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SDS Polyacrylamide Gel Electrophoresis of Proteins

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1. Introduction

SDS-PAGE is the most widely used method for qualitatively analyzing protein mixtures. It is particularly useful for monitoring protein purification, and because the method is based on the separation of proteins according to size, the method can also be used to determine the relative molecular mass of proteins (*see* **Note 14**).

1.1. Formation of Polyacrylamide Gels

Crosslinked polyacrylamide gels are formed from the polymerization of acrylamide monomer in the presence of smaller amounts of *N*,*N*'-methylene-*bis*-acrylamide (normally referred to as "*bis*-acrylamide") (**Fig. 1**). Note that *bis*-acrylamide is essentially two acrylamide molecules linked by a methylene group and is used as a crosslinking agent. Acrylamide monomer is polymerized in a head-to-tail fashion into long chains, and occasionally a *bis*-acrylamide molecule is built into the growing chain, thus introducing a second site for chain extension. Proceeding in this way, a crosslinked matrix of fairly well-defined structure is formed (**Fig. 1**). The polymerization of acrylamide is an example of free-radical catalysis, and is initiated by the addition of ammonium persulfate and the base *N*,*N*,*N*',*N*'-tetramethylenediamine (TEMED). TEMED catalyzes the decomposition of the persulfate ion to give a free radical (i.e., a molecule with an unpaired electron):

$$S_2O_8^{2-} + e^- \rightarrow SO_4^{2-} + SO_4^{-\bullet} \quad (1)$$

If this free radical is represented as $R^\bullet$ (where the dot represents an unpaired electron) and M as an acrylamide monomer molecule, then the polymerization can be represented as follows:

$$\begin{aligned} R^\bullet + M &\rightarrow RM^\bullet \\ RM^\bullet + M &\rightarrow RMM^\bullet \\ RMM^\bullet + M &\rightarrow RMMM^\bullet, \text{ and so forth} \end{aligned} \quad (2)$$

In this way, long chains of acrylamide are built up, being crosslinked by the introduction of the occasional *bis*-acrylamide molecule into the growing chain. Oxygen "mops up" free radicals, and therefore the gel mixture is normally degassed (the solutions are briefly placed under vacuum to remove loosely dissolved oxygen) prior to addition of the catalyst.

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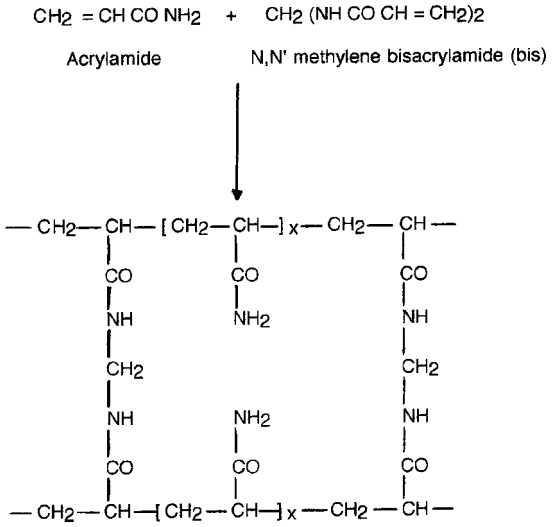

Fig. 1. Polymerization of acrylamide.

