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#01 Polyacrylamide Slab Gel Electrophoresis of Proteins

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INTRODUCTION

Description

Electrophoresis involves the transport of charged molecules through a solvent in an electrical field. Many practical laboratory techniques have been developed to apply the principles of electrophoresis. Polyacrylamide gel electrophoresis (PAGE) is a powerful analytical technique used by biochemists and molecular and cell biologists to characterize and assay the purity of various biological macromolecules. The method is based on the principle that macromolecules can be separated according to their unique size and charge when placed in a gel matrix under an electrical field. In this experiment, the student will become familiar with one of the more popular versions; *the sodium dodecyl sulfate (SDS) PAGE.*

Student Audience

High school seniors and college juniors or seniors

Goals for the Experiment

- Understand the principle of gel polymerization (polyacrylamide formation)
- Perform the gel polymerization for the experiment
- Understand the principle of gel electrophoresis
- Perform the SDS-polyacrylamide gel electrophoresis for protein separation
- Use the results of the experiment for the determination of molecular weight of an unknown protein

Recommended Placement in the Curriculum

Based on the importance of the technique in academic research and industry, it is highly recommended that this experiment is included (even if just as a demonstration) in the high school and college curriculum.

STUDENT HANDOUT

Polyacrylamide Slab Gel Electrophoresis of Proteins

Purpose

The primary objective of the experiment is to understand the principle of polyacrylamide gel electrophoresis (PAGE). Students will work in a group to prepare polyacrylamide gel from monomers and use the gel to separate proteins. A mixture of six standard proteins will be placed in one lane and the other lane will contain an unknown protein. After loading the protein sample and connecting the current through the gel, the proteins will migrate as bands. Because each protein is made up of unique amino acids which differ in shape, size, and net charge, it is likely that each *protein* also has a unique "shape, size, and charge" and therefore, a unique migration rate in an electric field. Separation of proteins occurs because each protein migrates toward the electrode at a different rate and, therefore, each protein moves a different distance in any given time. The migration of the protein in SDS-PAGE will depend only on the size of the proteins, because in the presence of SDS proteins have the same charge/mass ratio. After staining the protein bands with dye reagent, the relative migration of the protein bands will be determined. By plotting the log of the molecular weight of standard proteins against the relative mobility, a standard curve will be generated. This curve will be used to determine the molecular weight of the unknown protein by comparing its movement to that of proteins of known molecular weight (standard proteins).

Scenario/Industrial Applications

The following are some of the applications of PAGE.

1. Both in the academic and industrial research laboratories, this technique is used to establish the purity of the isolated proteins. Generally the number of protein bands on the gel indicate the number of proteins in the sample tested.
2. SDS-PAGE is used to estimate the molecular weight.
3. SDS-PAGE is also used to determine if the protein is a monomeric protein or if it has more than one subunit structure.
4. PAGE is used as one of the methods of protein purification. It is often used to separate proteins from one another or proteins from other macromolecules.
5. PAGE is used in DNA sequencing, where oligonucleotides are separated by PAGE before their sequencing.
6. PAGE and other PAGE-based modified techniques are extensively used in clinical laboratories. Analysis of human serum proteins by PAGE has been used to investigate various liver and kidney diseases and inflammatory conditions. Analysis of cerebrospinal fluid has been used to investigate multiple sclerosis and various neurological diseases. Analysis of urine samples provide critical diagnostic information such as elevated levels of b_2-microglobulin protein, which strongly suggest the presence of myeloma cancer. Similarly, disappearance of bands corresponding to Bence-Jones proteins from urine samples indicates progress of the cytostatic drugs.
7. PAGE is also being used to monitor the change in the protein content in body fluids such as blood and milk from living and extinct species to assist in tracing the evolutionary trees.

Safety, Handling, and Disposal

Some of the reagents used in this experiment may be hazardous if used improperly. All the students, stockroom personnel, and instructors involved in this experiment should review the MSDS for acrylamide, bisacrylamide, N,N,N´,N´-tetramethylethylenediamine (TEMED), and b-mercaptoethanol. Notify the instructor of any accidental spills, inhalation, or ingestion of the hazardous materials.

- Both acrylamide and bisacrylamide are potent *neurotoxins.*
- N,N,N´,N´-tetramethylethylenediamine (TEMED) is a *carcinogen.*
- Wear disposable plastic gloves and goggles at all time when handling these solutions or the gel itself. Remember, acrylamide is most dangerous in the monomeric form. It is less dangerous when polymerized because the polymers are difficult to be absorbed through the skin.
- Never mouth pipette any reagent. If weighing out solid acrylamide, always wear a nose mask.
- Contact with operating electrophoresis apparatus may result in electrical shock, therefore, extreme care should be taken while handling the electrical connections.
- The staining dye should be handled carefully; spilling of the dye may spoil benches, clothing, and the laboratory.
- The leftover acrylamide, bisacrylamide solutions, and the gels should be disposed of properly and according to the departmental/institutional policies.

