BCMB 351 Biochemistry I Laboratory Fall 2007
Ursinus College

Introduction

This laboratory is designed to give you extensive exposure to methods whose purpose is to isolate, purify, and characterize proteins from a biological source. This lab is part of an upper division course; meaning significant expectations for your thought, input, and work exist.

The first phase of the laboratory consists of introducing you to protein concentration and enzyme activity assays and gel electrophoresis. These are techniques you will use throughout the semester (and next semester!). The second phase is a group project where you will attempt to purify and characterize an enzyme.

What You Can Expect

This is a capstone course and you should not expect to come into lab and follow a cookbook procedure. You will be given appropriate guidance and information but it will often be up to you to design a specific experimental protocol. You will be required to look in the primary literature and your laboratory book, *Protein Methods*, for assistance in experimental designs. <u>Therefore it is absolutely required that you be thinking at all times and planning ahead.</u> Before you do an experiment, make sure that you completely understand what you are about to do and why! As in the real scientific world, an hour in the library or in discussion with your lab partner can save a day in the lab! This laboratory will require you to spend significant time outside of the 3-hours every Tuesday or Wednesday afternoon *thinking about, planning, discussing and writing about* your experiments.

What I Expect

I expect that every member of each group will fully participate in the planning, execution, and data analysis of each experiment. I also expect that every member of each group will have an equal hand in writing about each and every experiment. If this is *not* the case within your group, notify me as soon as possible. I will work with your group to reach a compromise or, in rare cases, to re-assign groups members.

Your group must write 4 complete lab manuscripts. A brief description of the essential components of each manuscript submitted is outlined later in the syllabus. You may reference prior laboratory manuscripts.

Grading of the Laboratory Section of BCMB351

The laboratory accounts for 26% of your final grade. Maintaining complete, detailed laboratory notebooks will be included as part of the participation grade.

Tentative Laboratory Schedule

Week of

8/27	Introduction
9/3	Protein Concentration Assays
9/10	SDS-PAGE (make solutions and pour lower gel)
9/17	SDS-PAGE (pour upper gel and run)
9/24	Staining SDS-PAGE & preparing solutions
10/1	Enzyme Activity Assay (LDH)
10/8	Group Project
10/15	Group Project – Wednesday class only
10/22	Group Project
10/29	Group Project
11/5	Group Project
11/12	Group Project
11/19	Group Project – Tuesday class only
11/26	Group Project (all project procedures completed today)
12/3	Mandatory Laboratory Clean-up

Lab Manuscript Due Dates

Manuscript 1:	Protein Concentration Assays	4%	due Sept 10th in lab
Manuscript 2:	SDS-PAGE	4%	due Oct 1st in lab
Manuscript 3:	Group Project 1st paper	9%	due Nov 5th by 5pm
Manuscript 4:	Group Project 2nd paper	9%	due Dec 4th by 5pm

Note: I will read a rough draft of two manuscripts and return it with comments. I will need drafts at least one week prior to the due date.

Laboratory Ground Rules

1) Safety regulations must be adhered to at all times in the lab (you MUST wear goggles in any lab, and **this includes the instrument lab**).
2) Reagents must be used sparingly and equipment must be treated with due care.
3) Lab begins *promptly at 1:30 p.m.*
4) There will be no lab work conducted outside of the lab times, except with my consent.
5) You must return equipment and reagents to their proper location.
6) If equipment malfunctions, you must tell me as soon as possible.
7) If you don't know how to use a piece of equipment, ask for directions.
8) You must keep a detailed, scientific notebook.
9) In order to be issued your participation points for lab, you must help to clean-up the entire lab on the last scheduled lab day.
10) Plagiarism/academic dishonesty of any kind will result in the harshest penalty that I can pursue under college regulations. If you are not sure what constitutes academic dishonesty in a specific setting, just ask and we can consider examples.

Failure to cite properly work from books, the literature, and your colleagues **IS** academic dishonesty!

Lab Manuscript Guidelines

You will write up complete journal-style lab manuscripts that should always contain background information from the literature that you have obtained. Analysis of your data may also require that you report on similar experiments from the literature or your peers. Data should be reported efficiently and in a manner that aids in communication of the science. These manuscripts will be graded on background information, completeness, accuracy, analysis of the data, presentation, grammar, punctuation, spelling, proper scientific voice/presentation, and discussion of results. Although this varies from field to field, manuscripts here should be written in the *third person passive voice*. Each manuscript should consist of (in order): title page; abstract; introduction; materials and methods; results; discussion; acknowledgements, references.

