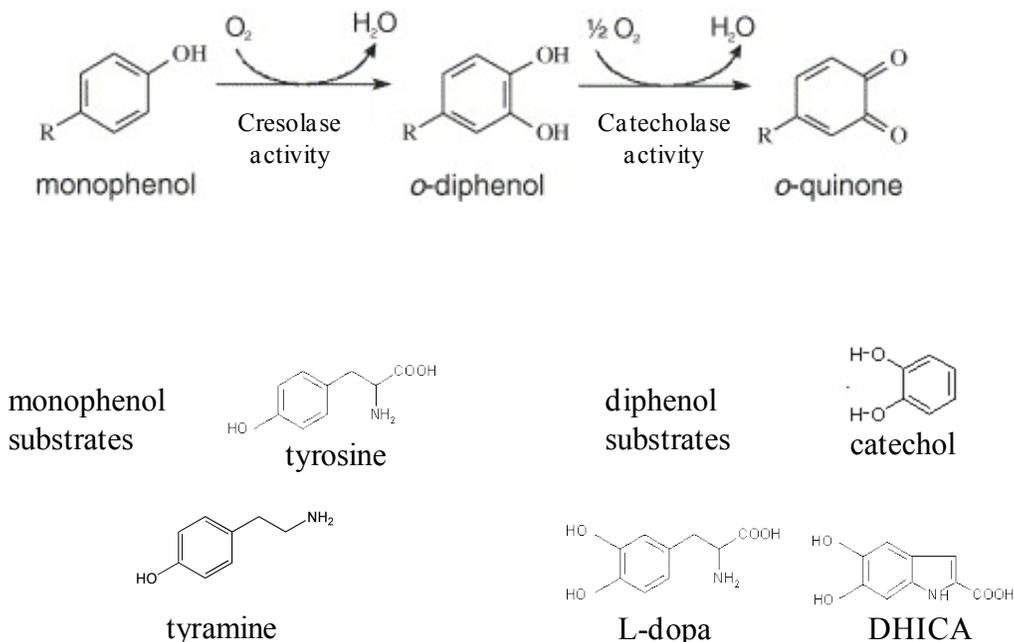


Characterization of Mushroom Tyrosinase Activity

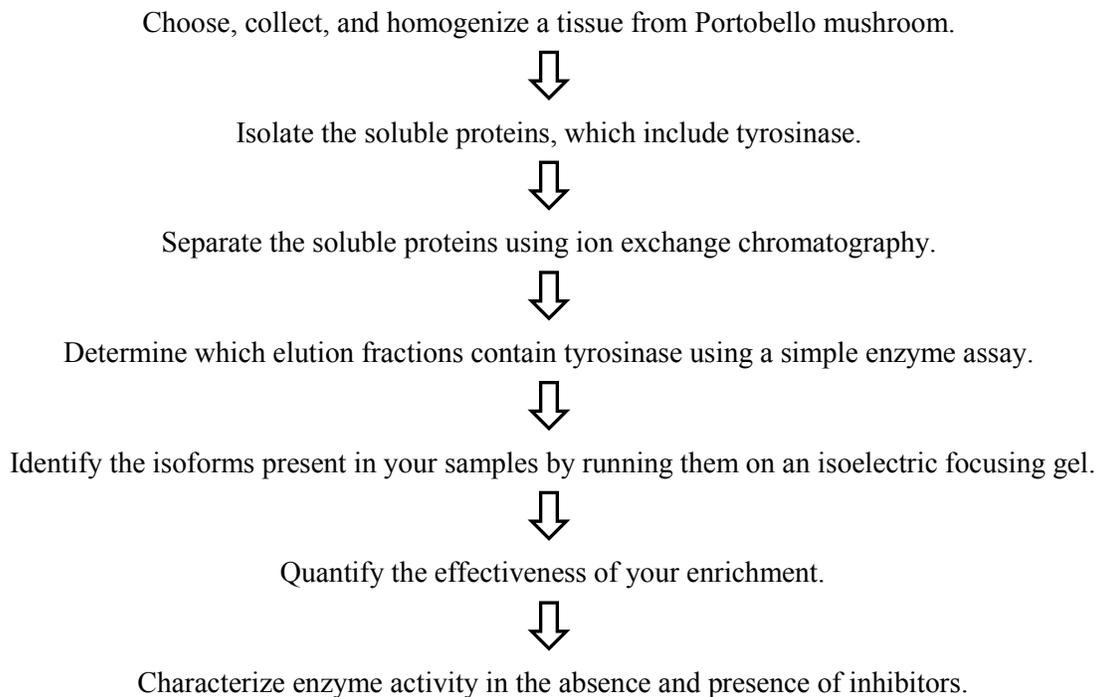
LAB OVERVIEW

If you let mushrooms sit in your refrigerator for too long, they will turn brown, with the most pronounced darkening occurring where they have been bruised or wounded. This is caused by the accumulation of brown pigments known as *melanins*. Melanins are formed through the enzymatic oxidation of phenols (such as L-tyrosine) followed by a series of spontaneous oxidations and polymerizations. In mushrooms, melanins help the organism protect themselves from biotic and abiotic stresses. For example, melanins provide cell wall resistance against hydrolytic enzymes. Melanins are also involved in the formation and stability of spores.

Tyrosinases are a group of oxidoreductases that catalyze the initial oxidation reaction in melanin formation. They are found in bacteria, fungi, plants, insects, and mammals. Tyrosinases have two distinct activities: (1) the reduction of monophenols (e.g. tyrosine), which is referred to as cresolase activity, and (2) the oxidation of diphenols (e.g. catechol), which is referred to as catecholase activity. Different tyrosinases will hydroxylate different substrates to varying degrees. For example, the diphenol DHICA is a substrate of human tyrosinase but not of mouse tyrosinase. Diphenols are better substrates of mushroom tyrosinases than monophenols.



Mushrooms have at least ten different isoforms of tyrosinase. While the reason so many isoforms exist is not known, it is known that they differ in enzymatic activity and tissue specificity. In this lab, you will identify and enrich the isoenzymes present in different tissues of the Portobello mushroom. You will take advantage of the fact that the isoforms differ in their isoelectric points to separate them via anion exchange chromatography. You will characterize the enzyme activity in an enriched sample containing 1 or more isoforms, and characterize the effect of various inhibitors on this activity. A flow chart for the lab is shown below.



SIX-DAY OUTLINE

Day 1: Isolate soluble proteins and prepare the anion exchange columns

To isolate soluble proteins, you will homogenize the mushroom tissue you have chosen by freezing, thawing, and grinding. You will then separate soluble proteins from insoluble cellular material by centrifugation followed by filtration. The result is called the clarified lysate. You will also prepare an anion exchange column that will be used on day 2.