Materials Needed

- It is recommended to purchase the pre-cast gels for demonstration experiments. This would avoid the use of many hazardous procedures and their disposal and also save significant amount of time. (If precast gels are used, many of the following items may not be needed).
- Complete mini gel electrophoresis system with a power supply (Source: Fisher Scientific, BioRad Laboratories, Pharmacia Biotech.; Approximate cost $800)
- Glass pipettes (1 mL, 2 mL, 5 mL, and 10 mL)
- 50-mL Erlenmeyer flask
- Air displacement pipettes (10 µl, 250 µl, and 1000 µl) with gel applicator tips
- Small transparent tray (glass or plastic) for staining and destaining of gel
- Standard molecular weight markers (Source: BioRad Laboratories, Pharmacia Biotech.; approximate cost $50-70)
- Pure protein whose molecular weight is within the range of the standard markers
- Acrylamide solution (purchase of prepared solution is recommended; Source: Fisher Scientific; BioRad Laboratories; approximate cost: $50)
- Bisacrylamide (if prepared acrylamide solution is purchased this may not be required)
- Microwave (optional)
- pH meter
- Tris
- Sodium dodecyl sulfate
- Coomassie Brilliant Blue R-250 (Source: Sigma chemicals)
- Bromophenol Blue
- β-mercaptoethanol
- Ammonium persulfate
- TEMED
- Methanol
- Acetic acid
- Glycerol
- Goggles

- Disposable gloves
- Nose masks
- Staining dye solution
- Destaining solution
- Electrophoresis, sample and other
- Distilled water

Procedure

Preparation of the gel

Set up the gel casting apparatus according to the instructions provided by the manufacturer of the equipment. Make sure the gel casting system (plates) do not leak. It is recommended to check for leaks by filling it with water before pouring the gel cocktail. Prepare the gels with 5 or 10 sample wells. A general setup of the apparatus for the slab gel electrophoresis is given in Figure 1. The setup may differ slightly from one manufacturer to another.

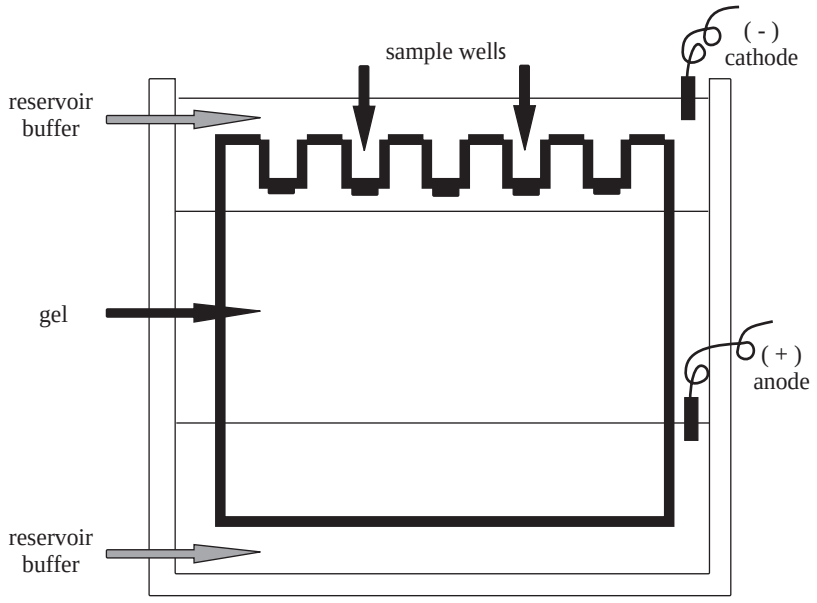

Figure 1 Apparatus setup for the slab gel electrophoresis

Dispense the required amounts of the solutions into a 50-mL Erlenmeyer flask. The concentration of acrylamide and the number of gels to be prepared will be given by the instructor. Acrylamide is a hazardous chemical and therefore gloves and goggles must be worn at all time while handling the solutions containing acrylamide. Often the gel will not polymerize as required; therefore, it is recommended that two gels be always prepare even if one gel is to be used by a group of students or the instructor. Table 1 gives the recipes for the preparation of various concentrations of gel cocktail. Note that less than 5.0 mL of the cocktail is needed to polymerize one 0.75 mm gel using the BioRad mini gel system.

