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Crossed Immunoelectrophoresis from Sodium Dodecyl Sulfate–Polyacrylamide Gels after Fixation and Staining and without Nonionic Detergent Intermediate Layers[1]

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A modified method is described for crossed immunoelectrophoresis in which the first-dimension separation has been carried out by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The described method does not require nonionic detergents and is carried out after fixation and staining of the polyacrylamide gel. This permits more precise alignment of immunoprecipitates with polypeptide bands as well as allowing direct testing of an individual polypeptide band for reaction with antibody.

The coupling of polyacrylamide gel electrophoresis with crossed immunoelectrophoresis has yielded hybrid techniques (1–4) with greater resolution, sensitivity, and specificity than either of these two powerful techniques independently. The electrophoresis of proteins from a polyacrylamide gel into antibody-containing agarose requires only a brief soak in the buffer used in the second-dimension electrophoresis (1), providing no ionic detergent has been used in the first-dimension (polyacrylamide gel) electrophoresis. Inclusion of an ionic detergent, usually sodium dodecyl sulfate (SDS),[2] as a solubilizing agent for membrane polypeptides or for molecular weight determination during the first-dimension electrophoresis results in nonspecific precipitation of serum proteins in the second dimension gel (2). This has been overcome by inclusion of nonionic detergent (Lubrol PX or Triton X-100) in an intermediate layer between the SDS–gel strip and the antibody-containing agarose (2). The nonionic detergent presumably incorporates free SDS from the first-dimension gel into mixed micelles thereby preventing nonspecific precipitation. Modifications of this technique allow better alignment of immunoprecipitates with SDS–gel bands (3) and overcome failure of membrane polypeptides to migrate out of the first-dimension gel by use of a mobilizing ionic detergent (4). The use of nonionic detergents (2–4) may be required for the maintenance of solubility of hydrophobic membrane polypeptides during electrophoresis in the second dimension. As demonstrated below, the electrophoresis of proteins from an SDS–gel strip into antibody-containing agarose may also be carried out after fixation and staining of the SDS–polyacrylamide gel. This allows for increased precision in alignment of immunoprecipitates in the agarose gel with stained bands in the acrylamide gel and also permits the examination of individual protein bands for reaction with antibody. Removal of free, interfering SDS is accomplished during fixation, staining, and destaining of the first-dimension gel.

MATERIALS AND METHODS

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–gel electrophoresis) was carried out on 10% polyacrylamide

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[2] Abbreviation used: SDS, sodium dodecyl sulfate.

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(BDH Biochemicals Ltd., Poole, England) slab gels using the buffer system of Laemmli (5). After electrophoresis, gels were fixed in isopropanol/acetic acid/water (25:7:68) (Solution A) for 30 min, stained overnight in 0.2% Coomassie blue R-250 (Biorad Laboratories, Richmond, Calif.) in solution A, and destained in several changes of solution A. The gels were immersed in distilled water for 2 to 3 h prior to photography, storage, or second-dimension electrophoresis.

Immunoelectrophoresis in the second dimension was carried out on either 50 × 50-mm or 83 × 102-mm glass plates. The latter plate size accomodated the entire length of the running gel from the first-dimension electrophoresis (100 mm). Use of the former conserved antisera while still permitting analysis of the portion of first-dimension gel of interest. One percent (w/v) agarose (Seakem HGT, Marine Colloids, Rockland, Maine) in barbital/HCl buffer (I = 0.02, pH 8.6) was used for all second-dimension gels. Agarose was cast on precoated glass plates (6) to give a volume-to-surface area ratio of 0.14 ml/cm^2. A schematic diagram showing the arrangement and dimensions of the acrylamide and agarose gels on each plate size is shown in Fig. 1. Although a 5-mm wide polyacrylamide gel strip is shown, strips as narrow as 2 mm were also used. Each strip was immersed in the barbital buffer for 5–10 min at approximately 14°C prior to casting of agarose around the strip. Electrophoresis in the second dimension was carried out at 70 V for 24 h in a Holm-Nielsen electrophoresis cell (Copenhagen, Denmark) a constant temperature of 14°C. Each reservoir of the cell contained 1 liter of the barbital/HCL buffer. Handi-Wipes cloths were used as wicks. After immunoelectrophoresis the nonantibody-containing layers were excised. The antibody-containing agarose layers were pressed, washed, and stained on the glass plate as previously described for conventional crossed immunoelectrophoresis (6).

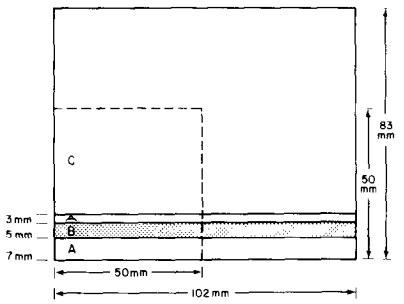

FIG. 1. A schematic illustration of the arrangement and dimensions of the gel layers for immunoelectrophoresis of polypeptides from a polyacrylamide gel strip. The polyacrylamide gel strip (B) is positioned as shown and agarose is poured on either side in the sections marked A. Either a brass bar or an agarose barrier can be used to form the agarose within area A (6). The antibody-containing agarose is then poured in section C. The dotted line shows the limits of the smaller plate as used for Fig. 2.

To test for the presence of antibody against individual polypeptide bands, 3.5 ml of antibody-containing agarose was cast on 50 × 50-mm plates and large (2 mm) wells punched as for rocket immunoelectrophoresis (6). Pieces (1 mm^2) of carefully excised bands from stained gels were placed in each well and covered with molten agarose. Electrophoresis, pressing, washing, and staining were carried out as detailed for the second-dimension gels above.

