

Chem 405 Biochemistry Lab I

Experiment 5 Purification of the MDH-GFP-6X His (MGH) Plasmid

INTRODUCTION: For the next five weeks, you will be working on purifying your fusion MGH protein. One of the colonies from your transformation was selected and grown up in a broth that included IPTG, a chemical to induce the bacteria to express (produce) the protein on the plasmid (MGH). These cultures have been centrifuged and cells lysed (using both sonication and enzyme membrane degradation) by your instructor. YOU will be responsible for making the buffers (from stock concentrations) planning the experiments (using the information, protocols and teg videos provided) save your samples, prepare them for the next chromatography and analyze at each step of the way. This handout contains the basic purification information, practical chromatography basics, logistical information, and instructions necessary for you to be able to carry out your purification. The specific protocols/guides for using the equipment, chromatography, preparation, assays and other pertinent information are found in the the protocols on your laboratory webpage. To start you off, your instructor will show you how to use the equipment (column adaptors, columns, fraction collector, dialysis buffers...) in class. Outside of class, you will watch the teg files to help remind you how to do this. The webpage has several helpful links to sites with additional theory and specific chromatographic detail.

BASICS FOR CHROMATOGRAPHY: Based on the tutorial, you will choose two different chromatographic medias (resins, beads, or techniques) and plan how to prepare your sample and run the proteins through the column. With each step of the purification, you will need to remember the following general flow of activity

1. Design Chromatography
2. Prepare column and buffers
3. Load and run the chromatography
4. Analyze the fractions for MGH and total protein.
5. Pool fractions (save portion)
6. Prepare pooled samples for next chromatography.

Do not forget to save at least 300 μ l of your lysates and the pooled fractions along the way. Saved samples are critical for proper analysis at the end of the experiment. FREEZE these samples. You will not freeze the pooled solutions along the way (the pooled samples and fractions will be saved in the coldroom).

INTRODUCTION TO YOUR FUSION PROTIEN

As already mentioned in an earlier handout, you are using a fusion protein consisting of the Malate Dehydrogenase from watermelon, Green Fluorescent Protein AND a 6XHis tag. We are using this fusion protein for a couple of different reasons. First, is that the His tag can be used for affinity purification (using nickel columns) and identification (using antibodies against the 6X His tag). Malate Dehydrogenase is an important enzyme found in cytosol, mitochondria and glyoxal fractions of cells. Take a moment to look up the role of this enzyme in your textbook and on the web. We will later assay and characterize you're the enzymatic nature of Malate Dehydrogenase. Finally the Green Fluorescent Protein is an important fusion protein that is easily detected and quantitated by it's fluorescence.

Green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* is a revolutionary reporter molecule for monitoring gene expression and protein localization *in vivo*, *in situ*, and in real time.



Fig 1. *The source of our fun.*

The GFP fluoresces bright green upon mere exposure to UV or blue light—unlike other bioluminescent reporters which require additional proteins, substrates, or cofactors to emit light. Upon mechanical stimulation *A. victoria* emits a green light. The chromophore itself is a p-hydroxybenzylidene-imidazolidone (green background). It consists of residues 65-67 (Ser - dehydroTyr - Gly) of the protein. The cyclized backbone of these residues forms the imidazolidone ring.

Although the amino acid sequence SYG can be found in a number of other proteins as well, it is

neither cyclized in any of these, nor is the Tyrosine oxidized, nor

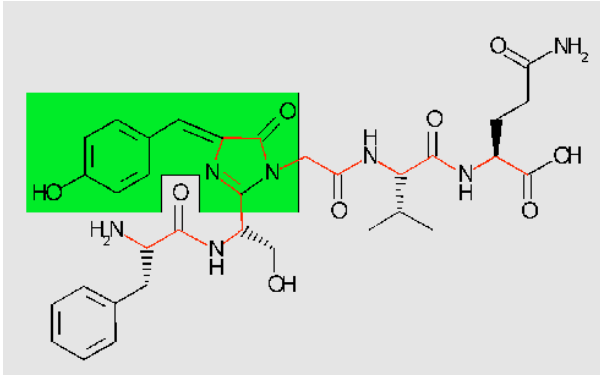


Fig 2. The chromophore for the GFP

are these proteins fluorescent. The protein has a very specific secondary structure and if not folded properly, the fluorescence is lost. The excitation spectrum of native GFP from *A. victoria* (blue) has two excitation maxima at 395 nm and at 470 nm. The fluorescence emission spectrum (green) has a peak at 509 nm and a shoulder at 540 nm.

