

Chem 405 Biochemistry Lab I

Denatured SDS-Polyacrylamide Gel Electrophoresis of MGH

Required Reading: *At the Bench*, Pages 386 - 390

Background

When an electrical field is applied across a solution the movement of the charged particles (proteins) is influenced not only by the charge but also the voltage, distance between electrodes, the size and shape of the molecule, temperature and time. Polyacrylamide gels are polymerized products of acrylamide and bisacrylamide (n,n'-methylene bisacrylamide). When ammonium persulfate ($-O_3S-O-O-SO_3-$) is added to water it breaks down forming unstable $\bullet SO_4^-$ free radicals, which can then initiate the polymerization reaction. TEMED (tetra methyl ethylene diamine) is a tertiary amine that reacts with these radicals to form TEMED free radicals, which in turn react with acrylamide to induce polymerization. The addition of bisacrylamide cross links the long acrylamide molecules creating of an average size. The size of the pore can be regulated by the concentration of acrylamide and bisacrylamide. Relatively small proteins will migrate faster through SDS-PAGE gels than larger proteins. Conversely, lower percent gels are more useful for larger proteins. In this case the smaller proteins will not be retarded by the size of the pores and have a similar mobility. that is they will run very close to each other at the bottom of the gel.

The gels we are running are discontinuous gels. These are gels that contain both a stacking gel and a resolving gel. When glycine from

the upper reservoir enters the low pH of the stacking gel, it will principally be in the neutral form. This prevents glycine from being an effective carrier of electrical current. The Cl^- ions now carry the current and migrate toward the anode. During this step the Cl^- ion concentration becomes lower at the top of the gel and higher at the bottom of the stacking gel. As electrophoresis continues, protein molecules (which are negative charged due to the SDS) will become greatly retarded, allowing the trailing protein molecules to catch up. The stacking gel is very low in percent to ensure there is little separation based on the pore size. This will ensure all of the proteins enter the resolving gel at the same time to get small tight bands.

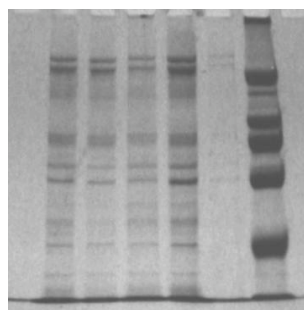


Fig 1: Coomassie Stained SDS PAGE

After running a 10% SDS-PAGE gels, it can be stained with coomassie blue. Coomassie blue is a dye that will bind to the acidic amino acids in proteins. This method of

staining will stain most proteins in the gel with as little as $0.1 \mu g$ of protein in a single band. Washing of the gel with destain will remove the dye from the gel but not the protein leaving behind several nice blue bands that show each protein.

Once the gel is destained, we can measure the relative mobility of the proteins and use molecular weight standards to determine the molecular weight of an unknown protein. The relative mobility (Rf) is calculated by dividing the distance the protein migrates by the distance the tracking dye migrates. See Table I for molecular weights of your standards.

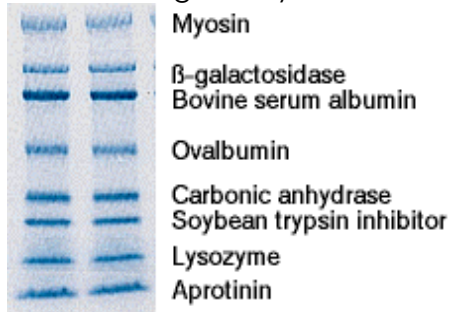


Fig 2 Prestained Molecular Weight Standards. Prestained standards are used in SDS-PAGE and western blotting applications. They provide a quick and easy way to assess blotting efficiency and allow continuous monitoring of protein separations during electrophoresis. Prestained standards can be used as a control for repetitive blotting experiments, or in locating proteins for excision from unstained preparative gels.

The tracking dye (bromophenol blue) is a small colored molecule that runs much faster than proteins and approximates the movements of small ions in the electric field. Therefore it is important not to run the dye off the bottom of the gel. A plot of the relative mobility for each standard protein vs. the log of the molecular weight of the standard proteins will be linear, and the molecular weight of the unknown protein can be determined from its position on the plot. To determine the relative mobility, measure the distance the protein has moved from the top of the resolving gel to the

center mass of the band of protein. Divide this value by the distance the dye traveled. This is the relative distance.