Day 2: Separate proteins by anion exchange chromatography

You will use a low-pressure flow-through chromatography system and a Sepharose-DEAE column to separate proteins into different fraction according to charge and test the resulting protein fractions for tyrosinase activity.

Day 3: Identify isoforms of tyrosinase

You will identify the isoforms of tyrosinase in your fractions by running them on isoelectric focusing (IEF) gels, which separate proteins according to their isoelectric points (pI). You will also characterize the enzymatic activity of a commercial sample of tyrosinase. Specifically, you will determine if the substrate dependent activity obeys Michaelis-Menten kinetics and determine the Michaelis-Menten constant, K_M if it does.

Day 4: Quantify your how well your enrichment worked and begin characterizing enzyme kinetics

You will determine how well your enrichment procedure worked by quantifying your enrichment level (fold increase in enzymatic activity per total protein) and yield (the percent of the initial enzymatic activity that remains in the sample). You will also begin characterizing the enzyme activity of choosen fractions containing one or more tyrosinase isoform.

Day 5: Continue enzyme assays and design inhibitor assays

After characterizing the enzyme activity of your isoform(s), you will design a series of experiments that will characterize the affects of a specific inhibitor on the tyrosinase activity of your sample.

Day 6: Perform inhibitor studies

DAY 1: ISOLATE SOLUBLE PROTEINS AND PREPARE THE ANION-EXCHANGE COLUMN

Preparing the mushroom homogenate

1. Choose and dissect a particular tissue from Portobello mushrooms. Possible tissue choices include the cap skin, cap, gills, and stem. Collect ~15 g of tissue and break into relatively small pieces. Create a table in your notebook to record the masses and volumes of your enriched samples throughout the lab, and record the initial mass of your mushroom sample.
2. Break the cells in your sample by freezing, thawing, and grinding

Place the tissue in the mortar, and freeze by filling the mortar with liquid nitrogen (N_2 , 73°K). Grind the tissue with a pestle until it is about the consistency of cocoa powder. Be careful to not let the mushroom-nitrogen slurry spill out of the mortar. Liquid N_2 has almost no friction on a smooth surface because of the *Leidenfrost effect*. Add more N_2 as it evaporates away.
3. Pre-weigh an empty 50 ml conical and record its mass in your table. Pour the mushroom powder- N_2 slurry into the pre-weighed conical. Adding more N_2 to the slurry makes it easier to pour, again because of the Leidenfrost effect. CAUTION: Do not screw the 50 ml conical lid on tightly. Because the the gas will expand ~1000-fold as it evaporates, this can create a “mushroom bomb.”
4. Wipe off any ice that has collected on the outside of the conical and weigh the conical plus mushroom powder. An accurate mass cannot be measured until most of the N_2 has evaporated, at which point the mass will remain fairly constant. Calculate and record the mass of the mushroom powder in your table.
5. Add to your sample 1.5 ml of *Tyrosinase Lysis Buffer* per g of powder.
6. Gently mix at 4°C for 20 min. Break up frozen clumps of sample by tapping the conical against a surface every 2-3 min. The resulting powder should be thoroughly mixed in the liquid, with no frozen clumps remaining. Continue shaking for 5 min if clumps remain.
7. Centrifuge the sample at 4°C for 30 min at 8000 g. When centrifuging, always include another 50 ml conical of equal mass (within 0.01 grams) to balance your sample. Use a 50 ml conical filled with water (or another group’s sample) for your balance, and place the two samples in mirror opposite positions in the rotor. The solid that sediments to the bottom of the conical is referred to as the *pellet*, and the liquid above the pellet is the *supernatant*. The pellet contains large mushroom chunks and insoluble cellular components while the supernatant contains soluble proteins including tyrosinase.
8. In a quick and smooth motion, pour the supernatant into a new 50 ml conical. Avoid disturbing the pellet. There may still be some particulate in the supernatant.
9. Measure the mass of the pellet and the volume of supernatant, and record these in your table.
10. Using a syringe and filter, remove the remaining particulate from the supernatant and collect the resulting sample, the *clarified supernatant*, in a new 50 ml conical. To filter the sample, remove the plunger from a 12 ml syringe and screw a 0.22 filter on its end. Do not reverse the order of these last two steps to avoid damaging the filter. Pour ~10 ml of your sample into the open end of the syringe. Place the plunger back into the syringe and apply gentle and continuous pressure to push the liquid through (excessive force may damage the filter). As

the filter becomes clogged, you will feel greater resistance against your pushing. Replace the filter as necessary. Filter your entire sample.

11. Collect 200 μL of the clarified supernatant for analysis (Days 3 and 4). Store at 4°C.

Preparing the anion exchange column

Column preparation and chromatography is performed using the computer controlled Bio-Rad Biologic Low Pressure (LP) chromatography system. In column chromatography the column bed is referred to as the *solid phase* and the solvents and buffers that flow through the column are the *mobile phase*. A detailed background of chromatography is provided on Day 2. The LP system consists of the components listed below:

1. A peristaltic pump that is capable of delivering flow rates from 0.05 to 40 ml/min.
2. A mixer module that allows two buffers to be mixed in various proportions. The mixed buffers make up the *mobile phase*.
3. A 6-way router that controls whether the *mobile phase* flows through the sample loop. In one configuration of the router, the sample loop is detached from the LP system flow (Figure 1). In a second configuration, the mobile phase flows through the sample loop before passing through the column (Figure 2). The router contains the *injection* port, through which sample is added to the sample loop.
4. A column that contains the stationary phase. For the tyrosinase enrichment, the column is packed with DEAE sepharose.
5. A UV optics module that measures absorbance at 280 nm (A280) just following the column. A280 is roughly proportional to protein concentration.
6. A conductivity detector that measures conductivity just following the column. Because conductivity is proportional to ionic strength, this measurement is useful for tracking changes in the salt concentration.
7. A fraction collector that allows for automated collection of fixed volume samples, which we refer to as fractions.
8. Tygon tubing that connects all the components.

The schematics on the next page show the two configurations of the LP system: (1) the sample-loading configuration and (2) the sample-injection configuration. The router is turned counterclockwise for the sample-loading configuration and clockwise for the sample-injecting configuration. The system should be stored in the sample-loading configuration. Study these figures and trace the path of the flow in each configuration.