The GFP fluorescence is stable, species independent, and can be monitored non-invasively in living cells. It has been expressed and shown to fluoresce in a variety of species. We can use the easy detection of the protein to follow its expression in bacteria, purification through a size exclusion chromatography and its subsequent analysis by SDS-PAGE and western blot.

BASIC CHROMATOGRAPHY: The chromatography tutorial has already introduced you to the concepts of several chromatographic resins and the application of selecting a separation technique, analyzing the resulting chromatography and selecting samples to pool and move onto the next step of purification. The information presented here are less about theory (there are several links for this on your laboratory webpage) and more about the practical aspects of purification. This is vital information you will not find in most textbooks.

As mentioned in the introduction, there are six basic steps in purification:

Step 1: Design the Chromatography – This is the step that will make or break most of your efforts. Too little attention here will result in frustration in preparing solutions and running the column. Take the time to be thorough in this step.

There are several components that need to be considered when designing your chromatographic purification; type of separation technique, sample preparation, size of column, flow rate, buffers needed to bind and wash unwanted proteins and the elution conditions. Before choosing a separation technique you should review the purification strategy found in the tutorial focusing on the choice of purification methods. In short, you should try to avoid using chromatographies that use the same chemistry to separate compounds. In other words, you will typically get a poor yield and purification from two ion exchange columns in a row or two size exclusion columns, one after the other. You should also consider how the sample is eluted from the column. Will the final buffer be amenable to directly load onto the next column or will you have to prepare the pooled fractions by dialysis or concentrate them by ammonium sulfate fractionation before continuing on.

Once you've picked your purification resin, you need to determine the size of column, volume of resin, how to load the sample, flow rate (how fast to run the column) how much buffer to run through the column, how to elute the sample and the collection method. You will be given three or four different chromatography resins to choose from. In the protocol section on the class webpage are basic protocols to help each step. You will be given the specifics on how to address the important questions listed above in these protocols. You should be aware of the advanced theory and practice of chromatography. There are many excellent websites which have this information, your class website has a few. Chapter 11 in *Principles and Techniques of Biochemistry and Molecular Biology (6th Ed.)* has much of this key information for you to look over. However, there are a few basics that you should understand before looking through the protocols.

1) Column size – A short thick column will have less backpressure than a taller thin column (that is how much resistance the fluid has as it is pumped through the column). Too much backpressure and your tubing, connections and pump will fail. A wide short column will have

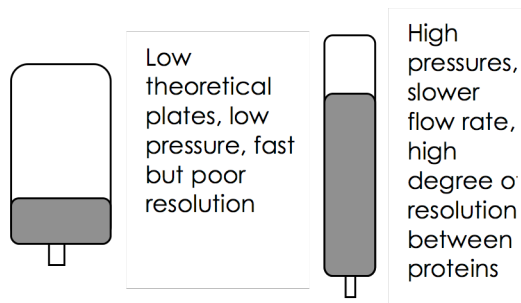
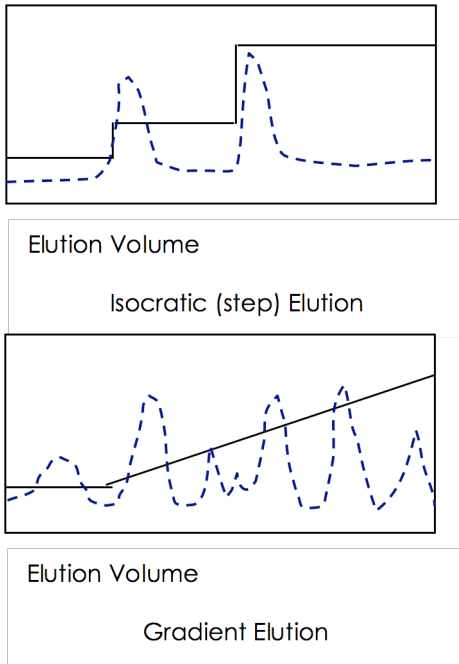


Fig. 3. Short wide vs. tall thin column

a faster flow rate. If you are going to simply bind wash and elute with what is called a different wash (step or isocratic gradient) and not a gradual change from one buffer to another (gradient elution) then you might consider using a wider short column. But you should also be aware that the eluted sample will be more dilute and less resolution (separation from contaminants). If you are going to use a gradient



elution or work with size exclusion resins, then you must have a taller thin column.