Guide for the Preparation of Gels of Different Acrylamide Concentrations									
	7.0% Acrylamide concentration			10% Acrylamide concentration			12% Acrylamide concentration		
	1 gel	2 gels	4 gels	1 gel	2 gels	4 gels	1 gel	2 gels	4 gels
Distilled water	2.5 mL	5.0 mL	10.0 mL	2.0 mL	4.0 mL	8.0 mL	2.5 mL	3.4 mL	6.7 mL
1.5 M Tris-HCl, pH 8.8	1.3 mL	2.5 mL	5.0 mL	1.3 mL	2.5 mL	5.0 mL	1.3 mL	2.5 mL	5.0 mL
SDS (10%)	50 mL	100 mL	200 mL	50 mL	100 mL	200 mL	50 mL	100 mL	200 mL
Acrylamide (30%)	1.2 mL	2.3 mL	4.7 mL	1.7mL	3.3 mL	6.7 mL	2.0 mL	4.0 mL	8.0 mL
Ammonium persulfate* (10%) fresh	25 mL	50 mL	100 mL	25 mL	50 mL	100 mL	25 mL	50 mL	100 mL
TEMED*	3 mL	5 mL	10 mL	3 mL	5 mL	10 mL	3 mL	5 mL	10 mL
Total volume	5 mL	10 mL	20 mL	5 mL	10 mL	20 mL	5 mL	10 mL	20 mL

*These items are added to the cocktail at the very last. Also make sure that the gel casting apparatus is ready for pouring of the gel cocktail before the addition of these items.

Casting the gels

Combine all the solutions except ammonium persulfate and TEMED.
Degas the solution under vacuum for at least 15 minutes.
Place the comb in the glass sandwich.
Add ammonium persulfate and TEMED to the degassed solution and use a Pasteur pipette to pour the solution down the spacer. Be sure to add the solution slowly to prevent bubble formation. Tap the gel assembly gently if bubbles do appear. Pour until the bottoms of all the teeth of the comb are covered. Then adjust the comb to its proper position. Continue adding the solution until the sandwich is completely filled.

Preparing and loading the sample

Samples are prepared by diluting the protein sample to at least 1:4 with the sample buffer and heating at 95^{o}C for 2-4 minutes. The samples may be prepared after the gel polymerization.
Let the gel polymerize for 45 minutes to 1 hour at room temperature.
Remove the gel from the gel casting apparatus and place the gel on the mini gel running apparatus as instructed by the manufacturer of the apparatus.
Fill the lower and the upper chambers of the apparatus with the running buffer.
Using an air displacement pipette equipped with a gel loading tip or micro-syringe, add the required amount of standard molecular weight marker in the second well from your left. Care should be taken not to spill the sample from the well. Load the unknown sample in the other appropriate well. (You may want to keep a gap of one or two wells to avoid contamination of samples.)
Put the cover on, connect the cables to the power pack, and turn on the power supply.
The power pack should be set to run at a constant voltage of 200 volts.

Staining and destaining the gel

The power should be turned off after 45 minutes either manually or the power supply could be programmed to turn off automatically.

Disassemble the apparatus and remove the gel. Using an appropriate object, separate the two glass plates from each other and carefully pick up the gel and place in a tray containing 50 mL of staining solution. Let the gel stain overnight. Next day, remove the staining solution, and add 50 mL of destaining solutions. Several changes of the destaining solution will be required before the protein bands are visible. Alternatively, the staining could be done by placing the gel in 50 mL of staining dye in a microwave for 30 sec at high power followed by gentle shaking for 10-15 minutes. The destaining could be done immediately following the staining by placing the gel in 50 mL of the destaining solution for 30 sec at high power followed by gentle shaking for 10-15 minutes. Repeat the destaining procedure if necessary every 30 minutes until clear.

Determination of Molecular weight (M_r) of the unknown protein

The pattern of protein bands expected in the experiments is depicted in Figure 2.

1. Wearing gloves, spread the slab gel on parafilm or cellophane. Measure the distance in millimeters (mm) from the top of the gel to the mid-point of each protein band in the lane for each protein. Also measure the distance of the tracking dye (bromophenol blue) front. Record the results in a table on the DATA SHEET.

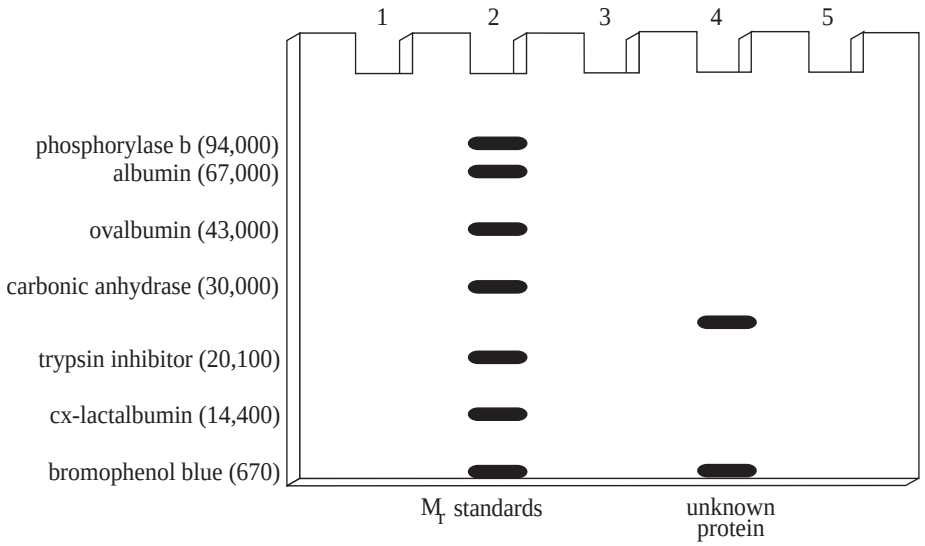

Figure 2 Electrophoretic mobility of proteins bands on SDS-PAGE. Lane 2 shows the protein bands of the standard molecular weight markers. The mobilities and the molecular weights of these bands are used to determine the molecular weight of the unknown protein in lane 4.

