Profiling and analysis of Plant Proteins using SDS-PAGE

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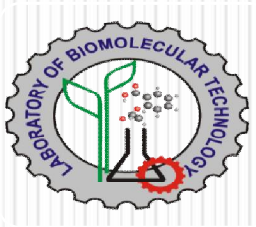

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Key Topics

- Introduction
- SDS-PAGE related equipment and accessories
- Chemicals and buffers for SDS-PAGE
- Steps of SDS-PAGE
- Procedure
- Observation
- Tips

Introduction

- The separation of macromolecules in an electric field is called electrophoresis.
- For protein separation, discontinuous buffer systems use. In this system a gel separated into two sections (a large-pore stacking gel on top of and a small-pore resolving gel) and different buffers in the gels and electrode solutions (Wheeler et al. 2004).
- In gel electrophoresis, protein do not all enter the gel matrix at the same time. Samples are loaded into wells, and the proteins that are closer to the gel enter the gel first.
- The discontinuous gel system was developed by Ornstein and Davis (Ornstein 1964, Davis 1964) for the separation of serum protein conformation, subunit interactions, and biological activity.
- In discontinuous system, protein first migrate quickly through the large-pore stacking gel and then are slowed as they enter the small-pore resolving gel. As they slow down, they stack on top of one another to from a tight band, which improves resolution.

- Due to discontinuous systems use ions in the electrophoresis buffer that sandwich the protein as they migrate through the gel, and this tightens the protein bands even more.
- Discontinuous buffer systems provide higher resolution than continuous systems, and varying the buffers used in the sample, gel, and electrode chambers creates a variety of discontinuous buffer systems that can be used for a variety of applications.
- To overcome the limitations of native PAGE systems, Laemmli (1970) incorporated the detergent SDS into a discontinuous denaturing buffer system.
- A polyacrylamide gel with acrylamide content above a critical density restrains large molecules from migrating as fast as smaller molecules.
- Because the charge to mass ratio is nearly the same among SDS denatured polypeptides, the final separation of proteins is dependent almost entirely on the differences in molecular weight (MW) of polypeptides.

Equipment and Accessories

- **Equipment**
- Weighing machine
- pH Meter
- Different range Pipettes and their autoclaved tips
- Dry bath incubator
- Vertical electrophoresis Unit
- Power supply
- Comb

- **Accessories**
- Spatula and Butter Paper
- Pre-chilled pestle and mortar & Spatula
- Tissue roll or Clean cotton cloth
- Different size Measuring slender
- Different size Beaker and Flask with cap
- Dropper
- Dark and Transparent Reagent Bottles
- MCTs or PCR tubes

List of Chemicals and buffers

Nitrogen gas	Acrylamide	Bromophenol blue
Tris base	Bisacrylamide	Coomassie Brilliant Blue R-250
NaCl	NaOH	Methanol
Sucrose	HCl	Glacial Acetic Acid
EDTA	Sodium dodecyl sulfate (SDS)	Sodium carbonate
Beta-mercaptoethanol	Ammonium per sulfate (APS)	Formaldehyde
PMSF (Phenyl methane sulfonyl fluoride)	TEMED (N,N,N',N'-Tetramethyl ethylene diamine)	Autoclave double distill water
Cysteine	Glycine	$AgNO_3$
Tween-20	Glycerol	Ethanol

Step-I:Protein extraction buffer (pH =7.0-9.0)

Ingredients	Final Concentration	stock solution (A)		Required Volume from stock solution for 100mL extraction buffer (B)	Required weight (gm) for 100mL extraction buffer (C)
TrisCl pH 8.0	200 mM	1M	12.114 gm in 100ml	20 ml	2.423
NaCl	100 mM	7M	4.09 gm in 10 ml	1.43 ml	0.5844
Sucrose	400 mM	-	-	13.5 gm	13.5
EDTA	10 mM	0.5M	14.71 gm in 100 ml	2 ml	0.00294
Beta-mercaptoethanol	14 mM	-	-	70 µl	70 µl
PMSF	1 mM	100 mM	1.74 gm in 100 ml	100 µl	0.0174
Cysteine	5 mM	-	-	0.087 gm	0.087
Tween-20	0.05%	-	-	50 µl	50 µl
Autoclaved ddH_2O		-	-	Make up to 100 ml	Make up to 100 ml

Storage condition of protein extraction buffer solutions

Solution	Reagent bottle	Autoclaved	Temperature
TrisCl pH 8.0	Transparent	Autoclave	4ºC
NaCl	Transparent	Autoclave	4ºC
Sucrose	Transparent	Autoclave	4ºC
EDTA	Transparent	Autoclave	4ºC
Beta- mercaptoethanol	Dark	-	4ºC
PMSF (Fresh prepared)	MCT	Dissolve in autoclaved water (DAW)	4ºC
Cysteine	Transparent	-	4ºC
Tween-20	Dark	-	RT
ddH_2O	Transparent	Autoclave	RT

Preparation of protein extraction buffer

- Mix Trsi-Cl, NaCl, Sucrose and EDTA and make up 100 ml with ddw
- Check pH and Autoclave
- After autoclave, cool then add cysteine and tween-20 in solution
- Vortex then store at 4ºC
- Beta-mercaptoethanol and PMSF, add in to the extraction buffer just before grinding of the tissue
- Vortex and Spin

- Proteins are highly heterogeneous, complex bio macromolecules consisting of one or more long chains of amino acids.
- Proteins are susceptible to degradation, denaturation and precipitation when taken out of their native environment.
- During protein extraction, proteins are exposed to different pH, ionic strengths, temperature and proteases in solution.

