

Denison University
BIOCHEMISTRY LABORATORY, Spring 2014

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 at all times.
- 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 re-copy 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, Horseradish Peroxidase (HRP).

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 HRP enzyme. 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.*

LAB SCHEDULE, CHEM 258-03

<u>Date</u>	<u>Lab activities</u>	<u>Assignment due this week</u>
<u>Experiment #1: Purification of Cytochrome c</u>		
Jan. 21	Introduction, aqueous equilibria involving acids, bases, and buffers; make buffers	
Jan. 28	"The Big Picture", protein quantitation	<i>hand in notes from week one</i>
Feb. 4	equilibria involving weak non-covalent interactions	<i>std curve, sample calculations</i>
Feb. 11	lysate prep, cation exchange	(test #1 this week)
Feb. 18	dialysis, purification table workshop	<i>lab notebook interviews this week</i>
Feb. 25	analysis of samples, plan SDS-PAGE	<i>dry run purification table</i>
Mar. 4	SDS-PAGE <i>and: Back to the Big Picture: what does it mean?</i>	(test #2 this week)
<u>Experiment #2: Kinetic Characterization of Horseradish Peroxidase</u>		
Mar. 11	Isolation of HRP	
Mar. 25	kinetic analysis of HRP	<i>cytochrome c purification analysis (40 pts)</i> <i>Hand in lab notes from first experiment (10 pts)</i>
Apr. 1	specific activity of isolated HRP	(test #3 this week)
Apr. 8	planning of independent projects	<i>HRP purification table and assay data lab plan for independent project</i>
Apr. 15	independent projects	(test #4 this week)
Apr. 22	independent projects poster planning and feedback	<i>hand in poster abstract and data tables for feedback</i>
Apr. 29	Chem/Biochem Core Assessment	
May 1, 6 PM (proposed)	Poster session	<i>Poster Presentation (50 pts)</i> <i>hand in lab notes for Experiment #2 (20 pts)</i>

EXPERIMENT I

PURIFICATION AND CHARACTERIZATION OF YEAST CYTOCHROME C

LAB DAY ONE

Objectives: Introduction to this manual
Explore aqueous acid/base equilibria
Make buffer

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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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 pH 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 will 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.

LAB DAY TWO

Objectives: Understand the Big Picture of Cytochrome *c* purification
Construct protein concentration standard curve
Determine concentration of unknown protein sample
Determine concentration of cytochrome *c* in an unknown sample

Procedure:

Part I. Using the Bio-Rad protein assay dye kit, construct a protein concentration standard curve and use it to determine the concentration of protein in the unknown sample.

Total protein concentration determination will be performed using the Bio-Rad protein assay dye kit and egg white lysozyme (0.1 mg/mL in 100 mM NaPi, pH 7.0) as a standard. The procedure is fairly simple but it is **very important** that you keep accurate records of each sample prep and analysis.

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 λ_{\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.

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.

ii. As with other absorbance-based assays, it is critical to develop a good standard curve to relate protein concentration to dye binding (and A₅₉₅). 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, we will 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 μL .

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.

SAMPLE	μL NaPi	μL lysozyme stock	μL dye
Blank	160	0	40
0.5 μg	155	5	40
1 μg	150	10	40
2 μg	140	20	40
3 μg	130	30	40
4 μg	120	40	40
5 μg	110	50	40
7.5 μg	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 μL (before addition of dye). Depending on the situation, this may be pure sample, or may be a mix of sample and buffer.

2. Add 40 μL dye concentrate to all microtiter wells (see notes on timing under step 4).

3. Pipet up and down slowly and 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 for all of your standard curve samples. Perform a linear regression to get the equation of the best fit line. Your linear regression should have an R^2 value of at least 0.95.

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,

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

The value of your unknown protein concentration should therefore be reported as:
calculated concentration in $\mu\text{g} \pm$ uncertainty in μg

(with both the calculated concentration and the uncertainty rounded to the appropriate number of digits)

Part II. Determine concentration of cytochrome *c* in unknown samples

1. Measure the absorbance of samples containing unknown amount of cytochrome *c* at 410 nm.
2. Using an extinction coefficient of $95.0 \text{ mM}^{-1} \text{ cm}^{-1}$ for cytochrome *c* at 410 nm, calculate the quantity of cytochrome *c* in each sample.