The specific amount of resin depends on the type of chromatography and the particular binding capacity of each resin. For resins that bind their analyte, the top 20% of the column should bind most of the protein, for SEC columns, the sample volume loaded should be no more than 3% of the bed volume (bed volume = the volume of resin in the column).

2) Flow Rate: For simple open columns, gravity will work just fine. If you are pumping buffers through the column, then the flow rate can vary. A fast flow rate may cause excess packing of the resin and the backpressure will build, causing the tubing and pump to leak and fail. At a high rate of flow, most peristaltic pumps (the kind with a tube stretched around a roller) will pulse back and forth, causing a dilution of the eluted proteins and mixing of unresolved proteins. Too slow and the proteins may lose activity waiting to elute. A simple rule of thumb is set the flow rate no faster than 2 or 3 times that of gravity for soft resins (agarose and sepharose) for 2-5 times that for more crosslinked resins.

A 1 cm diameter column can easily be run at 0.5 to 2 ml per min. A 2.5 cm diameter column can handle a flow rate of up to 4 or 5 ml per min. Don't forget that if you flow the sample (analyte) too fast, you may get reduced binding kinetics to the resin, dilution of the analyte as it elutes or excessive rates can result in a poor elution & low yields.

3) Buffer Selection and wash & elution volume:
 Buffer composition – Buffers: You MUST include a weak acid base (buffer) in each solution. The pH should be within one pH unit of the pKa for a buffer to be effective. For most uses, a 10 – 25 mM concentration of buffers will do just fine. If you add a metal chelator such as EDTA to maintain stability of the protein, appropriate concentrations run from 0.1 to 2 mM. Reducing Agents: Most purification require that a reducing agent keep the protein native. Dithiothreitol is typically used at a 0.1 to 1 mM concentration. Use this fresh. The DTT will quickly be oxidized by the dissolved oxygen in water and thus will only last for a few hours. 1M DTT is typically stored in a small aliquot in the freezer and used each time you run purification. Protease Inhibitors are also commonly used. Simple metal chelators like EDTA are used at a 0.1 – 5 mM concentration to inhibit metalloproteases as EDTA binds the metal that is necessary for the protease activity. EDTA is stable at room temp. and is used from a stock solution. Phenylmethylsulfonyl fluoride (PMSF) is very unstable in aqueous solutions. PMSF is relatively inexpensive and routinely used by many laboratories. A 100 mM stock solution is made in isopropanol and stored in the dark at -20 degrees. Must be warmed (37 degrees) to get into solution. Use at 1 mM in solution but is only stable for two or so hours and thus must be used immediately. There are other cocktails of inhibitors that inhibit a wide range of proteases. If the protein you are purifying is a target for proteases, you should look into using a mix or proteases.

Loading – Make certain you do not have interfering compounds in your buffer. You must inspect the components of your pooled sample to see if each component will interfere with the purification of your protein. Common problems include: Including EDTA (a metal chelator) when loading onto a Nickel column. EDTA will compete with the His tagged protein to bind to the metal on your resin. Another common problem encountered in purification is the salt concentration. Ion exchange resins do not bind

many proteins if the salt concentration is 50 mM or greater (NaCl or KCl). Hydrophobic resins will not bind unless there is more than 200 mM salt in the buffer.

Column equilibria – Each time you use or pour a column, the resin should be washed from proteins that may still be bound from a prior use. For most purifications, a 1M NaCl in your buffer will suffice. SEC chromatography does not need this step. Check the elution conditions for each column you use and wash two or three column volumes of this through the column prior to use. At this point your resin will not bind your protein unless the salts and other components are removed and the column equilibrated in your loading (equilibration) buffer. Therefore, after washing your column with a high salt wash, you must wash 5 to 10 column volumes of equilibria/loading buffer through the column prior to loading the column.

Wash and Elution Volumes – Loading a column – Once you've initially pumped your sample onto the column, it is important to realize that the material still in the tube, pump or in the column needs to be washed through the column. Washing more of the equilibrium buffer through the column is important to remove unbound protein before eluting the protein. Typically a two to five column volume of equilibrium buffer will do just fine. Washing a column – After loading you may want to wash off weakly bound proteins or use a buffer with a sub-optimal concentration of ligand, salt or pH to remove other proteins, leaving your protein on the column. To do this, simply stop the column, switch the tube into a new beaker or flask containing your wash buffer and develop the column with 3 to 5 column volumes of the new buffer. By this time nearly all the protein that will elute has eluted. Eluting the protein from the resin - Most of a protein eluted in an isocratic gradient will come off in two or three column volumes, less for a gradient. It is a general rule of thumb to use a 8-10 column volume elution when using isocratic washes. When using a gradient, a steep gradient (a large change in buffer concentration or short volume of gradient {1-5 column volumes for the total gradient}) will result in a tight concentrated elution of your protein, but the resolution from contaminating proteins will be less than ideal. Using a larger more shallow gradient (one with 8 to 10 column volumes of buffer for the total gradient) will result in a much better resolution but will also dilute your protein. If the dilution effect is significant your protein may be too dilute to detect. A 5 to 10 column volume gradient is a good starting point.