Abstract: This section must concisely state the problem being studied, the major techniques used, the results, and a brief interpretation of the major results. Abstracts are usually given in one paragraph and restricted to 150-300 words. The abstract is frequently the last section written in a paper. Highlight your major findings or the name of major techniques you used, but do not go into minute detail here in terms of data or experimental.

Introduction: This section should include a review of what was previously known about what you have studied (this means references, no Wikipedia!!!). Also, the introduction should define the questions that your studies were designed to address and the reasons for conducting the work. Don't re-state the lab manual or other papers, but give a nice framework of what is known already and how does your experiment fit within this. The final paragraph of this section should set-up the rest of the paper by describing what was done in the experiment. This section will necessarily contain references to books and journals in most cases. The internet is *not* peer-reviewed and as such references from the internet are of less value!

Materials and Methods: This section, though tedious to compile, should be complete such that an outsider *familiar* with the techniques could repeat your work. It should include the name and settings of all instrumentation used, the composition of all key reagents (and their manufacturer or supplier), and any key parameters such as temperatures, times, etc. This section is written in paragraph form in the past tense. If a method was used from a previous paper, this method can be cited and any modifications you made noted as opposed to re-writing an entire procedure that appears in text. However, if your modifications were substantial, consider rewriting the procedure for clarity.

Results: This section should contain both a written and graphical presentation of your observations and numerical results. Data may be best presented in tables, graphs, photographs, or molecular drawings. Each figure must be discussed and referred to in the narrative portion of the results. Do not editorialize on your results in this section.

Present them clearly and concisely and use the discussion section to analyze them. Look at different graphing styles and consider tables for numerous data. The main thing you want to do in this section is present your data so that the reader can quickly and accurately see your results. Make all figure legends and table titles descriptive and complete.

Discussion: In this section your creative and critical thinking skills should be employed. What conclusions can be drawn from your data? How does this compare with the results in the literature of other investigators or your colleagues (be sure to reference properly in this section)? Can you explain something that did not work in terms of the science done? This section should be probative of your results and the results of others (in class and from the literature). Don't simply repeat your results, but discuss them in the context of what they do/do not tell you about your experiment. You should be supportive of your data where your data is strong and you should point out inconsistencies in your data where you see them. The last thing you want as an author is other people pointing out errors in your analysis.

Bibliography: This section should contain a complete listing of literature you used in the report. It is expected that a *minimum of four references* be included. Follow a consistent citation method for each book and journal (look to journals such as *Biochemistry*, *Proceedings of the National Academy of Sciences USA*, *Science*, *Cell*, or *Nature* for citation styles). You may list your sources numerically (with associated numbering within the text of your paper) or you can list your references alphabetically in this section (referring to them by author and year in the text). Most biological journals use the latter system and this is fine. Most chemical journals use the former system and this is also fine. Just <u>be consistent</u> within your paper as to method and style and use the same method and style for every paper you write in lab. I have a preference for more complete references, meaning the title of the article and *all* authors are listed in the reference.

<u>Some helpful tips on what to include (minimally) in each lab report:</u>

Report on Protein Concentration Assays:
- The theory behind each assay, sensitivities, benefits, drawbacks, etc.
- Results of two different assays that your group carried out AND results of another assay carried out by another lab group.

Report on SDS-PAGE:
- The theory behind SDS-PAGE, coomassie staining and silver staining (sensitivities, benefits, drawbacks).
- Results of two gels (one stained with coomassie, one with silver)

Reports on Group Project (how this is split up is up to timing):
- LDH (biological role, reaction catalyzed, etc)
- Theory behind protein purification in general
- Theory behind specific purification protocols used
- Theory behind enzyme assay used (inhibition if appropriate)

Results of purification protocols, enzyme assays, protein concentration assays, SDS-PAGE, specific activity at each purification step, % yield of each purification step, calculations of K_m, V_{max}, k_{cat}, inhibitor calculations
Discussion of everything!

Group Project

The Purification of Lactate Dehydrogenase

Originated by John Tymoczko
Updated by Rebecca Roberts, Tom Ruttledge, and Amy Brown

This semester in Phase II, the students in BCMB 351L (you!) will undertake the isolation, purification, and characterization of an important enzyme, namely Lactate Dehydrogenase (or Lactic Acid Dehydrogenase, LDH).