Tris Cl

- Buffer can cope up with sudden changes in pH. If the protein extraction lysis solution is not well buffered then proteins can easily and irreversibly denature.
- Most buffers show a pH-dependence with temperature.
- Tris as a buffering agent in the lysis solution (proteases are often least active at basic pH).
- Tris buffers, for example, have a pH that can be strongly affected by temperature and concentration.
- The pKa of Tris buffers changes from 8.06 at 25°C to 8.85 at 0°C.
- Tris possesses a potentially reactive amine and participates in various enzymatic reactions.
- The pH of a Tris buffer is affected by the concentration. The pH decreases 0.1 unit upon a tenfold dilution.
- Buffers are usually used above 20 mM concentration to ensure sufficient buffering capacity.

NaCl

- Maintain ionic strength of medium.
- NaCl to help keep proteins soluble and to mimic physiological conditions.
- Too high or too low concentration of salts can precipitate or denature the protein.

Sucrose

- Osmolytes are also known to aid protein folding.
- They are used as additives to enhance solubility and stability of the proteins in solutions.
- Sucrose is function like the bearer.
- it increase the viscosity of solution and so when centrifuge it will maintain the protein in the supernatant when discarding the cell debris.
- The other function is retention of isotonic osmolarity.

Proteases and peptidases

- Proteases and peptidases are enzymes that cleave the proteins at specific site and degrade them.
- There are four known classes of proteases including serine proteases, cysteine proteases, aspartic proteases and metallo proteases.

EDTA

- Reduce oxidation damage, chelate metal ions
- Work as protease inhibitor, inhibit of metallo proteases

PMSF and Cysteine

- Work as protease inhibitor, inhibit of serine proteases and cysteine proteases
- PMSF (phenylmethylsulfonyl fluoride) has a short half-life time in aqueous solutions.
- A stock solution of 100 mM in isopropanol should be made and diluted into buffer immediately before use.
- Pefablock is a less toxic and more soluble and stable alternative for PMSF.

Beta marcaptoethanol

- ßME is added to prevent oxidation of cysteines and to break up disulfide bonds.
- It is thiol reducing agent, disrupt intramolecular and intermolecular disulfide bonds and are used to achieve complete protein unfolding and to maintain proteins in their fully reduced states.
- It is volatile, evaporates from solution, and reduces protein disulfide bonds by disulfide exchange.
- There is an equilibrium between free thiols and disulfides, so ßME is used in large excess in sample buffers to drive the equilibrium reaction toward completion.
- If the concentration of ßME drops and proteins reoxidize, fuzzy or spurious artifactual bands may result.

Tween-20

- Detergents disrupt hydrophobic interactions between and within proteins.
- Some proteins, especially membrane proteins, require detergents for solubilization durin isolation and to maintain solubility.
- Zwitterionic detergents provide higher solubilization efficiency, especially for integral membrane proteins.

Extraction of protein from plant tissue

- Weigh 150 mg of sample tissue
- Sample tissue freeze in liquid nitrogen
- Grind plant tissue in pre-chilled pestle and mortar to homogenous powder
- Add homogenized powder to buffer containing MCT with the pre-chilled spatula
- Vortex to mix it
- 
  Centrifuge the MCT at 10000 rpm for 30 minutes at 4ºC
- Collect the supernatant in a fresh MCT and discard the pellet
- Centrifuge the supernatant again at 12000 rpm for 15 minutes at 4ºC
- Collect the supernatant in a fresh MCT and discard the pellet

Cell Disruption

Different biological materials require different cell disruption strategies. Use chemical inhibitors and controlled temperature to minimize the activity of proteases and other enzymes that may modify the protein composition of the sample.

Protein Solubilization

For successful PAGE, proteins must be well solubilized. Use solubilization solutions that contain chaotropic agents, detergents, reducing agents, buffers, and salts as needed and that are compatible with the electrophoretic technique used.

Contaminant Removal, Desalting, Concentration (as needed)

Remove interfering substances that can negatively impact SDS-PAGE (salts, detergents, denaturants, or organic solvents). Use either buffer exchange (desalting) or protein precipitation (which can also help concentrate the sample if needed).

Quantitation

Determine the concentration of protein in a sample by protein assay. Adjust the concentration as necessary for analysis by PAGE.