4) Collection Method (picking a fraction size): When to collect a fraction or not... This is a typical and

challenging question to answer. The more fractions the more test tubes you will have to assay to find your protein. However, on the other hand, the more fractions, then the more likely you will be able to isolate your protein from other contaminant proteins.

First, if you know that your protein is NOT in a given wash, then it is wise to collect that wash as a single fraction. The same goes for the flow through fraction (this is the material that does not bind to the column during a load step). Each of these steps could easily be collected in a beaker. One for each step and analyzed as one. Alternatively, you could also collect fractions from the beginning to the end of a purification.

When collecting fractions the volume collected for each fraction depends on the number of tubes you have in your fraction collector and the volume of your wash and elutions. Again, it is important to consider how many fractions you can practically collect and measure yet maintain reasonable purity. For larger columns (>10 ml column volume) a starting point is to collect fractions 1/5th to 1/10th the size of the column. For a 1-2 ml column, I recommend using 0.5 ml fractions. For a 2-10 ml column, fractions of 1 or 2 ml would suffice.

Use the purification check-sheet in the protocols section of your laboratory web page to help you through this section. Each type of chromatography resin will have a short protocol and guide found in the protocol section of the laboratory web page.

Step 2 Prepare the column and buffers -

Now that you've planned your chromatography, you need to make the buffers and pour the columns. Prepare at least 25% more buffer than you calculate you need. The handout for each type of chromatography has specific suggestions on buffers and preparing the resins. All stock solutions are found in the laboratory. Use these items to prepare your buffers. Store buffers in the cold room across the hall. Plan to make buffers one day and running the column on a different day.

Do NOT throw any resin or columns. All of these materials are reusable and expensive. There are recepticals for used chromatography resin in the hood.

Packing the column – Columns should be packed in one step or pour to avoid distinct layers of the resin.

- Pour enough resin in a small beaker to use and let settle.
- Decant the clear buffer from the beads and add enough equilibria buffer to make a 50% slurry.
- Attach the glass or plastic column to a stand, cap the bottom of the column with a small closure or turn the stopcock to the off position. Fill the column 1/3 with buffer.
- Resuspend the beads by swirling and pour the beads into the column.
- Once the beads have settled open the bottom of the column to drain excess buffer. Cap when the buffer is just to the top of the resin.
- If using a pump, fix the flow adaptor to the top of the column, being careful not to introduce air into the tube or column. If using a gravity fed column, you are now ready to equilibrate.
- DO NOT LET THE COLUMN RUN DRY. This will result in cracking and the buffer and proteins will flow around and not through your column. If the column does run dry, empty and repour. If this happens during a run, when your samples are already loaded, gently use a glass pipet and stir the beads with a minimal amount of equilibria buffer.

Specific Instructions for using the flow adaptors are found on the protocols and teg web links.

Important note: Do not use mechanical methods to empty columns or transfer chromatography beads. Using glass rods or scoops will result in crushed beads causing particulate mater to interfere with column flow and purification. Use buffer or water to mix the container or column to resuspend any beads. Alternatively, a slight pressure of air introduced to the bottom of the column (using the lab air or a syringe) will usually push the resin out of a column.

Step 3 Load and run the chromatography -

This may take longer than you think. Plan to spend at least 30 min gathering materials and getting the column and pump set up. Do NOT start until your sample is ready to load and the buffers are all prepared.

Calculate how long the column will run. Plan an appropriate time to start and stop the column. Remember other people will be using the equipment so sign up for a time and be polite by keeping to your schedual. Make certain to collect your fractions and set them aside for later assay right away!

TIPS FOR RUNNING A SUCCESSFUL CHROMATOGRAPHY.

