

Electrophoretic Methods for the Separation and Identification of Muscle Proteins

Marion L. Greaser*

Lawrence D. Yates¹

Karol Krzywicki²

Daniel L. Roelke

Introduction

Electrophoresis is a method by which charged molecules are caused to move by the application of an electrical field. The velocity of movement depends on the molecule's net charge and frictional resistance (size and shape dependent) and on the strength of the electrical field applied. This technique has been used most extensively with proteins, although other charged macromolecules can be separated in a similar fashion.

Protein electrophoresis received a major impetus in 1964 with the description of a polyacrylamide gel procedure for the separation of serum proteins (Davis, 1964). Unfortunately, the muscle myofibrillar proteins are mostly insoluble under these conditions of electrophoresis. A new approach was later used by Weber and Osborn (1969) with the inclusion of sodium dodecyl sulfate in the sample and gel solutions. This detergent was found to bind to most proteins in a relatively constant weight ratio. As a result, the proteins have a high negative charge and a migration rate inversely proportional to the logarithm of their subunit weights. SDS dissociates proteins to the subunit level if they are not covalently linked. All of the myofibrillar proteins are soluble in SDS without the normally required high salt concentration. While Weber and Osborn used phosphate buffer in their gel system, most researchers now utilize the stacking gel procedure described by Laemmli (1970). A useful book on protein electrophoresis has been edited by Hames and Rickwood (1981).

The procedure described below has been used successfully in our laboratory. It is a modification of the Laemmli system including changes proposed by Thomas (1978). The major alterations are: (1) doubling the buffer concentration in the separating gel and reservoirs, (2) changing the acrylamide: Bis ratio to 20:1 in the stacking gel, (3) changing

the acrylamide: Bis ratio to 200:1 in the separating gel. The latter change in particular markedly improves band resolution. In fact the high acrylamide: Bis ratio used by Porzio and Pearson (1977) probably accounts in large part for the good resolution obtained with their system. The elaborate purification procedures for the different reagents outlined in this paper appear to be of minor importance if the usual electrophoresis chemicals are employed.

Figure 1.

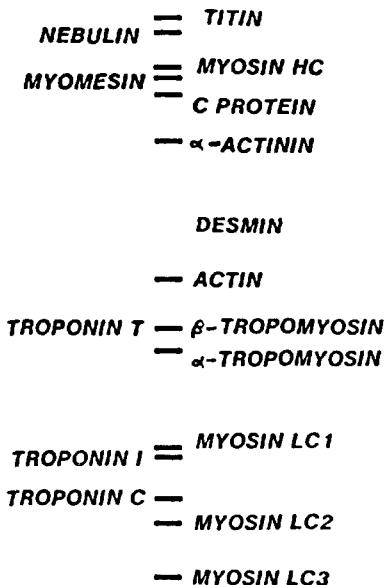


Figure 1. Migration positions of the major myofibrillar proteins on a 15% SDS polyacrylamide gel.

A schematic profile of the major myofibrillar proteins is shown in Figure 1. Desmin is only visible with extremely high protein loads; most of the other proteins are usually observed. The titin and nebulin bands are normally not clearly resolved on 15% gels but require a lower percent acrylamide and an altered buffer system (Wang, 1982). Electrophoresis of whole muscle gives additional complexity because of the presence of the sarcoplasmic proteins.

*M. L. Greaser, Muscle Biology Laboratory, University of Wisconsin, Madison, WI 53706

¹L. D. Yates — current address: Department of Physiology and Biophysics, University of Washington, Seattle, WA 98195

²K. Krzywicki — current address: Meat and Fat Research Institute, Poznan, Poland

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Apparatus

1. Vertical Slab Gel Unit — Hoefer Scientific Instruments, 610 Fifth Street, San Francisco, California, 94107 or Bioread Laboratories, 2200 Wright Avenue, Richmond, California, 94804.
2. A constant current power supply — available from above companies as well as other sources.

Reagents

Biorad

Acrylamide (electrophoresis grade)
Bis (N'N — methylene-bis acrylamide)

Sodium Dodecyl Sulfate

TEMED

Coomassie Brilliant Blue R-250

Sigma Chemical Company, Box 14508, St. Louis, MO
63178

Tris (Tris [hydroxymethyl] aminomethane) — trizma base

Glycine (free base, ammonia free)

Thiourea

2-mercaptoethanol

Bromphenol blue

Ammonium persulfate

All other chemicals are reagent grade.