Assignment:

- 1) Use a spreadsheet program (such as Excel) to generate a standard curve for determining protein concentration. In the spreadsheet should be the labeled original data (μg of lysozyme and related absorbance in triplicate).
- 2) Using the equation of the standard curve, calculate the amount of protein in your unknown sample(s) and list this in the spreadsheet.
- 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 professionally labeled and titled graph of your standard curve (see the handout on "good graphing"). Mark the absorbance of your unknown sample(s) on the graph with a labeled point (*not* by hand).
- 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 procedure you followed, and found an absorbance value of 0.45, how much protein was in her sample?
- 6) What is the concentration of cytochrome *c* in each of your unknown samples?

LAB DAY FOUR

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

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. 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 1 mL to store at 4°C for use in determining total protein concentration and cytochrome *c* concentration. Set aside another 1 mL sample and store 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 ensure 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⁺ form; from Bio-Rad) with two washes of 100 mM NaPi, pH 7.0.
2. Dilute the homogenate with six volumes of Millipore purified water 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 inserting a glass wool plug into a 20 ml syringe. 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 bucket. Remember to make observations as to color, cloudiness *etc.* at all points. Decant the supernatant into a separate beaker. Save 1 mL at -20°C for later analysis by SDS-PAGE. 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_i, pH 7.0, containing 3 mM 2-mercaptoethanol.
7. Elute the cytochrome *c* with a minimum of 100 mM NaP_i, 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.

Eluted samples should be kept at 4°C and saved for analysis in two weeks

LAB DAY FIVE

Objectives: Begin dialysis of cation exchange eluate
Learn how to construct a purification table

Procedure:

Part I. Dialysis

(You will need ~1 L of 100 mM NaPi, pH 7.0 buffer for this step. If you do not have it, make it while you are waiting during step 1.)

1. Equilibrate a piece of dialysis tubing by soaking for 30 min (without stirring) in ddH₂O containing a pinch of EDTA. The tubing should be cut to hold approximately 2 - 3 times the volume of your eluate sample.
2. Remove 1.00 mL of the eluate from last week's cation exchange and place it in a clearly labeled 1.5 mL microcentrifuge tube. Store this sample at 4°C for analysis next week.
3. Put the remainder of the eluate 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: Finish constructing the purification table for cytochrome *c* using the sample 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.

LAB DAY SIX

Objectives: Quantify protein from purification
Plan SDS-PAGE gel

Procedure:

Part I. Quantification of Total Protein and Cyt C using absorbance assays

1. Using a disposable plastic pipet, remove your sample from the dialysis bag.
2. Clarify the sample by centrifugation.
3. Determine the total protein content in the three samples you have collected (clarified yeast homogenate, cation exchange eluate, dialysate) using the Bio Rad protein assay. You must also generate a new standard curve (triplicate samples) as done on *Lab Day Two*. You will have to dilute your experimental samples (with NaPi 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 below. Calculate the quantity of protein in each sample (total volume) remembering to figure in any dilution factors that were made.
4. Using an extinction coefficient of $95.0 \text{ mM}^{-1} \text{ cm}^{-1}$ for cytochrome *c* at 410 nm, you should also be able to calculate the quantity of cytochrome *c* in each sample.
5. Store the dialyzed sample at -20°C for analysis next week by SDS-PAGE.

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

- A. You will need at least 350 μL of each sample to complete the assay below.
- B. **For each sample** (e.g., yeast homogenate, cation-exchange eluate),
 - a. put a 100 μL aliquot in a 1.5 mL centrifuge tube. Add 900 μL phosphate buffer (with βME) and mix gently but thoroughly. Mark this tube "1/10".
 - b. Take 100 μL of your 1/10 dilution, transfer to a new 1.5 mL tube, and add 900 μL buffer as before. Mix gently but thoroughly. Mark this tube "1/100".
 - c. Repeat step (b) to generate dilutions of 1/1000, 1/10⁴, and 1/10⁵.
- C. Centrifuge all samples for 2 minutes at maximum *rpm* to precipitate suspended debris.