Preparation for PAGE

Dilute the sample in the appropriate sample buffer to a final sample buffer concentration of 1x.

Step-II: Component of SDS-PAGE Gel

Ingredients	Final Concentration		Stock solution		12% Separating gel / resolving Gel (10 ml)	5% Stacking (5 ml)
Autoclave ddH_2O	-		-	-	3.3	3.4
Acrylamide and Bisacrylamide (29:1) (30% acrylamide solution)	12 %	5%	-	29 gm Acrylamide and 1 gm bis-acrylamide in 100 ml	4.0	0.850
TrisCl pH 8.8	375 mM		1.5M	18.171 gm in 100ml	2.5	-
TrisCl pH 6.8	125 mM		1M	66.67 ml 1.5M	-	0.625
SDS	0.1 %		10%	100 mg in 1 ml	0.100	0.050
APS	0.1 %		10%	100 mg in 1 ml	0.100	0.050
TEMED	0.005%		0.10%	-	0.004	0.005

Storage condition of SDS-PAGE solutions

Solution	Reagent bottle	Autoclaved	Temperature
DDW	Transparent	Autoclave	RT
Acrylamide and Bisacrylamide (29:1) (30% acrylamide solution)	Dark	Dissolve in autoclaved water (DAW)	4ºC
TrisCl pH 8.8	Transparent	Autoclave	4ºC
TrisCl pH 6.8	Transparent	Autoclave	4ºC
10% SDS	MCT	DAW	4ºC

Role of different Chemicals in SDS-PAGE Gel formation

- **Staking gel** – Tris –HCl staking gels are prepared at pH 6.6-6.8.
- This discontinuous buffer system relies on the stacking effect of a moving boundary formed between the leading ion (Chloride) and the trailing ion (glycinate).
- In SDS-PAGE, the stacking gel usually contains **chloride ions**, which migrate faster than proteins, while the buffer usually contains glycine, which migrates more slowly. This causes the protein to be sandwiched in one band between the chloride and the glycine.
- The stacking gel also has a larger pore size than the resolving gel, so large and small proteins can go through it at a similar rate.
- Electrophoresis is run at low voltage during this period as well, so that everything migrates toward the resolving gel slowly and does not get separated.

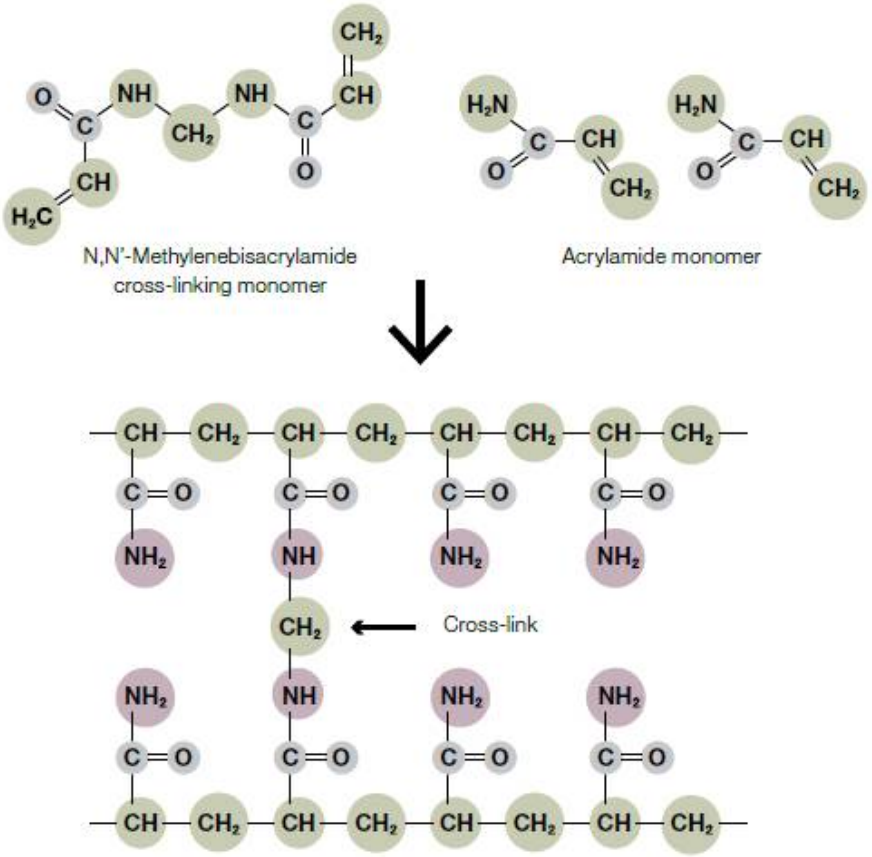
- The stacking gel has a low pH relative to that of the resolving gel, so that the glycine becomes deprotonated as it enters the resolving gel, and the "sandwich" is broken up.
- Then when the single band of protein hits the resolving gel, voltage can be increased, and proteins will be separated by size as they migrate through the smaller pores.