Immunoblotting (western blots) use specific interactions of antibodies to detect a protein of interest. Western blotting can be divided into two steps: transfer of the protein from the gel to the matrix (paper) and detection of the epitope with antibodies. We will be using the wet method to transfer the proteins, in which the gel and the immobilizing paper are sandwiched between buffer wetted filter paper through which a current is applied for 60 min. or overnight.

Safety: Coomassie stain is not harmful but care should be taken as not to stain your clothing. Take it from me, it is very easy to spill a little bit onto yourself. I have a few shirts that are stained blue. The SDS-PAGE Sample Buffer contains β -mercaptoethanol. This is harmful to breath in large quantities. Unless the bottle of BME is spilled there is little to no risk other than the stink. Try not to get any on you cloths. It will make them smell rotten eggs for quite some time. The solution A in the Gel preparation contains unpolymerized acrylamide. Until it is reacted with the APS and TEMED it is a neurotoxin. Do not get any on your hands. If you do get any on you, wash them with soap and water. The TEMED stinks and is a potential teratrogen. Once these chemicals are mixed together, there is no safety concern, however treat them carefully prior to mixing. Electrical currents used are very high so use the equipment as directed.

Table I : Calibrated BioRad PreStained Low Range Molecular Weights of Prestained SDS-PAGE Standards

Phosphorylase B	107,000
BSA	76,000
Ovalbumin	52,000
Carbonic anhydrase	36,800
Soybean trypsin inhibitor	27,000
Lysozyme	19,000

Lab Notebook:

- Will include an outline of today's experiment as well as any changes to the protocol.
- You will enter in the volumes of protein and sample buffers you've mixed together
- Include a table of volumes of proteins loaded and which sample was loaded for each lane
- Show all calculations used to prepare buffers and any dilution.
- Copies of the pictures of the gels with figure legend.
- Graphs of the relative mobility of the protein
- Determination of the molecular weight of your MGH

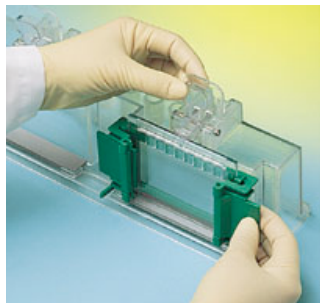
Part I – Running the SDS PAGE Gel

We will be preparing stacking gels for one gel that will be cut vertically down the middle. One half of the gel will be stained for total protein and the other will be transblotted for later immunodetection. The general order of things will be to:

- 1st prepare the stacking gel, the fractions for running the gel.
- Prepare the running buffer and then as the gel is running,
- prepare the transfer buffer and the transfer apparatus.

Gel Preparation: The resolving portion of a **12% SDS-Page gel** have already been poured. You will prepare the stacking gel. See "putting together the Mini-Protean 3" page.

1. **Prepare the stacking gel solution** according to the directions on the accompanying page. The amounts are enough for two minigels. Be certain not to add the APS until you are ready to start the gel. The gel will start to polymerize within 3 or so minutes so be certain everything else is in hand and ready to start.



2. **Remove all water and butanol** that was overlaid on the resolving gel. Turn upside down, and rinse with deionized water. Use a paper towel to wick off the remaining water
3. **Add APS to the solution** and immediately pour the stacking gel into the gel. Fill to near the top but leave a small of space to place the comb into the gel. Then insert the comb into the set stacking gel.

4. When the gel is polymerized, **the comb may be removed** gently, and the gel sandwich can be loaded into the electrophoresis apparatus.
5. **Prepare 500 ml of 1 X running buffer** from the 10 X running buffer solution. Record your calculations in your laboratory book.

Loading and running the samples: Load between 5 and 20 μg of protein per lane. Too much protein will distort the bands, too little protein load will be difficult to detect by comassie staining.

Samples to prepare for running a gel.

Lane 1: SDS PAGE Low Range Molecular Weight Standards (load 5 μl)

Lane 2: Lysate (load 10 μl)

Lane 3: Chromatography pool 1 (load 30 μl)

Lane 4: Chromatography pool 2 (load 30 μl)

Lane 5: Blank – load 20 μl sample buffer only

Lanes 6-10 repeat lanes 1 – 4 from above. Use 10 μl of the standards for lane 6.