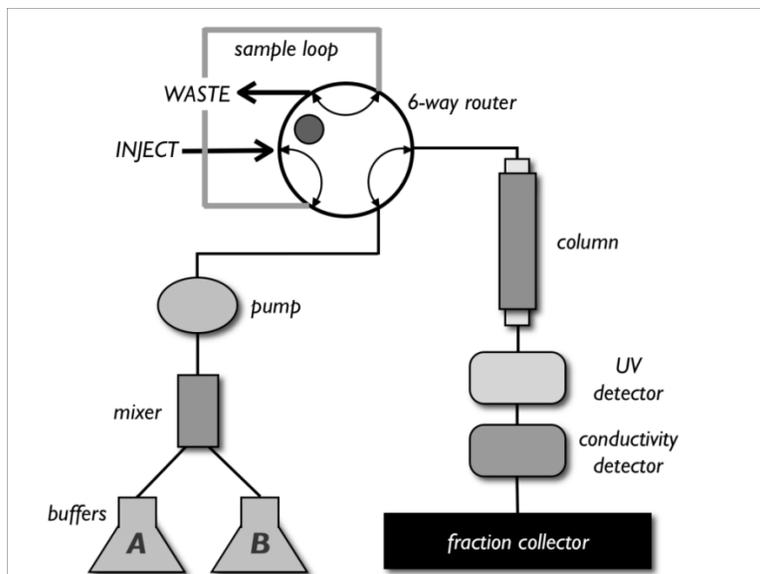


FIGURE 1 - Sample-loading configuration. The router is in the counterclockwise position.

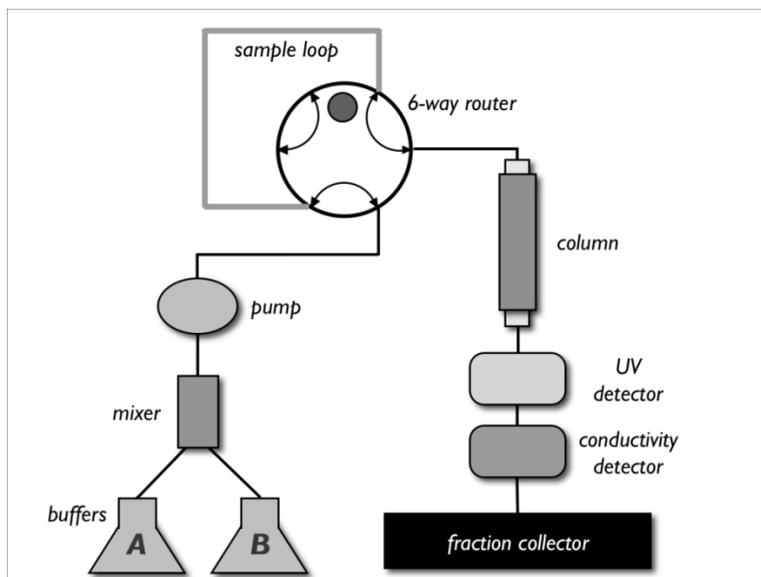


FIGURE 2 - Sample-injection configuration. The router is in the clockwise position.

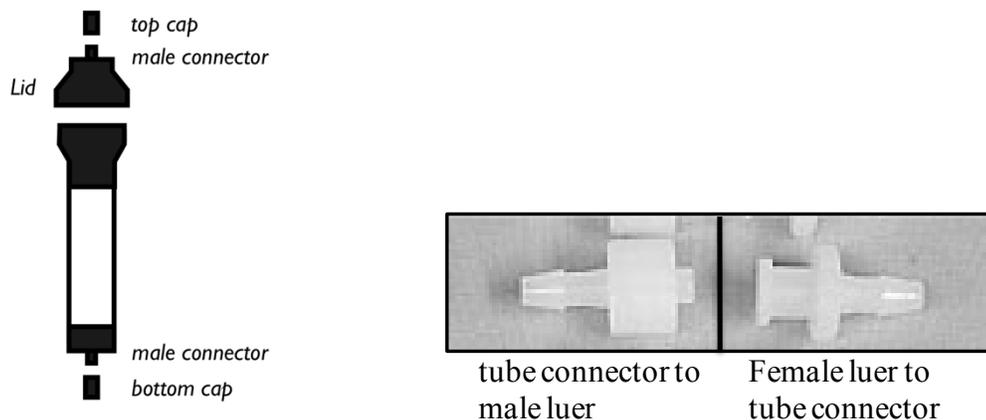


FIGURE 3 - Reusable glass column and luer adaptors. Tubing is attached to components of the LP system through male and female luer adaptors.

The on/off switch is on the front left of the LP system. The three most common tasks you will be required to perform when using the LP system are described below.

To start a flow: Push the MANUAL key followed by the PUMP key. Push the key below FLOW on the digital display, type in the flow rate, and push the key below OK. Push START.

To stop a flow: Push the MANUAL key followed by the PUMP key. Push the key below STOP on the digital display.

To select a buffer: Push the MANUAL key followed by the PUMP key. Push the button below BUFFER on the digital display. Use the arrow keys to select buffer A or B. Push the key below OK.

Step 1: Preparing the DEAE-Sepharose column

Sepharose is a bead-form of agarose to which various exchangers such as diethylaminoethyl (DEAE) can be crosslinked. A description of DEAE anion exchange chromatography can be found in the background to day 2.

1. Gently mix the DEAE-sepharose to ensure the slurry is homogeneous. It takes some time to get the sepharose to re-suspend, so be patient. Whenever mixing the sepharose, avoid introducing air bubbles. The slurry is stored in 20% ethanol and sepharose settles *very* rapidly.
2. Pipette 8 ml of slurry into a 15 ml conical. This is enough for a 6 ml column volume.
3. Let the exchanger settle to the bottom of the conical (~10 min).
4. Pipette off most of the ethanol without disturbing the exchanger. Add filtered water to the slurry until the total volume reaches 12 ml. 12 ml is just less than the total volume of the column.
5. Mix by gently inverting the conical.

6. Place a glass column upright in a lab stand, and place the lab stand on the LP system platform. Be sure that the column is perfectly upright and vertical to prevent a slanted surface at the top of the column bed.
7. Attach the bottom cap to the column (Figure 3).
8. Unscrew the lid from the column and the top cap from the lid (Figure 3).
9. Pour the slurry into the column in a single, smooth motion. You will have to remix the DEAE-water mixture before pouring.
10. Using a squirt bottle, add water to the column until the level of liquid rises to the very top. This will ensure that, when the top cap is placed on the column, no air is introduced.
11. Let the exchanger settle in the column until ~3 cm of the liquid at the top of the column is completely transparent. This will take a few minutes.

Step 2: Packing the column with flow

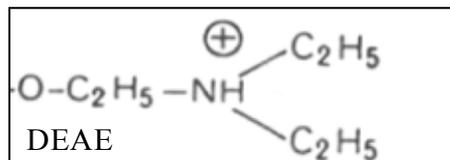
You will now pack the ion exchanger into the column using flow. Be sure the LP system is in its sample-loading configuration (Figure 1) before continuing. Specifically, make sure the 6-way router is in the appropriate position (rotated counterclockwise). The system should always be stored in the sample-loading configuration when not in use. Carefully review the entire protocol before starting, since some steps must be performed quickly.