- If your sample is not clear and has precipitate in it, centrifuge the sample in the swinging bucket rotor found in the cold room for 10 – 15 min.
- When using the pump to flow buffer through the column, make certain all tubes are placed in the bottom of your buffer beaker and the beaker. Tape may be needed to ensure the hose doesn't slip.
- Practice a few ml run with the pump/column/fraction collector to ensure thngs are set up right before loading on your fraction.
- Plan to stick around and watch the load and flow. ALWAYS watch the first few fractions go through the fraction collector. NEVER assume it will just work right.
- Label ALL tubes.
- Store fractions covered with tinfoil or saran wrap in the cold room. Don't forget to label with your name and lab section.
- Save 300 µl of the lysate and each pooled sample you take along the way. FREEZE these samples in well labeled microfuge tubes. Record the volumes of each pool for later assay.

Save fractions and run assay as soon as possible! Your laboratory notebook should have all the observations and settings from this chromatography. You should be able to give this notebook to anyone in the lab and expect them to repeat the experiment without additional instructions from you.

Step 4 Analyze the fractions for MGH and total protein –

In the purification tutorial, you clicked on a button to run an assay to determine which fractions had your enzyme. You could also look at the chromatograph to see where all of the protein eluted from the column. This is where using the GFP portion of the fusion MGH protein comes in handy. We will use the fluorescence nature of GFP to quantitate how much MGH is in each fraction using a fluorescent plate reader. You will then conduct a protein assay on EACH fraction to determine the total amount of protein in the fractions. When you are finished you will prepare a chromatograph using two Y axis (one for protein concentration in mg/ml and the other for MGH concentration in Relative Fluorescent Units – RFU) . From this graph you will determine which fractions to save.

It is vital that you realize one normally uses an enzyme assay or western blot to detect the protein and not fluorescence. We are able to take advantage of the fluorescent nature of the GFP portion of the fusion protein.

Total fusion protein is quantitated by multiplying the relative fluorescent units by any dilution used in measuring fluorescence times the volume of the sample (fraction or pooled sample). This should be included in your lab book and will be used in preparing the final purification table. Protocols for using the plate reader and conducting a protein assay are found on the laboratory webpage.

Step 5 Pool fractions (save portion) – This is a critical step. As you learned in the tutorial, if you save all of the fractions to get a high yield, the result is a low purification step. Take just the fractions with the highest activity and you may not achieve a reasonable yield. In a perfect world, you would run an SDS PAGE gel and a western blot each time. BUT this takes lots of time and money. Instead, you have to use your judgement based on experience. A rule of thumb is to save 70% of the peak. If there is extensive tailing (non-gaussian curves) then you may need to adjust.

SAVE 0.3 ml OF YOUR POOLED FRACTION. RECORD THE VOLUME OF YOUR POOLED FRACTION IN YOUR NOTEBOOK. You do not need to save samples from the other fractions.

Wash your test tubes and prepare for the next chromatography.

Step 7 Prepare pooled samples for next chromatography – At this point, you should plan which chromatography to use next. Now is the time for you to determine if your sample needs to be concentrated (ammonium sulfate precipitation or ultrafiltration) or dialyzed to change buffer and/or desalt your sample. Then repeat your chromatography steps as above. Save all of your final pooled sample and store.

STORAGE OF PURIFIED PROTEINS: Now that you have spent so much time and hard work into your protein, the last thing you want to do is forget about storing the protein to maintain activity. Long term storage of proteins (> 24 hours) at extreme pH or pH near the pI or with buffers with imidazole will cause your protein to denature and ... well we will not even think about it...

For short term storage (up to 24 hrs) most proteins can be kept at 4°C. Keep the sample covered! Bacteria and mold will easily contaminate an open protein solution.

For long term storage after purification (>24 hours) it is important to freeze your protein. To stabilize your protein, you should add enough glycerol to make the solution 10% glycerol. You may want to add DTT at a final concentration of 0.1 mM. Alternatively, longer storage can be done by precipitating the protein with ammonium sulfate and storing at 4°C. This is extremely stable. Another long-term storage solution is to freeze-dry or lyophilize the sample and store at 4°C.

LOGISTICS & IMPORTANT INFORMATION:

Updates and ordering materials – Timely information and notices will be found on the webpage and/or the blackboard at the front of the laboratory. It is YOUR responsibility to check both.

Ensure that you write any chemicals or buffers that are running low on the chalkboard. Don't wait until a container is empty.

Sign up – Each group has their one glass column, column adaptor and a plastic column. The pump and fraction collector must be shared with both sections. Therefore, there will be a sign up at each bench for the pump and fraction collector. Ensure that you plan to sign up for an appropriate length of time in advance. If you need to cancel, be professional and make the indication on the sheet. Extra sign up sheets will be found on the web.

