

**Denison University**  
**BIOCHEMISTRY LABORATORY, Fall 2013**

Chemistry / Biology 302; Room 001, Ebaugh Laboratories

**SAFETY RULES**

- 1) Know the location of all safety equipment such as fire extinguishers, eye-wash fountains, safety showers, and fire blankets.
- 2) ALWAYS WEAR EYE PROTECTION. Safety glasses or other eye protection must be worn in lab at all times. Chemicals used in Biochemistry, for the most part, are not hazardous. However, we will use strong acids and bases as well as oxidizing and reducing reagents, which can be very dangerous. When called for, goggles or face shields will be made available for protection against UV lamps and more hazardous compounds.
- 3) Never mouth pipet or taste anything in lab.
- 4) No smoking, eating, or drinking is allowed in the laboratory.
- 5) Long hair should be tied back out of the way of flames and apparatus.
- 6) **No open-toed shoes are allowed in the laboratory**
- 7) **No above-knee skirts, dresses, OR SHORTS are allowed in the laboratory unless you wear them underneath a lab coat. You should wear clothing that covers you fully from shoulders to knees at all times in the laboratory.**
- 8) Extra clothing, books, and bags should be stored on the shelves provided.
- 9) Report all injuries, no matter how trivial.
- 10) Never work in the laboratory alone or perform unauthorized experiments.
- 11) An untidy or dirty laboratory is an inherently unsafe laboratory. You will be held responsible, both as individuals and as a class, for ensuring that your lab bench, the common-use areas of the lab, and all pieces of apparatus are clean and well-organized when you leave the lab each day.
- 12) Protective gloves will be available to wear when handling potentially caustic materials. It is recommended that you don't wear expensive clothing to lab in case of spills, or that you wear a laboratory coat.

## LABORATORY RECORDS

You'll be working in small groups for the semester. Each person in a lab group may do different parts of a larger experiment. To ensure that each member of a group has equal access to all the data collected by the group, notebooks will be provided in which ALL data collected by the group is to be placed. The notebooks will be three ring. *Your group will need to provide notebook paper.*

The data collected in the notebooks are the raw data and any commentary that you wish to add. The data should be well organized, neatly recorded and labeled with your group identification, the name of the person who collected the data, the experiment title and subtitle, and the date the data were collected. If your handwriting is a bit on the unreadable side you **MUST** recopy the data you collect and place it in the group's notebook during the same lab period in which the experiment is done. If you choose to recopy any notes, you must of course include ALL original data records in case an error is made in copying the data.

Notebooks are **NEVER** to leave lab except for photocopying. Anyone who removes a notebook from lab for an extended period of time without permission from their lab partners and/or the instructor will earn a failing grade for the lab assignment in question.

If you have any questions about how to record your data in the group's notebook, please speak with the instructor.

## LABORATORY ASSIGNMENTS

There will be two laboratory investigations during the semester:

- 1) Purification and Characterization of Yeast Cytochrome *c*.
- 2) Kinetic Analysis of an Enzyme, Tyrosinase.

Several assignments will be required as a part of each investigation. See the rest of the lab manual and subsequent handouts for specific instructions. At the end of the second project your group will present a poster on your work on the kinetic analysis of the enzyme tyrosinase. More detailed instructions on this aspect of the lab will be provided later in the semester.

***SPECIAL NOTE:*** *Although data will be collected in small groups, some assignments must be synthesized and written individually, and all group members are expected to be responsible for all the material at all times. The level of responsibility that you show in your lab group will be reflected in your subjective assessment score.*

# EXPERIMENT I

## PURIFICATION AND CHARACTERIZATION OF YEAST CYTOCHROME C

### LAB ONE

Objectives: Understand the Big Picture  
Understand how to make measurements in the biochemistry laboratory  
Introduction to this manual  
Make buffer

Pre Lab - overview of purification and characterization procedure

Procedure:

Make approximately one liter of phosphate buffer - 100 mM NaPi, pH 7.0

#### General guidelines for making solutions:

1. Decide the volume of solution you want to make.
  2. Assemble the dry reagents that will be used to make your solution.
  3. Using the actual molar mass of the dry reagents you will use, calculate the mass of each ingredient you will need, given the volume from step #1 and your target concentration.
  4. Weigh out each ingredient in a carefully labeled weigh-boat or clean, dry beaker.
  5. Combine dry ingredients in a clean beaker that is more than big enough to accommodate your target volume. Add solvent to about 80% of target volume (graduations on beaker are usually accurate enough for this).
  6. FULLY dissolve all solutes.
  7. Adjust pH, etc as needed.
  8. Transfer solution to graduated cylinder. Don't transfer stir bars to the cylinder. Bring up to final volume by repeatedly adding a small amount of solvent to the beaker used in step #5, swirling to rinse, then adding to graduated cylinder.
  9. Pour into a carefully labeled, clean storage container. If storage container is not dry to start with, be sure to rinse it with a small amount of your solution (and then discard the rinse solution) before pouring the bulk of the solution into the container. This will minimize dilution of your solution with the water in the container.
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## LAB TWO

Objectives: Construct protein concentration standard curve  
Pour gel filtration column, and determine void volume

Procedure:

### Part I. Construct protein concentration standard curve

Total protein concentration will be determined using the Bio-Rad protein assay dye kit.  
Full instructions can be found in Appendix B of this manual.

### Part II. Pouring a gel filtration column, and measuring the void volume

1. Obtain an 88 ml column (40 x 1.7 cm) and clamp it onto a ring stand with two clamps. Check that the column is vertically true, then attach a stopcock to the outlet.
2. Obtain enough Sephadex G-50-150 slurry to fill your column.
3. With the stopcock closed, pour approximately 25 mL of NaPi buffer containing 3 mM 2-mercaptoethanol into the column. ALWAYS WORK WITH THE NEAT 2-MERCAPTOETHANOL IN THE HOOD, NOT AT YOUR BENCH. Be sure that no air bubbles are trapped in the column or the stopcock. You can drain some buffer through the stopcock to remove any bubbles.
4. Fill your column with the slurry and let the column pack by gravity until a bed of at least 1 cm is evident. Once this packed bed is established, open the stopcock and **continually** add slurry until the column is packed to about 3 cm below the colored column top. Once it is packed, rinse the column for 15 min with buffer at a flow rate of between 1 and 2 mL/min.

*HELPFUL HINT: So you don't have to baby-sit the column as it rinses and to maintain an even flow rate for future runs you will want to set up a top tubing connector. This is done by cutting a 200  $\mu$ L micropipet tip with a razor blade so that the large end fits snugly over the column cap inlet port. To the pipet tip is connected a length of small, clean Tygon tubing. Buffer is then drawn up into the tubing, and while maintaining a filled tube the column top is replaced on the column. The other end of the tubing is kept in a flask of buffer, and the flask is raised or lowered to the appropriate height to maintain a 1 mL/min flow rate. When the column is stored, the stopcock is closed, and Parafilm should be wrapped around the top and bottom of the column. The column will then be ready for use in the future. You are strongly advised to check your column every few days in order to ensure that there is always a layer of buffer on top of the column to prevent it from going dry. If your column runs dry, you'll have to re-pour it, and repeat any measurements you have made with it up to that point in the semester.*

5. Follow the instructions in Appendix C for running your Gel Filtration Column.

Note:

You will only need 30 glass tubes for this run.

Your sample will be a 1 mg/mL solution of blue dextran.

Because your sample is strongly colored, you'll know when it has eluted. There's no need to continue collecting fractions after all the blue dextran is off the column.

Because Blue Dextran is blue, it can be quantified by the absorbance of light at a wavelength of about 600 nm.

6. Graph your results and determine the void volume of your gel filtration column.

Record this value in your notebook.