1. Place line (A) into filtered water. Place the *WASTE* lines into a waste container.
2. Select buffer [A] (see directions above).
3. Push the key corresponding to PURGE on the digital display. This will pump water at high flow rates (6.5 ml/min) through the system and remove all air. If you do not see PURGE on the digital display, push the MANUAL key followed by the PUMP key.
4. After 4 min, stop the purge by pushing the STOP key on the digital display.
5. Set the flow to 2 ml/min - Push the FLOW key, type in 2, and push OK.
6. Start the flow.
7. Detach the male and female luer connectors adjacent to the 6-way router. Water will slowly flow from the female connector (Figure 3).
8. Attach the female luer to the male connector on the column's lid. Water will now drip from the top cap.
9. Screw the lid to the top of the column. Excess water will spill down the side of the column making this a messy procedure. Make sure that no important buffers are underneath the column.
10. **Quickly**, remove the bottom cap of the column before too much pressure builds up. This will make a mess as water starts dripping from the bottom of the column.
11. Disconnect the other set of male-female luer connectors, and attach the female connector to the bottom of the column. The system is now in its *sample-loading* configuration (Figure 1).

12. The flow will pack the exchanger. Pack the column until the exchanger is completely settled and the liquid above the column is translucent (~ 5min). While the column is packing, check that the top of the settled exchanger is flat and perpendicular to the column. Adjust the column in the stand as necessary to prevent an uneven column bed.
13. Using the marking on the side, estimate the volume of the column bed (column volume, CV) and record this in your table.
14. Detach the column without introducing air onto the column. Perform step #1 from the *Protocol for attaching a column or introducing a new buffer*, page 85.

DAY 2: SEPARATE PROTEINS BY ANION EXCHANGE CHROMATOGRAPHY

Chromatography is the most commonly used techniques for separating proteins. A sample consisting of multiple proteins is allowed to interact with a **mobile phase** and a **stationary phase**. In column chromatography, the stationary phase is packed into a column and the mobile phase (a solvent or buffer) is flowed through the column. The affinity of a protein for a column depends on the chemical properties of both the protein and the stationary and mobile phases. In ion-exchange chromatography, the stationary phase is charged. Thus, when the mobile phase is of low ionic strength, the degree to which a protein adsorbs to the stationary phase is dependent on the protein's charge. We will use Sepharose-DEAE as our stationary phase. DEAE (diethylaminoethyl) is a weak anion exchanger (meaning it binds anions).



Before sample is loaded onto the column, the column is pre-equilibrated at pH 7 with a low ionic-strength buffer (*DEAE loading buffer*, 10 mM sodium phosphate, pH 7). All proteins that are negatively charged at pH 7 will bind the column under these conditions. The isoelectric point (pI) of a protein is the pH at which the protein is uncharged. *What do you know about the pI values of proteins that bind the column at pH 7?*

The column is then washed with *DEAE loading buffer* to ensure removal of all uncharged or positively charged proteins. Next, the proteins are eluted with a gradient of sodium chloride ranging from 0 to 1 M NaCl. The chloride ion compete with the proteins for the DEAE binding sites. *Would you expect proteins with higher pI values to elute at higher or lower NaCl concentrations?*

When describing the amount of buffer passed over the column, we will use the column bed volume (CV) determined on Day 1 as our base unit of volume. Before continuing, prepare the fraction collector. Fill the fraction collecting rack with 13x100mm glass culture tubes, and place pre-numbered 1.5 ml Eppendorf tubes in each. Only number every 5th tube. Make sure the collection rack is placed properly in the collector (ask an instructor to check!). Place the arm of the fraction collector directly over the tube for fraction 1.

Protocol for attaching a column or introducing a new buffer:

There will be multiple instances when you need to either attach a column or introduce a new buffer to the LP system. Both of these actions introduce air into the tubing, and care must be taken to not allow this air to be introduced onto the column. Air bubbles cause the sample to travel unevenly down the column, and thus compromise the ability of the column to separate proteins. If you are introducing a new buffer to the system and there is a column attached to your system, first you must detach the column from the system, which is described in the first step. If there is no column attached to your system, start at Step 2.

1. To detach the column without introducing air onto the column, perform the following steps:
 - a. Start a 3 ml/min flow of the buffer in which the column will be stored.
 - b. Remove the female luer connector that is on the bottom of the column. Liquid will drip from the bottom of the column.
 - c. Attach the bottom cap to the column (Figure 3).
 - d. **Quickly**, remove the female luer connector on the column's top before too much pressure builds.
 - e. Attach the top cap to the column. Store the column upright at 4°C.
 - f. Find the short piece of tubing with 2 male connectors on either end, and attach it to the 2 female connectors you detached from the column.
2. Put the system in the *sample-loading* configuration (Figure 1). Make sure the 6-way router is in the counterclockwise position. Place the column in a stand, checking that it is completely upright and vertical. Place the stand on the LP system platform, and place lines A and B in their buffers.
3. Remove all air from the LP system by purging lines A and B with buffer. To do this, select either buffer A or B. Push the PURGE key to start a 6.5 ml/min flow. You may see air bubbles make their way through the system tubing. After 3 min, the system should be purged of all air bubbles. Switch the pump to the other buffer, and continue purging. When the system is purged of all air, push the STOP key.
4. Start a 3 ml/min flow of buffer A. Disconnect the female-male luer connectors adjacent to the 6-way router (Figure 1). Buffer will drip from the female connector.
5. Unscrew the lid from the column and remove the top cap from the lid (Figure 3). Attach the female connector from which buffer is dripping to the column lid.
6. Using water from a squirt bottle, fill the column until the liquid reaches the very top. Screw the lid back onto the column. Excess buffer will spill down the sides of the column, so make sure no buffers are underneath it.
7. **Quickly**, remove the bottom cap of the column before too much pressure builds. This will make a mess, so do not place any buffers underneath your column.
8. Disconnect the other set of male-female luer connectors (Figure 1), and attach the female connector to the bottom of the column. The system is now in its *sample-loading*

configuration (Figure 2). You have now attached the column to the LP system flow without introducing air into the column bed.

Step 1: Pre-equilibrating the column

1. Turn the LP system on (the black switch on the bottom left).
2. Start the LP Data View Software, which records time traces of conductivity and absorbance at 280 nm from the LP system. Double-click the LP Data View icon on the computer desktop.
3. On the front panel of the LP system, push the MANUAL key and then the RECORDER key. Start the chart recorder output by pushing RECORD. LP Data View will automatically start recording. Traces of conductivity and UV absorption will begin to scroll across the screen, and the *Record* button along the top of the Data View window will appear depressed.
4. The ranges of the x- and y-axes can be altered using the computer controls along the bottom of the window or drawing a square with the mouse.
5. Put the column online using the protocol above. Put line A in *DEAE loading buffer* and line B in *DEAE elution buffer*.
6. Set the flow rate to 6 ml/min.
7. Equilibrate the column with 3-5 CV of *loading buffer* (Buffer A). The column is equilibrated when UV and conductivity readings stop changing.