Goals:

1) In depth work with an enzyme, from tissue to partial purification.
2) Understanding the techniques and theory behind a variety of protein purification techniques, including: preparation of solutions; tissue homogenization; ammonium sulfate precipitation; pouring and running gel filtration columns (i.e. Sephadex), ion-exchange columns (i.e. DEAE), and affinity columns (i.e. Cibacron Blue); protein quantification, protein assays (i.e. LDH activity); enzyme kinetics (i.e. Km, Vmax); inhibition assays (i.e. sodium oxalate, gossypol), and protein purity analysis (i.e. SDS-PAGE).
3) Exposure to the scientific literature and searching capabilities.
4) Development of writing strategies for scientific papers.
5) Development of your ability to work as part of a collaborative group.
6) Development of your ability to write as part of a collaborative group.
7) Development of experimental techniques related to proteins.
8) Development of you as a scientist (meaning the ability to develop experimental protocols, troubleshooting those protocols, and the analysis of your experimental data).
9) Have some fun!!!!

Brief Description

This project will last for the remainder of the semester. Your work one week will determine what you will do the following week. There are some inherent pitfalls with this type of approach to lab. One is that if you are not careful with your biological materials (i.e. tissue and enzyme solutions), all could be lost. You must be sensitive to how you handle and store all materials. Second, this type of lab requires YOU to plan ahead for the next week. Your group must come into lab having all of the solutions ready for that day. You must be prepared to do the experiments *and* understand when they are not going well so that you can make corrections on the fly. Third, your group must have clear organization and an understanding what *everybody* in the group and in other groups will do. Fourth, this type of lab runs the high risk of failure at any point. Failure is not so important to me, it is your *analysis* of that failure in scientific ways that will be important. Similarly, your successes must also be accounted for scientifically. I am looking for you to make connections between theory and practice and your reports must reflect that! I do not want simple re-statements of lab procedures followed by less-than-inspired data

analysis. I want literature precedents, literature reviews, concise statements of your materials and methods, and awe-inspiring critical analyses of your data. Not much, huh?

The rewards of this type of lab are numerous. They include strong connections between theory and practice, development of scientific methods, the rewarding of creative thought, the rewarding of organization and attention to detail, and critical thought so necessary to science. I additionally hope that people will develop good habits of working in groups toward a common goal and the appreciation of individual differences in the attainment of that goal.

Estimated Time Line of Project (*Individual results may vary*)

Week 1: Tissue homogenization and 45% ammonium sulfate precipitation
Week 2: 60% ammonium sulfate precipitation; pour Sephadex column, pour DEAE column
Week 3: Run Sephadex, DEAE columns; pour affinity column.
Week 4: Run affinity column; protein and activity assays of various fractions.
Week 5: Kinetic experiments; Pour separating gel.
Week 6: Inhibition experiments; pour spacer gel and run gel
Week 7: Finish kinetic and inhibition experiments; stain gel.

The Enzyme (Kopperschlager, G. and Kirchberger, J. (1996))

Lactate dehydrogenase (E.C. 1.1.1.27), L-(+)-lactate:NAD+ oxidoreductase (LDH) is an ubiquitous enzyme among all vertebrate organisms. It catalyzes the final reaction of glycolysis, the formation of L-(+)-lactic acid. LDH is also present in many invertebrates, plants, and diverse microorganisms.