Running gel

- Tris-HCl resolving gels are preparaed at pH 8.6-8.8.
- At this basic pH, polyacrylamide slowly hydrolyzes to polyacrylic acid, which can compromise separation.
- For this reason, Tris-HCl gels have a relatively short shelf life.
- In addition, the gel pH can rise to pH 9.5 during a run, causing proteins to undergo deamination and alkylation.
- This may diminish resolution and complicate postelectrophoresis analysis.

Polymerization

- Acrylamide and Bisacrylamide- They are use for polyacrylamide gel preparation. Acrylamide works as free radical polymerization and bis acrylamide works such as a comonomer cross-linker.
- APS -APS function is to initiate the polymerization.
- TEMED - TEMED function is to accelerate the reaction means acting as a catalyst. It is a free radical stabilizer. Free radicals promote acryl amide polymerization.
- Polymerization speed depends on various factors like monomer and catalyst concentration, temperature, and purity of reagents and must be carefully controlled because it generates heat and may lead to nonuniform pore structures if it is too rapid.

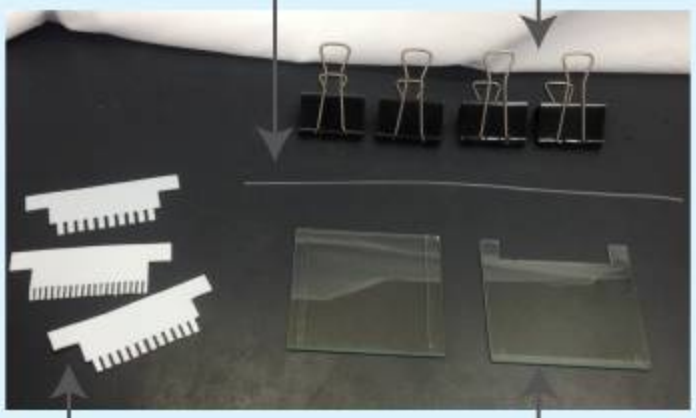


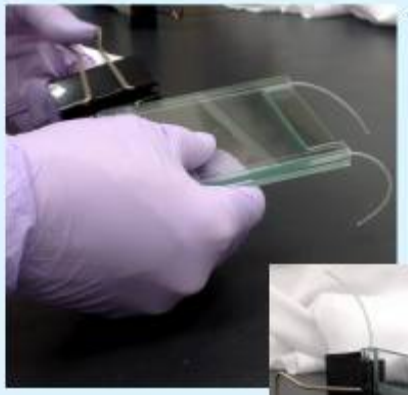
SDS

- SDS is an anionic detergent, meaning its molecules have a net negative charge.
- It binds to most soluble protein molecules in aqueous solutions over a wide pH range.
- Polypeptide chains bind amount of SDS that are proportional to the size of molecules.
- The negative charges on SDS destroy (denature) most of the complex (secondary and tertiary structures) of proteins and are strongly attracted towards an anode (positive charged electrode) in an electric field.
- Presence of SDS, Protein become fully denatured and dissociate from each other.
- Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is used to separate proteins with relative molecular mass no smaller than 10 KD.
- Very small proteins (<10 KD) are difficult to resolve due to low ability of binding to SDS, which can be solved by gradient gels or using different electrophoresis conditions, like Tricine-SDS-page.

Preparation of SDS polyacrylamide gel

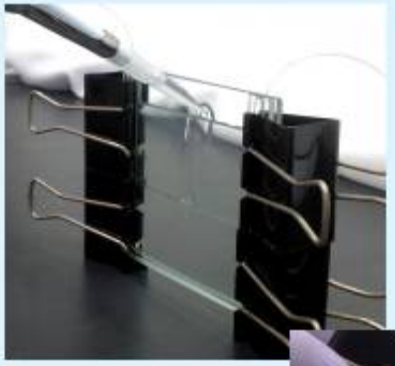
- Thoroughly clean and dry the glass plates and spacers, then assemble them properly
- Hold the assembly together with binder clips. Clamp in an upright position.
- Prepare a sufficient volume of running gel mixture (12% running gel) as describe above.
- Quickly pour the gel solution in the chamber between the glass plates approx length of 3/4.
- Layer of chilled isopropanol on top of the gel and leave to set for 30-60 minutes.
- After 60 minutes, Remove isopropanol layer and prepare stacking gel (5%) as described above
- Quickly pour the stacking gel mixture between remaining place of the chamber of the glass plates
- Quick after that, slowly place ¾ part of the comb in the stacking gel and allow the gel to set (30-60 minutes)
- After the stacking gel has polymerized, remove the comb without distorting the shape well.



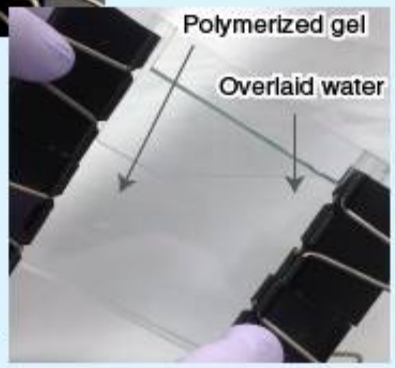


◀ Assemble the gel casting mold, taking care not to leave any space.