Step 2: Loading sample on the column

Load sample into the sample loop by following the steps below. In the system's sample-loading configuration, the sample loop path is completely detached from the LP system's flow (Figure 1). When sample is injected through the *injection* port of the 6-way router, liquid in the sample loop exits to the *waste* port. Thus, sample can be loaded into the loop while the column is still being equilibrated. An 8 ml sample loop is provided. To prevent introduction of air onto the column, always overfill the loop. This is especially important because the 6-way router and the luer connections to the sample loop add additional volume to the sample loop path. Thus, for the 8 ml sample loop, plan on loading 9 ml of sample. As you load the sample, it may be difficult to tell when the loop is filled and when sample has begun to enter the waste line. To make this easier, before injecting the sample, use an empty syringe to push air into the loop. This clears out any liquid and makes it easier to see when sample has completely filled the loop. If you would like to make a different volume sample loop, calculate the length of Tygon tubing required for your desired volume. $\text{Volume} = [\text{tube-length (cm)}] * [\text{Volume/cm}]$. $\text{Volume/cm} = 0.020 \text{ ml/cm}$ for the tubing provided to you. Cut the tubing, and attach male luers to each end. Label the volume of the loop so that others can use it.

1. Fill a syringe with your sample. The end of your syringe should be a female luer connector. To help you collect all the sample, attach a small length of Tygon tubing containing a male luer connector to the syringe (this has been provided for you). Remove this tubing after filling the syringe.
2. Remove all air from the syringe, and screw it to the *injection* port of the 6-way router (Figure 2). Slowly depress the plunger. Fill the loop with sample until sample can be seen exiting the *waste* port. Do not depress the syringe completely, or you may accidentally introduce air into the loop. Leave the syringe attached to the router for the remainder of the protocol.
3. Inject your sample onto the column:
 - a. Stop the 6 ml/min flow.
 - b. Switch the LP system to the *sample-injection* configuration by rotating the 6-way router clockwise (Figure 3). Buffer now flows through the sample loop before entering the column.
4. Restart the flow at 3 ml/min.
5. From this point on, collect *everything* that passes through the column. Prior to elution, collect sample that passes over the column in 50 ml conicals. The sample will be coming out of the *waste* line following the conductivity meter.
6. Once the sample is injected, switch the router back to the *sample-loading* configuration by rotating counterclockwise (Figure 2). It is not necessary to stop the flow. Though you will not affect the sample elution if you forget to do this, you will add an additional 9 ml of volume to your LP system, thus increasing the length of time to complete the anion exchange chromatography protocol.

Step 3: Washing the column

Wash the column with 3-5 CV of loading buffer at a flow rate of 3 ml/min. The washing is complete when the UV and conductivity readings reach baseline values. You can set up the program for step 4 during the wash, but **do not hit run**.

Step 4: Eluting your sample from the column

1. Push the PROGRAM key and select NEW METHOD.
2. The digital display will ask whether you want to use units of *time* or *volume* for your method. Choose *volume*.
3. Select ADD to add a new step to your method.
4. Push the GRAD key to create a gradient step.
5. Set the beginning %B to zero and the final %B to 100. You can move between these two settings using the two arrow keys. Push OK.
6. The digital display asks for the “Length of Step” (i.e. the volume duration of the gradient). The gradient must be slow enough to allow for separation of different proteins but fast enough to not dilute the protein sample. Five CV is a good volume for this gradient (for

example, if your column volume is 4 ml, perform the gradient over $3 \times 4 = 12$ ml. Enter the volume, and push OK.

7. The digital display asks for flow rate (ml/min). Enter 3, and push OK.
8. Select ADD to add a new step to your method.
9. Use the arrows to select buffer B, and push OK. Enter a length of step corresponding to 6 CV, and push OK. This step will continue to wash the column in elution buffer once the gradient has ended.
10. Select OK to go to the methods list.
11. Push COLLECTOR to set-up fraction collection. When the digital screen asks you for the mode, select ALL to collect fractions throughout the gradient.
12. Type in a fraction size of 1.25 (ml), and push OK.
13. Select OK to return to the method list.
14. Select DONE. The display will ask you if you want to save the method. Select NO SAVE to run the method without saving it.
15. Push RUN once the wash is complete to start the method. The liquid coming off the column is immediately routed to the fraction collector (instead of the *waste* line). For the first few fractions – carefully watch the fraction collector and make sure the sample is dripping directly into the eppendorf tubes. If the sample is missing the tubes, correct the problem by “jiggling” the rack containing the fractions or adjusting the arm of the collector. Check that tube 2 or 3 actually contains about 1.25 ml. The first tube always contains less.
16. Once the run is complete – place the elution fractions into the cold room.

Step 5: Cleaning up the LP system

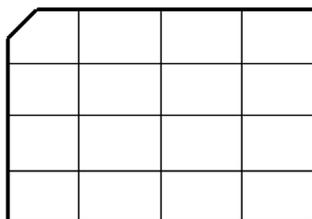
1. Remove the column from the LP system by disconnecting the male-female luer connections at either end of the column. To the two detached female connectors, reattach the small length of tubing with male luer connectors at its ends. Your system will now be back in its sample-loading configuration (Figure 1).
2. Wash the LP system. Place lines A and B in 20% ethanol, and purge both lines with ethanol for 3 min.
3. Wash the sample loop by filling a syringe containing a female luer connector with 20% ethanol. Attach the syringe to the *injection* port on the 6-way router, and push the ethanol through the sample loop. Repeat this at least three times.
4. Recap any buffers you used, and clean the stage of the LP unit. Leave everything as you found it.

Using a catechol blot to assay your fractions for tyrosinase activity

A catechol blot assay allows you to quickly identify which fractions contain tyrosinase activity.

Wear gloves throughout this procedure.

1. Count the number of fractions that you want to test for tyrosinase activity and determine the number of blots you will need. A blot holds 16 samples. You should only test fractions with a significant protein concentration. (>0.05 AU). In order to identify these fractions, you must take two factors into account:
 - a. LP Dataview marks on the chromatogram the start and end points of each fraction, and then displays the number of the fraction above the end point. For example, fraction # 2 is located in the fraction that is *before* where the number “2” is displayed on the chromatogram.
 - b. There is a lag of ~ 2 ml between the UV detector and the fraction collector. As a result, the value of A280 on the chromatogram is actually the measurement for the fraction that will be collected 2 ml later. As a result of this lag, the A280 measurement for fraction 2 actually appears on the chromatogram above fraction 1. Make sure you take this lag into account when you select your fractions.
2. For each blot, cut out a piece of nitrocellulose membrane just less than the size of a 1 ml pipette-tip box. The nitrocellulose membrane is stored as a roll of two sheets. The membrane is white and a protective paper is blue. Cut both the membrane and the paper together, and use the blue paper to protect the underside of the membrane when you place it on your bench. Nitrocellulose is a material that non-specifically adsorbs proteins. It will adsorb your proteins if you are not wearing gloves
3. On one side of the membrane, use pen or a pencil to draw out a 4x4 grid. Cut one corner of the paper to define an orientation of your blot. Also be sure to label each nitrocellulose membrane so that you can distinguish them.
4. For each blot, draw the diagram below in your notebook, and label each square with the fraction that you will blot in the square. Again, be sure to label each blot in a way that lets you distinguish them.