Stock Solutions

1. Resolving gel acrylamide — 30% acrylamide, 0.15% Bis. Weigh out 30g of acrylamide and 0.15g Bis into a beaker, add about 50 ml of water, stir till dissolved, dilute to 100 ml. Filter through a 0.45 micron filter (such as a Millex-HA, Millipore Corporation, Bedford, Massachusetts, 01730). Store in a brown bottle in the cold room (4°C). Danger! Avoid skin contact.
2. Stacking gel acrylamide — 10% acrylamide, 0.5% Bis. Treat as above.
3. 3M Tris, pH 8.8 — Dissolve, adjust to final pH with concentrated HCl. Be sure to use a pH electrode suitable for Tris. Store in the cold.
4. 0.5M Tris, pH 6.8 — Dissolve, adjust to final pH with concentrated HCl. Make final pH adjustments with the buffer at room temperature (the pH of Tris has a strong temperature dependence). Store in the cold.
5. 10% (W/V) SDS — Dissolve, filter through 0.45 micron filter. Store at room temperature.
6. Reservoir buffer concentrate (5X) — 0.25 M Tris — 1.92 M glycine — 0.5% SDS. Store in the cold.
7. Ammonium persulfate — Prepare a 100 mg/ml solution in water; make fresh each day.
8. Sample buffer A — 8M urea — 2M thiourea — 0.05M Tris (pH 6.8) — 0.7M 2-mercaptoethanol — 3% SDS. The thiourea should be deionized by passing a 2M solution through a mixed bed ion exchange resin and subsequently lyophilizing it. The urea and thiourea occupy almost 50% of the final volume of the total solution; therefore, add water with care. You may need to warm the solution slightly to get all the urea-thiourea to dissolve. Prepare this buffer fresh or store frozen and thaw when needed. Avoid boiling or extended heating — the urea will form cyanate which

reacts with proteins and alters migration rates.

9. Sample buffer B — 0.05M Tris (pH 6.8) — 0.7M 2-mercaptoethanol — 3% SDS — 10% glycerol.
10. Bromphenol blue — 0.1% bromphenol blue — 30% glycerol.
11. Staining solution — weigh out 0.45g of Coomassie Brilliant Blue R-250 and add 900 ml of 50% methanol — 9.2% acetic acid. Stir till dissolved completely (about 5-10 minutes). Store at room temperature. Can be used for 10-15 slab gels; discard if a scum appears on top of the solution.

Gel Preparation

1. Resolving gel (15% acrylamide) — Into a 125 ml filtering flask, add the following:
25 ml of 30% acrylamide — 0.15% Bis
12.5 ml of 3M Tris, pH 8.8
0.5 ml of 10% SDS
0.125 ml of 100 mg/ml ammonium persulfate
11.9 ml of water
Swirl to mix thoroughly, attach to a water aspirator to degas the solution (1 minute).
2. Add 0.01 ml of TEMED, swirl to mix, immediately transfer the solution to the previously assembled slab gel apparatus, insert separating gel former on top of the acrylamide and avoid trapping any air bubbles. Allow to polymerize. The optimal time is 10 to 20 minutes. If the gel sets more quickly, decrease the amount of ammonium persulfate; if the gel takes longer, increase the ammonium persulfate. There is considerable batch to batch variation in the acrylamide and ammonium persulfate so the levels need to be readjusted. Also the degree of degassing affects the polymerization rate — dissolved oxygen retards polymerization. The acrylamide is gelled when a clear line can be observed 1 to 2 mm below the gel former.
3. Remove separating gel former, rinse off unpolymerized upper layer with water.
4. Stacking gel (3% acrylamide) — Into a 50 ml filtering flask, add the following:
6 ml of 10% acrylamide — 0.5% Bis
5 ml of 0.5M Tris (pH 6.8)
0.2 ml of 10% SDS
0.1 ml of 100 mg/ml ammonium persulfate
8.7 ml of water
Swirl to mix, degas 1 minute, add 0.01 ml TEMED, swirl to mix, immediately transfer to the top of the slab gel. Insert the sample combs, avoid trapping air bubbles. Allow to polymerize.

5. After polymerization, remove the sample combs and rinse out the wells with water.

Sample Preparation

- A. Whole Muscle — Portions (25 to 35 mg) of muscle tissue are weighed into a pre-weighed 5 ml glass homogenizing tube. Add 140 mg of sample buffer A per mg tissue, homogenize with a Teflon pestle, sonicate for 10 minutes, and then heat in a boiling water bath for 1 minute. Cool to room temperature, repeat the Teflon homogenization. Samples should be prepared immediately before application to the gels. If specimens must be stored before analysis, it is better to keep the tissue at -20°C rather than storing the proteins in sample buffer. Sonication may

- be omitted without seriously affecting the solubilization efficiency.
- Myofibrils — Washed myofibril suspensions are centrifuged at 15,000 xg for 15 minutes and the supernatant solution decanted off. Weigh approximately 15 mg of the pellet into a pre-weighed 5 ml glass homogenizing tube. Add 0.2 ml of sample buffer A per mg of myofibril pellet (the protein content of the pellets is about 0.25-0.30 mg/mg). Treat the myofibrils as described for whole tissue.
 - Soluble proteins — Pipette 0.1 ml of protein solution (1 to 5 mg/ml) into a small glass test tube. Add 0.05 ml of sample buffer B, mix. Heat for 1 minute in a boiling water bath or 2 minutes in a block heater.

