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Polyacrylamide Gel Electrophoresis (PAGE) of Egg Proteins

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Background

Charged groups or ions will migrate in an electric field. Proteins carry a charge (except at their isoelectric point), and they too will migrate in an electric field with the rate of migration dependent upon the ratio of charge to mass. The application of an electric field to a mixture of proteins will result in different proteins moving at different rates within the field. In PAGE, a supporting medium (polyacrylamide) is used so that the proteins can be fixed in their final migration positions and analyzed.

Acrylamide monomer polymerizes into long chains and is cross-linked by N,N´-methylene bisacrylamide (bisacrylamide) reacting with free functional groups at the ends of the chains to form polyacrylamide gel.

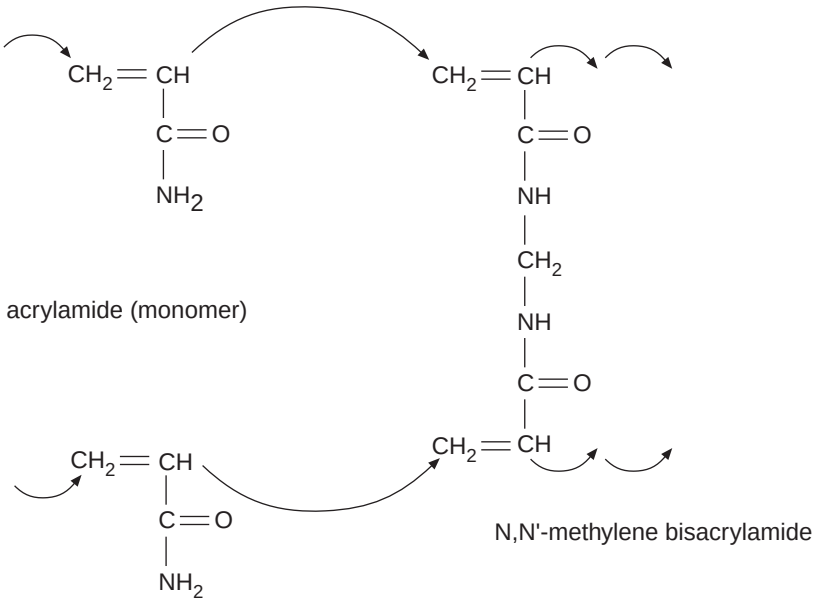

Figure 1: Acrylamide bisacrylamide structure

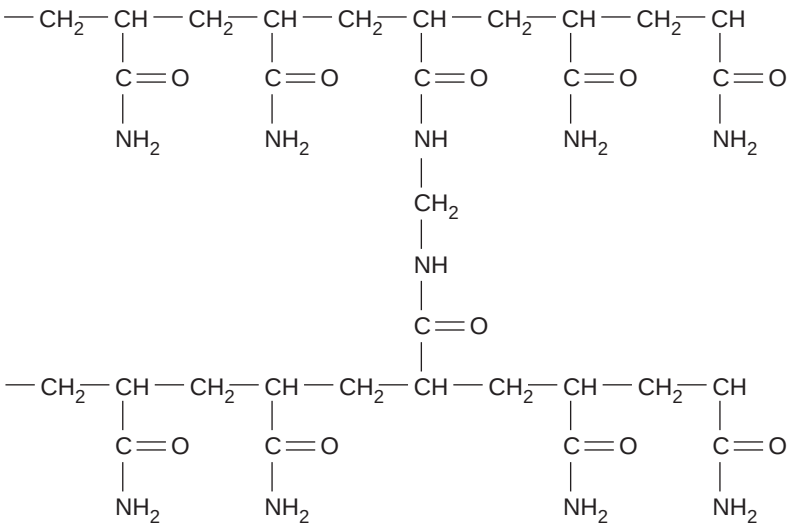

Figure 2: Polyacrylamide gel structure

The effective pore size of the gel is determined by the total acrylamide concentration used during polymerization. The pore size decreases as the acrylamide concentration increases. An acrylamide concentration of at least 2.5% is needed to provide molecular sieving effects for molecules with molecular weights above 10^6, such as with DNA and RNA. Gels with this low an acrylamide concentration are almost fluid and require the addition of agarose. Polyacrylamide gels can also be formed with as high an acrylamide concentration as 30%. Polypeptides with molecular weights as low as 2,000 can be separated. Above a concentration of about 30%, the polyacrylamide gel becomes very brittle and difficult to handle. The choice of acrylamide concentration is critical for proper protein separation. In general, proteins with molecular weights ranging from 10,000 to 100,000 can be separated on gels with 11% acrylamide concentration, and proteins from 30,000 to 200,000 molecular weights can be separated on 7% gels.