**Assignment:**

1) Use a spreadsheet program (such as Excel) to generate a standard curve for determining protein concentration. In the spreadsheet should be the original data ( $\mu\text{g}$  of lysozyme and related absorbance in triplicate).

2) Using the equation of the standard curve, calculate the amount of protein in each standard sample and list this in the spreadsheet. In other words, calculate the value indicated by the absorbance of that sample using the equation of the straight line, as opposed to the amount measured out from the stock lysozyme solution.

3) Turn in this well-organized and -labeled spreadsheet. Pay special attention to the number of significant figures you are reporting; do not report more digits than are justified by your data.

4) Turn in a labeled and titled graph of your standard curve.

5) Suppose that one of your classmates ran a standard curve and computed an equation for the line of  $y = 0.065x + 0.34$ . If this student also measured the absorbance of an experimental sample, combined with dye in the same manner as the lysozyme standards, and found an absorbance value of 0.45, how much protein was in her sample?

## LAB THREE

Objectives: Yeast lysis - done for you prior to lab  
Homogenate preparation  
Bulk cation exchange

**WARNING - This lab may run longer than the assigned time so please plan your schedule accordingly. You will get this time back later in the semester when we have some short labs. If you have a commitment that requires that you leave lab by 4:20 pm, please make arrangements in advance with your instructor and your lab partners.**

**VERY IMPORTANT NOTE:** If you do not remove a sample for analysis after each step of the purification process your **entire** study and all its results will be meaningless. You should also accurately measure the volume of all samples resulting from the various purification steps. Again if you do not do this your data cannot be interpreted fully.

Procedure:

### Part I. Yeast homogenization (done prior to lab)

Approximately 200 g of wet packed *Saccharomyces cerevisiae* (Baker's yeast, Fleischmann's Yeast Company) was weighed out and suspended in an equal volume of 100 mM NaPi, pH 7.0, containing 1.0 M NaCl, 3 mM 2-mercaptoethanol, and a protease inhibitor cocktail (see Appendix D for details). An equal volume of glass beads was added and the yeast were homogenized for 4 min on ice (alternating 30 s on/30 s off) in a Bead Beater (Biospec Products, Bartlesville, OK).

You should keep this homogenate and all subsequent fractions **on ice at all times**.

### Part II. Homogenate clarification

To get rid of insoluble cell contents (what might these include?) centrifuge the lysate at 4,000 x g for 10 min at 4°C. Collect the supernatant, measure its total volume and set aside 5 mL for use in determining total protein concentration and cytochrome *c* concentration. Keep the unused portion of the sample at -20°C for later analysis by SDS-PAGE. *Note: you will be held responsible for all samples stored at -20°C in this and subsequent steps, so take the necessary steps to insure that they are labeled and secure.*

### Part III. Bulk cation-exchange

1. Equilibrate ~10 g of Bio-Rex 70 cation exchange resin (100-200 mesh, Na<sup>+</sup> form; from Bio-Rad) with two washes of 100 mM NaPi, pH 7.0.
2. Dilute the homogenate with six volumes of distilled deionized water (ddH<sub>2</sub>O) containing 3 mM 2-mercaptoethanol, and add the suspension to the equilibrated resin.

3. Stir the mixture at 4°C for at least 30 min. Be sure that the stir bar is rotating evenly and at a moderate speed -- just enough to create a small vortex and evenly suspend the resin.
4. Set up a small syringe column by cutting a Teflon frit to the proper size and inserting it into a 20 ml syringe (smooth side of frit towards the resin). You can use a cork borer to cut the frit, then clamp the column onto a short ring stand. The instructor or lab assistant will help you assemble this column.
5. When the 4°C incubation is complete, turn off the stirbar and let the resin settle to the bottom of the beaker. Remember to make observations as to color, cloudiness *etc.* at all points. Decant the supernatant into a separate beaker. Measure the volume and save 5 mL on ice for later use. Rinse the resin into your syringe column and let the column run dry.
6. Rinse the resin with one column volume of 100 mM NaP<sub>i</sub>, pH 7.0, containing 3 mM 2-mercaptoethanol.
7. Elute the cytochrome *c* with a minimum of 100 mM NaP<sub>i</sub>, pH 7.0 containing 0.8 M NaCl and 3 mM 2-mercaptoethanol. Your goal should be to capture all of the pink liquid in one tube, but as little unpigmented liquid as possible. Accurately measure the volume of the eluate. Keep a portion (~50 µL) of this eluate at -20°C for SDS-PAGE analysis.

*If you are out of time at this point,  
you may postpone the analysis of your samples until next week.  
Samples saved for analysis next week should be kept at 4°C*

8. Determine the total protein content in the three samples you have collected (clarified yeast homogenate, cation exchange decant, cation exchange eluate) using the Bio Rad protein assay. You must also generate a new standard curve (triplicate samples) as done in *Lab Two*. You will have to dilute your experimental samples (with NaP<sub>i</sub> buffer) some unknown amount in order for the values to register on the standard curve. This is a trial and error process, but you can try several dilutions at once, as described in Appendix E. Calculate the quantity of protein in each sample (total volume) remembering to figure in any dilution factors that were made.

HELPFUL HINT: *To avoid interference by particulates in your samples, microcentrifuge the samples for 2 min at maximum rpm prior to analysis using the Bio-Rad protein concentration kit and UV/vis spectroscopy.*

9. The same samples must now be examined for cytochrome *c* concentration. Using an extinction coefficient of  $95.0 \text{ mM}^{-1} \text{ cm}^{-1}$  for cytochrome *c* at 410 nm you should be able to calculate the quantity of cytochrome *c* in each sample.
10. Store eluate at  $4^{\circ}\text{C}$  and other samples you wish to keep for SDS-PAGE analysis at  $-20^{\circ}\text{C}$  (this should include a  $50\mu\text{L}$  sample of your eluate).

*NOTE: It is a good idea to take some time during this lab period to calculate your total protein and cytochrome *c* concentrations and determine how they are used in constructing the purification table. After generating a first set of numbers you may find that some results are erroneous. If this is the case you may need to repeat some measurements and/or explain the results in a convincing way. That is, your instructor must agree with your interpretation. If you leave without first going over these data you may be left in a tight situation when it comes to the assignment if you find that you cannot calculate the numbers correctly or if some mistake or misinterpretation has been made.*

**Assignment:** Fill out the sheet in the appendices entitled "Ten Questions" (Appendix F).

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## LAB FOUR

Objectives: Begin dialysis of sample  
Learn how to construct a purification table

Procedure:

### Part I. Sample dialysis

1. Equilibrate a piece of dialysis tubing by soaking for 30 min (without stirring) in ddH<sub>2</sub>O containing a pinch of EDTA. The tubing should be cut to hold approximately 2 - 3 times the volume of your eluate sample.
2. Place the eluate from the cation exchange into the dialysis bag, with one end sealed, then seal the other end leaving enough room for influx of buffer during the dialysis. Try to minimize air bubbles trapped in the dialysis bag. The instructor or lab assistant will help you with this procedure. Dialyze at 4°C against one liter of 100 mM NaPi, pH 7.0 containing 3 mM 2-mercaptoethanol, with gentle stirring. The dialysis buffer should be replaced with one liter of fresh buffer at least twice over the next week with no less than 5 h between changes.

### Part II. A Purification Table

1. Your instructor will walk you through the construction of a purification table, using data for a hypothetical cytochrome *c* purification.

**Assignment:** Construct a purification table for cytochrome *c* using the table of data distributed in class (the format of your table should follow the example given) and answer the following questions.

*Which step in the sample data purification table purifies the cytochrome *c* the greatest amount?*

*Which purifies it the least amount?*

*Briefly explain your answers.*

NOTE: you should check on your stored gel filtration column during this class period to ensure that it is still sealed and properly preserved for next week's lab.

