0273-2289/93/4202-03—0201$03.00

Protocols in Biotechnology

Polyacrylamide Gel Electrophoresis of Proteoglycans in Large-Pore Gradient Gels

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ABSTRACT

A procedure is described for preparing large-pore polyacrylamide gradient gels (1.2–16% T, 10–4% C) that allow the separation of large proteoglycans of molecular mass up to several millions.

Index Entries: Polyacrylamide gel electrophoresis; proteoglycans; high-molecular-mass proteins.

INTRODUCTION

SDS-PAGE in the discontinuous buffer system according to Laemmli (*1*) has become a standard laboratory technique for the rapid determination of the molecular weights of proteins. The lowest concentration of acrylamide that can be used to prepare the stacking gel, which also forms the sample wells, is about 3%. The upper limit of apparent molecular weight of the molecules that enter these gels is approx 1 million daltons. The separation range can be extended by preparing the stacking gel from a polyacrylamide-agarose composite that also forms the sample wells and stabilizes the very loose gel at the top of the separating polyacrylamide gradient gel (*2*). The pore size of the composite gel (approx 1.1% T, 5% C, 0.4% agarose) is large enough to allow even very large molecules (of molecular weight up to several million daltons) to pass; these then enter the separating gradient gel (1.2–16% T, 10–4% C), where they separate according to their sizes.

MATERIALS

All stock solutions containing acrylamide as well as stock solutions of separating gel buffer and sample gel buffer are to be stored at 4°C. All other solutions are stored at room temperature. Remember that acrylamide is toxic. Wear gloves, and do not pipet solutions by mouth.

Separating Gel

1. Light acrylamide stock solution: 3.24% acrylamide/0.36% *N,N*-methylene-*bis*-acrylamide.
2. Dense acrylamide stock solution: 46.08% acrylamide/1.92% *N,N*-methylene-*bis*-acrylamide.
3. Separating gel buffer: 0.75*M* Tris-HCl, pH 8.8.
4. 10% Sodium dodecyl sulfate (SDS), w/v (aqueous).
5. 10% Sodium persulfate w/v. Always make fresh before use.
6. *N,N,N′,N′*-tetramethylethylenediamine (TEMED).
7. Covering solution: 0.375*M* Tris-HCl, pH 8.8/0.1% SDS.

Stacking Gel

1. Solution of acrylamide: 2.96% acrylamide/0.16% *N,N*-methylene-*bis*-acrylamide.
2. Stacking gel buffer: 0.25*M* Tris-HCl, pH 6.8.
3. 0.06% Sodium persulfate w/v. Always make fresh before use.
4. High-gelling-temperature agarose.

Electrophoresis

1. Electrode buffer pH 8.4: 30.3g Tris, 144 g glycine, 10 g SDS, make up to 1 l. Dilute fivefold before use; this gives 0.05*M* Tris/0.38*M* glycine in the working solution of the electrode buffer.
2. Sample buffer (reducing): 0.06*M* Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.01% Bromophenol blue, 5% 2-mercaptoethanol.

Staining and Drying

1. Stock solution of dyes: 0.5% Coomassie Blue R in 40% ethanol/8% acetic acid; 1% Alcian Blue in 40% ethanol/8% acetic acid. The stock solution of Coomassie Blue R has to be prepared fresh after about 2 wk, since the dye slowly precipitates from the solution. The stock solution of Alcian Blue is stable over the course of several months.
2. Washing solution: 40% ethanol/8% acetic acid.
3. "Drying" solution: 40% methanol/3% glycerol.

Equipment: Vertical electrophoresis unit, gradient former.

METHOD

The reagent volumes given below are used to cast one gel of volume 30 mL (dimensions measuring approx 140×130×1.5 mm). To prepare a gel of different dimensions, the volumes of individual solutions must be changed proportionately.

Casting of the Separating Gel

1. Connect the gradient former to the assembled gel mould via a peristaltic pump. Level the gradient former on the magnetic stirrer. Level the gel mould.
2. Briefly mix 5 mL dense acrylamide stock solution, 7.5 mL separating gel buffer, 2.25 mL H_2O, 0.15 mL 10% SDS, and 10 μL TEMED, and pour the solution into the mixing chamber of the gradient former. Open the connection tube between the mixing and the reservoir chamber briefly to fill it with a solution, and then close it again.
3. Briefly mix 5 mL light acrylamide stock solution, 7.5 mL separating gel buffer, 2.15 mL H_2O, 0.15 mL 10% SDS, 0.2 mL 10% sodium persulfate, and 20 μL TEMED, and pour the mixture into the reservoir chamber of the gradient former.
4. Begin to stir the contents of the mixing chamber. Add 0.1 mL 10% sodium persulfate. Open the connection tube between the mixing and the reservoir chamber and an outlet of the mixing chamber. Start pumping. Dispense the gradient into a gel mould by means of a peristaltic pump at an initial rate of about 15 mL/min; for the final one-third of the gradient volume, lower the dispensing rate to about 3 mL/min to prevent remixing of the solution in the gel mould. Stop the pump before any air bubbles leave the tubing.
5. Let the gel polymerize at room temperature for 2–4 h. Then overlay it with the covering solvent and leave it overnight.

Casting of the Stacking Gel

1. Prepare a mixture of 5 mL stacking gel buffer, 0.7 mL H_2O, and 100 μL 10% SDS solution, and add it to 40 mg agarose in an Erlenmeyer flask. Cover the flask with aluminum foil, and heat it, with constant stirring, until the agarose melts. Be prepared to remove the flask from the hotplate immediately if boiling occurs.
2. While the agarose is heating, remove the covering solvent from the top of the gel, insert a well-forming comb between the glass plates, and transfer the gel to an oven preheated to 50°C.