1.2. The Use of Stacking Gels

For both SDS and buffer gels samples may be applied directly to the top of the gel in which protein separation is to occur (the separating gel). However, in these cases, the sharpness of the protein bands produced in the gel is limited by the size (volume) of the sample applied to the gel. Basically the separated bands will be as broad (or broader, owing to diffusion) as the sample band applied to the gel. For some work, this may be acceptable, but most workers require better resolution than this. This can be achieved by polymerizing a short stacking gel on top of the separating gel. The purpose of this stacking gel is to concentrate the protein sample into a sharp band before it enters the main separating gel, thus giving sharper protein bands in the separating gel. This modification allows relatively large sample volumes to be applied to the gel without any loss of resolution. The stacking gel has a very large pore size (4% acrylamide) which allows the proteins to move freely and concentrate, or stack under the effect of the electric field. Sample concentration is produced by isotachophoresis of the sample in the stacking gel. The band-sharpening effect (isotachophoresis) relies on the fact that the negatively charged glycinate ions (in the reservoir buffer) have a lower electrophoretic mobility than the protein–SDS complexes. which in turn, have lower mobility than the Cl^- ions if they are in a region of higher field strength. Field strength is inversely proportional to conductivity, which is proportional to concentration. The result is that the three species of interest adjust their concentrations so that [Cl^-] > [protein-SDS] > [glycinate]. There are only a small quantity of protein–SDS complexes, so they concentrate in a very tight band between the glycinate and Cl^- ion boundaries. Once the glycinate reaches the separating gel, it becomes more fully ionized in the higher pH environment and its mobility increases. (The pH of the stacking gel is 6.8 and that of the separating gel is 8.8.) Thus, the interface between glycinate and the Cl^- ions leaves behind the protein–SDS complexes, which are left to electrophorese at

their own rates. A more detailed description of the theory of isotachophoresis and electrophoresis generally is given in **ref. *1***.

1.3. SDS-PAGE

Samples to be run on SDS-PAGE are first boiled for 5 min in sample buffer containing β-mercaptoethanol and SDS. The mercaptoethanol reduces any disulfide bridges present that are holding together the protein tertiary structure. SDS (CH_3-$[CH_2]_{10}$-$CH_2OSO_3^-Na^+$) is an anionic detergent and binds strongly to, and denatures, the protein. Each protein in the mixture is therefore fully denatured by this treatment and opens up into a rod-shaped structure with a series of negatively charged SDS molecules along the polypeptide chain. On average, one SDS molecule binds for every two amino acid residues. The original native charge on the molecule is therefore completely swamped by the SDS molecules. The sample buffer also contains an ionizable tracking dye usually bromophenol blue that allows the electrophoretic run to be monitored, and sucrose or glycerol which gives the sample solution density, thus allowing the sample to settle easily through the electrophoresis buffer to the bottom when injected into the loading well. When the main separating gel has been poured between the glass plates and allowed to set, a shorter stacking gel is poured on top of the separating gel, and it is into this gel that the wells are formed and the proteins loaded. Once all samples are loaded, a current is passed through the gel. Once the protein samples have passed through the stacking gel and have entered the separating gel, the negatively charged protein–SDS complexes continue to move toward the anode, and because they have the same charge per unit length they travel into the separating gel under the applied electric field with the same mobility. However, as they pass through the separating gel the proteins separate, owing to the molecular sieving properties of the gel. Quite simply, the smaller the protein, the more easily it can pass through the pores of the gel, whereas large proteins are successively retarded by frictional resistance owing to the sieving effect of the gel. Being a small molecule, the bromophenol blue dye is totally unretarded and therefore indicates the electrophoresis front. When the dye reaches the bottom of the gel the current is turned off and the gel is removed from between the glass plates, shaken in an appropriate stain solution (usually Coomassie brilliant blue) for a few hours, and then washed in destain solution overnight. The destain solution removes unbound background dye from the gel, leaving stained proteins visible as blue bands on a clear background. A typical large format gel would take about 1 h to prepare and set, 3 h to run at 30 mA, and have a staining time of 2–3 h with an overnight destain. Minigels (e.g., Bio-Rad minigel) run at 200 V. Constant voltage can run in about 40 min, and require only 1 h staining. Most bands can be seen within 1 h of destaining. Vertical slab gels are invariably run since this allows up to 20 different samples to be loaded onto a single gel.

2. Materials

1. Stock acrylamide solution: 30% acrylamide, 0.8% *bis*-acrylamide. Filter through Whatman No. 1 filter paper and store at 4°C (*see* **Note 1**).
2. Buffers:
   a. 1.875 *M* Tris-HCl, pH 8.8.
   b. 0.6 *M* Tris-HCl, pH 6.8.