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## LAB FIVE

Objectives: Concentrate sample  
Standardize gel filtration column

Procedure:

### Part I: Sample concentration

1. Using a disposable plastic pipet, remove your sample from the dialysis bag.
2. Clarify the sample by centrifugation.
3. Determine the levels of total protein and cytochrome *c* in this sample as before. You must repeat the standard curve determination (in triplicate) as in *Lab Two*.
4. Keep a portion (50  $\mu$ L) of this sample at -20°C for analysis by SDS-PAGE.
5. In order to load your entire sample onto your gel filtration column it must be concentrated to a volume of < 2 mL. To do this you will be using a vacuum concentrator. Your sample should be placed in a series of microcentrifuge tubes and labeled with your group's name. The samples will then be placed in the apparatus and concentrated. You will be shown how to correctly operate the instrument. Once concentrated to the appropriate volume, the sample can be stored at 4°C.  
**Note that concentrating a large sample down to < 2 mL may take several days, so plan your group's time accordingly.**

### Part II: Standardize the gel filtration column

1. Follow the instructions in Appendix C for running your Gel Filtration Column.  
Note:  
You will need 95 glass tubes for this run.  
The standard solution contains 3 mg each of aprotinin (6.5 kDa), bovine cytochrome *c* (12.5 kDa) and carbonic anhydrase (29 kDa) in a total volume of 2 mL.  
Continue collecting fractions until the  $A_{280}$  profile is flat and roughly equal to the background absorbance level.
2. Analyze all fractions for protein (absorbance at 280 nm) and cytochrome *c* (absorbance at 410 nm) levels using the plate reader. Think about why your data display the trends they do, and be prepared to explain these trends to your instructor next week.

**Assignment:** Graph the results of your gel filtration standardization so that you can discuss your data with your instructor in lab next week. There are instructions in Appendix G that show you how to extract three clean peaks from your absorbance data. Please bring a graph similar to that shown for step 4 of the process to lab next week.

## LAB SIX

Objectives: Gel filtration of concentrated sample  
Plan SDS-PAGE gel

Procedure:

### Part I. Run sample on gel filtration column

1. Follow the instructions in Appendix C for running your Gel Filtration Column.

Note:

You will need 95 glass tubes for this run.

Before loading your sample on the column, bring the volume to 2.0 mL with NaPi buffer, then add 5  $\mu$ L of 2-mercaptoethanol and allow the sample to sit for five minutes. Microcentrifuge it for 2 min, carefully remove the supernatant (be sure not to disturb the pelleted debris) and load it onto your column using a plastic pipet. Continue collecting fractions until the  $A_{280}$  profile is flat and roughly equal to the background absorbance level.

2. Analyze all fractions for protein (absorbance at 280 nm) and cytochrome *c* (absorbance at 410 nm) levels using the plate reader. Take 50  $\mu$ L of the fraction containing the highest absorbance at 410 nm and store at  $-20^{\circ}\text{C}$  for SDS-PAGE analysis.

3. Graph the gel filtration results, and combine the fractions that represent each of the peaks of protein, being particularly cautious with the peak of cytochrome *c*. Most of your cytochrome *c* will be found in the fractions with absorbance levels greater than 50% of the peak value. Be sure to record the total volumes of each of these combined fractions.

4. Perform total protein and cytochrome *c* content assays on the pooled fractions as previously described. Save these pooled fractions at  $-20^{\circ}\text{C}$  for SDS-PAGE analysis.

**Assignment:** Once you have determined the levels of total protein and cytochrome *c* in your pooled gel filtration samples, you should prepare a final version of your purification table. This should be done keeping three things in mind:

- 1) neatness, presentation, and organization count
- 2) significant figures are extremely important in the values you give
- 3) although you need only hand in one table for your group, each person *must* know how to do all of the calculations because there will be some aspects of these calculations on the lab test!

## Part II. Plan samples for SDS-PAGE

1. Determine what samples you wish to run on your SDS-PAGE gel. There will be ten lanes per gel, one of which must be a molecular mass standard (provided by instructor) while a second must be reserved for a sample of pure cytochrome *c* ( $1 \mu\text{g}/\mu\text{L}$ ; also provided by the instructor). You will have two gels per group; thus you have as many as sixteen lanes for your samples.
  2. The method of protein visualization that you will use -- Coomassie Brilliant Blue dye -- typically requires 0.1 to 1  $\mu\text{g}$  of protein *per band* for a strong signal. In order to ensure that you get a lane that contains this level of protein, it's typical to run multiple dilutions of one's samples on an SDS-PAGE gel -- think of it as hedging your bets. The procedure described on the next page outlines a way to get three dilutions of each sample on your gel. Bear in mind that this is a *recommendation*, not a *requirement*. For instance, if you think that one of your samples is very dilute (such as a sample that you expect to contain cytochrome *c* but which is very pale pink), it may make sense to only run a concentrated sample on your gel.
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## LAB SEVEN

Objectives: SDS-PAGE

Procedure:

### Part I: SDS-PAGE

1. Set up two gels per group. General instructions for running SDS-PAGE gels are provided in Appendix H. Your instructor or lab assistant will help you set up the gel apparatus.
2. As one group member prepares the apparatus the others should be preparing the samples.

*NOTE: It takes most groups at least 60 min to set up the gel, prep the samples, and load the gel. It takes 60 min to run the gel and 60 min to go through the first staining step. This totals 3 h. Thus you must be organized and efficient to ensure that the lab is completed within a reasonable amount of time.*

3. Remove 50  $\mu$ L of your sample and place in a small microcentrifuge tube that contains 50  $\mu$ L of 2x sample buffer (provided by instructor). This will provide enough volume to run three amounts of each sample -- 20  $\mu$ L in one lane, 5  $\mu$ L in another, and 2  $\mu$ L in a third -- and still leave enough to run a second gel if something goes awry.
4. Heat all samples, except the molecular weight standards, for 3 min at 100°C in the heating block. After heating, microcentrifuge the samples for 2 min at the highest spin setting. The samples are then ready for loading.
5. Load your samples. Save any extra sample at 4°C until you are sure that the gel was a success.
6. Run the gel at ~150 V for ~45 minutes, until the dye front is within 1 cm of the bottom of the gel.
7. Stain and destain the gel according to the directions in Appendix H. This will take several days. Next lab period we'll take digital photographs of the gels for use in your final assignment.

**Assignment:**

1) Gel filtration analysis.

From your elution profile of the gel filtration standards (absorbance: y-axis vs. elution volume: x-axis) generate a gel filtration standard curve (log MW: y-axis vs.  $v_e/v_0$ : x-axis).

From the standard curve and the sample gel filtration graph (absorbance: y-axis vs. elution volume: x-axis) calculate the molecular weight of *your* cytochrome *c* sample.

**Turn in**

- an elution profile of your experimental sample with indications of the void volume and the elution volumes of your standards (this is most commonly done with vertical arrows). In order to be most useful, this graph should present both  $A_{280}$  and  $A_{410}$  data.

- a gel filtration standard curve graph that shows the measured molecular weight of your cytochrome *c* sample on the standard curve graph.

Both graphs should be properly presented with appropriate labels and titles. In choosing your labels, think about what pieces of information are most relevant -- for instance, protein name vs. molar mass.

2) SDS-PAGE analysis.

From the migration of your molecular weight standards, generate an SDS-PAGE standard curve (log MW: y-axis vs.  $R_f$ : x-axis).

From the standard curve and the migration of your purified cytochrome *c* pool, calculate the molecular weight of *your* cytochrome *c* sample.

**Turn in**

- the SDS-PAGE standard curve graph that shows the measured molecular weight of your cytochrome *c* sample on the graph

- a professional-quality figure (with legend) for your SDS-PAGE results. Be sure to include in your figure every sample that you refer to in your answers to the questions below. This figure should be executed in the style of a figure in a *Journal of Biological Chemistry* article.

3) Answer the following questions.