- D. To determine total protein and cytochrome *c* content, set up a 96-well plate as follows:
- lysozyme dilutions for a standard curve, PLUS buffer, PLUS 40 uL Bio-Rad dye, as you did in lab day two (in triplicate) – **Columns 1-3**
 - sample dilutions for total protein concentration, a column for each sample: 160 uL undiluted or 160 uL of each dilution PLUS 40 uL Bio-Rad dye – **Columns 4-5**
 - sample dilutions for cyt *c* concentration, a column for each sample: 200 uL undiluted or 200 uL of each dilution – **Columns 6-7**

Your plate might look like this:

	1	2	3	4	5	6	7
A	assay blank plus buffer and dye	assay blank plus buffer and dye	assay blank plus buffer and dye	160 uL lysate plus dye	160 uL eluate plus dye	200 uL lysate	200 uL eluate
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	160 uL lysate 1/10 plus dye	160 uL eluate 1/10 plus dye	200 uL lysate 1/10	200 uL eluate 1/10
C	1 ug lysozyme plus buffer and dye	1 ug lysozyme plus buffer and dye	1 ug lysozyme plus buffer and dye	160 uL lysate 1/100 plus dye	160 uL eluate 1/100 plus dye	200 uL lysate 1/100	200 uL eluate 1/100
D	2 ug lysozyme plus buffer and dye	2 ug lysozyme plus buffer and dye	2 ug lysozyme plus buffer and dye	160 uL lysate 1/1000 plus dye	160 uL eluate 1/1000 plus dye	200 uL lysate 1/1000	200 uL eluate 1/1000
E	3 ug lysozyme plus buffer and dye	3 ug lysozyme plus buffer and dye	3 ug lysozyme plus buffer and dye	160 uL lysate 1/10 ⁴ plus dye	160 uL eluate 1/10 ⁴ plus dye	200 uL lysate 1/10 ⁴	200 uL eluate 1/10 ⁴
F	4 ug lysozyme plus buffer and dye	4 ug lysozyme plus buffer and dye	4 ug lysozyme plus buffer and dye	160 uL lysate 1/10 ⁵ plus dye	160 uL eluate 1/10 ⁵ plus dye	200 uL lysate 1/10 ⁵	200 uL eluate 1/10 ⁵

- E. Read the absorbances from the plate at 595 nm for the Bradford assay (Columns 1-6), and then read the absorbances at 410 nm for the cytochrome *c*.

*NOTE: It is a good idea to take some time during this lab period to calculate your total protein and cytochrome *c* concentrations from your absorbance values 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 lab 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.*

Part II. Plan SDS-PAGE Analysis (during lab if time permits, or for homework)

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). Thus you have as many as eight lanes for your samples.
 2. The method of protein visualization that you will use -- Coomassie Brilliant Blue dye -- typically requires 0.1 to 1 μg 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. This is described further under step three of next week's lab procedure. 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 DAY SEVEN

Objectives: SDS-PAGE

Procedure:

Part I: SDS-PAGE

1. Set up your gel. General instructions for running SDS-PAGE gels are provided on the next page. 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 μ l of your sample and place in a small microcentrifuge tube that contains 50 μ l of 2x sample buffer (provided by instructor). This will provide enough volume to run three amounts of each sample -- 20 μ L in one lane, 5 μ L in another, and 2 μ 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 on the next page. This will take several days. Next lab period we'll take digital photographs of the gels for use in your final assignment.