2. Calculate the electrophoretic mobility for each protein using the following equation.

$$\text{Relative mobility} = \frac{\text{Distance of protein migration}}{\text{Distance of tracking dye migration}}$$

3. Using the distance measured from the protein mixture, plot the mobility of the standards in mm on the abscissa (x) axis and the log of the known molecular weights on the ordinate (y) axis, as indicated in Figure 3. Attach the plot to your Lab report. Connect the data points and label the plot clearly. If the graph is not linear, give a possible explanation for the results. **Indicate the mobility of the unknown protein and estimate the molecular weight from the graph.**

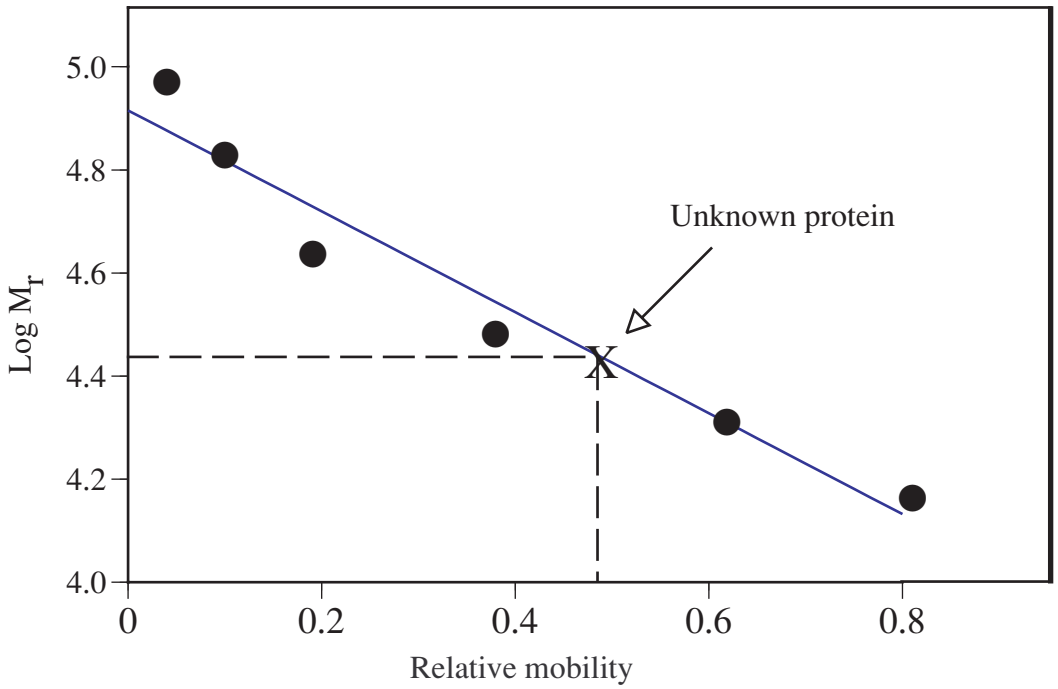

Figure 3: A plot of log M_r of the standard marker proteins versus relative migration. This plot allows the M_r of the unknown protein to be read from the graph.

DATA SHEET

Name: _____

Partner(s):_____

Results					
Protein	Protein Migration (mm)	Dye Migration (mm)	Mobility	M_r*	(log M_r)
Phosphorylase B				106,000	
Bovine Serum Albumin				80,000	
Ovalbumin				49,500	
Carbonic Anhydrase				32,500	
Soybean Trypsin Inhibitor				27,500	
Lysozyme				18,500	
Unknown					

* The molecular weights (M_r) are according to pre-stained protein markers from BioRad Laboratories.

Molecular Weight of Unknown Protein _____

Attach the graph of standards and unknown protein.

Questions

1. A total of how many amino acid(s) does it take to synthesize the proteins that exist in nature?
2. Which optically active form of the amino acid is required for living organisms?
3. If a protein molecule has a net charge of zero at the pH in which the PAGE is being carried out, towards which electrode will the protein molecules migrate?
4. If the acrylamide concentration is increased in the gel, will the pore size of the gel matrix increase or decrease?
5. Will a gel of higher acrylamide concentration be suitable for the separation of high or low molecular weight proteins?
6. Macromolecules that are too large to enter the pores of polyacrylamide gel such as double stranded DNA having molecular weight greater than 100×10^6 Dalton are generally separated by which type of electrophoresis?
7. Which of the following, pH, ionic strength, temperature, or current, is likely to have the greatest effect on the separation of proteins? Explain why.