A. Did the purification work? Compare the results of your purification table and your gel to answer this question. Do the numbers from your purification table agree with your visual assessment of your SDS-PAGE results? For instance, does the sample which appears to have the fewest bands on PAGE show up in your table as the most pure? Which do you think is a more accurate representation of the true purity of the sample? Explain briefly.

B. Which purification step worked best, and which worked least, as determined by overall analysis? Explain briefly, making reference to both the purification table and the gel.

C. What is the apparent molecular weight of your cytochrome *c* as determined by SDS-PAGE and how does it compare to your value from gel filtration? If they are different, which is more credible and why? (More points will be given for a more rigorous answer.)

## EXPERIMENT II

### KINETIC ANALYSIS OF AN ENZYME, TYROSINASE

Adapted from "Modern Experimental Biochemistry," second edition, by Rodney F. Boyer, The Benjamin/Cummings Publishing Company, Inc., 1993.

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#### LAB ONE

Objectives: Understand the Big Picture  
Determination of pH optimum for tyrosinase activity

Pre Lab - overview of tyrosinase activity, general kinetics overview, and spec use.

Procedure:

#### Part I. pH Profile of Tyrosinase Activity

##### 1. Solutions (provided)

- 100 mM acetate, pH 5.0
- 100 mM citrate, pH 6.0
- 100 mM NaP<sub>i</sub>, pH 7.0
- 100 mM NaP<sub>i</sub>, pH 8.0
- 100 mM borate, pH 9.0
- 2 mg/ml L-dopa (in 100 mM NaP<sub>i</sub>, pH 7.0)
- Tyrosinase (concentration will be provided, in 100 mM NaP<sub>i</sub>, pH 7.0)

##### 2. Experimental Procedure

The general procedure for spectroscopically measuring tyrosinase activity is described in Appendix I.

Your samples should be prepared according to the table below.

NOTE: the value "x" in the table below refers to the proper amount of tyrosinase, which will be determined by your instructor prior to each lab. This value will be written on the white board in the front of the room.

Buffer pH	5.0	6.0	7.0	8.0	9.0
Buffer	2.50-x*	2.50-x	2.50-x	2.50-x	2.50-x
L-dopa	0.50	0.50	0.50	0.50	0.50
Tyrosinase	x	x	x	x	x

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\*units are milliliters

**Assignment:** Generate a figure and legend for the data you collected on the pH profile of tyrosinase with L-dopa. Examples of such a figure can be found in many *Journal of Biological Chemistry* articles. For this figure you should provide both the level of detail and the quality of presentation that you would expect to find in a professionally produced figure and figure legend. Think carefully about your choice of graph form, and be sure that your figure legend includes the optimal pH value you determined.

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## LAB TWO

Objectives: control  $K_M$  determination  
Inhibitor test

Today you will use the general procedure in Appendix I to measure the activity of tyrosinase at several concentrations of substrate, in the presence and absence of an inhibitor. You'll calculate the  $K_M$  and  $V_{max}$  for tyrosinase under these conditions by graphical fitting of your initial velocity values to the Michaelis-Menton equation. Laptops will be available for you to process your data on the fly in lab.

### Part I. Control $K_M$ Determination for Tyrosinase

Using the quantity of tyrosinase that yields linear product formation for 3 min. ("x" below, determined by instructor) use the following table to prepare your solutions for determination of initial velocities. You will have to make the appropriate buffer, the one you determined in Lab 1 to have the pH that supports optimal tyrosinase activity. Feel free to make arrangements with your instructor to come in early to make the appropriate buffer.

	Assay #					
	1	2	3	4	5	6
Buffer	2.90-x*	2.80-x	2.60-x	2.40-x	2.20-x	2.00-x
L-dopa	0.10	0.20	0.40	0.60	0.80	1.00
Tyrosinase	x	x	x	x	x	x

\*units are milliliters

### Part II. Inhibitor Analysis

Having established the kinetic parameters for tyrosinase activity by itself, you will now test its activity in the presence of an inhibitor. Some groups will use 100 mM sodium benzoate (in the appropriate buffer) as their inhibitor, and others will use a 100 mM solution of thiourea.

1. First determine the amount of inhibitor necessary to reduce the enzyme activity with 0.40 mL of L-dopa to approximately 20% of the activity found for the uninhibited reaction (the volume of buffer *plus inhibitor* should equal 2.60-x mL).
2. Once you have determined how much inhibitor to use, you should follow the general procedure outlined above for the determination of  $K_M$ , except that another substance (the inhibitor) will be in the assay mixture, added into the blank, as shown in the table on the next page. Note that you will be determining  $K_{M\text{apparent}}$  and  $V_{\text{max apparent}}$  -- the kinetic parameters for the enzyme under the influence of the inhibitor.

NOTE: the values x and y in this table refer to:

- x) the tyrosinase quantity necessary to give an absorbance change of ~0.2 units per min. (determined by the instructor)
- y) the quantity of inhibitor necessary to reduce the rate of activity to ~20% of the uninhibited rate (from step 1)

Reagent	Assay #					
	1	2	3	4	5	6
buffer	2.90-x-y*	2.80-x-y	2.60-x-y	2.40-x-y	2.20-x-y	2.00-x-y
L-dopa	0.10	0.20	0.40	0.60	0.80	1.00
Inhibitor	y	y	y	y	y	y
Tyrosinase	x	x	x	x	x	x

\*units are milliliters

3) Email an Excel spreadsheet containing your kinetic data (both with and without inhibitor) to your instructor. Please ensure that your data are clearly labeled and well organized. Your instructor will pool the data from multiple groups and share them with the full class so that you can complete the assignment below.

**Assignment:**

1) Generate a professional-caliber figure and legend for the kinetic data on the behavior of tyrosinase with L-dopa, with and without the inhibitors sodium benzoate and thiourea.

This assignment requires you to produce a Lineweaver-Burk figure with three lines on one graph. Examples of such figures can be found in many *Journal of Biological Chemistry* articles. Please provide both the level of detail and the quality of presentation that you would expect to find in a professionally produced figure and figure legend. In your figure legend, be sure to give the  $K_M$  and  $V_{max}$  values you determined (paying close attention to significant figures) as well as the type of inhibition displayed by each inhibitor, as determined by your analysis of the lines on the graph.

2) Based on your graph, what kind of inhibitor do you think sodium benzoate is? Please explain briefly. Be sure to take into account the uncertainty in your numbers when you make your analysis. Now do the same for thiourea.

3) Can you rationalize your answer to #2 based on the structure of each inhibitor? Please explain briefly.

## LABS THREE and FOUR

Objectives: Carry out an independently-designed experiment

These experiments **MUST** be laid out before coming to lab. You should discuss the details of your experiments with your instructor during lab two, and will ideally execute the lab during weeks three and four. In some cases, it may be necessary to use lab three to make solutions, order special reagents, or build special apparatus, followed by the "real" experiments in week four. All experiments should be carefully controlled and should involve replicate samples as far as is practical.

**Assignment:** The results from these last two weeks of analysis of tyrosinase will be presented next week (the last week of classes) as a poster presentation. Guidelines are presented in Appendix J. Think about your presentation and prepare ahead of time since you will have many other assignments from this and other classes to fit into your schedule during the last week.

The lab period of the final week of classes will be used for critical review of the ideas and data that you will present on your poster. This is an opportunity for you to receive critical feedback from your instructor and lab assistants before you submit your poster for a grade. You are strongly encouraged to make full use of this review process!



## APPENDIX A

### Procedure for using the Hanna pH 211 Microprocessor pH Meter

*If the pH meter is turned off...*

- Plug the pH meter into the wall.
- Plug the electrode into the pH meter.
- Remove the protective cap. Don't be alarmed if salt deposits are present. This is normal and they will disappear when rinsed with water. Immerse probe in the storage buffer solution.
- Switch the instrument on by pressing **ON/OFF**. The meter automatically defaults to pH measurement mode.