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

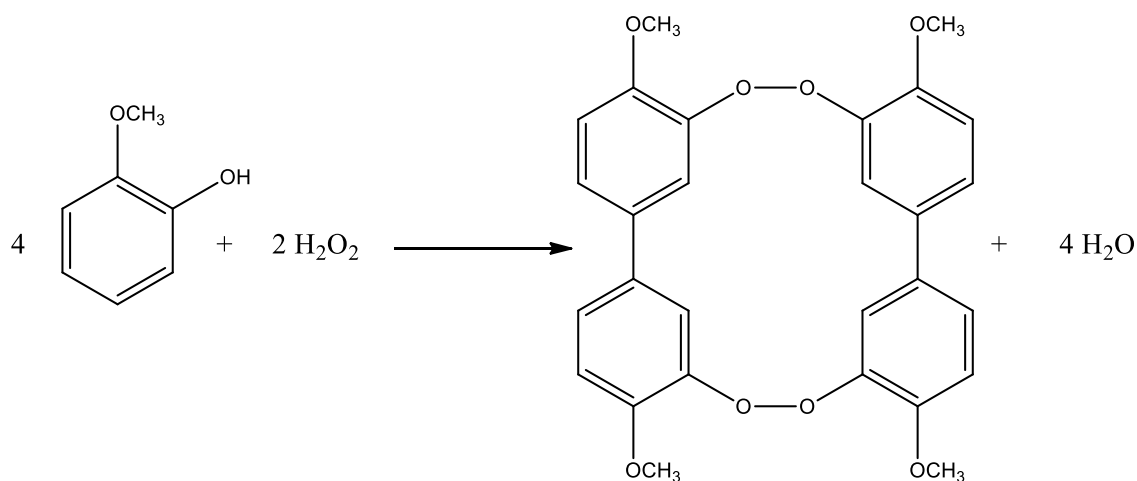
Super Duper Destain:

- 75mL acetic acid
- 25mL acetone

EXPERIMENT II

KINETIC ANALYSIS OF AN ENZYME, HORSERADISH PEROXIDASE

Peroxidases are a general class of enzymes that convert peroxides, such as hydrogen peroxide (H_2O_2) into activated oxygen species that then react with oxygen-containing organic molecules. For example, the reaction between H_2O_2 and 2-methoxyphenol (guaiacol) in the presence of Horseradish Peroxidase (more commonly known by its acronym *HRP*) produces the multi-ring structure tetraguaiacol:



In this reaction, neither the enzyme HRP nor the substrates guaiacol and peroxide absorb light at 470 nm while the product tetraguaiacol does absorb light at this wavelength ($\epsilon = 26,600 \text{ M}^{-1} \text{ cm}^{-1}$). That means that to follow the rate of this reaction we can simply follow the development of the reddish color as measured by absorbance at 470 nm.

However, since there are two substrates in this reaction we must use conditions where we can measure the effect of each substrate separately. We will measure the rate of reaction with regards to substrate concentration for each component individually, keeping the other substrate concentration constant, and relatively large. In this way, we will perform this reaction under conditions to create pseudo-first order kinetics so that we can perform a Michaelis-Menten analysis on each substrate.

LAB DAY EIGHT

Objective: Isolate HRP from horseradish root

Procedure:

Part I. Horseradish root homogenization (done prior to lab)

~200 g of horseradish root was cubed and mixed with 500 mL of 100 mM phosphate buffer pH 6.0. Blended for 4 minutes on ice, filtered through cheesecloth, and clarified by centrifugation at 4,000 x g for 10 minutes. The supernatant is the HRP lysate.

Part II. Ammonium sulfate precipitation

You will be given ~50 mL of the HRP lysate. Remove a 1.0 mL sample into a microcentrifuge tube and store in the deli fridge. Measure the remaining volume of HRP lysate accurately (to the nearest mL).

First precipitation

1. Using the chart provided, determine the amount of ammonium sulfate necessary to bring the HRP lysate to **35%** (instructor will provide further details).
2. Add the ammonium sulfate slowly (5-10 batches over ~15 min) to your lysate while stirring at room temperature. Slow addition is required to limit local concentrations. After all the ammonium sulfate has been added continue to stir for 30 minutes in the refrigerator.
3. After stirring, centrifuge the solution for 10 minutes at 11,000 rpm.
4. Remove the supernatant – **KEEP THIS SUPERNATANT** – and discard the pellet. Remove 500 μ L of supernatant to a microcentrifuge tube and store in deli fridge.