3. 10% Ammonium persulfate. Make fresh.
4. 10% SDS (*see* **Note 2**).
5. TEMED.
6. Electrophoresis buffer: Tris (12 g), glycine (57.6 g), and SDS (2.0 g). Make up to 2 L with water. No pH adjustment is necessary.
7. Sample buffer (*see* **Notes 3** and **4**):

0.6 *M* Tris-HCl, pH 6.8	5.0 mL
SDS	0.5 g
Sucrose	5.0 g
β-Mercaptoethanol	0.25 mL
Bromophenol blue, 0.5% stock	5.0 mL

   Make up to 50 mL with distilled water.
8. Protein stain: 0.1% Coomassie brilliant blue R250 in 50% methanol, 10% glacial acetic acid. Dissolve the dye in the methanol and water component first, and then add the acetic acid. Filter the final solution through Whatman No. 1 filter paper if necessary.
9. Destain: 10% methanol, 7% glacial acetic acid.
10. Microsyringe for loading samples. Micropipet tips that are drawn out to give a fine tip are also commercially available.

3. Method

The system of buffers used in the gel system described below is that of Laemmli ***(2)***.

1. Samples to be run are first denatured in sample buffer by heating to 95–100°C for 5 min (*see* **Note 3**).
2. Clean the internal surfaces of the gel plates with detergent or methylated spirits, dry, then join the gel plates together to form the cassette, and clamp it in a vertical position. The exact manner of forming the cassette will depend on the type of design being used.
3. Mix the following in a 250-mL Buchner flask (*see* **Note 5**):

	For 15% gels	For 10% gels
1.875 *M* Tris-HCl, pH 8.8	8.0 mL	8.0 mL
Water	11.4 mL	18.1 mL
Stock acrylamide	20.0 mL	13.3 mL
10% SDS	0.4 mL	0.4 mL
Ammonium persulfate (10%)	0.2 mL	0.2 mL

4. "Degas" this solution under vacuum for about 30 s. Some frothing will be observed, and one should not worry if some of the froth is lost down the vacuum tube: you are only losing a very small amount of liquid (*see* **Note 6**).
5. Add 14 µL of TEMED, and gently swirl the flask to ensure even mixing. The addition of TEMED will initiate the polymerization reaction and although it will take about 15 min for the gel to set, this time can vary depending on room temperature, so it is advisable to work fairly quickly at this stage.
6. Using a Pasteur (or larger) pipet transfer this separating gel mixture to the gel cassette by running the solution carefully down one edge between the glass plates. Continue adding this solution until it reaches a position 1 cm from the bottom of the comb that will form the loading wells. Once this is completed, you will find excess gel solution remaining in your flask. Dispose of this in an appropriate waste container **not** down the sink.

7. To ensure that the gel sets with a smooth surface **very carefully** run distilled water down one edge into the cassette using a Pasteur pipet. Because of the great difference in density between the water and the gel solution the water will spread across the surface of the gel without serious mixing. Continue adding water until a layer of about 2 mm exists on top of the gel solution (*see* **Notes 7** and **8**).
8. The gel can now be left to set. As the gel sets, heat is evolved and can be detected by carefully touching the gel plates. When set, a very clear refractive index change can be seen between the polymerized gel and overlaying water.
9. While the separating gel is setting prepare the following stacking gel (4°C) solution. Mix the following in a 100-mL Buchner flask (*see* **Notes 8** and **9**):

0.6 *M* Tris-HCl, pH 6.8	1.0 mL
Stock acrylamide	1.35 mL
Water	7.5 mL
10% SDS	0.1 mL
Ammonium persulfate (10%)	0.05 mL