1. Add the following mixtures in a microfuge tube
 - For the bacterial lysate, mix 30 μl of the sample , 20 μl of of water and 20 μl of 5x sample buffer.
 - For the fraction samples, add 80 μl of each fraction and 20 μl of 5 X sample buffer.
2. Boil the samples in a heat block at 90 degrees C for 5 min.
3. Put the gel apparatus together and fill the inner reservoir to the top and pour the remaining buffer in the bottom
4. Prestained Molecular Weight Standards
 - For the coomassie protein stained gel, load 5 μl of molecular weight standards into lane 1. For the rest of the lanes load 30 μl of sample. Don't forget to record the lanes and fractions in your lab book.
 - For the gel that you will western blot, add 10 μl of prestained weight standards.
5. Apply the power source to your gel box and turn on to 200 V until the dye is just at the bottom. **DO NOT STOP THE GEL TOO EARLY.** The results will be difficult to obtain because the proteins will be best resolved when the gel has run all of the way.
6. Using the glass plate, carefully cut the gel in half (Vertically). It helps if the glass plate is wet. Lanes 1-4 will be stained in the next step, lanes 6-10 will be transferred in Part II in the westernblot.
7. Place one gel in the coomassie stain and rock for 30 min. or overnight. Then rinse with water once and then destain and fix the proteins in the gel with about 1/2 inch of destain solution. We will leave it in destain until next week. LABEL your sample.

Part II - Western Blotting

Step 1. Protein Transfer.

- **Prepare 300 ml transfer buffer.** 30 ml of methanol plus 10X transferbuffer. QS to 300 ml.
- **Prepare the transfer membrane and filter paper.**
 - If using immobilon (PVDF) transfer paper, pre-wet the paper for 30 seconds in methanol, rinse with distilled water and place in a shallow dish with transfer buffer. If you are using nitrocellulose, rinse briefly in transfer buffer.

If you are using nitrocellulose just rinse the paper in TTBS for 1 min.

- **Prepare the polyacrlamide gel for transfer.**

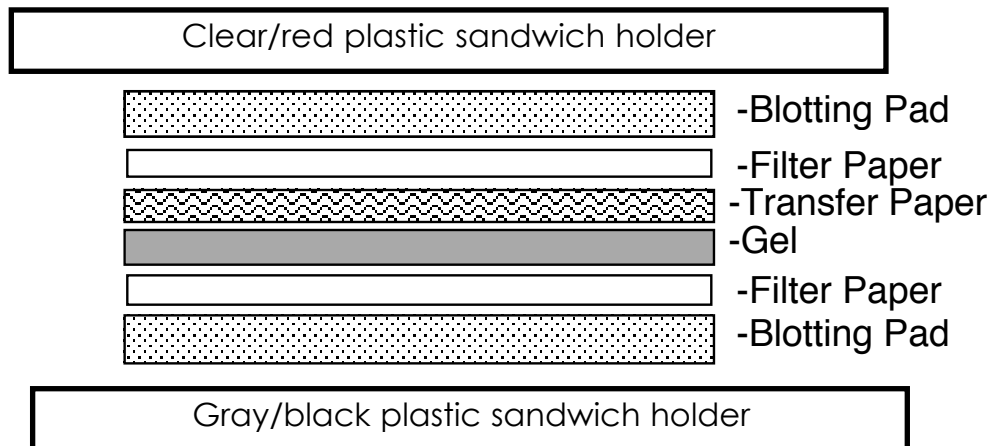
Soak the gel intransfer buffer for ten minutes to remove salts that may result in poor transfers.

- **Prepare the blotting pads.**

Soak the pads in transfer buffer until they are saturated. Remove air bubbles by squeezing the pads while immersed in the buffer. Any remaining bubbles will block the transfer of the proteins.

- **Assemble Blot Apparatus**

Place 1 blotting pad on the black part of the sandwich holder (**build on black**). Place the filter paper on the gel. Bubbles can be displaced with your fingers. On top of the blotting pad, place one sheet of filter paper followed by the transfer paper, gel and another filter paper.



