 solution</td>
<td>A50: 250 µL series A master mix + 5 µL Benz50 solution</td>
<td>A0: 250 µL series A master mix + 5 µL Benz0 solution</td>
<td></td>
</tr>
<tr>
<td><strong>Series B</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B0: 250 µL series B master mix + 5 µL Benz0 solution</td>
<td>B10: 250 µL series B master mix + 5 µL Benz10 solution</td>
<td>B25</td>
<td>B50</td>
<td>B0</td>
<td></td>
</tr>
<tr>
<td><strong>Series C</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C0</td>
<td>C10</td>
<td>C25</td>
<td>C50</td>
<td>C0</td>
<td></td>
</tr>
<tr>
<td>etc.</td>
<td>etc.</td>
<td>etc.</td>
<td>etc.</td>
<td>etc.</td>
<td></td>
</tr>
</tbody>
</table>
*Benz0 (ctrl) is a Benz0 set to which 0.002 N HCl will be added instead of enzyme (i.e. no-enzyme control reactions).

SUGGESTED LAYOUT FOR YOUR REACTIONS IN A 96-WELL PLATE.

![96-well plate layout diagram]

It would be helpful to have a copy of this plate layout pasted along side your Absorbance readout in your lab notes for ease of reference.

**Treatment of Michaelis-Menten data**

You will determine initial rates of hydrolysis for each reaction, and plot a series of Lineweaver-Burk (L-B) lines as indicated below. If time permits, plot your raw data before you leave the lab (you may use Absorbance values instead of converting to molar units for this quick in-lab plot.)

**Lab Report:** How do your values for $K_M$ and $K_{cat}$ compare to those reported by Barman (1969) in the *Enzyme Handbook*?
The plot on the right reminds you what a Lineweaver-Burk plot looks like and how it is derived from the standard Michaelis-Menten equation. You should consult a textbook for more information on the transformation of a hyperbola into a straight line. Modern labs will fit the data directly to a hyperbolic curve and calculate the kinetic parameters from that curve.

**Double-reciprocal (Lineweaver–Burk) plot.** This plot is derived from a linear transformation of the Michaelis–Menten equation. Values of $1/v_0$ are plotted as a function of $1/[S]$ values. (Moran et al. (2011) *Principles of Biochemistry* 5th ed. p. 147)

The graphical sketches below illustrate two possible theoretical outcomes for this type of M-M experiment plotted as L-B transformations:

- Line 1 = A0, B0, C0, etc.
- Line 2 = A10, B10, C10, etc.
- Line 3 = A25, B25, C25, etc.
- Line 4 = A50, B50, C50, etc

The dashed lines in grey indicate extrapolation of the plotted data.

**Lab Report:** How do you use these slopes to determine $K_i$? What does $K_i$ tell you about an inhibitor?
TRYPSIN

(Hydrolyses peptides, amides, esters, etc. at bonds involving the carboxyl group of L-arginine or L-lysine)

**Molecular weight**

<table>
<thead>
<tr>
<th>Source</th>
<th>Value</th>
<th>Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine pancreas</td>
<td>25,281</td>
<td>sequence studies</td>
</tr>
<tr>
<td>Porcine pancreas</td>
<td>25,400</td>
<td>pH 3.2</td>
</tr>
</tbody>
</table>

**Specificity and Kinetic constants**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$k_2$ (sec$^{-1}$)</th>
<th>$k_m$ (M)</th>
<th>Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>benzoyl-L-arginine ethyl ester</td>
<td>22</td>
<td>5x10$^{-5}$</td>
<td></td>
</tr>
<tr>
<td>benzoyl-L-argininamide</td>
<td>0.18</td>
<td>2.1x10$^{-3}$</td>
<td>pH 7.8-8.0, 25°</td>
</tr>
<tr>
<td>p-toluenesulphonyl-L-arginine methyl ester</td>
<td>147</td>
<td>5x10$^{-5}$</td>
<td></td>
</tr>
<tr>
<td>L-lysine-p-nitroanilide</td>
<td>0.003</td>
<td>3.64x10$^{-4}$</td>
<td>pH 8.5-8.8, 15°</td>
</tr>
<tr>
<td>benzoyl-L-arginine p-nitroanilide</td>
<td>0.611</td>
<td>9.39x10$^{-4}$</td>
<td></td>
</tr>
</tbody>
</table>

The data given are those of the enzyme isolated from bovine pancreas.

**Light absorption data**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$k$(max)</th>
<th>$k$ (substrate/product)</th>
</tr>
</thead>
<tbody>
<tr>
<td>benzoyl-L-arginine ethyl ester</td>
<td>255</td>
<td>808 M$^{-1}$ cm$^{-1}$ (pH 5-10)</td>
</tr>
<tr>
<td>p-toluenesulphonyl-L-arginine methyl ester</td>
<td>247</td>
<td>540 M$^{-1}$ cm$^{-1}$ (pH 8.1)</td>
</tr>
<tr>
<td>benzoyl-L-arginine p-nitroanilide</td>
<td>410</td>
<td>8800 M$^{-1}$ cm$^{-1}$ (pH 8.2)</td>
</tr>
</tbody>
</table>

**References**

3. Hill, R.L. (1965) *Advances in Protein Chemistry*, 20, 64

Enzyme kinetics 10