*If the meter is turned on...*

- Rinse the probe thoroughly with DI water whenever moving from one solution to another.
- For calibration:
  - Immerse the probe approximately 1 ½ in into the pH 7 solution and stir gently. Press **CAL**.
  - Check that BUFFER pH 7.01 appears on the screen. The “NOT READY” indication will blink until the reading has stabilized.
  - When the reading is stable and close to the selected buffer, the “READY” indication will appear and “CFM” will blink. Press the **CFM** button to confirm the calibration. (If the reading does not stabilize, ask your instructor for assistance.)
  - Rinse the electrode and move it into a second calibration buffer solution (either pH 4 or pH 10) and stir gently.
  - The LCD display should display the second buffer value. If necessary, use the **arrow keys** to select a different buffer value.
  - When the reading is stable, press **CFM** to confirm the calibration.
  - The meter stores the reading and returns to the normal mode.
- To measure the pH of a solution, submerge the tip of the electrode 1 ½ in. into the sample. Allow time for the electrode to stabilize. The pH reading is displayed on the primary LCD.
- When the electrode is not in use, be sure that it is immersed in storage solution.

*For long-term storage...*

- To prevent damage to the electrode, remove the pH electrode from the solution before turning the meter off. If the meter is off, detach the electrode from the meter before immersing the electrode in storage solution. Replace the solution in the protective cap with a few drops of storage solution and affix the protective cap.

## APPENDIX B

### Bio-Rad/Bradford assay for total protein (modified from Bradford (1976), *Analytical Biochemistry* 72:248)

#### *Preliminary notes:*

- i. The Bio-Rad protein assay dye kit is a commercial implementation of the tried-and-true Bradford procedure. The molecular basis of the procedure involves the binding of the dye *Coomassie Brilliant Blue G-250* to basic and aromatic amino acids in proteins. Upon binding to the protein, the absorbance characteristics of the dye molecules change; for an acidic solution, the  $\lambda_{\text{max}}$  shifts from 465 nm to 595 nm and the absorbance at 595 nm primarily reflects protein-bound dye molecules rather than free dye molecules.

Unfortunately, because the binding of Coomassie to proteins is mediated by aromatic and basic amino acids (especially Arginine), and because different proteins have different numbers of basic and aromatic amino acids exposed to the dye, different proteins bind different numbers of dye molecules. For instance, equal amounts of two of the most commonly used "generic" proteins, lysozyme and albumin, give  $A_{595}$  values that differ by a factor of about 2.

However, *to a first approximation*, the binding of dye (and thus the absorbance at 595 nm) is proportional to the number of amino acids and thus to the mass of the protein. Always include a blank (no protein) to determine the level of absorbance resulting from the free dye, and if you want to know the true range of error of the assay, use multiple proteins as standards.

- ii. As with other absorbance-based assays, it is critical to develop a good standard curve to relate protein concentration to dye binding (and  $A_{595}$ ). Because of different amino acid compositions of different proteins, it would be ideal to use the protein that you are studying to generate your standard curve, but this is usually impractical, so egg white lysozyme and bovine serum albumin (BSA) are commonly used as generic protein standards. The procedure is simple but it is very important to keep accurate records of each sample prep and analysis. For the most reliable results, prepare every sample in triplicate, and run a new standard curve for *every* set of experimental samples.
- iii. When making up dilutions of standards and samples, it is vital that
  - buffer used for the standards reflect the buffer used for the experimental samples
  - attention be paid to compounds that interfere with the Bradford assay. A detailed list is included in the Bio-Rad assay kit instructions. SDS above 0.001% may affect linearity; above 0.002%, definitely.
  - tubes (or microplates) be clean (this is NOT the time to save money by reusing tubes!)
- iv. The Bio-Rad dye reagent contains Coomassie, phosphoric acid, and methanol. The Coomassie dye will stain skin and clothing and neither the phosphoric acid nor the methanol is good for your health. Treat it with appropriate care.

*The Assay:*

1. Prepare dilutions of standards and experimental samples; the total volume of each well should be 200  $\mu\text{L}$

A. Standards. You will use egg white lysozyme (0.1 mg/mL in 100 mM NaPi, pH 7.0) as your protein standard. Prepare samples **in triplicate** according to the following table. Add the lysozyme and buffer, then proceed to step #2.

<u>SAMPLE</u>	<u><math>\mu\text{L}</math> buffer</u>	<u><math>\mu\text{L}</math> lysozyme stock</u>	<u><math>\mu\text{L}</math> dye</u>
Blank	160	0	40
0.5 $\mu\text{g}$	155	5	40
1 $\mu\text{g}$	150	10	40
2 $\mu\text{g}$	140	20	40
3 $\mu\text{g}$	130	30	40
4 $\mu\text{g}$	120	40	40
5 $\mu\text{g}$	110	50	40
7.5 $\mu\text{g}$	85	75	40

B. Experimental samples. In order to keep the final volume equivalent to that of your standards, all samples should have a volume of 160  $\mu\text{L}$  (before addition of dye). Depending on the situation, this may be pure sample, or may be a mix of sample and buffer. See Appendix E for an example of how to set up dilutions of experimental samples collected during cytochrome *c* purification.

2. Add 40  $\mu\text{L}$  dye concentrate to all microtiter wells (see notes on timing under step 4).
3. Pipet up and down gently but very thoroughly. Good initial mixing is extremely important for a successful assay, but *be sure to avoid creating bubbles in the wells*, since this will adversely affect absorbance readings.
4. Incubate for at least five minutes at room temperature (for best results, don't go over an hour). Color development is somewhat time sensitive, so all samples should be incubated approximately the same amount of time from addition of dye to spectrophotometry.
5. Read absorbance at 595 nm in the microplate reader. Instructions are posted by the instruments.
6. Use a spreadsheet program to generate a standard curve from your lysozyme concentration standards. To do this, plot quantity of protein on the x-axis versus absorbance at 595 nm on the y-axis.
7. Compute the amount (or concentration) of protein in experimental samples based on the standard curve. To a first approximation, the uncertainty in any protein determination derived from your standard curve will equal the computed amount of protein in your experimental sample times the fractional uncertainty of the slope of your standard curve (the uncertainty on the slope divided by the magnitude of the slope). That is,

$$\frac{\text{uncertainty in } \mu\text{g protein}}{\mu\text{g protein}} = \frac{\text{uncertainty in slope}}{\text{slope}}$$

## APPENDIX C

### General Instructions for Running your Gel Filtration Column

1. Prepare enough glass tubes to collect all the fractions you anticipate needing (30 should suffice for void volume, up to 95 for other experiments) and place them in the fraction collector. Connect a piece of tubing to the outlet of the stopcock and the arm of the fraction collector (use a pipet tip as before). The instructor or lab assistant will help you set up your fraction collector.
2. Rinse your column for 15 min with buffer (100 mM NaPi , pH 7.0, containing 3 mM 2-mercaptoethanol). Measure the flow rate of your column (in mL / min) and check to see that it agrees with your previous flow rate determination (unless this is your first run). If it does not agree, adjust the height of the buffer reservoir to correct the flow rate. Make sure that you are measuring the flow rate through the tubing attached to the fraction collector.
3. Remove the buffer inlet from the column top and open the stopcock to let the buffer drain until it is just at the bed level then quickly close the stopcock.
4. Carefully load 2 mL of your sample onto your column using a plastic transfer pipet, being careful not to disturb the bed surface.
5. Open the stopcock and begin collecting 1.0 mL fractions (collected by elution time).
6. When the sample has entered the column resin carefully add 2.0 mL of buffer using a plastic pipet (continue to collect fractions throughout this step).
7. When the buffer has entered the column resin carefully fill the column with buffer using a plastic pipet (continue to collect fractions throughout this step).
8. Replace the column top without the tubing attached.
9. Attach the buffer-filled tubing to the column top. The column should now flow at a rate of between 1 and 2 mL / min (as you previously determined), and you can collect fractions until you are sure that all analyte has eluted from the column.
10. After all components have eluted, stop the flow of the column by closing the stopcock, and carefully store your column on its ring stand on the side bench of the lab (be sure to label it and to wrap Parafilm *carefully* around the top and bottom).
11. Measure accurately the volume of your fractions (one fraction in the middle should be enough) using your pipetman. Record this volume for later use in graphing.
12. Transfer aliquots of all fractions to a microtiter plate for spectroscopic analysis at the appropriate wavelength(s).