Transfer Protein

Close the sandwich holder and place assembly into the gel box. Be certain to align the black with black and clear side with the red side of the transfer apparatus. Fill the inner portion with transfer buffer, place the stir bar into the box and add the ice cooler container. Transfer the protein at 100 V (constant) for 1 hours. *Rule of thumb* = smaller proteins < 50 kDa will be transferred after 0.8 hours larger proteins > 100 kDa will take 1.5 hours). Alternatively the blot can be transferred overnight at 30V in the cold room.

Step 2 Blotting and Detection

Wash - Rinse the blot for 5 min in TBS to reduce spotting in the developed blot

Blocking -Block for one hour to 30 min with 10 ml of 5% blocking buffer (TTBS and 5% dry milk [0.5 g dry milk per 10 ml TTBS]) on a rocking platform. This is a good stopping point. Seal it and leave the blot overnight in the cold room.

A Good example a figure with both chromatograph and the Gel results. You will not combine the data as these authors did, but you can get a good idea on how the gel and chromatograph should be displayed and what a proper figure legend should look like.

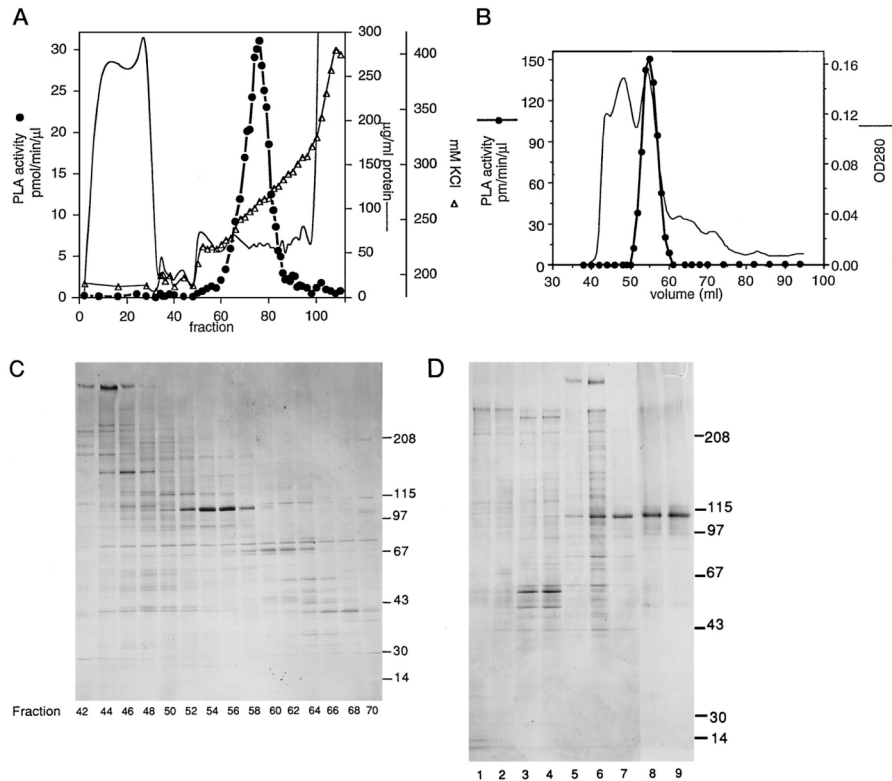


Figure 1: Chromatographic elution profiles and SDS-PAGE evaluation of PA-PLA purification. Elution profiles from Mono Q (A) and Superdex 200 (B) chromatography are shown. Elution volumes for markers on Superdex 200 (molecular mass in kDa in parentheses) were as follows: blue dextran 2000 (2,000,000), 43 ml; thyroglobulin (669), 47 ml; ferritin (440), 55 ml; catalase (232), 65 ml; aldolase (158), 67 ml; phosphorylase B (97), 68 ml; bovine serum albumin (67), 75 ml; ovalbumin (43), 83 ml; chymotrypsinogen (25), 91 ml. C, silver-stained gradient gel SDS-PAGE of the Superdex 200 fractions (2 μ l each). D, silver-stained gradient gel SDS-PAGE of the pools from each of the stages of purification. In lanes 1-7, 1 μ g of protein was loaded. In lanes 8 and 9, 50 ng of protein was loaded. Lanes 8 and 9 were stained separately in order to bring out minor bands. Lane 1, ammonium sulfate precipitate; lane 2, PEG precipitate; lane 3, SP pool; lane 4, concentrated SP pool; lane 5, Mono Q pool; lane 6, concentrated Mono Q pool; lane 7, Superdex 200 pool; lane 8, phenyl-Superose pool; lane 9, phenyl-CL-4B pool.