The effective pore size, brittleness, and swelling properties of the gel at any one monomer concentration also vary with the proportion of cross-linking agent used. If polymerization occurs without cross-linker, a viscous solution is obtained. As the amount of cross-linker increases, the effective pore size decreases until the bisacrylamide represents about 5% of the total monomer. If larger amounts of bisacrylamide are used, the polymer chains become cross-linked into increasingly larger bundles with large spaces between them, and thus, the effective pore size begins to increase again.

Originally, PAGE was carried out in cylindrical glass tubes or rods, but now flat slabs of 0.75- to 1.5-mm thickness are preferred. A major advantage of slab gels is that many samples, including marker proteins, can be electrophoresed under the same conditions in a single gel. Rod gels are rarely identical due to minor variations in polymerization efficiency. Additional advantages to slab gels include the fact that they are able to accommodate up to 25 samples on a single gel; any heat produced during the electrophoretic run is more easily dissipated in a thinner slab gel, thus reducing heat distortion; their rectangular cross-section makes densitometry and photography straightforward; and they can be dried easily for storage.

The polymerization reaction is initiated by a catalyst such as ammonium persulfate or riboflavin. N,N,N´,N´-tetramethylenediamine (TEMED) is also added as a polymerization accelerator. With the ammonium persulfate-TEMED system, TEMED catalyzes the formation of free radicals from the persulfate which initiate polymerization. Increasing either the ammonium persulfate or the TEMED concentration increases the rate of polymerization. The riboflavin-TEMED system requires light to initiate polymerization. The photodecomposition of riboflavin causes the production of free radicals. Oxygen inhibits polymerization, and therefore, gel solutions should be degassed prior to use.

The majority of protein electrophoresis in polyacrylamide gels utilizes a buffer system designed to dissociate the proteins into their individual polypeptide subunits. Sodium dodecyl sulfate (SDS), an ionic detergent, is the most common dissociating agent. Proteins are denatured in the presence of a thiol reagent (which cleaves disulfide bonds) and excess SDS by heating to 100°C. Most polypeptides will bind the SDS in a constant ratio by weight. Consequently the SDS-polypeptide complexes have essentially the same charge densities, since the charges intrinsic to the polypeptide are masked by the charges from the bound detergent. The SDS-polypeptide complexes migrate in the polyacrylamide gel according to polypeptide size. It is thus possible to determine the molecular weight by referencing the mobility of known molecular weight marker proteins under the same electrophoretic conditions. SDS-polyacrylamide electrophoresis (SDS-PAGE) requires only microgram quantities of sample proteins. Its speed and simplicity have made SDS-PAGE the most widely used method for determining molecular weight and complexity of constituent polypeptides in protein samples.

Continuous buffer systems employ the same buffer ions throughout the sample, gel, and electrode vessel reservoirs. The protein sample is loaded directly onto the separation gel. Discontinuous buffer systems have different buffer ions in the electrode reservoirs and in the gel. The sample is loaded onto a large-pore stacking gel, which has been polymerized on the top of the small-pore separating gel. The major advantage of a discontinuous system is that large volumes of dilute samples of proteins can be applied to the gels with good resolution of sample components. Proteins are concentrated into narrow zones (or stacks) while migrating through the stacking gel prior to entering the separating gel. In the original Ornstein-Davis system, the sample and the stacking gel contain Tris-HCl buffer, while the upper electrode reservoir contains Tris-glycine buffer. At the pH of the sample and stacking gel (pH 6.7), glycine has a low mobility and chloride ions have a higher mobility. The mobility of the proteins is intermediate between the glycine and chloride. When the voltage is applied, the chloride ions migrate away from the glycine ions until a steady state is reached in which the products of the voltage gradient and mobility for glycine and chloride are equal. The glycine and chloride move at the same velocity with a sharp boundary between them. A low-voltage gradient is in front of the boundary, and a high-voltage gradient moves behind the boundary. Any proteins in front of the boundary are in a low-voltage environment and move more slowly than the chloride-glycine boundary. Any proteins behind the boundary are in a high-voltage environment and move more rapidly than the boundary. As the boundary moves, it overtakes the more slowly migrating proteins and sweeps up the more rapidly moving proteins. Thus, the proteins are concentrated into thin “stacks” within the chloride-glycine boundary. Since the pore size is large, no molecular sieving occurs. As this moving boundary enters the separating gel, the pH value increases, changing the mobility of the glycine. The glycine migrates directly behind the chloride ions, and the proteins are retarded, since the pore size decreases. The proteins can be separated by the molecular sieving effect.