As soon as you finish a chromatography run, transfer your test tubes to a test tube rack and return the parts to your table for the next group.

Plan for 1-2 hours to prepare buffers for each chromatography, 2-3 hours to run a column chromatography and 2 hours to analyze each run.

Buffers provided and location – Stock buffers will be located at the table and cabinet at the front of the classroom. Please indicate any needed buffers on the blackboard when the buffer is nearing empty. Stock buffers that will be provided for you will be:

- 1M Tris-Cl pH 8.0 (make pH adjustments of your buffers)
- 1M Potassium Phosphate Buffer pH 7.0. NOT THE SAME AS PBS
- 10 X Phosphate Buffered Saline (PBS)
- 0.5 M EDTA
- PMSF

Where to find chemicals and materials –

- Dry chemicals such as NaCl, ammonium sulfate and Imidazole will be located in the cabinet at the front of the classroom.
- BSA for protein assays – 20cC freezer on the top shelf.
- Bradford Assay Reagent – 1X ready to use Bradford reagent will be in the repipettor in the east hood. 5X concentrated Bradford reagent will be in the refrigerator. Only use this when there is no remaining 1X. Prepare 500 ml at a time.
- Black 96 well plates for fluorescence assays will be on the table at the front of the classroom. Wash and return when finished.

Storage in the cold room – There is a labeled shelf in the cold room for each laboratory section. Ensure that you only use this space for storing your fractions and samples. Do not store your materials in the fridge. There is not enough room.

What to find on the web –

- Protocols/guides for each type of chromatography resin available for you to choose.
- Protocols for performing protein assays and measuring MGH fluorescence.
- Instructions and teg files to help you with the equipment.
- Tegrity files to give you the overview of purification.
- Examples of a properly prepared chromatograph.
- Signup sheets for the pump and FC.
- Sequence of MGH for you to use if you wish to find the molecular weight, structure or calculate the pI.
- Purification Table Preparation Guide

YOUR JOB:

- 1) Read and prepare yourself for the purification.
- 2) Work with your partner to design the purification. Choose which two resins to use. Use the check-sheet to help you along.
- 3) Make buffers and purify your protein.

When you are ready, show your plans to your instructor. Once approved, you will be given 10 ml of lysate from bacteria expressing MGH protein. This will be in a buffer containing 10 mM Tris-Cl pH 7.5, and 0.1 mM PMSF (a protease inhibitor). If you lose the sample in handling there is a very limited amount to replace. This will cost you points in the purification.

Following the purification, you will perform an SDS PAGE and western blot on your samples as well as assay the enzyme activity of your pure protein.

ALL BUFFERS MUST BE LABELED WITH THE FOLLOWING:

- Name of buffer and be specific
- Components of buffer
- Your name and date
- Which lab section Tues or Thurs

DEAE Gradient II Buffer (10 mM Tris-Cl pH 8.5, 0.1 mM EDTA, 300 mM NaCl) Provost 1/Oct/06 Tues Lab Section.

CHROMATOGRAPHIC RESINS AVAILABLE:

- Ion Exchange – DEAE and CM
- Size Exclusion - S-100, S-200, S-300
- His Tag Affinity - Ni-Agarose
- Affinity Dye – Reactive Blue Agarose
- Hydrophobic Interactions – Phenyl-Sepharose
- Hydroxyapatite

REFERENCES

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MDH

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- Musrati et al. (1998) Malate dehydrogenase: distribution, function and properties. *Gen Physiol Biophys* 17(3):193-210.

Protein Purification

- Guide to Protein Purification Ed M. P. Deutscher *Methods in Enzymology* Vol 182 Academic Press (one of my favorites for many years)
- Principles and Techniques of Biochemistry and Molecular Biology 6th Ed. K. Wilson & J. Walker Ed. Cambridge University Press (A great source for theory and advance information on chromatography and most things biochemical)
- Modern Experimental Biochemistry 3rd Ed. R. Boyer Benjamin Cummings (has good introductions plus nice experiments)

LAB NOTEBOOK:

Accurately record the procedures conducted during lab. This will include:

- Observations of the experiment
- Planning and calculations for each chromatography. Include volumes of buffers prepared and key info for each column.
- Data and calculations for quantifying your protein.

Your final report (paper not the lab notebook) should include some of the following points

- The key information on each chromatography used and an intro to chromatography
- Final chromatograph
- Purification table.