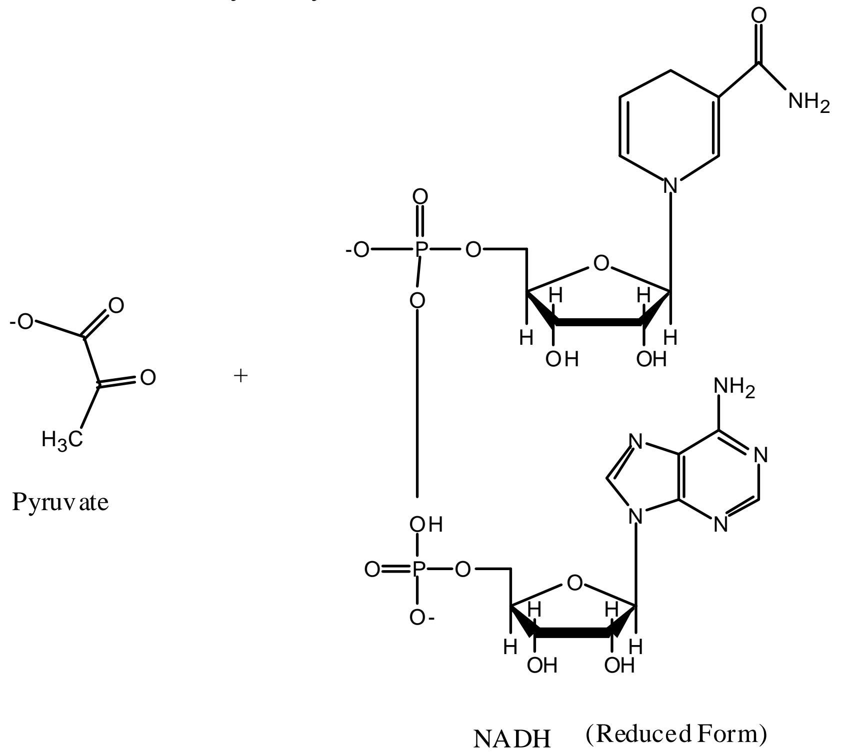
LDH catalysis was discovered first in cell-free muscle extracts which were found to oxidize L-lactate to pyruvate. The enzyme was finally purified by Straub in 1940, while the first crystals were obtained by Kubovitz and Ott in 1943.

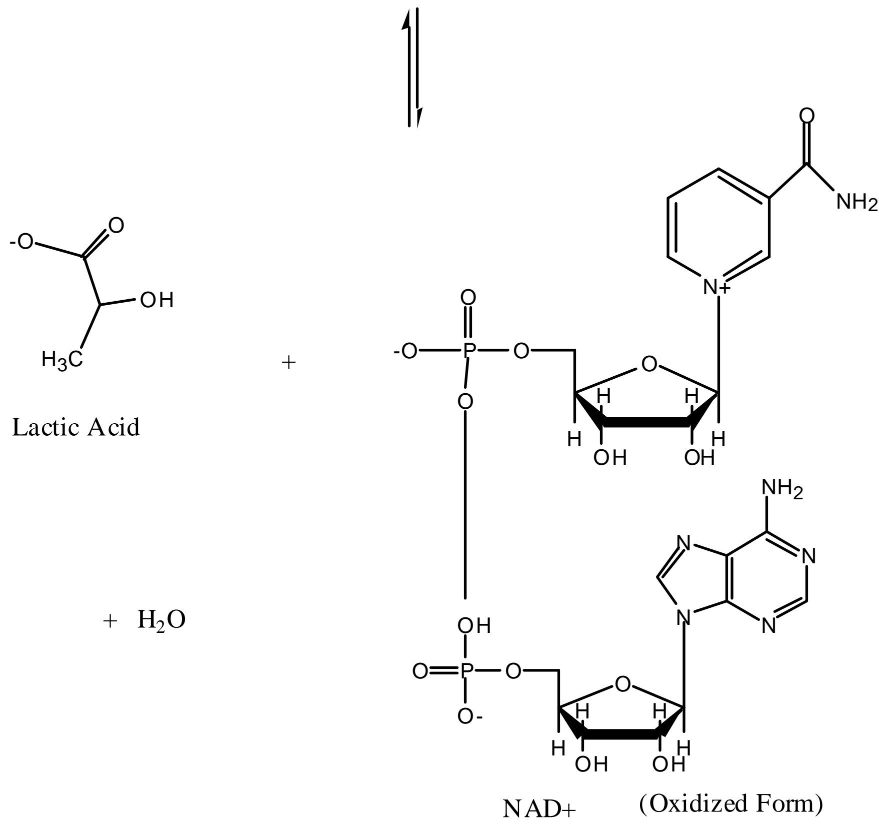
LDH has received great attention because of its significant metabolic role. The widespread occurrence of this enzyme is due to its function in the glycolytic metabolism. It permits organisms to overcome a temporary oxygen debt in the form of accumulated L-lactate to be later discharged by the reoxidation to pyruvate when oxygen becomes available.