Suggested Reading

1. Andrews A.T. “Electrophoresis: Theory, Techniques and Biochemical and Clinical Applications”; Second edition, Clarendon Press, Oxford, 1993.
2. Hames, B.D.; Rickwood, D. Eds. “Gel Electrophoresis of Proteins: A Practical Approach”; Second edition, IRL Press, Oxford, 1994.
3. John F Robyt.; White, B.J. “Biochemical Techniques: Theory and Practice” Waveland Press, Inc., Prospect Heights, IL, 1987. Chap 5, pp 129-157.
4. Wilson, K and Walker, J., Eds. “Principles and Techniques of Practical Biochemistry” Fourth edition, Cambridge University Press, Somerset, U.K. Chap 9, pp 425-460.

INSTRUCTOR NOTES

Polyacrylamide Slab Gel Electrophoresis of Proteins

Time Required

- 3 1/2 hours for the complete lab
- 1/2 hour for pre lab discussion
- 3 hours for preparation and running of the gel. If microwave is not used for destaining, students will be required to return at a later day to perform destaining and recording of the data.
  A demonstration of the experiment by the instructor may take only 2 hours provided the gels are polymerized ahead of time.

Group Size

Suggested group size of 3-4 students

Materials Needed

- It is recommended to purchase the pre-cast gels for demonstration experiments. This would avoid the use of many hazardous procedures and their disposal and also save significant amount of time. (If precast gels are used, many of the following items may not be needed).
- Complete mini gel electrophoresis system with a power supply (Source: Fisher Scientific, BioRad Laboratories, Pharmacia Biotech.; Approximate cost $800)
- Glass pipettes (1 mL, 2 mL, 5 mL, and 10 mL)
- 50-mL Erlenmeyer flask
- Air displacement pipettes (10 µl, 250 µl, and 1000 µl) with gel applicator tips
- Small transparent tray (glass or plastic) for staining and destaining of gel
- Standard molecular weight markers (Source: BioRad Laboratories, Pharmacia Biotech.; approximate cost $50-70)
- Pure protein whose molecular weight is within the range of the standard markers
- Acrylamide solution (purchase of prepared solution is recommended; Source: Fisher Scientific; BioRad Laboratories; approximate cost: $50)
- Bisacrylamide (if prepared acrylamide solution is purchased this may not be required)
- Microwave (optional)
- pH meter
- Tris
- Sodium dodecyl sulfate
- Coomassie Brilliant Blue R-250 (Source: Sigma chemicals)
- Bromophenol blue
- β-mercaptoethanol
- Ammonium persulfate
- TEMED
- Methanol
- Acetic acid
- Glycerol
- Goggles
- Disposable gloves
- Nose masks

Solutions to be prepared

It is recommended that these solutions be prepared ahead of time by the stockroom personnel or by the instructor; otherwise the experiment will not finish in suggested time.

Acrylamide solution

(30% acrylamide, 1.5% bisacrylamide)

- 30.0 g acrylamide
- 1.5 g bisacrylamide
- dissolve in 100 mL of distilled water

It is highly recommended, especially for instructional purposes, to purchase the prepared solutions of acrylamide. This will tremendously reduce the risk of acrylamide inhalation while weighing. Please also note that the pre-cast gels of various concentrations are also available from various manufacturers and could be used when adopting the experiment.

TEMED (use as purchased)

Ammonium persulfate (prepare freshly)

- 10 mg ammonium persulfate
- dissolve in 1.0 mL distilled water

Buffers

1.5 M Tris, pH 8.8

45.4 g of Tris

- dissolve in 150 mL distilled water
- adjust pH to 8.8 with 6 M HCl
- fill to 250 mL with distilled water

10x Electrophoresis Running Buffer

(250 mM Tris, 1.92 M glycine, 1% SDS)

- 30.3 g Tris
- 144.0 g glycine
- l0.0 g SDS
- fill to 1 liter with distilled water
- to make 1 liter of lx running buffer, add 900 mL of distilled water to 100 mL of l0x running buffer

Sample Buffer with SDS

(60 mM Tris, 2% SDS, 5% b-mercaptoethanol, 10% glycerol, 0.025% bromophenol blue)

- 25.0 mL distilled water
- 6.58 mL 0.5 M Tris, pH 8.8
- 5.26 mL glycerol
- 10.5 mL of 10% SDS
- 2.63 mL of 0.5% bromophenol blue
- fill to 50 mL with water in a volumetric flask
- to prepare fresh working sample buffer, add 50 µl of b-mercaptoethanol to 950 µl of the stock sample buffer before adding to the sample

Protein Staining and Developing Solutions

Staining Dye

(0.2% Coomassie Brilliant Blue-R250, 20% methanol, 10% acetic acid)

- 0.4 g Coomassie Blue-R-250
- 40 mL methanol
- 20 mL concentrated acetic acid
- fill to 200 mL with distilled water

Destaining Solution

(10% acetic acid, 20% methanol)

- 100 mL concentrated acetic acid
- 200 mL methanol
- fill to 1 liter with distilled water

Safety, Handling, and Disposal

Some of the reagents used in this experiment may be hazardous if used improperly. All the students, stockroom personnel, and instructors involved in this experiment should review the MSDS for acrylamide, bisacrylamide, N,N,N´,N´-tetramethylethylenediamine (TEMED), and b-mercaptoethanol. The instructor should be notified of any accidental spills, inhalation, or ingestion of the hazardous materials.