Discontinuous systems provide very high resolution. The most commonly used SDS-discontinuous system was originally described by Laemmli and consists of the discontinuous Ornstein-Davis system with SDS present.

Since the proteins cannot be seen on the gel, a method of visualizing the protein subunits must be employed. Commonly, immediately following electrophoresis, the proteins are fixed in their final positions to prevent diffusion throughout the gel. The entire gel is then stained with a dye such as Coomassie® Brilliant Blue, which binds to the proteins. The gel is destained to remove any unbound dye. The proteins appear as discrete bands in the gel.

In order to determine molecular weight, marker proteins of known molecular weight are co-electrophoresed with the protein samples. The relative mobilities of the known molecular weight markers are compared to the relative mobility of an unknown protein. Relative mobility is measured by calculating the ratio of the distance the protein moved on the gel to the distance the electrical front moved on the gel. The electrical front is easily followed by adding a small amount of a dye such as bromophenol blue to the protein samples. The dye is not retarded on the gel, and thus it moves with the electrical front. A graph is prepared by plotting the log of molecular weights of the known proteins versus their relative mobilities. The relative mobility of the unknown is then used to determine its molecular weight from the graph.

Materials

- SDS Molecular Weight Marker Kit MW-SDS-70L from Sigma Chemical Company, St. Louis, MO. The kit contains bovine albumin (66,000 molecular weight), egg albumin (45,000), glyceraldehyde-3-phosphate dehydrogenase (36,000), carbonic anhydrase (29,000), trypsinogen (24,000), trypsin inhibitor (20,100), and β-lactalbumin (14,000). The kit also contains one vial of a mixture of the seven proteins.
- electrophoresis apparatus (Bio-Rad Laboratories Mini-PROTEAN II)
- 1,000-volt power supply
- glass plates, spacers (1.5 mm), combs (1.5 mm) and gel casting apparatus
- electrophoresis grade:
  - acrylamide
  - bisacrylamide
  - TEMED
  - Tris
  - ammonium persulfate
  - sodium dodecyl sulfate
  - glycerol
  - glycine
  - Coomassie Brilliant Blue
  - 2-mercaptoethanol
  - bromophenol blue
- hydrochloric acid
- methanol
- isobutyl alcohol
- glacial acetic acid
- deionized water
- sonicator
- micropipetor
- electrophoresis tip

- razor blade
- chicken egg
- freezer that cools to -20°C

Safety, Handling, and Disposal

It is your responsibility to specifically follow your institution's standard operating procedures (SOPs) and all local, state, and national guidelines on safe handling and storage of all chemicals and equipment you may use in this activity. This includes determining and using the appropriate personal protective equipment (e.g., goggles, gloves, apron). If you are at any time unsure about an SOP or other regulation, check with your instructor.

Acrylamide and bisacrylamide are neurotoxins. Weigh them out only under a hood while wearing a dust particle mask. Do not inhale any of the acrylamide particles, and avoid skin contact. After acrylamide and bisacrylamide are in solution, do not allow any skin contact. The solutions can be absorbed through the skin. Use gloves when handling polymerized gels, since some unpolymerized monomer may still be present. TEMED and 2-mercaptoethanol should be pipetted under a hood and the solutions kept tightly capped. Never pipet by mouth.

Methods

(adapted from Sigma Technical Bulletin No. MWS-877L)

Reagents:

1. Separating gel buffer: Dissolve 36.3 g Tris and 0.23 mL TEMED in water and dilute to approximately 90 mL. Adjust the pH to 8.9 with concentrated HCl. Bring the volume to 100 mL with water. The solution is stable for two weeks when stored at 0–5°C.

2. Stacking gel buffer: Dissolve 5.98 g Tris and 0.46 mL TEMED in water and dilute to approximately 80 mL. Adjust the pH to 6.7 with concentrated HCl. Bring the volume to 100 mL with water. The solution is stable for two weeks when stored at 0–5°C.