3. Mix 3.6 mL acrylamide stock solution for the stacking gel with 75 μL TEMED, and place it briefly on the hotplate.
4. Take the gel mould out of the oven and level it.
5. Mix the warm solution of acrylamide with the solution of hot melted agarose. Add 375 μL 0.06% sodium persulfate and mix again briefly. Pipet the hot solution quickly onto the surface of the gel so that the comb's teeth are submerged to a depth of about 5 mm.
6. Wait until the remaining agarose in the Erlenmeyer flask solidifies (about 30 min), and then fill the upper part of the gel mould to submerge the stacking gel with electrode buffer using a syringe and thin needle. Transfer the gel to a refrigerator for 2–3 h.
7. Carefully remove the comb.

Electrophoresis

1. Place the gel into an electrophoretic tank, fill the reservoirs with an electrode buffer (stock solution diluted fivefold), and load the samples.
2. Apply a current of 5 mA/gel until the samples enter the gel. For a fast run, apply a current of 25 mA/gel. Alternatively, run the gel overnight at 7 mA/gel.
3. Stop the current when the tracking dye reaches the end of the gel. Dismantle the gel sandwich carefully to prevent the stacking gel from tearing off the upper end of the gradient gel.

Staining and Drying

1. Wash the gel four times for 60 min with 200 mL 40% ethanol/8% acetic acid to remove SDS.
2. To stain, place the gel in 200 mL 40% ethanol/8% acetic acid. Add 50 μL each of the stock solutions of each dye, and leave the gel to shake gently overnight.
3. Equilibrate the stained gel with a solution of 40% methanol/3% glycerol for 4 h. Place the gel between two cellophane sheets soaked in the same solution. Mount the sandwich into a plastic frame, and leave it to air-dry for 2 d.
4. Plot the R_f of the molecular weight standards vs the log molecular weight to construct a calibration curve.

NOTES

1. It is somewhat tricky to remove the comb from the stacking gel without damaging the wells. Preparing a 10-well gel is easier, but with a little practice, a 20-well comb can be used successfully.

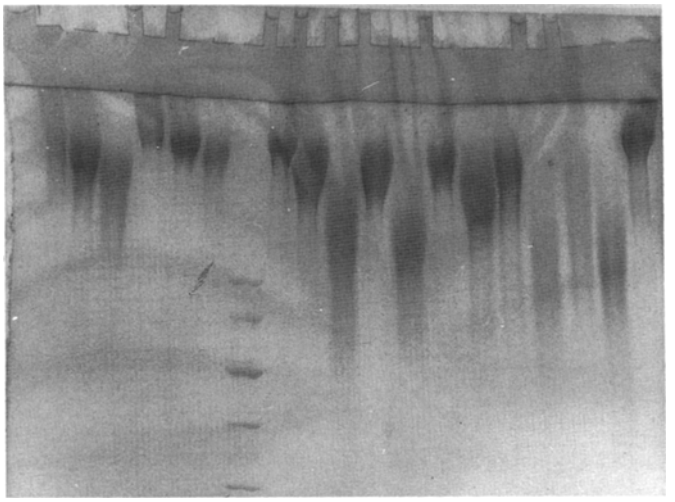

Fig. 1. SDS-PAGE (1.2–16% gradient) of various populations of aggrecan previously isolated by gel chromatography from extracts of bovine nasal and human articular cartilage. Molecular weight standards (lane 7 from the left) are myosin (212,000), α_2-macroglobulin (170,000), β-galactosidase (116,000), transferrin (76,000), and glutamic dehydrogenase (53,000).

Making the sample wells as shallow as possible significantly facilitates the removal of the comb. The presence of the buffer between comb teeth during its removal is also helpful.

2. Any more-or-less steep gradient (e.g., 1.2–10%, 1.2–20%) can be prepared using a protocol analogous to that described here. The gradient described here resolves all main proteoglycans present in cartilage, i.e., several high-molecular-weight populations of aggrecan (Fig. 1), and also biglycan, decorin, and fibromodulin. The gel also has good mechanical stability and can be used to prepare immunoblots. However, for proteoglycans present in other tissues, using a different gradient may be advantageous.
3. Molecular weight standards on the order of millions daltons are not commercially available. Populations of large proteoglycans, however, can be compared to each other or to an appropriate proteoglycan standard, with results similar to those achieved with analytical gel chromatography. Compared to gel chromatography, PAGE requires smaller sample amounts and is much faster, since up to 20 samples may be compared directly in one run.
4. The migration of large proteoglycans in gradient gels does not depend on SDS binding, likely being mediated rather by their own negative charges. However, usage of SDS is necessary if one wants to employ protein molecular weight standards, and

allows these large-pore gradient gels to be used routinely in the same way as any other SDS-PAGE system.

5. Cracking of the gel during drying is most probably caused by insufficient equilibration with glycerol. The time of equilibration with the "drying" solution can be prolonged; alternatively, the concentration of glycerol in the "drying" solution may be increased to up to 10%.
6. When stained with Coomassie Blue and Alcian Blue, the amount of sample loaded on the gel is not very critical. Protein amounts in the range 1–10 μg/track and proteoglycan amounts corresponding to 2–6 μg of chondroitin sulfate/track can usually be analyzed without significantly influencing a resolution. The amount of each dye added to the gel when staining can also be varied (i.e., increased for shorter staining times).
7. The concentration of the electrode buffer recommended here is that used by Fisher et al. (3) for SDS-PAGE of proteoglycans, i.e., twice as high compared to the Laemmli buffer system.

ACKNOWLEDGMENT

Development of this technique was supported by grant 0227-3 from the Internal Grant Agency of the Ministry of Health, Czech Republic.

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