- Both acrylamide and bisacrylamide are potent *neurotoxins.*
- N,N,N´,N´-tetramethylethylenediamine (TEMED) is a *carcinogen.*
- Wear disposable plastic gloves and goggles at all time when handling these solutions or the gel itself. Remember, acrylamide is most dangerous in the monomeric form. It is less dangerous when polymerized because the polymers are difficult to be absorbed through the skin.
- Never mouth pipette any reagent. If weighing out solid acrylamide, always wear a nose mask.
- Contact with operating electrophoresis apparatus may result in electrical shock, therefore, extreme care should be taken while handling the electrical connections.
- The staining dye should be handled carefully; spilling of the dye may spoil benches, clothing, and the laboratory.
- The leftover acrylamide, bisacrylamide solutions, and the gels should be disposed of properly and according to the departmental/institutional policies.

Points to Cover in the Pre-Lab Discussion

Polymerization of the Gel

In PAGE, the gels are prepared by a free radical-induced polymerization of acrylamide and N,N´-bisacrylamide in appropriate buffers. The polymerization of the monomers proceeds by a free-radical mechanism, which is initiated by a catalyst. The most commonly used catalyst is ammonium persulfate, which produces the free radicals (SO_4^{-}•) initiated by the addition of a base. The most commonly used base is an aliphatic amine, N,N,N´,N´-tetramethylethylenediamine (TEMED). The polymerization reaction produces long chains of polyacrylamide, incorporating a small proportion of bisacrylamide molecules which cross-links with other polyacrylamide chains, resulting in a three-dimensional network, gel matrix. The basic reaction of polyacrylamide gel polymerization is summarized in Figure 4. Both the length of the polymers and the extent of cross-linking determine the pore size, elasticity, and the mechanical strength of the gel.

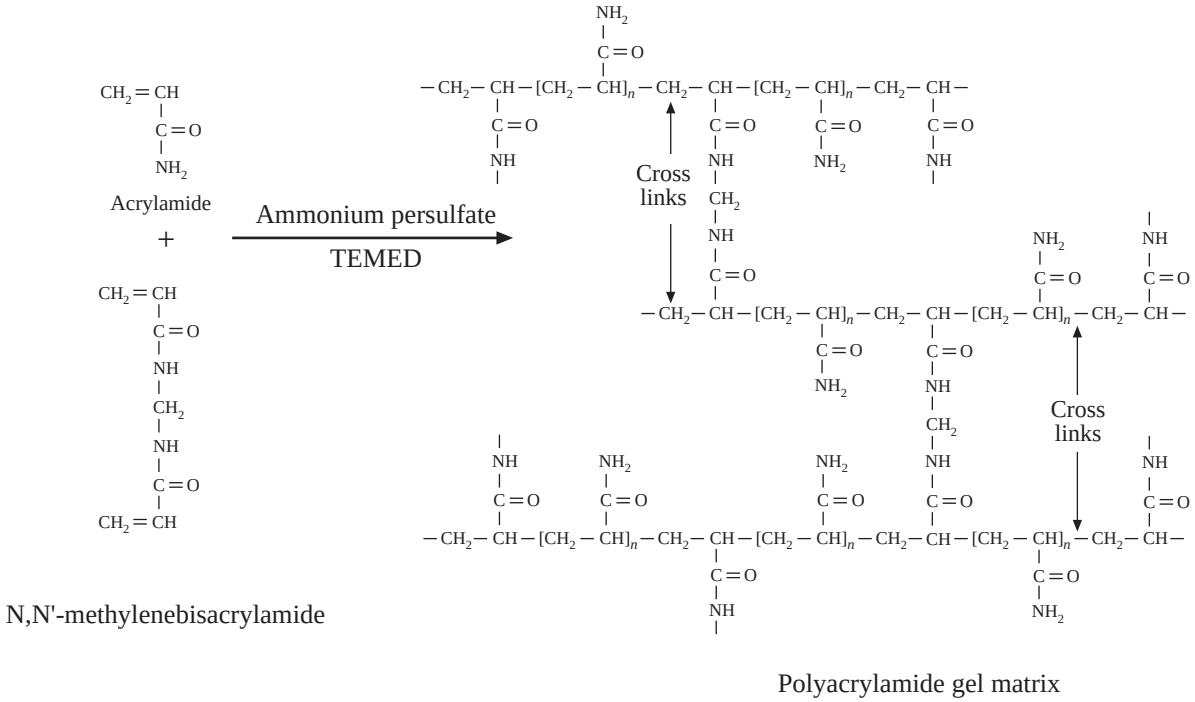

Figure 4: Formation of polyacrylamide gel matrix from acrylamide monomers and N, N'-bisacrylamide cross-linking agent in presence of ammonium persulfate (a source of free radicals (SO4–•)) and TEMED (a free radical stabilizer).