3. Separating gel solution: Dissolve 28.0 g of acrylamide and 0.74 g bisacrylamide in water and dilute to a final volume of 100 mL. The solution is stable for at least one month when stored in a dark bottle at 0–5°C.

4. Stacking gel solution: Dissolve 10.0 g of acrylamide and 2.5 g of bisacrylamide in water and dilute to a final volume of 100 mL. The solution is stable for at least one month when stored in a dark bottle at 0–5°C.

5. SDS solution: Dissolve 0.21 g SDS in water and dilute to a final volume of 100 mL. Care should be taken not to shake the solution, since the SDS detergent will foam. The solution is stable at room temperature for two weeks. The solution can be stored in the refrigerator. The solution may become cloudy but will become clear again when warmed to room temperature.

6. 2X sample buffer: Dissolve 1.51 g of Tris and 20.0 mL glycerol with 35 mL of water. Adjust the pH to 6.75 with concentrated HCl. Add 4.0 g SDS, 10.0 mL 2-mercaptoethanol, and 0.002 g bromophenol blue. Dilute to a final volume of 100 mL. The solution is stable for at least one month when stored frozen at -20°C.

7. Electrode buffer: Dissolve 6.05 g Tris, 28.8 g glycine, and 2.0 g SDS in water and dilute to a final volume of 2 L. The final pH should be about 8.3. Take care not to shake the solution, since SDS will foam.

8. Fixative solution: Combine 400 mL methanol, 70 mL glacial acetic acid, and 530 mL water.

9. Staining reagent: Dissolve 1.25 g Coomassie Brilliant Blue in 500 mL fixative solution. This solution is stable for several months when stored at room temperature tightly capped.

Preparation of Samples

Marker proteins: Dilute the sample buffer 1:1 with water. Reconstitute the molecular weight protein markers from the Sigma kit as per the Sigma technical bulletin instructions listed below.

Protein	Amount Diluted Sample Buffer
bovine albumin	35 mL
egg albumin	25 mL
glyceraldehyde-3-phosphate dehydrogenase	7.5 mL
carbonic anhydrase	10 mL
trypsinogen	25 mL
trypsin inhibitor	5 mL
β-lactalbumin	10 mL
SDS-7 (mixture)	1.5 mL

The proteins must be heated at 100°C for 1 minute in sample buffer. The proteins may be stored at -20°C. (They are stable for at least one year at this temperature.)

Preparation of Egg Samples

Separate the yolk and white of an ordinary chicken egg. (Separation is best achieved while the egg is still cold from the refrigerator.) Measure 1 mL egg white by using a small graduated cylinder and a Pasteur pipet to transfer. Add 9 mL sample buffer prepared according to the Sigma procedure. Carefully pour the mixture into a small beaker, stir, and heat to 100°C for 1 minute. Store the sample below -20°C until ready for use.

Prepare the egg yolk sample in the same manner as you did the egg white. Store frozen until ready to use.

Preparation of Electrophoresis Gels

Assemble the gel sandwich plates according to the Mini-PROTEAN cell instructions (or for the appropriate apparatus being used). The gel slabs will be poured between two glass plates fitted with two 1.5-mm spacers. The gel sandwich must be placed in the gel casting apparatus so that there will be no leaks during gel casting. Insert the comb that will form the wells for the samples. Make a mark with a lab marker on the outside of each glass sandwich about 0.5 cm below the comb.

To prepare two 11% slab gels for use with the Mini-PROTEAN system, measure each of the following and degas in a sonicator for 60 seconds:

- 6 mL separating gel buffer
- 19 mL separating gel solution
- 23 mL SDS solution containing 34 mg ammonium persulfate prepared just before use

Carefully mix the three solutions and immediately transfer to the casting apparatus using a Pasteur pipet. Make sure to prevent air from being blown into the mixture by evacuating the air from the pipet before placing it into the mixture. Pipet the mixture directly between the glass plates for both gels. The mixture should be pipetted up to the mark made on the glass sandwich so that there will be room for the stacking gel and comb on top. Layer about 0.5 mL isobutyl alcohol on the top of the separating gel. This will eliminate the meniscus on top of the gel. Allow the gels to stand undisturbed until polymerization is complete. This will take about 1 hour.