Electrophoresis

Assemble the electrophoresis unit and fill the lower and upper reservoirs with diluted reservoir buffer (0.05M Tris — 0.384 M glycine — 0.1% SDS). Insert the samples through the reservoir buffer and into the wells with a microsyringe equipped with a long needle or with a small piece of polyethylene tubing attached to the end of a Pipetman pipette tip. Be sure to rinse the syringe or tubing between samples to avoid cross-contamination. Add 5 microliters of bromphenol blue to each sample well. Attach unit to the power supply — be sure to connect the positive lead to the lower chamber. Apply 24 milliamp constant current until tracking dye reaches the bottom of the gel (about 8-9 hours). Alternatively use 10 to 12 millamps for 16 hours. Use a continuous flow of tap water to cool the unit during electrophoresis.

After completion of the run, turn off the current and disassemble the glass plates. Use disposable plastic gloves to handle the gel. Staining should be done immediately to avoid band broadening by diffusion.

Staining

- Rapid — Place gel and stain solution in a Pyrex baking dish. Heat on a hot plate to 50°C, remove dish from the heat and allow to stand for 10 minutes.
- Standard — Place gel and stain solution in a Pyrex dish. Incubate at 40°C in an oven for 2 hours. This procedure is preferable if gel densitometry is contemplated.

Destaining

Gels may be destained by soaking in several changes of 10% methanol — 7.5% acetic acid until the background is clear. Alternatively, more rapid destaining can be accomplished in a gel destainer (Biorad) which circulates the methanol — acetic acid past the gel. A solution of 25% ethanol — 5% acetic acid removes the excess Coomassie stain even more rapidly, but care must be taken to prevent destaining of the protein bands as well. If a gel becomes over-destained or fades, the staining can be repeated.

Storage

Stained gels may be stored in Ziploc sandwich bags or dried onto Mylar sheets using a gel dryer (Biorad).

Additional Comments:

- We normally use 1.5 mm thick gels. The band separation is improved with 0.75 mm gels, but the thinner gels are more easily broken during staining — destaining.
- The high molecular weight proteins (>100,000

daltons) are better resolved on 5% acrylamide. Simply reduce the volume of acrylamide in the separating gel and replace it with water. The stacking gel composition can remain the same.

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Discussion

Question:

What should I do if the sample contains high salt?

Answer:

High salt (>0.5M) will definitely cause problems in electrophoresis. You might want to homogenize the sample in buffered 20 mM NaCl and centrifuge the homogenate at 1000 xg to precipitate the myofibrils. Another possibility would be to make a homogenate and then dialyze it against a low salt buffer. In both cases, the sample manipulation may result in protein changes (such as proteins coming off the myofibril or degradation by proteases) that may complicate the interpretations.

Question:

Does freezing meat or separated proteins affect the electrophoresis?

Answer:

Most proteins are unaffected by freezing. However, the myosin heavy chain and especially titin may irreversibly aggregate.

Question:

Which system is better, slab or tube gels?

Answer:

For most applications, slab gels are superior. First, the bands from all of the samples applied are lined up. Second, there is much less labor involved in working with one slab with 20 sample wells than 20 separate glass cylinders. Third, the protein bands are usually narrower in slab gels than tube gels, allowing better resolution. Fourth, the time necessary to stain and destain is less in a 0.75 or 1.5 mm slab compared to a 6 mm tube gel. One real advantage with tube gels is the possibility of using larger volumes of sample if the sample protein concentration is too low. Another advantage is that cross-contamination between different tube gels is virtually impossible if good techniques are used while with slab gels this is a common occurrence.

Question:

Is there some condition where the position of tropomyosin can be altered?

Answer:

Yes. Sender (1971) showed that including 5 M urea in the separating gel caused tropomyosin bands to have apparent molecular weights in the 50,000 dalton range while all the rest of the myofibrillar proteins migrated normally. This trick may be particularly useful in looking for changes in troponin T because it moves the tropomyosin out of the interfering region of about 35,000 daltons.

Question:

Are there differences in SDS gel patterns between different beef muscles?