Significance of Gel Pore Size or Acrylamide Concentration

Since the separation of molecules in PAGE depends on the size and charge of the proteins, molecular sieving through the pore size of the gel plays a role in the separation process. Therefore, the pore size or the acrylamide concentration required to provide optimal separation depends on the size of the proteins to be separated. Table 2 provides an approximate guide for appropriate gel or acrylamide concentration for the various molecular weight ranges of the protein.

Percent of Polyacrylamide Gels for Various Molecular Weight Range Proteins

% Acrylamide Concentration	Molecular Weight Range of Proteins
3% to 5%	above 100,000 Daltons
5% to 12%	from 20,000 to 150,000 Daltons
10% to 15%	from 10,000 to 80,000 Daltons
15% or more	below 15,000 Daltons

The Principle of Protein Separation by PAGE in Presence of SDS

Proteins possess a charge because their composition includes basic and acidic amino acids which have ionizable side groups. In electrophoresis, only the net charge is significant. For proteins, the net charge is a function of the amino acid composition and pH. If an electric field is applied, the charged protein will move toward the oppositely charged electrode. The greater the net charge on the protein, the faster it moves through the electric field. The rate of movement and the velocity is directly proportional to the net charge.

Because each protein has a unique amino acid sequence, it is likely that each protein also has a unique "size and charge" and, therefore, a unique migration rate in an electric field. Separation of proteins occurs because each is migrating toward the electrode at a different rate, and therefore each moves a different distance in any given time.

Because proteins actually differ in shape, the effect of size upon the migration rate is not a simple function. However, in practice, the problem can be surmounted by adding sodium dodecyl sulfate (SDS) to the solution. SDS is an anionic detergent, which binds to the hydrophobic regions of proteins, separates most of them into their component subunits, and unfolds the polypeptide conformation. Hydrodynamic studies suggest that the SDS-protein complex is a rod-like particle, the length of which varies uniquely with the molecular weight of the protein moiety. SDS binding also imparts a large negative charge to the denatured, randomly coiled polypeptide. This charge largely masks any charge normally present in the absence of SDS; therefore, SDS binding also minimizes the charge difference between proteins. In the absence of SDS, proteins in solution migrate according to size, shape and charge in an electric field. In the presence of SDS, proteins have the same rod-like shape and the same negative charge/mass ratio, therefore, protein migration in an electric field is essentially a function of size of the protein or its molecular weight.

Determination of Molecular Weight

In the present experiment, SDS polyacrylamide gel electrophoresis will be applied simultaneously to a typical research question using slab gel techniques. The major objective of this experiment is to determine the molecular weight of an unknown protein. This will be conducted in a manner similar to the Weber and Osborn empirical determinations (6).

In 1969, Weber and Osborn empirically demonstrated that the mobility-size relationship was a logarithmic function using a poly-acrylamide gel support. When the mobilities were plotted against the logarithm of the known molecular weights, a straight line was obtained, Figure 3. This standard curve has been used successfully to determine the molecular weights of a variety of unknown proteins within an accuracy of ±10%.

Basic Structure and Properties of Proteins

Any biochemistry textbook could be used to explain the basic structure of proteins and properties of some of the amino acids, especially ones with the ionizable groups. These amino acids determine the charge on the proteins at different pH. The net charge in turn influences the mobility of the proteins in PAGE.

Use of Tracking Dye

Since most of the proteins are colorless, they are not visible during the progress of the

electrophoresis, and therefore it is difficult to determine the time length for which the electrophoresis should be carried out for optimal separation. To overcome this problem bromophenol blue (a pH indicator dye) is added to the sample buffer. This dye has a molecular weight of 670 gm/mol and blue color in slightly alkaline conditions. Since the molecular weight of this dye is much smaller than the most proteins, it will travel ahead of the proteins as a sharp blue color band. Figure 2 shows the bands of standard and unknown proteins and that of bromophenol blue after electrophoresis.