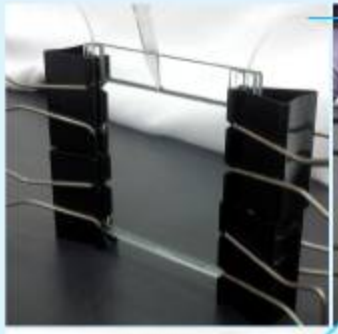
Assembled gel casting mold ▶

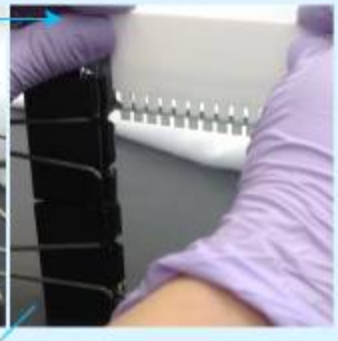


◀ Pouring acrylamide solution for a separating gel



Polymerized gel ▶





Pour acrylamide solution for a stacking gel, and gently insert a comb parallel to a separating gel. Allow acrylamide to polymerize.

Tris glycine SDS buffer/ Electrode buffer/ Running buffer

Tris buffer (25mM)	3.025 gm
Glycine (192mM)	14.4 gm
SDS (0.1%)	1.0 gm
Autoclave double distil water	Make up to 1000 ml
Total	1000 ml

Sample treatment buffer/Loading buffer (6x)

Ingredients	Final Concentration	Stock solution (A)		Required Volume from stock solution for 100mL extraction buffer (B)
Tris-Cl, pH 6.8	0.35 M	0.5M		7.0 ml
SDS	0.35 M	-	-	1.0 gm
Glycerol	30 %	-	-	3.0 ml
Bromophenol blue	0.715 M	-	-	0.2 mg
Autoclave ddH_2O	-	-	-	Make up to 10 ml

Preparation of loading Sample

Mixing protein extract with sample treatment buffer (5:1) in PCR tube or MCTs

Heat tem to 100 ºC (in a boiling water bath) for 5 minutes to denature the proteins.

Cool the sample then use for run.

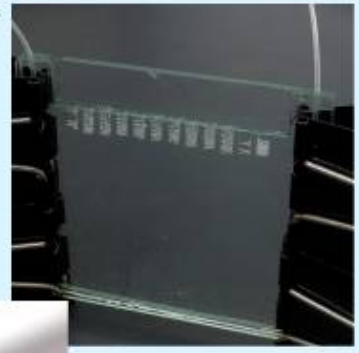
Loading dye or buffer

- To prevent protein sample diffusing in the electrode buffer, adding an equal volume of 40% sucrose or 50% glycerol to increase the density would be a good choice.
- Glycerol is much more dense than water and is added to make the sample fall to the bottom of the sample well rather than just flow out and mix with all the buffer in the upper reservoir.
- To observe the mobility of protein samples, it's better to add bromophenol blue dye or some other tracer dyes into the sample.
- These colored substances can migrate faster than any macromolecules. As long as the dye dose not move out of the gel, there would be no danger for the sample.

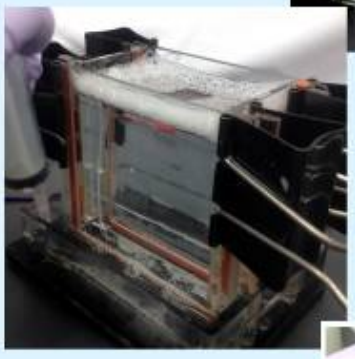
Vertical Gel electrophoresis set up

- Carefully place the gel after removing the clips in the electrophoresis apparatus
- Fill both reservoir (upper and lower) with electrode buffer.
- Prepare samples load upto 18-22 µl in a pre-determined order into the bottom of the wells
- Attach the electrophoresis apparatus to an electric power supply.
- Apply a voltage of 80V to the gel and run the gel untill the bromophenol blue reaches the bottom of the resolving gel. Then turn-off the power suppy
- Remove the glass plates from the electrophoresis apparatus and place them in gel staining tray.
- Use an extra gel spacer to carefully pry the plates aparts.
- Important step- Mark the orientation of the gel by cutting a corner of the gel that is closest to the left most well

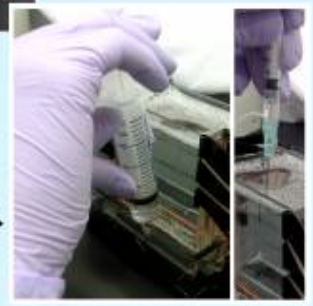
The comb is removed from the gel assembly.