Answer:

Yes. The general pattern of the positions of the myofibril proteins shown in Figure 1 applies only to so-called white fiber muscle. There are different isoenzymes for the myosin light chains and troponin subunits in red muscle which have slightly different molecular weights. Since most muscles have varying mixtures of red and white fibers, the resulting protein patterns may be quite complex. Young and Davey (1981), Young (1982) and Salviati et al. (1982) have dealt with this problem by doing electrophoresis on single muscle fibers. An additional complication is that the myosin light chain 3 is present in variable amounts and may not be visible at all.

Question:

Can you distinguish animal species by SDS gel electrophoresis?

Answer:

No. The patterns for beef, pork, lamb and rabbit look very much the same. Isoelectric focusing, however, may be useful to detect different species.

Question:

Are there any advantages with gradient gels?

Answer:

Yes. Some people use a 5% to 20% gradient of acrylamide concentration. This allows the large proteins (myosin heavy chain, C protein, etc.) to be separated more widely and also the proteins in the lower molecular weight regions (15,000-30,000). The major problem with gradient gels is that it is difficult to reproduce the same gradients and gel patterns from day to day. Most people find the slightly better resolution is not worth the bother.

Question:

What causes uneven bands?

Answer:

The main problem is the surface between the stacking and resolving gel. If the gel polymerizes too rapidly, this surface may be rough. Most slab gel kits use a plastic spacer (the separating gel former) on top of the separating gel during the polymerization. We sometimes use water saturated insobutanol on top of the acrylamide during the polymerization for improved performance.

Question:

Why do I get streaking on my gels?

Answer:

Streaking may result when some of the sample is not fully dissolved. It may also occur when the gel pulls away from the glass (due to improper cleaning, too rapid polymerization, or inadequate degassing).

Question:

Why are my gel bands so wide?

Answer:

There may be several causes. First, if you are not using a stacking system, the bands are generally wider. This is particularly true if you use large sample volumes. Second, all the lower molecular weight proteins are usually wider than

the high molecular weight ones. Third, if you don't transfer the gel into the fixing-staining solution quickly after the current is turned off, the bands broaden due to diffusion. Fourth, the bands tend to get wider if the sample is stored in sample buffer too long.

Question:

Are there alternatives to Coomassie blue R-250 for staining?

Answer:

Yes. Several others have been used. In the early work, Amido black was employed, but it has much less sensitivity than Coomassie and is difficult to destain. Some people use Coomassie blue G-250 in trichloroacetic acid (Chrambach et al., 1967). This procedure allows more rapid visualization of the bands but is less sensitive. Fast green can also be used, especially for quantitative work, because it gives a more linear response to protein concentration than Coomassie (Gorovsky et al., 1970; Bertolini et al., 1976). For very high sensitivity, staining methods using silver have been employed; we have adapted a procedure which has the sensitivity to stain the myosin light chains from a 0.2 mm long segment of a single muscle fiber (Giulian et al., 1983). The method is too complex for routine use unless sample size is limiting. Further details concerning the relative attributes of different stains can be found in a recent review (Wilson, 1983).

Question:

Do all proteins bind the same ratio of dye?

Answer:

No. There is at least a 2 or 3 fold difference between different proteins. This means that if you want to determine the concentration of a certain protein, you must prepare a standard curve with that specific protein.

Question:

How good is the gel densitometry technique?

Answer:

There are definite problems with it. First, with tube gels, the circular geometry makes it difficult to pass the light beam through the very center of the gel. With slab gels, this isn't a problem but another one occurs — the increasing width of the bands perpendicular to the migration direction the greater the distance of migration. Second, you must be sure that the stain response versus protein load is in the linear region. Third, you must be very careful with the staining and destaining protocols to insure reproducible results. Fourth, there is usually difficulty in determining the true baseline. The net result of all these factors is a degree of inconsistency that masks small differences. We have recently used an alternative approach, namely amino acid analysis of the stained bands, for protein quantitation (Yates and Greaser, 1983).

Question:

Are there other alternatives to protein quantitation besides densitometry and amino acid analysis?

Answer:

Everett and coworkers (1983) have described a couple of new procedures. One is to radioiodinate the Coomassie dye and then count the separate bands. Another is to use an RIA or ELISA procedure if you have antibodies against the specific protein of interest.

Question:

What is a normal protein load to apply?

Answer:

That depends on the degree of heterogeneity of the sample. If you have a single pure protein, 1 or 2 µg will give a nice band with Coomassie staining. If you use whole muscle, then 50 to 100 µg should be applied to see most of the myofibrillar proteins.

Question:

Should I measure protein by Kjeldahl before deciding on the protein load?

Answer:

That is unnecessary. You can adequately guess the protein amount by assuming 20% protein for lean muscle samples. With myofibrils which have been centrifuged, they are 25% to 30% protein by weight. If you are working with solubilized proteins, a biuret or dye binding method should get you a reasonable estimate. The electrophoresis technique is quite tolerant of variations in protein load.

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