Animal LDH was found to be a tetromeric molecule which exists in different isoenzymes. These forms are generated by association of two genetically distinct subunits to homo- and heterotetramers. The oligomeric enzyme can be dissociated by freezing and thawing, by high salt concentrations, or by treatment with denaturing agents. Sequence similarities allow association of subunits from different species *in vitro* to form functional tetramers. At least two gene duplication events have led to three genetically distinct subunits, LDH-A or M(muscle)-type, LDH-B or H(heart)-type and LDH-C or X-type in vertebrates and each species has diverged substantially in physio-chemical properties, biological function and developmental regulation. LDH-A_4, LDH-B_4, and their three hybrid forms (LDH-A_1B_3, LDH-A_2B_2, and LDH-A_3B_1) are present in all vertebrates and the characteristic tissue-specific expression of each has remained relatively unaltered throughout their evolution. The relative abundance of the five isoenzymes in each cell type is entirely dependent upon the numbers of subunits of each type available to combine.

KEEP EVERYTHING ON ICE OR IN THE COLD ROOM AT ALL TIMES!!!!!!!!!!!

__**The Reactions Catalyzed by LDH**__





USEFUL BIOCHEMISTRY LABORATORY PROTOCOLS

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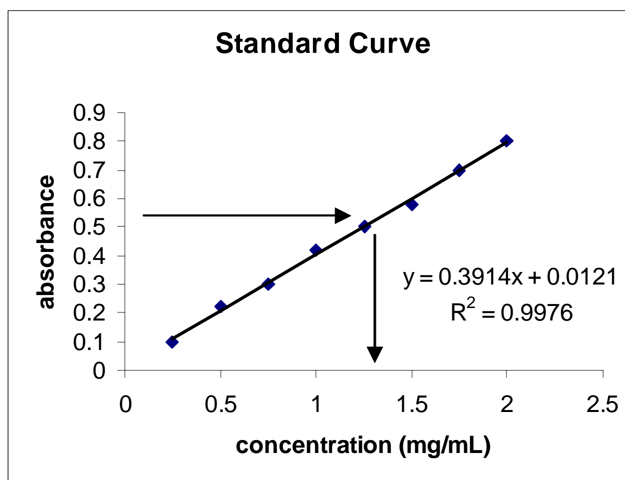
Protein Concentration Assays

Four different protein concentration assays are described below. Each group must perform two assays during the second week of lab. For your lab manuscript you will discuss these two assays as well as an additional assay performed by another group in the laboratory. As such, groups should discuss which assays they will perform.

For each assay you will determine the concentration of an unknown sample(s). First, however, you must construct a standard curve. This is done using different concentrations of an inexpensive protein, for example bovine serum albumin (BSA). Once the standard curve is obtained, you can use it to determine the concentration of unknown samples. Absorbance of unknown samples must fall within the standard curve!



Preparation of BSA Stock Solutions

Note that you must construct a BSA standard curve for any assay that you perform (every time you perform it!!).

Prepare a 2mg/ml BSA stock solution in water (total of 10 mL). Make several dilutions for the standard curve. Choose concentrations that are relatively evenly spaced out between 0.1mg/ml and 2mg/ml (you may choose to make lower concentrations depending on the sensitivity of the assay).

*****What follows are general instructions – refer to your Protein Methods text and information on Blackboard for more detailed instructions*****

(this is a big hint that for your first lab manuscript you need to go to the text and to Blackboard and print out the appropriate reference material! You also should get some primary literature references. These may take a while from interlibrary loan so you should do this ASAP.)

Direct Measurement Assay

UV Spectroscopy

Perform the UV spectroscopy assay on the same day that you prepare the sample. Always perform in triplicate.

1.) Record absorbances at 280 nm (absorption maximum for aromatic amino acids) with the Deuterium lamp of a UV-visible spectrophotometer and using semi-micro quartz cuvettes OR micro cuvettes made of special plastic (ask Amy)
2.) Determine if you need to DILUTE samples and or standards and repeat #1.
3.) Plot absorbance (Y axis) versus concentration (X-axis).