5. Blot 5 μ L of sample on the appropriate space on the nitrocellulose. As you finish a piece of nitrocellulose, place it in a gel box to dry.
6. Once the samples have dried (5 min), pour 10-15 ml catechol staining solution (5 mM catechol in 10 mM sodium phosphate, pH 7) over each blot. Catechol is very pungent and irritating to your lungs; do not put your face directly over the bottle or gel box.
7. Portions of the membrane containing tyrosinase turn brown almost immediately – though a good staining of dim samples may require ~ 10 -15 min. When you are satisfied with the staining, dump the catechol in the *Catechol Waste Container*. Rinse the box with water, and

let the nitrocellulose dry on paper towels. Once it is dry, you can record the data and tape the blot into your notebook.

Questions (answer in your notebook):

1. Do the fractions with high levels of tyrosinase activity correlate to those with high protein concentration (high UV readings in the chromatography trace)?
2. Does activity correlate with the color of the fraction?
3. Do you think you collected multiple isoforms? Why or why not?

Picking fractions to study further

The A280 trace collected during the anion exchange column elution will likely contain multiple peaks. These peaks correspond to one (or more) protein(s) that eluted at a specific salt concentration due to its negative charge at pH 7. Using the results from your catechol blot assays, you can determine if any of these peaks correspond to fraction with tyrosinase activity. If you observe that more than one UV peak contains tyrosinase activity, you can conclude that you have separated tyrosinase isoforms that have different charges at pH 7. Since they have different charges at pH7, they are likely to have different pI values although this is not always the case. As you will discover on day 3 our ability to distinguish isoforms with similar pI values is limited. We cannot assume that a single elution peak that contains tyrosinase activity contains only one isoform.

For each tyrosinase-containing A280 peak, choose the elution fraction with the highest levels of tyrosinase. Print out your chromatogram and label with the fractions you have chosen. On day 3, you will determine how many isoforms each chosen fraction contains by running these samples on an isoelectric focusing (IEF) gel. On Days 4-6, you will characterize the enzyme kinetics of the fraction with the least number of isoforms.

DAY 3: IDENTIFY ISOFORMS OF TYROSINASE

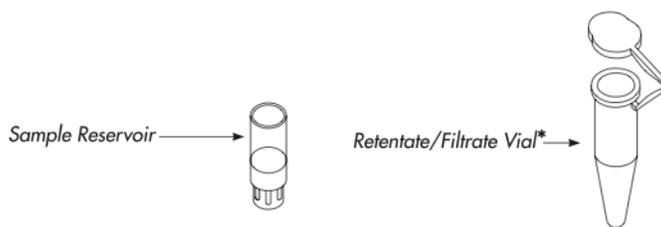
An isoelectric focusing gel (IEF) gel can be used to separate proteins with different pI values. The pI is the pH at which a protein has a net charge of zero, and it depends on the number of acidic and basic residues in the protein. *If you had two proteins that are identical except that one has asparagine at position 52, while the other has aspartate, which would have the higher pI? If you had two proteins that are identical except that one is phosphorylated (has a PO_4^{3-} attached) which has a higher pI?*

You will run two identical gels. One will be stained for total protein and the other for tyrosinase activity. Each gel contains 10 lanes. Your samples will include (1) the clarified lysate, (2) the fractions you selected from Day 2, and (3) the IEF standard. Before beginning, record in your notebook what sample you will run in each lane of your gels. You will concentrate you eluate samples before loading onto the gel. The protocol for IEF gels is in Appendix 3.

Step 1: Concentrating the samples

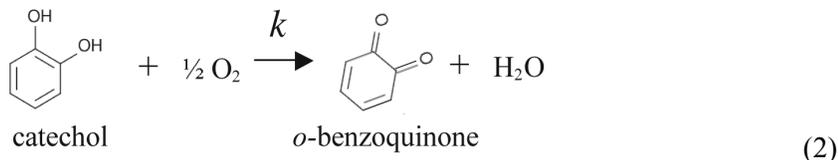
In order to see the different tyrosinase isoforms on the IEF gel, you will need to make a concentrated sample of each fraction. To do this, you will use Microcon YM-30 centrifugal filter units from Millipore. These filters will allow proteins of size 30 kDa or smaller to pass through. Because tyrosinase is bigger than this cutoff, it will not pass through the filter.

1. For each sample that you will concentrate, you will need 1 Sample Reservoir and 2 Filtrate Vials. Label each vial with the sample name.



2. Place a Sample Reservoir into a Filtrate Vial, and pipette 500 μ L of the fraction into the reservoir. The narrower, white plastic piece on the Sample Reservoir should be facing down. Do not touch the pipette tip to the filter. Seal the attached cap over the reservoir.
3. Place the unit into a bench-top centrifuge, and spin for 10 minutes at maximum speed (14k rpm). Liquid and smaller proteins will pass through the filter into the vial. Your tyrosinase will remain in the Sample Reservoir.
4. Place the sample reservoir upside down in a new vial.
5. Spin at 1000 rpm for 3 minutes to recover the sample in the reservoir. You should have 25-50 μ l of sample.

reactions downstream of the catalyzed reaction since they cannot occur until *o*-benzoquinone has been formed. This greatly simplifies our model to the following:



Over time, however, the concentrations of *o*-benzoquinone and the other products will increase until we can no longer ignore the numerous other rates in the pathway. Given enough time, the entire system will reach equilibrium, and the rate of *o*-benzoquinone formation will go to zero.

If the concentration of tyrosinase is much greater than the concentration of catechol and the O₂ concentration remains constant, the model in equation (2) predicts that the rate of *o*-benzoquinone formation will be proportional to the concentration of catechol:

$$\frac{d[P]}{dt} = k[C]
 \tag{3}$$

In the equation above, *P* and *C* denote *o*-benzoquinone (the product) and catechol, respectively.