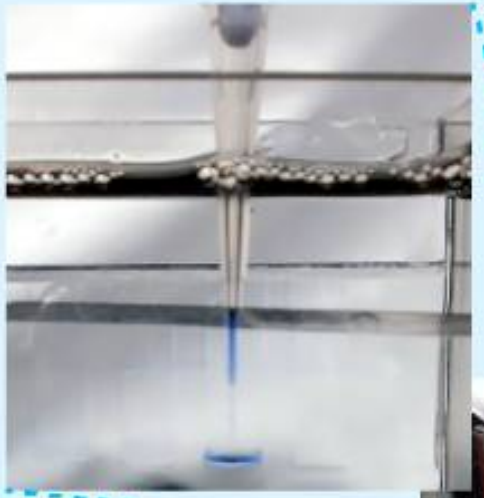
Remove the spacer and binder clips.



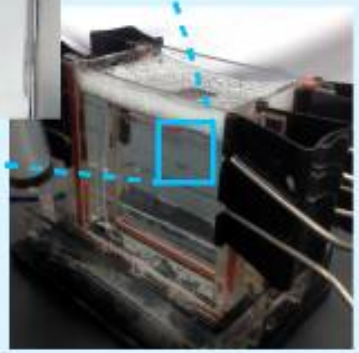
The gel is mounted on the electrophoresis apparatus, and running buffer is poured.

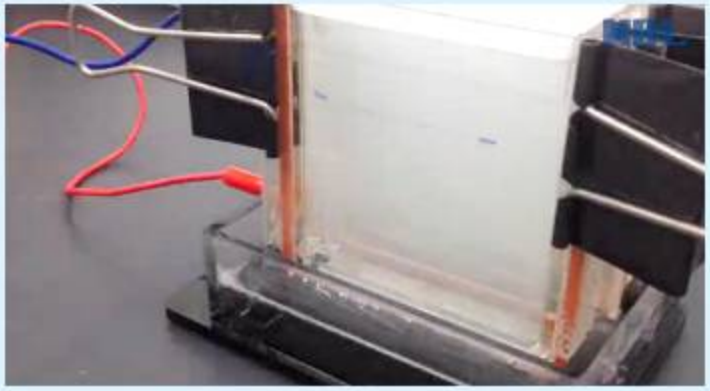


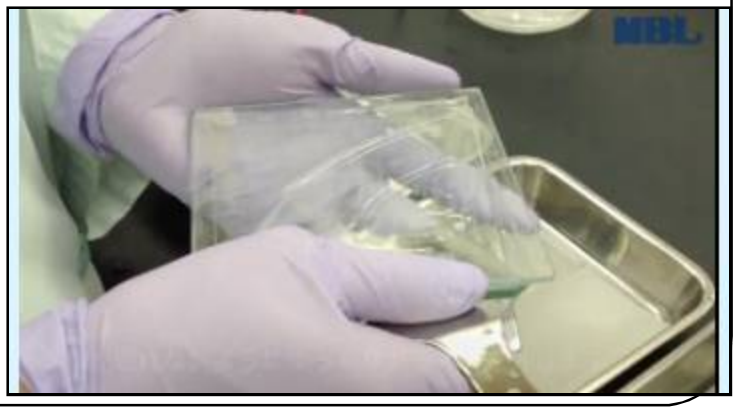
Remove air bubbles and small pieces of gel using a syringe.



Loading a sample in a well.