Procedural Tips and Suggestions

- All the solutions, buffers and reagents should be ready before the start of the lab.
- To save time and reduce the safety risks, it is recommended to purchase the prepared solution of acrylamide or even the pre-cast gels.
- If the experiment is adopted as a demonstration experiment, it is recommended that the instructor polymerizes a set of gels and has everything else ready before the lab meeting time. At the beginning of the lab, load the gels with the sample and run the gel for 40-50 minutes. While the gel is running, demonstrate the polymerization of the gel using another set of a gel casting apparatus. At the end of ~50 minutes, the staining process of the experimental gel could be continued.
- Use of ammonium persulfate that has been stored more than one year should be avoided.
- While polymerizing the gel, fresh solutions of ammonium persulfate should be prepared.
- Polymerization of gel (addition of ammonium persulfate and TEMED) should be started only after the gel casting apparatus is fully set up.
- Many manufacturers now provide pre-stained standard marker proteins. If these markers are use in the experiment, students can appreciate the separation of proteins during the progress of the experiment.
- Samples are prepared by diluting the protein sample to at least 1:4 with the sample buffer and heating at 95^{o}C for 2-4 minutes. The samples may be prepared after the gel polymerization.

Sample Results

Figures 2 and 3 could be used as a sample result for the experiment.

Plausible Answers to Student Questions

1. There are only 20 amino acids used by the cells to synthesize all the proteins; these are referred as standard amino acids. It is important to emphasize that only these 20 amino acids are responsible for the synthesis of proteins in mammals, as well as in any other species such as birds, fishes, bacteria, fungi, and plants. If a protein upon analysis indicates the presence of an amino acid other that the standard amino acids, that amino acid must either be added or modified after the proteins is synthesized (post-translation modification).
2. All biological molecules with chiral centers occur naturally in only one steroisomeric form, either D or L. It is remarkable that the amino acids presents in proteins are all L stereoisomers. This unique ability of cells to specifically synthesize the L-isomeric forms of all amino acids reflects one of the many astonishing properties of protein.

3. If the protein molecule has no net charge at the pH in which the electrophoresis is being carried out, the protein is not expected to move towards either electrode. The protein is said to be at its isoelectric point (pI is the pH at which the molecule has no net charge). Having equal number of negative and positive charge(s), the protein molecule will not be attracted to either electrode. Note that the total charge on the protein molecule changes with change in pH. At pH lower than its pI, a protein is expected to have a net positive charge, where as at pH higher than its pI, a protein is expected to have a net negative charge. The following example (Figure 5) of alanine (one of the 20 amino acid) demonstrates the change in charge on the amino acids with change in pH. The pI of this amino acid is 6.0.

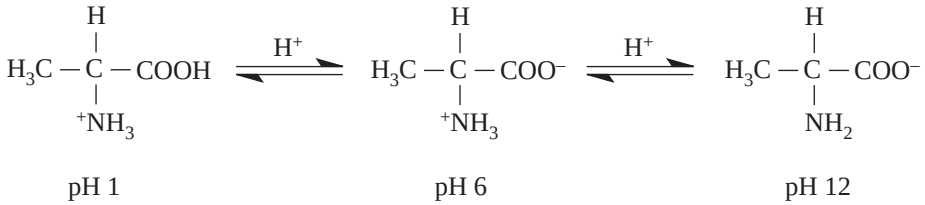

Figure 5: Alanine

4. As the acrylamide concentration in the gel is increased, the pore size of the gel matrix will be reduced because of the formation of extensive polymerization and cross-linking of the acrylamide polymers by bisacrylamide.
5. A high acrylamide concentration gel is expected to have smaller pore size. Smaller pore size gel would not allow larger size proteins to enter the gel matrix, and therefore larger proteins will not migrate in a gel with a pore size smaller that its size.
6. Double stranded DNA molecules are relatively bigger in size (M_r_100 x 10^6). This size molecule is not expected to enter the largest pore size polyacrylamide gels. Separation of such large molecules is generally achieved on agarose gel electrophoresis. The pore size of agarose gels allows DNA molecules of such lengths to migrate under the influence of an electric current yet still remain rigid enough to provide a support medium.
7. Although all these factors are expected to influence the mobility of the proteins, several factors are expected to have a similar effect on the proteins in the sample. However, a change in pH is expected to have the greatest effect. As mentioned above change in pH will significantly change the magnitude of the charge carried by a protein molecule. One might also predict that ionic strength might have the greatest effect on the separation as it will shield the counterions and reduce the net charge. The magnitude of the ionic strength effect will depend on the nature and charges of the ionizable groups on the surface of the proteins. pH on the other hand effects the ionizable amino acids inside as well as on the surface of the protein molecule.

Extensions and Variations

- It is recommended that when adopted at the high school levels, this experiment should be restricted only to explain the principle of macromolecule separation by PAGE. In this case, the instructor's demonstration is the best option. However, if adopted to the four- year college courses, students must be required to use the data of the PAGE to determine the molecular weight of an unknown protein.

- If required, the PAGE could be modified to do the electrophoresis with discontinuous buffer where two gels (stacking and resolving) are made on top of each other in two different buffers. The resolution of protein bands is significantly increased in discontinuous-PAGE.
- For increased resolution, the PAGE could be modify to be performed in two dimensions, a technique referred to as two-dimensional gel electrophoresis.

References

For theory and practice of PAGE, refer to the following books/articles.

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