## Product Information

### Protease Inhibitor Cocktail for general use

Catalog Number **P2714**

Storage Temperature  $-20\text{ }^{\circ}\text{C}$

#### Product Description

Crude cell extracts contain a number of endogenous enzymes, such as proteases and phosphatases, which are capable of degrading the proteins present in the extract. The best way to improve the yield of intact proteins is to add inhibitors of those enzymes known to be present. This protease inhibitor cocktail has been optimized and tested for general use. It is a mixture of water-soluble protease inhibitors with a broad specificity for the inhibition of serine, cysteine, and metallo-proteases.

Specific inhibitory properties of the components are:

- AEBSF – [4-(2-Aminoethyl)benzenesulfonyl fluoride hydrochloride] – serine proteases, e.g., trypsin, chymotrypsin, plasmin, kallikrein and thrombin
- Aprotinin – serine proteases, e.g., trypsin, chymotrypsin, plasmin, and kallikrein; human leukocyte elastase, but not pancreatic elastase.
- Bestatin hydrochloride– aminopeptidases, e.g., leucine aminopeptidase and alanyl aminopeptidase.<sup>1,2,3,4</sup>
- E-64 – [N-(trans-Epoxy succinyl)-L-leucine 4-guanidinobutylamide] – cysteine proteases, e.g., calpain, papain, cathepsin B, and cathepsin L.
- EDTA – metalloproteases
- Leupeptin hemisulfate salt– both serine and cysteine proteases, e.g., plasmin, trypsin, papain, and cathepsin B.

#### Recommended Usage

One ml of the cocktail solution is recommended for the inhibition of endogenous enzymes equivalent to 1 mg of USP pancreatin. One bottle is recommended for the inhibition of proteases present in a maximum of 20 g of cell extract.

**Note:** Not all extracts contain the same levels of endogenous enzymes, and it may be necessary to adjust the volume of cocktail required.

#### Reagent

Supplied as a lyophilized powder

#### Precautions and Disclaimer

This product is for R&D use only, not for drug, household, or other uses. Please consult the Material Safety Data Sheet for information regarding hazards and safe handling practices.

#### Preparation Instructions

One bottle prepares 100 ml of cocktail solution. The powder dissolves quickly in a minimal volume of water or buffer, and may be prepared as a concentrate and diluted as needed.

The product is packaged in a 10 mL serum vial with a crimp cap. Dissolve the contents of the vial in 10 ml of water, then transfer to another container for dilution to 100 ml.

#### Storage/Stability

Store the lyophilized powder at  $-20\text{ }^{\circ}\text{C}$ . The product, as supplied, is stable for 4 years when stored at  $-20\text{ }^{\circ}\text{C}$

#### References

1. Umezawa H., *Ann. Rev. Microbiol.*, **36**, 75-99 (1982).
2. Aoyagi, T., et al, *Biochem. Int.*, **9**, 405-411 (1984).
3. Aoyagi T., and Umezawa, H., *Acta Biol. Med. Ger.*, **40**, 1523-1529 (1981).
4. Mumford, R. A., et al, *Biochem. Biophys. Res. Comm.*, **103**, 565-572 (1981).

AP,NDH,PHC 02/06-1

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## APPENDIX E

### Dilution series for Bio-Rad/Bradford assay and cytochrome c quantification

1. **For each sample** (e.g., yeast homogenate, cation-exchange decant, cation-exchange eluate),
  - a. put a 100 uL aliquot in a 1.5 mL centrifuge tube. Add 900 uL phosphate buffer (with  $\beta$ ME) and mix gently but thoroughly. Mark this tube "1/10".
  - b. Take 100 uL of your 1/10 dilution, transfer to a new 1.5 mL tube, and add 900 uL buffer as before. Mix gently but thoroughly. Mark this tube "1/100".
  - c. Repeat step (b) to generate dilutions of 1/1000, 1/10<sup>4</sup>, and 1/10<sup>5</sup>.
2. Centrifuge all samples to precipitate suspended debris.
3. To determine total protein and cytochrome *c* content, set up the following samples in a 96-well plate:
  - lysozyme dilutions for a standard curve, PLUS buffer, PLUS 40 uL Bio-Rad dye, as you did in week two (in triplicate)
  - a row for each sample: 20 uL undiluted and 20 uL of each dilution PLUS 140 uL of buffer PLUS 40 uL Bio-Rad dye
  - a second row for each sample: 200 uL undiluted and 200 uL of each dilution

For example:

	1	2	3	4	5	6	7	8	9
<b>A</b>	assay blank plus buffer and dye	assay blank plus buffer and dye	assay blank plus buffer and dye	20 uL lysate plus buffer and dye	20 uL decant plus buffer and dye	20 uL eluate plus buffer and dye	200 uL lysate	200 uL decant	200 uL eluate
<b>B</b>	0.5 ug lysozyme plus buffer and dye	0.5 ug lysozyme plus buffer and dye	0.5 ug lysozyme plus buffer and dye	20 uL lysate 1/10 plus buffer and dye	20 uL decant 1/10 plus buffer and dye	20 uL eluate 1/10 plus buffer and dye	200 uL lysate 1/10	200 uL decant 1/10	200 uL eluate 1/10
<b>C</b>	1 ug lysozyme plus buffer and dye	1 ug lysozyme plus buffer and dye	1 ug lysozyme plus buffer and dye	20 uL lysate 1/100 plus buffer and dye	20 uL decant 1/100 plus buffer and dye	20 uL eluate 1/100 plus buffer and dye	200 uL lysate 1/100	200 uL decant 1/100	200 uL eluate 1/100
<b>D</b>	2 ug lysozyme plus buffer and dye	2 ug lysozyme plus buffer and dye	2 ug lysozyme plus buffer and dye	20 uL lysate 1/1000 plus buffer and dye	20 uL decant 1/1000 plus buffer and dye	20 uL eluate 1/1000 plus buffer and dye	200 uL lysate 1/1000	200 uL decant 1/1000	200 uL eluate 1/1000
<b>E</b>	3 ug lysozyme plus buffer and dye	3 ug lysozyme plus buffer and dye	3 ug lysozyme plus buffer and dye	20 uL lysate 1/10 <sup>4</sup> plus buffer and dye	20 uL decant 1/10 <sup>4</sup> plus buffer and dye	20 uL eluate 1/10 <sup>4</sup> plus buffer and dye	200 uL lysate 1/10 <sup>4</sup>	200 uL decant 1/10 <sup>4</sup>	200 uL eluate 1/10 <sup>4</sup>
<b>F</b>	4 ug lysozyme plus buffer and dye	4 ug lysozyme plus buffer and dye	4 ug lysozyme plus buffer and dye	20 uL lysate 1/10 <sup>5</sup> plus buffer and dye	20 uL decant 1/10 <sup>5</sup> plus buffer and dye	20 uL eluate 1/10 <sup>5</sup> plus buffer and dye	200 uL lysate 1/10 <sup>5</sup>	200 uL decant 1/10 <sup>5</sup>	200 uL eluate 1/10 <sup>5</sup>