By using a sufficient amount of catechol and measuring the rate of *o*-benzoquinone formation rapidly, you can further simplify equation (3) by assuming that the catechol concentration remains constant. Equation (3) then predicts that the concentration of *o*-benzoquinone will increase linearly with time:

$$[P] = k[C]_0 t
 \tag{4}$$

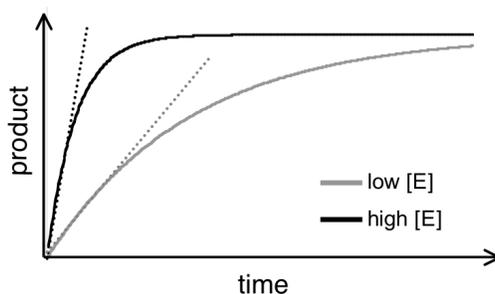
Here, $[C]_0$ is the initial catechol concentration and *k* is the rate constant for the catalyzed reaction.

Equation (4) only holds for short periods of time. Eventually, both the formation of product and the depletion of catechol will make our assumptions invalid. Your goal is to identify experimental conditions for which equation (4) appropriately describes the formation of *o*-benzoquinone from catechol in the presence of tyrosinase. This includes determining how long following initiation of the reaction equation (4) remains valid.

To find the optimal data collection condition, you will keep $[C]_0$ constant and vary the tyrosinase concentration ($[E]$). Although $[E]$ is not explicitly in equation (4), varying the concentration of tyrosinase changes the rate constant *k*. As you decrease $[E]$, *k* decreases and *o*-benzoquinone formation is slowed. This is advantageous because it takes longer for the concentration of *o*-benzoquinone to reach a level where we can no longer ignore its presence. On the other hand, if

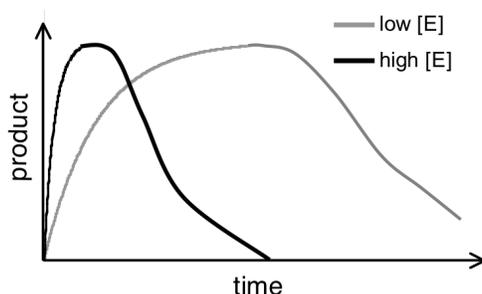
the rate of product formation is too slow, the *o*-benzoquinone that is formed will have time to undergo downstream reactions, invalidating our assumption that we can ignore these reactions. This sets up a Goldilocks dilemma in which you must find the optimal enzyme concentration between these two extremes.

The figure below shows plots of product formation over time for high and low enzyme concentrations. Dashed lines are added to make clear the range of times where the plots are linear. Notice that the time range is longer for the lower $[E]$.



There is one further complication that you must take into account. As melanins polymerize to form larger granules, they become insoluble and slowly sediment during the reaction. This poses a two-fold problem. First, these large granules will scatter light, thus artificially increasing your absorbance signal. Second, as the melanin granules sediment, the concentrations of the various downstream products will change, further complicating the reaction pathway. The formation of sedimenting granules occurs more rapidly at higher enzyme concentrations due to the more rapid formation of *o*-benzoquinone and the subsequent downstream products.

The figure below shows the same time traces as the previous figure, but with the sedimentation of melanin granules taken into account.



Step 1: Optimizing the tyrosinase concentration

To optimize data collection conditions for characterizing tyrosinase activity, you will look at the activity of a commercially available tyrosinase (Sigma, Catalog# T3824) in the presence of

catechol. You will track formation of *o*-benzoquinone through its absorbance at 410 nm using a spectrophotometer.

All reactions are performed in the presence of 5 mM catechol and a buffering system (10 mM sodium phosphate, pH 7) which maintains a constant pH. Repeat the following protocol with three or four different enzymes concentrations. Start with a dilution of about 1:10 of the commercial stock, and determine subsequent dilutions based on your results.

Before beginning, prepare small squares of parafilm (~1/2 inch on a side) and set the measurement wavelength to 410 nm on the Genesys spectrophotometer. Remember, you will want to work as rapidly as possible so that you can observe the formation *o*-benzoquinone at early time points, when it is linear with time. Develop a team plan with your lab partner(s) on how to make these measurements as rapidly as possible.

1. Prepare a stock of 5 mM catechol in 10 mM sodium phosphate.
2. Fill a disposable cuvette with 1 mL of the catechol stock.
3. Add 25 μ l of your diluted tyrosinase sample.
4. Mix quickly by covering the top of the cuvette with a piece of parafilm, placing your thumb over the parafilm to form a seal, and inverting the cuvette. Start the timer as soon as the cuvette is inverted.
5. Place the cuvette in the spectrophotometer and immediately record both the A410 and the time of the measurement.
6. Record the absorbance every 15 seconds for 5 minutes.
7. Plot A410 as a function of time using Excel. Over what time range does A410 increase linearly with time? Are you close to optimal data collection conditions? Do you think the concentration should be higher or lower?

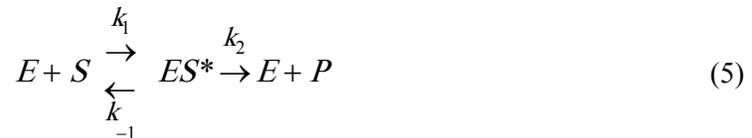
Ideally, you would like to observe a linear increase in product that lasts as long as possible before flattening out. Aim for a linear portion of at least 60 seconds so that you can trust your measurement of the initial product formation rate. Also, absorption is not linear with respect to concentration over 0.8 AU for most spectrophotometers. Make sure that the linear region of your time trace does not exceed this value.

8. Decide on a tyrosinase dilution that you think will be more optimal, and repeat the measurement.

Review of Michaelis-Menten kinetics

The Michaelis-Menten model describes the rate of enzyme-catalyzed reactions and their dependence on enzyme and substrate concentrations for many enzymes. The model postulates that an enzyme (E) and its substrate (S) are in fast equilibrium with a complex (ES*), which then dissociates to yield the free enzyme and product (P). Leonore Michaelis and Maud Menten proposed this theory, though G.E. Briggs and J.B.S. Haldane derived the kinetic equations with which we are familiar. Their derivations are based on the quasi-steady state approximation, which assumes that the concentration of intermediate complexes does not change.

This Michaelis-Menten model can be written as follows :

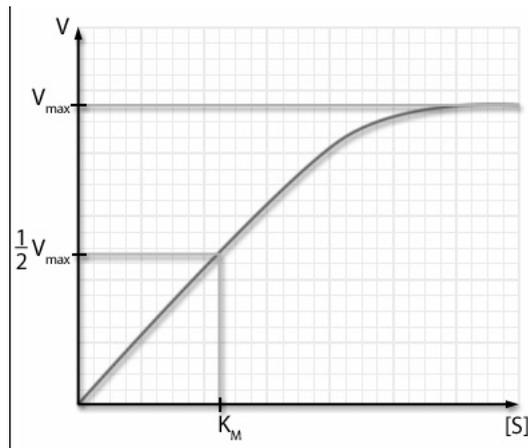


Solving for the rate of product formation (v) using the quasi-steady state approximation yields the relations:

$$v = \frac{dP}{dt} = k_2[E_o] \frac{[S]}{K_M + [S]} = V_{\max} \frac{[S]}{K_M + [S]} \quad (6)$$

$$K_M = \frac{(k_{-1} + k_2)}{k_1} \quad (7)$$

where $[E_o]$ is the concentration of starting enzyme. The substrate dependence of this rate has the following appearance:



This curve can be described completely by the two constants: V_{\max} and K_M , both of which are shown in the plot above. V_{\max} is the maximum rate of product formation, and, according to

equation (6), is equal to $k_2[E_0]$. When product is formed at this rate, the enzyme sites are saturated with substrate. K_M , referred to as the Michaelis constant, is defined in equation (7) is the substrate concentration at which the rate of product formation is half its maximum value ($v = V_{\max}/2$).