Colorimetric Assays

Lowry Assay

Solutions for Lowry Protein Determination

Lowry A 2% Sodium Carbonate in 0.1 N Sodium hydroxide solution.
Lowry B 0.5% $CuSO_4 \cdot 5 H_2O$ in 1% Sodium Citrate.
Lowry C Has to be prepared fresh 5:0.1 ratio of a and b respectively

1) Prepare standard BSA solutions. Note appropriate solvent for Lowry et al. assay is dilute NaOH.
2) Add 5 ml of reagent C to each assay tube vortex and maintain at room temperature for 10 minutes.
3) Add 0.5 ml of 1 N Folin-Ciocalteu phenol agent to each tube; vortex and maintain at room temperature for 30 minutes.
4) Record absorbances at 660 nm with the Tungsten lamp of a spectrophotometer using plastic or glass cuvettes.
5) Plot absorbance vs. concentration.

Adapted from Lowry, *et al.* **1951**. Protein measurement with the Folin phenol reagent. J. Biol. Chem. *193*: 265-75.

Bradford Assay

Bradford Assay (the old fashioned way)

1) Prepare BSA stock solutions for comparison.
2) Prepare Coomassie Blue Solution:
   100 mg Coomassie Brilliant Blue G-250 dissolved in 50 ml of 95% ethanol to which 100 ml of 85% phosphoric acid has been added. Dilute entire mixture to 1 L with water.
3) In a test tube, place 100 µl of protein solution (containing 10-200 mg).
4) Add 5.0 ml of the Coomassie Blue solution.

5) After 5 minutes, the absorbance at 595 nm is read (plastic cuvette).

Bradford Assay (using Sigma BradfordReagent)

1) Prepare BSA stock solutions.
2) Combine 3 mL of Bradford Reagent and 100µL of sample.
3) Mix several times by gentle inversion.
4) Maintain at room temperature for 5-45 minutes.
5) Record absorbances at 595 nm with the Tungsten lamp of a spectrophotometer. (plastic cuvette)
6) Plot absorbance vs concentration.

Bradford 96-well Plate Assay (using Sigma Bradford Reagent)

1) In each well add 5ul of sample.
2) Add 250ul of Bradford Reagent.
3) Mix gently by shaking for 30 seconds.
4) Maintain at room temperature for 5-45 minutes.
5) Measure absorbance at 595 nm. With the plate reader located in Thomas 126.
6) Plot absorbance versus concentration.

Modified from Sigma Bradford Reagent Technical Bulletin (see Blackboard).

BCA Assay (using Sigma BCA Kit)

Standard 2.1 mL BCA Assay Protocol

1) Prepare required amount of BCA Working Reagent needed. BCA Working Reagent is prepared by mixing 50 parts of Reagent A (BCA solution) with 1 part of Reagent B (Copper (II) Sulfate Pentahydrate 4% solution). Mix until a uniform, light green color.

Example calculation: for a total of twelve 2.1mL tubes mix 25mL Reagent A and 0.5 mL reagent B.

2) Combine 2mL of BCA Working Reagent with 100µL of sample. Vortex or invert to mix.
3) The following incubation parameters may be used:
60°C for 15 minutes OR
37°C for 30 minutes OR
25°C for 2 hours to overnight
4) Cool tubes to room temperature
5) Transfer solution to cuvet and measure at 562 nm. (note: color development continues slowly after cooling to room temperature so read all tubes within 10 minutes)
6) Plot absorbance versus concentration.

Adapted from Sigma BCA Protein Assay kit Technical Bulletin (on Blackboard).

NOTES: Colorimetric Assay Sensitivity

The BCA protein assay is a highly sensitive, flexible protein quantification procedure that does not require the precisely timed addition of reagents and vortexing, as the Lowry procedure. In addition, other advantages include: "compatibility with ionic and non-ionic detergents, a stable working reagent, less protein-to-protein variation than with other methods, broad linear working ranges with excellent sensitivity and the ability to chance the protocol which provides flexibility.

The main reagent in the patented BCA protein assay is Bicinchoninic acid (BCA) as a water-soluble salt. It is a sensitive, stable and highly specific reagent for cuprous ions. Color development appears to be dependent upon cysteine, cystine, tryptophan and tyrosine. The sensitivity of the BCA assay is affected by both time and temperature

SDS-PAGE

Refer to *Protein Methods* for detailed instructions and recipes. Recipes for preparing the small mini-gel should be multiplied by 4 to have enough volume for the larger gel rigs.

We will run 12.5% separating gels in this class. In the past we've found that running these gels at 60-70V for 16-17 hours gives good separation and is therefore a good starting point.