Second precipitation

5. Measure the volume of the supernatant from step #4 accurately (to the nearest mL). Using the chart provided determine the amount of ammonium sulfate necessary to bring the supernatant to **90%**.
6. Add the ammonium sulfate slowly (~15 min) to your lysate while stirring at room temperature. After all the ammonium sulfate has been added continue to stir for 30 minutes in the refrigerator.
7. After stirring, centrifuge the solution for 10 minutes at 11,000 rpm. Remove the supernatant.
8. Resuspend the pellet in a minimal amount (5-10 milliliters) of phosphate buffer (pH 7.0). Record the volume. Remove 500 μ L of resuspension to a microcentrifuge tube and store in deli fridge.
9. Dialyze the resuspended protein three times against 1.0 L of 100 mM phosphate buffer (pH 7.0).

How to use the ammonium sulfate concentration table.

Select your initial concentration of ammonium sulfate in “%” from the left hand column. Select the desired target ammonium sulfate concentration from the “%” row. The intersection of column and row will give you the *grams of ammonium sulfate needed to change the concentration of one liter of solution*. Be sure to scale the amount to your measured volume.

%	10	15	20	25	30	33	35	40	45	50	55	60	65	70	75	80	85	90	95	100	
0	56	84	114	144	176	196	209	243	277	313	351	390	430	472	516	561	610	662	713	767	
10		28	57	86	118	137	190	183	216	251	288	326	365	406	449	494	540	592	640	694	
15			28	57	88	107	120	153	185	220	256	294	333	373	415	459	506	556	605	657	
20				29	59	78	91	123	155	189	225	262	300	340	382	424	471	520	569	619	
25					30	49	61	93	125	158	193	230	267	307	348	390	436	485	533	583	
30						19	30	62	94	127	162	198	235	273	314	356	401	449	496	546	
33							12	43	74	107	142	177	214	252	292	333	378	426	472	522	
35								31	63	94	129	164	200	238	278	319	364	411	457	506	
40									31	63	97	132	168	205	245	285	328	375	420	469	
45										32	65	99	134	171	210	250	293	339	383	431	
50											33	66	101	137	176	214	256	302	345	392	
55												33	67	103	141	179	220	264	307	353	
60													34	69	105	143	183	227	269	314	
65														34	70	107	147	190	232	275	
70															35	72	110	153	194	237	
75																36	74	115	155	198	
80																	38	77	117	157	
85																		39	77	118	
90																				38	77
95																					39

LAB DAY NINE

Objectives: Conduct kinetic characterization of HRP

Procedure:

1. You will be provided solutions of:
 - 30.0 mM hydrogen peroxide
 - 45.0 mM guaiacol
 - 3.0 mg/L HRP (HRP has a MM of 40 kDa)All compounds are dissolved in 100 mM NaP_i, pH 7.0.
2. To a plastic 3 mL cuvette,
 - add the appropriate amounts of buffer, hydrogen peroxide and guaiacol (see table)
 - blank the spectrophotometer with this mixture
 - remove the cuvette, add HRP, cover with parafilm, invert 3 times and replace in the spectrophotometer
 - measure the reaction rate for 2.0 minutes at 470 nm
 - record the reaction velocity reported by the instrument
3. Repeat the procedure with differing amounts of reagents as described in the tables below.

Trial #	Buffer (mL)	H₂O₂ (mL)	Guaiacol (μL)	HRP (μL)
1	1.600	1.000	200	200
2	1.500	1.000	300	200
3	1.400	1.000	400	200
4	1.300	1.000	500	200
5	1.200	1.000	600	200
6	1.100	1.000	700	200
7	1.000	1.000	800	200

Trial #	Buffer (mL)	H₂O₂ (μL)	Guaiacol (mL)	HRP (μL)
8	1.790	10.0	1.000	200
9	1.780	20.0	1.000	200
10	1.770	30.0	1.000	200
11	1.760	40.0	1.000	200
12	1.750	50.0	1.000	200
13	1.740	60.0	1.000	200
14	1.730	70.0	1.000	200

4. After collecting the velocity data it must be converted into units of $\mu\text{M}/\text{min}$. If you do not enter a "factor", the spectrophotometer will provide a rate (listed as "I.U.") given in $\Delta\text{Abs}/\text{min}$. Using Beer's Law and the information given above, you should be able to calculate the conversion from $\Delta\text{Abs}/\text{min}$ to μM tetraguaiacol/ min .
5. The calculated velocity combined with the substrate concentration can then be used to construct a set of graphs. Note that the concentration of substrate is the concentration IN THE CUVETTE.