SDS-PAGE Gel Instructions

The following recipes are more than enough for two 1.5 mm Gels
The buffers/solutions do not need to be degassed if using a minigel

	% Resolving Gel					
	6%	8%	10%	12%	14%	16%
Soln A (ml)	2.25	3.00	3.75	4.50	5.25	6.00
Soln. B (ml)	3.75	3.75	3.75	3.75	3.75	3.75
H ₂ O (ml)	9.00	8.25	7.50	6.75	6.00	5.25
TEMED (μ l)	10	5	5	5	5	5
10% APS (μ l)	75*	75*	75*	75*	75*	75*

* Add the APS last. Once added it will start to polymerize

4% Stacking Gel		10X Electrophoresis Buffer	1X Conc.
Solution A (ml)	0.60	30.0 g Tris Base	-- 25 mM
Solution C (ml)	1.50	144.0 g Glycine	--192 mM
H ₂ O (ml)	3.96	10.0 g SDS	--0.1%
10% APS (μ l)	40.0	QS to 1 liter, pH should be 8.3	
TEMED (μ l)	10.0		

Working Solutions:

Solution A: 40% (w/v) acrylamide, 1.09 % (x/v) bis-acrylamide

194.8 g acrylamide
5.2 g bis-acrylamide
QS to 500 ml w/ H₂O

5X Sample buffer, 10 ml

0.6 ml 1 M Tris-HCl (pH 6.8)
5 ml 50% glycerol
2 ml 10% SDS
0.5 ml β Mercaptoethanol
1 ml 1% Bromophenol blue
0.9 ml H₂O

Solution B: 4X Separating gel Buffer

125.0 ml 2.0 M Tris-Cl (pH 8.8) -- 1.5 mM
10.0 ml 10% SDS -- 0.4%
52.5 ml H₂O

Solution C: 4X Stacking Buffer

125.0 ml 1.0 M Tris-Cl (pH 6.8) -- 0.5 mM
10.0 ml 10% SDS -- 0.4%
115.0 ml H₂O

10% APS

0.5 g Ammonium persulfate
5.0 ml H₂O (stable frozen)

Gel well capacity:

At 1.0 mm gel thickness 10 wells = 32 μ l and 15 well = 18 μ l

At 1.5 mm gel thickness 10 wells = 48 μ l and 15 well = 27 μ l

Coomasie Gel Stain, 1 liter

1.0 g Coomasie Blue R-120
450 ml methanol
450 ml H₂O
100 ml Glacial Acetic Acid

Coomasie Gel Destain, 1 liter

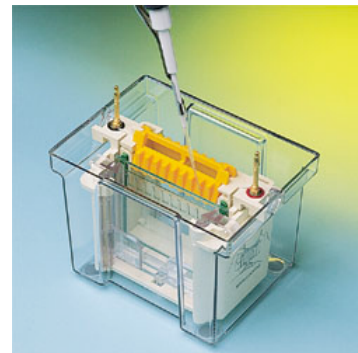
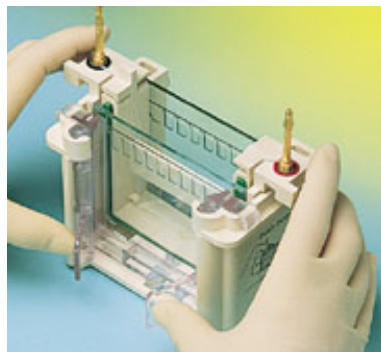
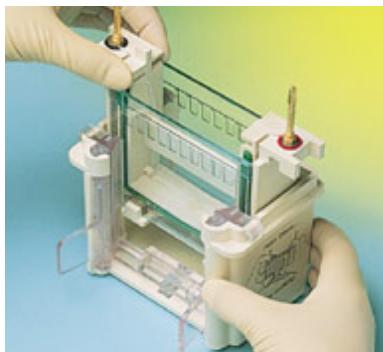
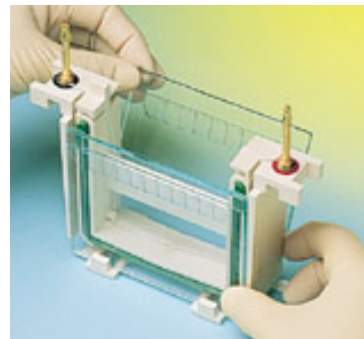
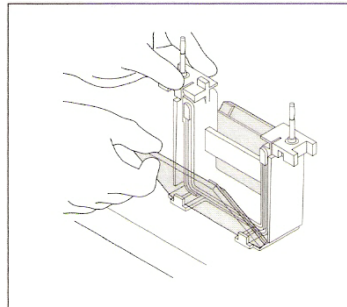
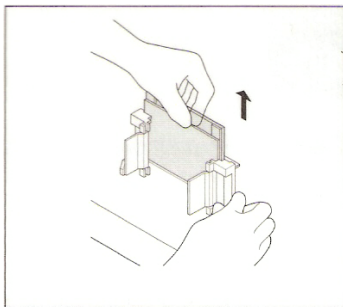
100 ml Methanol
100 ml Glacial Acetic Acid
800 ml H₂O