Staining the gel with Coomassie Stain:

1. if you are using Coomassie Stain put gel into Coomassie Stain following the run (*Protein Methods* Chapter 5 Section F).
2. make sure that the container is covered to avoid evaporation and gel drying.
3. modifications to *Protein Methods* protocol:
    - can leave in Comassie Stain for several days
    - can leave in destain for a day (although need to be careful because you can completely destain gel and therefore lose bands!)
    - you can change the destain several times to speed up process
    - transfer gel to water in a sealed container

Silver Staining the gel:

1. follow the protocol from Pierce (available on Blackboard)
2. after gel has run, put gel into fixative over night
3. transfer to water in a sealed container
4. continue protocol next week in lab

LDH Activity Assays

Over the course of this semester, you will be isolating and purifying the enzyme lactate dehydrogenase (LDH). As such, you will need to identify its presence and how much is there. This is typically done with an enzyme activity assay. This basically involves the placement of the enzyme into a buffered medium and supplying it with starting materials. One can then look spectroscopically for either the formation of product of the loss of starting materials!

To do this assay, you need to know what things are required. Obviously, enzyme is one. That will be supplied to you as a pure enzyme for this first assay. Later, you will use the biological material from your prep as the source of enzyme. The assay also requires a buffered solution and some salts and we will use sodium bicarbonate and sodium chloride in TrisHCl. Lastly, the assay requires NAD^+ and Lactate. Make only enough for what you need that day. Do not waste reagents.

You need to make the following stock solutions:

Lactate Stock: 120 mM lithium lactate in 10 mM Tris-HCl, pH 8.8

NAD^+ Stock: 12 mM NAD^+ in 10 mM Tris-HCL, pH 8.8

Bicarbonate Stock: 0.5 M NaCl containing 18 mM $NaHCO_3$.

Enzyme Stock: A final concentration of enzyme around 3 μg/mL. It should be diluted with 10 mM Tris-HCL, pH 8.6 (yes **8.6**).

In general, an assay will involve the addition of 0.6 mL of lactate stock, 0.4 mL of the NAD^+stock and 0.2 mL of the bicarbonate stock (total 1.2 mL). To this, you will add 10 μL of the diluted enzyme stock to the reaction, **in the cuvette (in the spec!)**, to start the reaction. The contents of the cuvette should be mixed after enzyme addition (how??, why??). The absorbance at 340 nm (what are you monitoring??) versus time is then monitored (how long??). Before the start of the assay, the enzyme should be kept away from the other solutions and all solutions should be kept on ice.

As you develop proficiency with this assay, you may want to try varying the concentration of lactate or the amount of enzyme you add. Also during the prep project later in the course, your prep material will substitute for the enzyme stock in the assay above!!

LDH Purification Protocols

Early procedures described herein are adapted mainly from Pesce, A., McKay, R.H., Stolzenbach, F., Cahn, R.D., Kaplan, N.O. (1964).

The procedures described below are only general guidelines for the lab. You should consult the literature and your text, *Protein Methods*, to develop a detailed procedure for yourself. Your detailed procedure must be in your notebook with all observations and results. **Your notebook will be turned in at the end of the semester and counted toward your participation grade.**

Week 1

You will be provided with chicken breast samples. You should cube these samples and remove any extraneous fat. You should homogenize approximately 50 grams of breast cubes. Homogenization should be done to minimize any increase in temperature and the temperature should be kept close to 5°C. Homogenization should be done in a blender (keep cold!!) and the homogenate then stirred in a beaker for 1 hr. (keep cold).

Homogenization Buffer (use 80 mL of buffer per 50 g of tissue):
20mM Tris-HCl, pH 8.6
1mM β-mercaptoethanol (BME)
1mM Phenylmethylsulfonyl fluoride (PMSF)

note: What is PMSF? Why is it important to include? Be careful – it's poisonous!

After stirring, you will need to filter a portion of the homogenate through two layers of cheesecloth into a 50mL centrifuge tube (on ice). Centrifuge the filtrate for 30 minutes at 14,000xg. The supernatant is used as the crude extract. *Please note that it is good practice to save all samples (superntants, pellets, etc.) and label them clearly. Store them in the freezer so that if an error is ever made, all is not lost! You also may want to run these samples on an SDS-PAGE gel later!*

You will then need to bring your supernatant to 45% saturation with ammonium sulfate. This can stand for a while in the cold room. After standing, centrifuge for 30 minutes at 14,000xg. After centrifugation, you may wash the pellet with 45% saturated ammonium sulfate and re-centrifuge. The supernatant from this should be combined with the previous supernatant. The pellet should be saved but will not be used (except in emergency!).