6. Read your plate twice, once at 595 nm for the Bradford assay, and then once at 410 nm for the cytochrome *c*.

## APPENDIX F

### Ten Questions for week three

- 1) What are two characteristics of a proper buffer that, when not taken into consideration, are likely to affect protein stability during the purification of a protein?
- 2) Suppose that you have a purified sample of bovine cytochrome *c* in a test tube. The total volume is 1.0 mL. You remove 100  $\mu\text{L}$  of this solution and add 400  $\mu\text{L}$  of buffer. You then take the absorbance of the diluted sample and find it to be 0.105 at a wavelength of 410 nm. How many milligrams (mg) of bovine cytochrome *c* were in the original 1.0 mL sample?
- 3) What is the purpose of adding  $\beta$ -mercaptoethanol to the buffer when purifying cytochrome *c*?
- 4) In diluting a protein sample a student does the following. She first removes 900  $\mu\text{L}$  of buffer from a stock solution using a P1000 pipetman and dispenses it into a labeled 1.5 mL microcentrifuge tube. She then removes 100  $\mu\text{L}$  of the protein solution using the P1000 and adds it to the microcentrifuge tube. She caps the tube and mixes the solution. What lab sin did she commit?
- 5) Suppose that you need to measure the protein quantity in a 5.0 mL sample. You have already constructed a Bio-Rad protein standard curve with lysozyme as the standard. The equation of your best-fit line for the standard curve is  $y = 0.0637x + 0.3257$ . You remove 100  $\mu\text{L}$  of sample and add 900  $\mu\text{L}$  of buffer, and then use 20  $\mu\text{L}$  of this dilution to find an absorbance of 0.455 at a wavelength of 595 nm. How many milligrams (mg) of protein were in the original 5.0 mL sample?
- 6) Yeast cytochrome *c* has a pI of approximately 9 and you used a cation-exchange resin to bind the protein during the purification. If a protein has a pI of 6.5, what type of resin would you use to bind it to help purify it in a buffer like you are using in lab?
- 7) Why don't we use the more conveniently-disrupted organism *Escherichia coli* rather than the cell wall-enclosed *Saccharomyces cerevisiae* as a source from which to purify cytochrome *c*?
- 8) What two characteristics of blue dextran make it ideal for determining the void volume of a gel filtration column?
- 9) Imagine that using the Bio-Rad protein assay reagent you measure the absorbance of a solution containing exactly 10  $\mu\text{g}$  of pure bovine cytochrome *c*. When you fit the absorbance to a well-constructed standard curve you find the apparent amount of cytochrome *c* in the sample is calculated to be 25  $\mu\text{g}$ . What might explain such a large discrepancy between the actual value and that calculated from the standard curve?
- 10) Why is the use of a cation-exchange column so effective in purifying cytochrome *c* from yeast?

## APPENDIX G

### De-convoluting Gel Filtration Data

A wealth of information is hidden inside even a poorly resolved Gel Filtration peak profile. As we've discussed, a longer column will typically give greater resolution of proteins in a gel filtration experiment. One of the lab groups in 2005 had some technical problems and ended up with an unusually short column, and so I've used their data to illustrate how to extract good information from what initially looks like a completely uninformative peak profile.

#### *Step One: The Raw Data*

First, looking at the raw data (panel 1 below), both absorbance profiles seem to indicate that only one protein has eluted, though the broad peak of absorbance at 280 nm doesn't quite line up with the fractions showing maximal absorbance at 410 nm.

#### *Step Two: Leveling the Field*

To begin to extract the hidden information, I first rescaled the data so that the absorbance at 280 nm and the absorbance at 410 nm have the same range of intensities (panel 2). This is done by dividing all the  $A_{280}$  values by the largest reading at 280, and separately dividing all the  $A_{410}$  values by the maximal reading at 410. The resulting graph shows that there is a slightly higher hump, just barely visible in the raw  $A_{280}$  data, that corresponds to the peak in absorbance at 410 nm.

Cytochrome *c* is a protein that contains aromatic amino acids as well as a heme group, so it absorbs light both at 280 nm and at 410 nm. Consequently, any fraction that contains cytochrome *c* should show absorbance at both wavelengths, in proportion to the amount of cytochrome *c* that's present. If only cytochrome *c* were present, then 100% of the  $A_{280}$  and 100% of the  $A_{410}$  would be attributable to cytochrome *c*. That's precisely what we're seeing where the blue line and the red line in panel 2 coincide -- at those points, all of the eluting protein is cytochrome *c*.

#### *Step Three: Removing Interference*

If the absorbance at 280 nm around  $V_e = 40$  mL is due to the cytochrome *c*, then the  $A_{280}$  to the left of the cytochrome *c* peak is probably due to carbonic anhydrase, and the  $A_{280}$  to the right of the cytochrome *c* peak is probably due to aprotinin (since we know those are the only three proteins present). But because many fractions contain more than one protein we're not seeing three clean peaks in the  $A_{280}$  profile. Remember, the raw  $A_{280}$  values are the result of aromatic amino acid absorbance from all the proteins in solution, and therefore they are the arithmetic sum of the absorbances of each of the three protein species individually. If we could separate out the individual protein species, we could resolve -- the jargon term is "deconvolute" -- the messy  $A_{280}$  profile into something cleaner.

Fortunately, we can do just that. As you know, the  $A_{410}$  profile shows us where cytochrome *c* is. So what we're doing in this step is simply subtracting the cytochrome *c*

peak out from the mix, leaving the  $A_{280}$  contributions of carbonic anhydrase and aprotinin. Up to this point, these had been pretty much hidden by the fact that the strong cytochrome c peak overlapped with them.

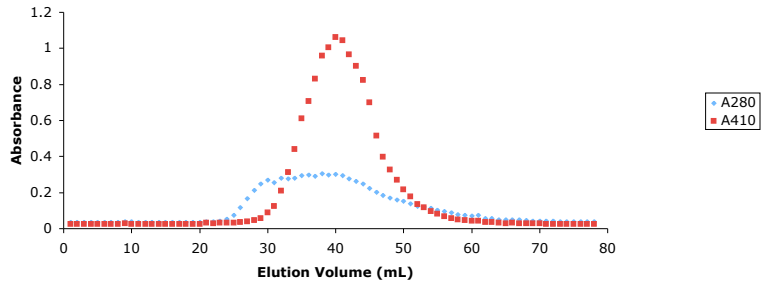
Computationally, this is done by making a new column of values of: (rescaled  $A_{280}$ ) minus (rescaled  $A_{410}$ ). The result is shown in panel 3 -- voila! -- there really ARE three clean peaks here!

***Step Four: Dressing it up***

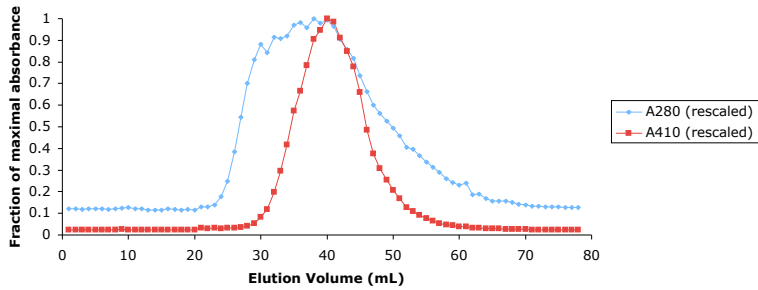
Finally, I simply prettied up the results of step 3 to make the three peaks clearer (panel 4).

You can easily do the same thing with *your* data, and remove all ambiguity about where exactly each of the three standards is eluting. This ought to make your gel filtration standard curve more accurate.