When $k_2 \ll k_{-1}$, the free enzyme and substrate are in rapid equilibrium with their complex, and product formation is rate limiting in the model in equation (5). Under these conditions, K_M reduces to k_{-1}/k_1 , and so K_M is the dissociation constant of the enzyme substrate complex (ES*).

You are going to compare the commercial tyrosinase sample and your enriched tyrosinase samples by measuring their K_M values.

QUESTIONS:

- (1) Why is it less useful to compare the V_{\max} values of your tyrosinase samples?
- (2) Why does K_M not depend on enzyme concentration?

Step 2: Measuring K_M of the tyrosinase sample

Your goal is to determine if the commercial tyrosinase stock obeys Michaelis-Menten kinetics. If it does, you will measure its K_M . To do this, you must create a plot that is analogous to the Michaelis-Menton plot shown above.

Because A410 is proportional to the concentration of the product, the rate at which A410 increases will be proportional to the rate at which product is formed. Thus, instead of measuring the rate of product formation, you will measure the rate at which A410 increases. By repeating this measurement at various substrate concentrations in the presence of a fixed amount of enzyme, you will create a Michaelis-Menten plot, though scaled by a constant factor. Luckily, this scaling will not affect the value of K_M that is found from this plot.

One approach to these measurements would be to collect a time trace of A410 for each substrate concentration. We would then perform a linear fit to the early time points of each time trace to determine their slopes. However, we can simplify the experiment greatly by taking advantage of your work from Step 1.

In Step 1, you identified an optimal tyrosinase concentration for which addition of 5 mM catechol resulted in an initial constant rate of product formation. This is the enzyme concentration you will use for these experiments.

1. Examine your plot of A410 as a function of time that you collected in Step 1 at your optimal tyrosinase sample concentration. Select a time point at which a measurable amount of

product has formed but which is still well within the linear portion of the trace. For example, you may want to choose the time point that is about halfway into the linear region.

For all your measurements from now on, you will measure the A410 at your selected time point. For example, if you picked 70 sec, you would call this A410(70). Since (1) the time period is entirely within the linear range, (2) the time period is identical for all measurements, (3) our rates are already in arbitrary units, we will make A410(70) representative of the rate of product formation. It will be used in lieu of the rate in all graphs of rate versus concentration.

To determine if the enzyme obeys Michaelis-Menten kinetics, measure the rate of product formation at different catechol concentrations in the presence of the optimized tyrosinase concentration. Graph the rate as a function of the catechol concentration. Does your sample appear to obey Michaelis-Menten kinetics? If so, continue on.

Make sure you have at least four points for which the rate is below its maximal value and at least two points for which it is extremely close to this value.

2. Generate a Lineweaver-Burk plot, which is a double-reciprocal plot, by plotting $1/\text{rate}$ as a function of $1/[\text{catechol}]$. If your enzyme obeys Michaelis-Menten kinetics, this plot should be linear. The x-intercept is equal to $-1/K_M$, the y-intercept is equal to $1/V_{\text{max}}$, and the slope is equal to K_M/V_{max} . A derivation of these results can be found in the Berg *et al.*

Fit the Lineweaver-Burk plot to a line, and determine the K_M of your tyrosinase sample.

DAY 4 – QUANTIFY THE ENRICHMENT AND ACTIVITY OF YOUR SAMPLES

Determining the success of your tyrosinase enrichment

In order to quantify how effective your tyrosinase enrichment was, determine the parameters listed in the table below for your clarified lysate and for your enriched tyrosinase fractions. Recreate the table below in your notebook, including an additional row for each of your enriched fractions. Following the table are brief descriptions of how to determine the various parameters.

Sample	Protein conc. (mg/ml)	Sample volume (ml)	Protein mass (mg)	Activity (A410 min ⁻¹ ml ⁻¹)	Total activity	Specific activity (A410 min ⁻¹ ug ⁻¹)	Yield (%)	Purification Level (-fold)
clarified lysate							100	1
fraction 1								

- *Protein concentration (mg/ml)*: You will determine the concentrations of all your samples using a bicinonic acid (BCA) assay. The BCA assay protocol is described in Appendix 3.
- *Activity (A410/min per volume)*: Repeat the optimization procedure from Step 1 of Day 3, measuring the increase in A410 as a function of time for your tyrosinase sample in the presence of 5 mM catechol. The activity is defined as the rate of A410 formation (i.e. the slope of the linear portion of the time trace) divided by the volume of your sample that you used.

NOTE – Using a catechol concentration of 5 mM is arbitrary. Any concentration would have sufficed, so long as the same concentration was used for all activity measurements.

- *Total activity (A410/min)*: This is calculated by multiplying the activity by the sample volume.
- *Specific activity (A410/min per μg)*: This is calculated by dividing the total activity by the total protein mass.
- *Yield*: This is calculated by dividing the total activity of the sample by the total activity for the lysate sample, and multiplying by 100.
- *Purification*: This is calculated by dividing the specific activity of the sample by the specific activity for the lysate sample.

Was your tyrosinase enrichment a success? On what values in the chart is your answer based?

Characterizing the activity of your enriched tyrosinase samples.

For one of your fractions, determine if the tyrosinase isoform(s) obey Michaelis-Menten kinetics. Calculate the K_M of your sample if it does. Follow the protocols from Step 2 on Day 3. From the measurements of activity for the table above, you should already know the optimal amount of the enriched sample to use.

DAY 5: CONTINUE ENZYME ASSAYS AND DESIGN INHIBITOR STUDIES

Complete the assays from Day 4 before continuing.

Designing inhibitor studies

Read through the section 8-5 (*Enzymes Can Be Inhibited by Specific Molecules*) from Biochemistry by Berg, Tymoczko, and Stryer. Choose two of the provided inhibitors of tyrosinase activity and design assays that will allow you to:

- 1) Determine if the inhibitor is competitive, non-competitive, or uncompetitive and
- 2) Measure the K_i of the inhibitor.

Continue these studies on Day 6.