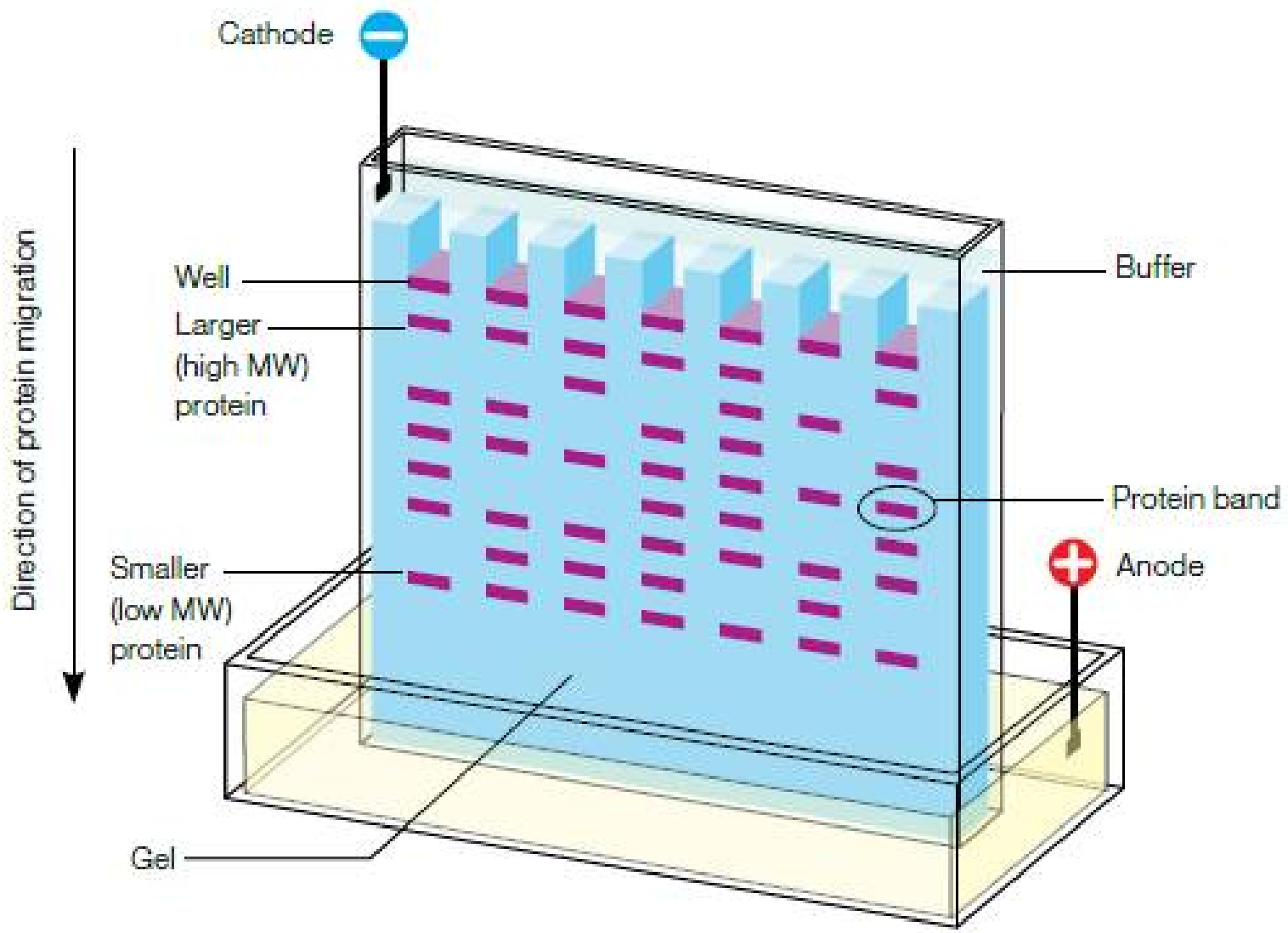


Fig. 2.1. Schematic of electrophoretic protein separation in a polyacrylamide gel. MW, molecular weight.

Step III- Staining Solution

(a-i) Coomassie staining solution

Coomassie Brilliant Blue R-250	625 mg
Methanol	125 ml
Glacial Acetic Acid	25 ml
Autoclaved double distill water	100 ml
Total	250 ml

Staining SDS-PAGE Gel

Immerse the gel in at least five times of gel volumes of staining solutions

⇩

Place on a slowly rotating/moving/rocking platform for a minimum of 30 minutes at room temperature.

⇩

Remove the stain and save it for future use

(a-ii) Destaining solution

Methanol	250 ml
Glacial Acetic Acid	50 ml
Autoclaved double distill water	200 ml
Total	500 ml

Destaining SDS-PAGE Gel

Immerse the stain gel in destaining solutions

⇩

Place on a slowly rotating/moving/rocking platform

⇩

Change the distaining solution 5-6 times till stain become light (If require, left over night at RT

⇩

Take photograph of stained gel or dry the gel

(1% Acetic acid used for reform dry gel)

(b) Silver staining solutions

Fixing solution		**Storage Condition**
Ethanol	30 ml	
Glactial acetic acid	10 ml	
ADW/ Deionized H_2O	60 ml	
Total	**100 ml**	**4°C**
30% Ethanol		
Ethanol	30 ml	
ADW/Deionized H_2O	70 ml	
Total	**100 ml**	**RT**
Fresh 0.1 % Sliver Nitrate solution		
Sliver Nitrate	50 mg	
ADW/ Deionized H_2O	50 ml	
Total	**50 ml**	
Fresh Developing solution		
Sodium carbonate	2.5 gm	
Formaldehyde	20 µl	
ADW/ Deionized H_2O	100 ml	
Total	**100 ml**	**4°C**