Preparation of Stacking Gel

Carefully rinse the isobutyl alcohol from the top of the separating gel using distilled water. Remove as much of the water as possible by inverting the gel casting apparatus and shaking gently. To prepare the stacking gel, measure out each of the following:

- 2 mL stacking gel buffer
- 4 mL stacking gel solution
- 8 mL SDS solution
- 2 mL deionized water with 16 mg ammonium persulfate prepared just before use

Degas each solution for 30 seconds in a sonicator. Carefully mix the solutions and dispense to the top of the gel sandwich using a Pasteur pipet as before. Carefully insert the 1.5 mm combs into the top of the gel sandwich, starting from one end and lowering down into the solution so that no air bubbles are trapped underneath the comb. Allow the stacking gel to stand and polymerize. The stacking gel will be cloudy when polymerization is complete.

Electrophoresis of Proteins

Remove the polymerized gels from the gel casting apparatus and place on the Mini-PROTEAN cell. Add electrode buffer to the lower buffer chamber, place the Mini-PROTEAN cell in the chamber, and fill the upper chamber with electrode buffer. Underlay 10 µL of each protein marker in separate wells on one of the slab gels using a micropipetor fitted with a electrophoresis tip. Underlay 10 µL of each of the egg white and egg yolk samples into separate wells. Place protein markers and egg white and yolk samples on the second gel until all wells are used. Be sure to record the order in which the samples are pipetted. Using a Pasteur pipet, pipet 1 mL bromophenol blue (tracking dye) to the upper electrode buffer reservoir to allow visualization of the electrical front. Place the top on the electrophoresis apparatus, being sure to line up the positive and negative electrodes. Attach the electrical leads from the electrophoresis apparatus to the power supply. Be sure to connect positive to positive and negative to negative (red to red and black to black). Turn the power supply on and set it for 20 mA per gel (or 40 mA total). The electrical front will be marked by the bromophenol blue dye. It will take approximately 1 hour under these conditions for the electrical front to move down the slab. Be sure to stop electrophoresis before the tracking dye runs off the gel. Turn off the power supply and disconnect the electrodes.

Remove the gel holder from the electrophoresis apparatus. Carefully remove the top glass plate from the gel by lifting the glass plate on one corner with a small spatula while rinsing the gel with water from a wash bottle. Place a small notch in the top right-hand corner of the separating gel to mark the right side. This will allow you to determine the order of proteins on the gel after staining. (The stacking gel will be very fluid and can be discarded.) Also use a sharp razor blade to cut the gel along the front edge of the electrical front. The tracking dye will disappear during the fixing and staining process. (Alternatively, mark the center of the tracking dye front with a small piece of fine wire.) Place the gels in fixative for at least 10 hours. The gels may be left in fixative overnight. Be careful not to tear the gel. Pour off the fixative and stain the gels in staining solution for at least three hours. Overnight staining will give better results. Destain the gels by diffusion with several changes of fixative. Destaining will take approximately 15 hours. The gels may be left overnight in fixative. Gels should then be transferred to 7% glacial acetic acid for storage. Gels will swell somewhat in about three hours.

Results

Record the migration distance of the tracking dye and of the protein bands from the top of the separating gel. Carefully measure the gels with a ruler to 0.01 cm. (If a densitometer is available, the gels may be scanned instead.) To determine the relative mobility (R_f) of a protein, divide its migration distance from the top of the separating gel to the center of the protein band by the migration distance of the tracking dye from the top of the separating gel.

$$R_f = \frac{\textit{distance of protein migration}}{\textit{distance of tracking dye migration}}$$

The R_f values are plotted on the *x* axis against the known molecular weight on semi-logarithmic paper (*y* axis). Estimate the molecular weight of the proteins in egg white and egg yolk from the calibration curve. Several proteins are found in the egg white and egg yolk. Estimate the molecular weight for each protein band in the unknowns.

References

Hames, B.D.; Rickwood, D. *Gel Electrophoresis of Proteins;* IRL: Oxford, England, 1981; pp 1–12.

Peacocke, A.R.; Harrington, W.F. *Electrophoresis: Theory, Techniques, and Biochemical and Clinical Applications*, 2nd ed.; Clarendon: Oxford, England, 1987.

Sigma Chemical Company. *SDS Molecular Weight Markers in a Discontinuous Buffer.* Technical Bulletin No. MWS-877L, 1988.