Assignment:

Generate a Michaelis-Menton plot (V_o vs. $[\text{S}]$, aka *Substrate Saturation Curve*) and a Lineweaver-Burk plot for each substrate. Turn in these professional quality figures with legends.

The figure legends for your Lineweaver-Burk plots should include your determination of V_{max} , K_M , k_{cat} and catalytic efficiency (paying close attention to significant figures) with respect to *each* substrate.

Procedure for using the SQ-2800 Single Beam Scanning UV/Visible Spectrophotometer for Kinetics

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 120 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 λ] and enter 470 nm.
7. In a 3 mL plastic cuvette, place all solutions **EXCEPT** the enzyme (all solutions should be at room temperature except the enzyme, 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 enzyme 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 (to use the whole time, set start time to "0" and stop time to "120"). You will also be prompted to enter a "factor", at which you should enter your conversion factor (for converting from $\Delta\text{Abs}/\text{min}$ to $\mu\text{M tetraguaiacol}/\text{min}$) and press [Enter] (to use a factor of "1" just press enter).
11. The I.U. readout on the right side of the screen is the reaction velocity.
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.

LAB DAY TEN

Objective: Calculate the Specific Activity of the HRP isolated two weeks ago from horseradish root

Procedure:

1. Remove your sample from the dialysis bag. Microcentrifuge the entire volume. Transfer liquid to a single 15 mL tube. Measure the volume to the nearest $1/10^{\text{th}}$ mL. Keep on ice. (This is your post-dialysis sample.)
2. Determine the total protein in the samples from *each* step of the purification using lysozyme as a standard with the Bradford / BioRad protein dye procedure (as performed in the cytochrome *c* lab).
3. Determine the total units of HRP activity in each sample:
 - a) Determine the velocity of the catalyzed reaction in the presence of each of your HRP purification samples
 - follow the same general procedure that you used before (see step two from *Lab Day Nine*)
 - use 1.000 mL of H_2O_2 , 1.000 mL of guaiacol and the appropriate amount of (phosphate buffer+HRP sample) to bring the volume to 3.00 mL
 - Note that the amount of enzyme sample needed is likely to be quite small. In fact, you may have to dilute your samples significantly.
 - Measure the activity for 2 minutes being sure that the line produced by the spectrophotometer is linear throughout the two minute run (if it isn't, then dilute the enzyme more).
 - b) Convert your velocity values into units of *activity*, where 1 unit of activity is defined as the amount of sample required to produce $1.0 \mu\text{mol}$ of tetraguaiacol in 1.0 minute.

Assignment:

Generate a purification table for your HRP isolation. An example is shown on the next page and you're encouraged to carefully examine table 5.1 in your textbook.. Your instructor will provide you with additional details of what should be included in what you hand in.

Sample HRP Purification table.

Sample	Total Protein (mg)	Total Activity	Specific Activity	Percent Recovery	Fold Purification
Lysate					
35% AS cut Supernatant					
90% AS cut Resuspended Pellet					
Post-Dialysis					

LAB DAYS ELEVEN THROUGH THIRTEEN

Objective: Design and carry-out an experiment using HRP kinetics as your analysis tool

Part 1:

Design an experiment, or short set of experiments, that allows you to test a hypothesis about HRP activity. Submit an outline of your experiment to have your design approved by your instructor.

- Your design must include collection of data for kinetic analysis (K_M and V_{max} determination) under at least two different experimental conditions.
- Your outline must include a list of any reagents or equipment you will need.

Part 2:

Perform your independently-designed experiment. Be sure to collect all data in your notebook.

Assignment:

The results of your independently-designed project will be presented as a poster presentation at a combined poster session with all sections of biochemistry. See your syllabus for date and time of the poster session.

In addition to a poster (details to follow), 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. More guidelines and recommendations are available online at <http://www.denison.edu/~kuhlman/courses/poster.info.html>