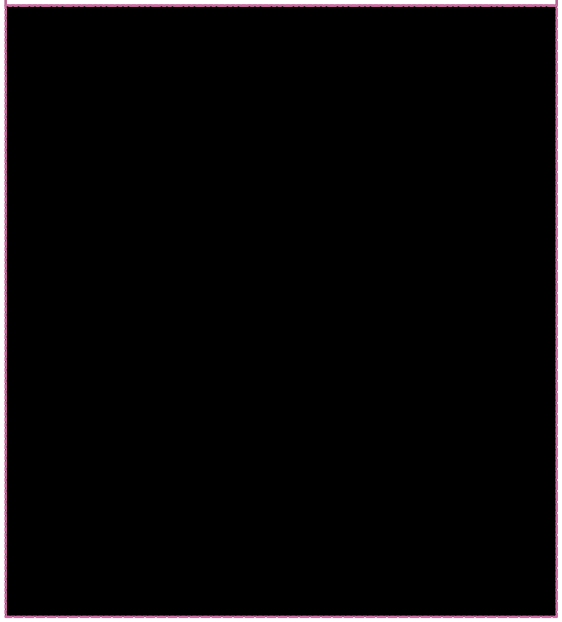
(b) Sliver staining

1. Fix the Protein on Gel

2. Ethanol treatment

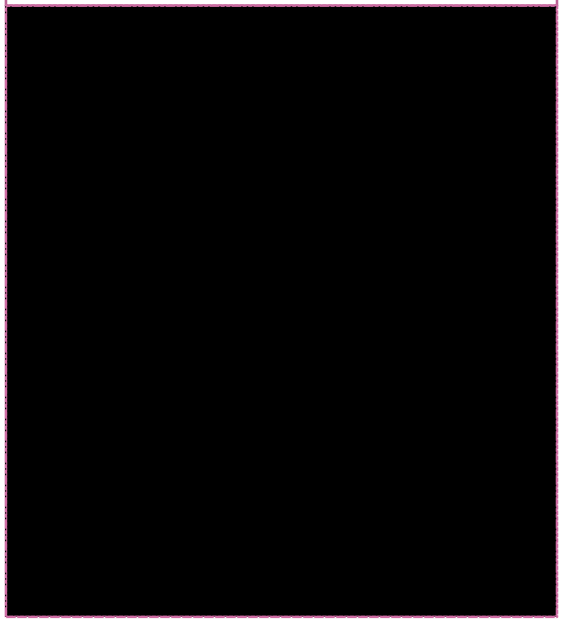
3. Washing

4. Sliver nitrate treatment
(Wearing gloves)



5. Washing

6. Staining of protein bands



Observations

- Observe the gel to see the protein of given plant sample. Count the number of bands observed and calculate R_f value foe each band.
- $R_f = \frac{Distance\ travaelled\ by\ protein\ molecules}{Distance\ travelled\ by\ dye}$
- A polyacrylamide gel with acrylamide content above a critical density restrains large molecules from migrating as fast as smaller molecules.
- Because the charge to mass ratio is nearly the same among SDS denatured polypeptides, the final separation of proteins is dependent almost entirely on the differences in molecular weight (MW) of polypeptides.
- In a gel of uniform density the relative migration distance of a protein (Rf) is negatively proportional to log of its MW. If proteins of known molecular weight are run simultaneously with the unknowns, the relationship between Rf and MW can be plotted and the MWs of unknown proteins determined.
- **1% Acetic acid (v/v; 1ml in100 ml) for used storage to gel.**

Tips for running good gels:

- After pouring the running gel, carefully overlay it with ethanol or another imiscible liquid. This will give you a nice flat surface. Also, since polymerization of acrylimide is inhibited by oxygen it will speed up polymerization.
- For the mini-gels we run the minimum protein loading per well (single band) is 0.1 µg for standard Coomassie staining and 2 ng for silver staining. I haven't tested it but my impression is that Simply Blue staining is within a factor of two as sensitive as standard Coomassie staining.
- The maximum protein loading per well (for a mixture of proteins of different sizes) is about 40 µg. If you exceed amount this your gel will look like crap.
- KCl causes SDS to precipitate. If you samples contain KCl you should dilute them or methanol precipitate them and resuspend them in 1X sample buffer. With low concentrations of KCl (<200 mM) you can run them on the gel but you should loaed every lane with sample buffer containing the same concentration of KCl (even if they are blanks). This will help the gel run a little less anomalously.
- If your sample buffer turns yellow, it is at the wrong pH. Add NaOH or HCl.

Tips

- Acrylamide as a monomer is highly neurotoxic; handle with extreme care.
- After pour acrylamide solution for a separating gel, Overlay with water to prevent contact with air (oxygen), which inhibits polymerization. After 30 minutes, Remove the overlaid water.
- Using a higher acrylamide concentration produces a gel with a smaller mesh size suitable for the separation of small proteins.
- In general, an acrylamide concentration between 6 and 15% is used.
- Gels with an acrylamide concentration gradient (gradient gels) are also used.
- The positive electrode should be connected to the bottom buffer reservoir.

Safety Notes:

- Acrylimide is extremely toxic, causing central nervous system paralysis. It can be absorbed through unbroken skin. If skin comes in contact with acrylimide solution or powder, wash immediately with soap and a lot of water.
- Unpolymerized acrylimide should be polymerized with excess catalyst and disposed of with solid waste.
- Do not pour unpolymerized acrylimide down the sink.
- Amonium Persulfate should be made up fresh or used from a relatively fresh stock. It goes bad after a week or two in the refrigerator. It can be disposed of by dilution with water and pouring down the sink.
- TEMED should be stored in the refrigerator in dark glass bottles. A bottle should be good for about a year, maybe longer.

Thanks