Putting together the Mini-Protean 3:

Pour the stacking gel. Once you've removed and rinsed the resolving gel, prepared stacking gel and added APS and TEMED, carefully pipet the solution into the glass sandwich until just below the top of the glass plate. Then add the comb. Wait until the gel polymerizes. Tilt the apparatus to see if the gel has set.

Prepare the assembly.

1. When the gel is done setting, remove the gel cassette sandwich from the casting frame.
2. Place the gel cassette sandwich into the electrode assembly with the short plate facing inward.
3. Slide gel cassette sandwiches and electrode assembly into the clamping frame.
4. Press down the electrode assembly while closing the two cam levers of the clamping frame.
5. Lower the inner chamber into the mini tank. Use the spacer guide to load your samples.



Molecular Weight Estimations and SDS PAGE

Often questions are posed regarding apparent discrepancies between protein size as determined by gels vs. other methods, such as sequence analysis. Two factors explain most of the observed variation.

The first factor is the amount of SDS bound to the protein. SDS is employed to disrupt secondary structure and give all proteins a constant charge/ mass ratio, which is assumed to be 1.2g SDS/g protein. However, as stated in a review by Hjelmeland and Chrambach¹; 'this assumption fails more frequently than is generally known.' The most common deviation from this assumption is probably a lower than normal amount of bound SDS. All else being equal, mobility would decrease, since the protein would have less of a negative charge.

A second source of error in molecular weight estimates, is that protein mobility in the gel is more a function of molecular size (which is a function of both weight and length) than of molecular mass. It's generally assumed that SDS proteins all exist in a random coil form, so the relationship between length and mass should be constant. Even assuming constant charge, if a protein has unreduced disulfide bonds or areas of incompletely disrupted secondary structure, it cannot unfold to full length and, it would tend to run faster than expected in a typical SDS gel.

These deviations from the ideal can combine in every conceivable way, making it difficult to predict a net effect on migration rate. Nevertheless, the effects can be large. Unreduced BSA will run with an apparent size of about 55kDa instead of 67kDa. Furthermore, in smaller proteins a non-ideal region will have a larger proportional effect than the same region in a large protein. For example, polypeptides of around 2kDa can give estimates which are off by a factor of 2 or more from actual size.

This is not to say that SDS derived molecular weights are invalid, just that they have limitations. Most proteins will give estimates within a few percent of their actual weight by comparing them to appropriate calibration markers. And possible deviations from 'true' molecular weight do not affect the utility of SDS gels in identification, because even 'unusual' proteins, if prepared in the same way each time should run reproducibly on a given gel type.

Finally, care should also be taken in inferring precise size based on published weights for calibration markers. Even common proteins may have several slightly different size estimates reported in the literature, depending upon the methods of molecular weight measurement. For a recent example see reference 2.

References:

1. Hjelmeland, L.M. and Chrambach, A., *Electrophoresis* 1981, 2, 1-11
2. Sallantin, M., Huet, J., Demartean, and Pernollet, J., *Electrophoresis* 1990, 11, 34-36