   Degas this solution as before.
10. When the separating gel has set, pour off the overlaying water. Add 14 μL of TEMED to the stacking gel solution and use some (~2 mL) of this solution to wash the surface of the polymerized gel. Discard this wash, and then add the stacking gel solution to the gel cassette until the solution reaches the cutaway edge of the gel plate. Place the well-forming comb into this solution, and leave to set. This will take about 20 min. Refractive index changes around the comb indicate that the gel has set. It is useful at this stage to mark the positions of the bottoms of the wells on the glass plates with a marker pen to facilitate loading of the samples (*see* also **Note 9**).
11. Carefully remove the comb from the stacking gel, and then rinse out any nonpolymerized acrylamide solution from the wells using electrophoresis buffer. Remove any spacer from the bottom of the gel cassette, and assemble the cassette in the electrophoresis tank. Fill the top reservoir with electrophoresis buffer, and look for any leaks from the top tank. If there are no leaks fill the bottom tank with electrophoresis buffer, and then tilt the apparatus to dispel any bubbles caught under the gel.
12. Samples can now be loaded onto the gel. Place the syringe needle through the buffer and locate it just above the bottom of the well. Slowly deliver the sample into the well. Five- to 10-μL samples are appropriate for most gels. The dense sample buffer ensures that the sample settles to the bottom of the loading well (*see* **Note 10**). Continue in this way to fill all the wells with unknowns or standards, and record the samples loaded.
13. Connect the power pack to the apparatus, and pass a current of 30 mA through the gel (constant current) for large format gels, or 200 V (constant voltage) for minigels (Bio-Rad). Ensure your electrodes have correct polarity: all proteins will travel to the anode (+). In the first few minutes, the samples will be seen to concentrate as a sharp band as it moves through the stacking gel. (It is actually the bromophenol blue that one is observing not the protein, but of course the protein is stacking in the same way.) Continue electrophoresis until the bromophenol blue reaches the bottom of the gel. This will take 2.5–3.0 h for large format gels (16 μm × 16 μm) and about 40 min for minigels (10 μm × 7 μm) (*see* **Note 11**).
14. Dismantle the gel apparatus, pry open the gel plates, remove the gel, discard the stacking gel, and place the separating gel in stain solution.
15. Staining should be carried out with shaking, for a minimum of 2 h. When the stain is replaced with destain, stronger bands will be immediately apparent, and weaker bands will appear as the gel destains (*see* **Notes 12** and **13**).

4. Notes

1. Acrylamide is a potential neurotoxin and should be treated with great care. Its effects are cumulative, and therefore, regular users are at greatest risk. In particular, take care when weighing out acrylamide. Do this in a fume hood, and wear an appropriate face mask.
2. SDS come out of solution at low temperature, and this can even occur in a relatively cold laboratory. If this happens, simply warm up the bottle in a water bath. Store at room temperature.
3. Solid samples can be dissolved directly in sample buffer. Pure proteins or simple mixtures should be dissolved at 1–0.5 mg/mL. For more complex samples suitable concentrations must be determined by trial and error. For samples already in solution dilute them with an equal volume of double-strength sample buffer. Do not use protein solutions that are in a strong buffer, that is, not near pH 6.5, since it is important that the sample be at the correct pH. For these samples, it will be necessary to dialyze them first. Should the sample solvent turn from blue to yellow, this is a clear indication that your sample is acidic.
4. The β-mercaptoethanol is essential for disrupting disulfide bridges in proteins. However, exposure to oxygen in the air means that the reducing power of β-mercaptoethanol in the sample buffer decreases with time. Every couple of weeks, therefore, mercaptoethanol should be added to the stock solution or the solution remade. Similarly protein samples that have been prepared in sample buffer and stored frozen should, before being rerun at a later date, have further mercaptoethanol added.
5. Typically, the separating gel used by most workers is a 15% polyacrylamide gel. This give a gel of a certain pore size in which proteins of relative molecular mass (M_r) 10,000 move through the gel relatively unhindered, whereas proteins of 100,000 can only just enter the pores of this gel. Gels of 15% polyacrylamide are therefore useful for separating proteins in the range of 100,000–10,000. However, a protein of 150,000 for example, would be unable to enter a 15% gel. In this case, a larger-pored gel (e.g., a 10% or even 7.5% gel) would be used so that the protein could now enter the gel, and be stained and identified. It is obvious, therefore, that the choice of gel to be used depends on the size of the protein being studied. If proteins covering a wide range of mol-wt values need to be separated, then the use of a gradient gel is more appropriate (*see* Chapter 12).
6. Degassing helps prevent oxygen in the solution from "mopping up" free radicals and inhibiting polymerization although this problem could be overcome by the alternative approach of increasing the concentration of catalyst. However, the polymerization process is an exothermic one. For 15% gels, the heat liberated can result in the formation of small air bubbles in the gel (this is not usually a problem for gels of 10% or less where much less heat is liberated). It is advisable to carry out degassing as a matter of routine.
7. An alternative approach is to add a water-immiscible organic solvent, such as isobutanol, to the top of the gel. Less caution is obviously needed when adding this, although if using this approach, this step should be carried out in a fume cupboard, not in the open laboratory.
8. To save time some workers prefer to add the stacking gel solution directly and carefully to the top of the separating gel, i.e., the overlaying step (**step 7**) is omitted, the stacking gel solution itself providing the role of the overlaying solution.
9. Some workers include a small amount of bromophenol blue in this gel mix. This give a stacking gel that has a pale blue color, thus allowing the loading wells to be easily identified.
10. Even if the sample is loaded with too much vigor, such that it mixes extensively with the buffer in the well, this is not a problem, since the stacking gel system will still concentrate the sample.
11. When analyzing a sample for the first time, it is sensible to stop the run when the dye reaches the bottom of the gel, because there may be low mol-wt proteins that are running