Week 2

The pooled supernatants from last week need to be subjected to a 60% ammonium sulfate fractionation now (bring them to 60% saturation, centrifuge, etc.). The pellet will be retained and used for further studies. Resuspend the pellet in a minimal amount of **Tris-ME buffer** (10mM Tris-HCl, 0.5 mM β-mercaptoethanol , pH 8.6). You might want to make up 200 mL or so of this buffer and put in the cold room). The supernatant from this fractionation will be saved but not used now.

You need to set-up and pour a Sephadex G-25 column. A 10mL G-25 column can desalt 3 mL of sample into 4 mL. You should use about 2.5g of Sephadex G25 swollen in 20 mL of Tris-ME buffer to pack the column. Store the column with buffer in it. Keep cold.

You need to set-up and pour a DEAE column. You should use about 3 mL of packed resin to complete the column. Store the column with buffer in it. Keep cold.

Week 3

Add about 3 mL of the re-suspended pellet to the Sephadex G25 column and have it enter the column. Collect the eluant as the first fraction. Add 4 mL of Tris-ME buffer to the top

and collect this 4 mL as the second fraction. Continue to add 4 mL aliquots until the eluant gets cloudy, all the while collecting 4 mL fractions. You might need to assay (for enzyme activity) the fractions to see which fraction contains the LDH.

A fraction containing LDH activity should then have a portion aliquoted into an Eppendorf tube and then frozen. The remaining fraction can then be loaded onto the DEAE column and eluted with 4x3mL aliquots of the Tris-ME buffer. These fractions may need to be assayed for LDH activity. Protein concentrations of all fractions containing LDH activity should be determined. This will allow you to calculate the specific activity and percent yield.

Prepare a 0.5 mL column of Cibacron Blue Agarose in a disposable column. Wash with 10 mL of Tris-ME buffer. Keep the column stored in buffer and cold!

Week 4

Load a portion of the DEAE-cellulose eluant (LDH active fraction) onto the affinity column. Have the eluant enter the column and then wash with 20 mL of Tris-ME. Collect 3 mL fractions. Now, wash with 5mL of **NADH buffer** (20 mM Tris-HCl, pH 8.6, 1mM NADH, 0.5 mM β-mercaptoethanol) and then with 10 mL of Tris-ME. Continue throughout to collect 3 mL fractions. All fractions may need to be assayed for LDH activity. Protein concentrations of all fractions containing LDH activity should be determined. This will allow you to calculate the specific activity and percent yield.

Week 5

The goal of this week is to plan and execute enzyme assays so that you can develop a complete kinetic profile of the enzyme. You will want, at a minimum, to determine the K_m, V_{max}, and k_{cat} for LDH. Your group should think ahead about how to do this and what experiments/data will be necessary to accomplish these calculations. As part of this kinetic profile, you have to know how much enzyme is contained in each fraction you work with. This will necessitate a protein (concentration) assay for all fractions as well (remember that you may have to dilute fractions so that eventual absorbance readings are in the range of 0-1 or so). So much to do, so little time. You also have to set-up and pour your SDS-PAGE separating gel. Store the gel in the cold room until you pour the spacer gel next week.

Week 6

You will given the choice of several inhibitors to use for inhibition studies. These inhibitors may include sodium oxalate and gossypol, among others. You will have to design and execute a series of assays in order to determine the type of inhibition displayed by the inhibitor as well as other effects or reaction kinetics. You also have to pour the spacer gel, load your samples, and run your gel. For this, your samples need to be prepared.

Determine the amount of each fraction that contains 50 μg of protein. Pipette this into an eppendorf tube. Make each of these samples 10% with respect to trichloroacetic acid (TCA). Once all the samples are 10% with respect to TCA, keep on ice for at least 15 minutes. Centrifuge (cold) and then pour off the TCA (use a pipettor to make sure the TCA is all removed). Rinse the pellet gently with ice-cold acetone and allow to air dry for a few minutes. Add the sample buffer and store samples or load onto gel. Run your gel for the appropriate time and then fix and rinse.

Week 7

This week is designed to give you time to finish any activity and inhibition experiments you may choose to do and to stain your gel if needed.

References

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