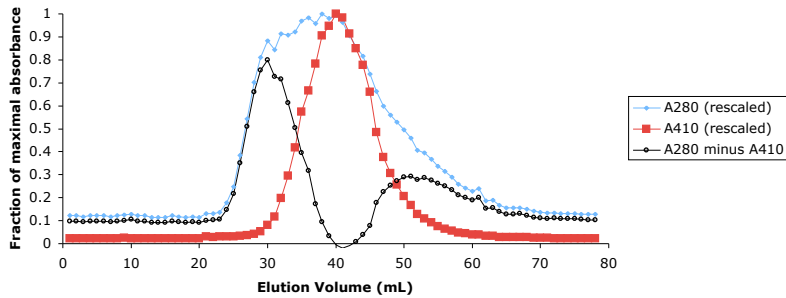
**Group 4's Gel Filtration standards, 27Sep2005**  
**Step 1: The raw data**



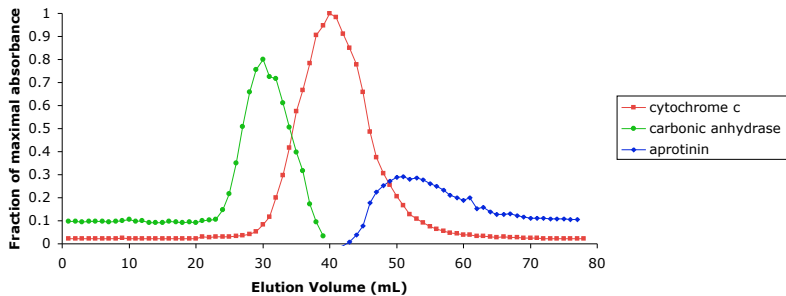
**Step 2: rescaled absorbance data**



**Step 3: the hidden peaks emerge...**



**Step 4: elution profiles of the three standards**



## **APPENDIX H**

### **SDS-PAGE FOR DETERMINATION OF MOLECULAR WEIGHT OF PROTEINS (LAEMMLI SYSTEM)**

Modified from *Laemmli, U. K., Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680-685, 1970.*

#### **REDUCING SAMPLE BUFFER:**

	<u>Volume</u>	<u>Conc. In 2X stock</u>	<u>Final conc.*</u>
0.50M Tris-HCl, pH 6.8	6.4mL	0.125M	0.0625M
10% SDS	10.0mL	4%	2%
Glycerol	5.0mL	20%	10%
0.5% Bromphenol Blue	1.0mL	0.02%	0.01%
2-Mercaptoethanol	2.5mL	10%	5%

\* Diluted with equal parts of sample or water

Store in brown bottle at 4°C. SDS may precipitate, but will go back into solution at room temperature. Boil samples 3 minutes before loading.

#### **RUNNING BUFFER:**

for one liter of 1X Tris-glycine:

- 3.0 g Trizma base (final concentration 0.025 M)
  - 14.4 g Glycine (final concentration 0.19 M)
- fill to 1000 mL with nanopure water  
add 10 mL 10% SDS (final concentration 0.1%)

Store at room temperature. Use ~300 mL per gel run.

#### **RUNNING THE GEL**

The Bio-Rad mini-gel rigs take ~110 V-hrs to run the dye front to the bottom of gel in a 10% gel.

#### **STAINING:**

This stain may be mixed as a stock. It may be reused multiple times. Place the gel in the Staining Solution for 30-60 minutes. If you stain for a longer period of time, destaining will take longer as well.

##### Stain Stock:

- 2.00g Coomassie Blue R-250
- fill to 200mL with nanopure water  
stir and filter using buchner funnel with aspiration

##### Staining Solution:

- 62.5mL Stain Stock
  - 250mL methanol
  - 50mL acetic acid
- fill to 500mL with nanopure water

## **DESTAINING:**

The procedure described above will stain the entire gel blue. To see protein bands, Coomassie that is not associated with proteins -- the "background" -- must be removed. During destaining, the background will lose dye before the protein bands, but incubating too long in Destain I can result in loss of too much stain, so proceed carefully.

To Destain:

1. Place the gel in Destain I with gentle agitation until the protein bands become clearly distinguishable from the background.
2. Destain the background with several changes of Destain II. Solution should be changed when the solution and the gel show the same intensity of blue color. The background should be almost completely clear after the destaining procedure.

To destain heavily stained gels or to destain the protein bands themselves, place the gel in Super Duper Destain.

Destaining can also be achieved by heating gel(s) in water in a microwave oven ~5 minutes (or until nearly boiling), pouring water off and repeating as many times as needed to achieve desired level of background stain.

### Destain I:

- 500mL methanol
  - 100mL acetic acid
- fill to 1.0L with nanopure water

### Destain II:

- 50mL methanol
  - 70mL acetic acid
- fill to 1.0L with nanopure water

### Super Duper Destain:

- 75mL acetic acid
- 25mL acetone



## APPENDIX I

### **Procedure for using the SQ-2800 Single Beam Scanning UV/Visible Spectrophotometer for Kinetic Experiments in Biochemistry Lab.**

*Note that the spectrophotometer requires 25 minutes of warm-up and calibration before any samples can be measured.*

1. Turn on the spectrophotometer
2. After the 15 minute warm up period, select [yes] to calibrate.
3. Once in the main menu, press [4] to access the kinetics package.
4. Press [F1] and set total time to 180 sec. Press [enter], then set delay time to 0 sec. Press [enter] again and press [3] to set time interval to 5 seconds. Press [enter].
5. To select units of measurement, press [F2] and select Abs, then press [enter].
6. To choose a wavelength, press [Set  $\lambda$ ] and enter 475 nm.
7. In a 3 mL plastic cuvette, place all solutions **EXCEPT** the tyrosinase (all solutions should be at room temperature except the tyrosinase, which should be kept on ice). Wipe down the cuvette with a Kimwipe.
8. Place the cuvette into the spectrophotometer and press [0 Abs / 100% T] to zero the instrument.
9. Add tyrosinase to the solution, cover the cuvette with parafilm, invert three times (**DO NOT SHAKE**), wipe with a Kimwipe, and quickly replace in the spectrophotometer. Press [Start].
10. When the time-scan stops and the spec has beeped 3 times, press [F3] and enter the start time and stop time of the linear region of the graph, pressing [Enter] after each command. You will also be prompted to enter a "factor", at which you should enter your "magic number" and press [Enter].
11. The I.U. readout on the right side of the screen is the "factor" x ( $\Delta A/\text{min}$ ).
12. The scale can be changed by the left and right arrow keys for the x-axis and the up and down arrow keys for the y-axis.

## APPENDIX J

### *Independently-designed Projects on Analysis of Tyrosinase Activity*

- on the lab day that you analyze tyrosinase inhibitors, your group should talk to your instructor about your plan for the next two weeks' lab periods. If you do not talk to me by Wednesday at the end of class, I can't guarantee that the reagents and equipment you need will be in place; even that may be too late depending on your needs. If you delay and the toys you need aren't in place in lab that week, you will still be expected to get your investigation done by December 9th.
- the results of your investigation will be presented at a poster session planned for 6 pm on Dec. 12th. You should prepare a brief (~3 minute) talk in which you can summarize your goals and your findings to your classmates and your instructor. You'll get a chance to check out the other groups' work and evaluate their results and presentations.
- your poster presentation should describe your experimental context, the hypothesis you were trying to evaluate, your results, and your interpretation of them. Your interpretation should include an attempt to make structure/function inferences about tyrosinase on the basis of your data **and** relevant published data. More guidelines and recommendations are available online at <http://www.denison.edu/~kuhlman/courses/poster.info.html>
- at lab check-out, your group should turn in an abstract of your poster and a summary data table, providing

	$K_M$	$V_{max}$	$K_i$
control sample(s) (multiple values if they differ substantially from day to day)			(not applicable)
benzoate			
thiourea			
your conditions/inhibitors			may or may not be applicable

(all of these values should be reported with measures of statistical significance)

- where relevant, posters that also present estimates of  $k_{cat}$  and/or the specificity constant for your reactions will be favorably received.