The antigen mixtures used for these experiments were Triton X-100 extracts (1% Triton X-100 in barbital/HCl, I = 0.1, pH 8.6) of *Streptococcus faecalis* strain 39-5 after induction of clumping response with cPDl, an extracellular pheromone. Details on the growth, characterization of the clumping response, and the pheromone are available elsewhere (7, 8). Isolation and characterization of the pheromone-induced antigen(s) are under current investigation. The 39-5 strain, cPDl, and the antiserum used in this study were the kind gifts of Drs. Y. Yagi, D. E. Lopatin, and D. B. Clewell (University of Michigan, Ann Arbor, Mich.). The antiserum was raised against glutaraldehyde-fixed whole cells of 39-5 after induction with cPDl.

All chemicals for which sources were not identified above were of reagent grade or better.

RESULTS AND DISCUSSION

Normal immunoprecipitates obtained from electrophoresis of a stained SDS-polyacrylamide gel into antibody-containing agarose in a direction perpendicular to the original electrophoretic direction are clearly shown in Fig. 2. Similar patterns were also obtained (a) with SDS-gel strips postfixation but prior to staining (a brief barbital buffer wash of the strip was added prior to second-dimension electrophoresis) or (b) by inclusion of a barrier gel (2,3) containing Triton X-100 between an SDS-gel strip (soaked 10 min in distilled water) and the antibody-containing agarose layer (immunoplates not shown). The differences in peak heights of the immunoprecipitates obtained by these two techniques compared to the one shown in Fig. 2 was minimal. Decreases in peak heights after storage of stained gels at 4°C in distilled water for 1 week were also small. Best results in alignment of stained bands in the SDS-polyacrylamide gel with immunoprecipitates were achieved by equilibrating the first-dimension gel at 14°C prior to pouring the second-dimension gel and then marking the location of major bands (or marker bands) on the underside of the glass plate with a diamond pencil. The use of an ink marker (3) would probably also be satisfactory. The equilibration at 14°C prevents distortions due to contraction of the first-dimension gel. Accordingly, all measurements on the original SDS-polyacrylamide gel should be made while the gel is at 14°C.

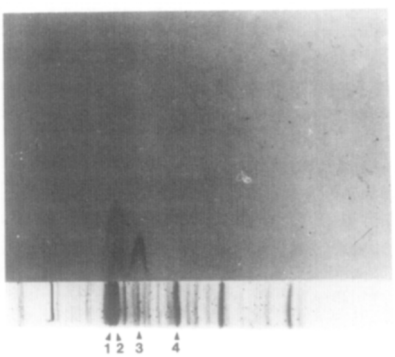

FIG. 2. Crossed immunoelectrophoresis of the constituents of a Triton X-100 extract of pheromone-induced *Streptococcus faecalis* 39-5. Approximately 16 μg protein was electrophoresed on a 10% polyacrylamide gel (running gel length = 10 cm) until the bromphenol blue dye marker reached the bottom of the gel. The gel was fixed, stained, destained, and washed as detailed under Materials and Methods. One-half of the top 5 cm was excised and electrophoresed at 70 V for 20 h into agarose containing 100 μl/ml antiserum raised against pheromone induced cells of strain 39-5. At the bottom is shown the SDS-polyacrylamide gel strip photographed prior to second-dimension electrophoresis. The top of the running gel is at the left. The immunoplate is positioned with the anodal portion at the top. Immunoprecipitates to bands 1, 2, and 3 can be seen. Band 4 is referred to in Fig. 3.

Confirmation of antigen-antibody reaction for a given polypeptide can be accomplished by excision of a small piece of the polypeptide band from a stained gel and electrophoresis of the contents of that piece into antibody-containing agarose in a manner similar to rocket immunoelectrophoresis (6). The results of such an experiment using the bands marked 2, 3, and 4 on Fig. 2 are shown in Fig. 3. As expected, rockets are present in the case of bands 2 and 3, while band 4 yields no reaction (as well as no artifacts).

The advantages of this modification and application are: (a) simplicity in set-up; (b) the quality of the SDS-gel separation is known before antiserum (often limited in supply) is used; (c) excellent alignment of SDS-gel bands and immunoprecipitates; and (d) ability to test individual polypeptides for reaction with antibody. This last advantage may be particularly useful with specific antibody and/or analysis of antibody combining sites or immunodeterminants on fragments of proteins. It may, however, be limited to water or buffer-soluble proteins since nonionic detergents or mobilizing detergents

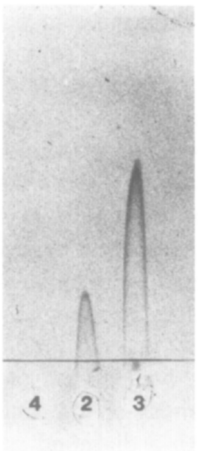

FIG. 3. Rocket immunoelectrophoresis of excised pieces of polyacrylamide gel. Pieces (1 mm^2) of bands 2–4 were excised and placed in wells of an immunoplate containing 100 μl/ml antibody. The wells were then filled in with molten agarose. Anode at top. Electrophoresis at 70 V for 20 h. From left to right: band 4, 2, and 3, respectively.

(4) may be required for maintenance of solubility and electrophoretic mobility of hydrophobic membrane polypeptides. Restaining of the first-dimension gel after second-dimension electrophoresis should be done to determine whether polypeptides migrate out of the first-dimension gel. In the present application, all visible polypeptide bands appeared to migrate out of the first-dimension gel. The results of second-dimension electrophoresis at lower voltage (50 V) for shorter time (18 h) indicated that some polypeptide bands of intermediate and high-molecular-weight migrated more slowly than others.

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