close to the dye, and these would be lost if electrophoresis was continued after the dye had run off the end of the gel. However, often one will find that the proteins being separated are only in the top two-thirds of the gel. In this case, in future runs, the dye would be run off the bottom of the gel, and electrophoresis carried out for a further 30 min to 1 h to allow proteins to separate across the full length of the gel thus increasing the separation of bands.

12. Normally, destain solution needs to be replaced at regular intervals since a simple equilibrium is quickly set up between the concentration of stain in the gel and destain solution, after which no further destaining takes place. To speed up this process and also save on destain solution, it is convenient to place some solid material in with the destain that will absorb the Coomassie dye as it elutes from the gel. We use a foam bung such as that used in culture flasks (ensure it is well wetted by expelling all air in the bung by squeezing it many times in the destain solution), although many other materials can be used (e.g., polystyrene packaging foam).
13. It is generally accepted that a very faint protein band detected by Coomassie brilliant blue, is equivalent to about 0.1 μg (100 ng) of protein. Such sensitivity is suitable for many people's work. However if no protein bands are observed or greater staining is required, then silver staining (Chapter 33) can be further carried out on the gel.
14. Because the principle of this technique is the separation of proteins based on size differences, by running calibration proteins of known molecular weight on the same gel run as your unknown protein, the molecular weight of the unknown protein can be determined. For most proteins a plot of $\log_{10}$ molecular mass vs relative mobility provides a straight line graph, although one must be aware that for any given gel concentration this relationship is only linear over a limited range of molecular masses. As an approximate guide, using the system described here, the linear relationship is true over the following ranges: 15% acrylamide, 10,000–50,000; 10% acrylamide 15,000–70,000; 5% acrylamide 60,000–200,000. It should be stressed that this relationship only holds true for proteins that bind SDS in a constant weight ratio. This is true of many proteins but some proteins for example, highly basic proteins, may run differently than would be expected on the basis of their known molecular weight. In the case of the histones, which are highly basic proteins, they migrate more slowly than expected, presumably because of a reduced overall negative charge on the protein owing to their high proportion of positively-charged amino acids. Glycoproteins also tend to run anomalously presumably because the SDS only binds to the polypeptide part of the molecule.

    To determine the molecular weight of an unknown protein the relative mobilities (*Rf*) of the standard proteins are determined and a graph of log molecular weight vs *Rf* plotted.

$$Rf = \text{(distance migrated by protein/distance migrated by dye)} \quad (3)$$

    Mixtures of standard mol-wt markers for use on SDS gels are available from a range of suppliers. The *Rf* of the unknown protein is then determined and the logMW (and hence molecular weight) determined from the graph. A more detailed description of protein mol-wt determination on SDS gels is described in **refs. *1*** and ***3***.

References

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