"High-Resolution" Mini-Two-Dimensional Gel Electrophoresis Automatically Run and Stained in <6 h with Small, Ready-to-Use Slab Gels

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Although two-dimensional (2-D) gel electrophoresis is one of the most powerful techniques for analyzing protein mixtures, its application in routine clinical laboratories is currently limited because it is time-consuming, complex, and relatively expensive. Here we describe a method for automatically running and staining "high-resolution" mini 2-D electrophoresis gels in <6 h, by using "ready-to-use" slab gels and a PhastSystem® electrophoresis apparatus. We present 2-D gel electrophoretograms of 25 nL of plasma, as well as their automatic computer analysis. For comparison, a conventional 2-D gel electrophoresis profile of 200 nL of a plasma sample is shown. The technique is easy to perform, highly sensitive, rapid, and potentially useful in semi-routine clinical chemistry laboratories.

Additional Keyphrases: proteins . isoelectric focusing . computerized gel . evaluation . SDS-PAGE

Two-dimensional gel electrophoresis (2-DGE)4 technology has improved dramatically since the original publications of O'Farrell (1), notably owing to the contributions of Anderson and Anderson (2) and of Tracy and Young (3). The reproducibility and sensitivity of the method have been defined. Merrill and Goldman (4) and Oakley et al. (5) have published very sensitive silver-staining methods; and various sophisticated computer programs have been developed for analyzing 2-DGE pictures (6–11).

Despite the great progress achieved and the important results obtained, this technique is still not generally applied in routine clinical chemistry, for various reasons (3), but mostly because 2-DGE is time consuming, complex, and relatively expensive. Isoelectric focusing (IEF), the first-dimension separation, takes generally >18 h and the second-dimension separation, sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS-PAGE) takes 5 to 6 h, depending on the size of the gels, even when a high current is applied. The slab gels are generally polymerized at least 24 h in advance, and most procedures for fixing and staining the gels take at least 24 h. Because the staining procedure is usually done the day after the second-dimension separation, the whole process, from sample load to picture visualization, can easily occupy two or three days.

In addition, the sophisticated computer programs available for analyzing the data are not efficient enough to be used routinely and on a large scale.

Because of the potential for this technique to be clinically important for analyzing diverse biological samples—e.g., plasma for apolipoproteins (12), in which numerous blood or biopsy specimens of a population must be analyzed—it would be desirable to develop a 2-DGE method that would be inexpensive, fast, and easy to perform. Such development would not only open up the field of semi-routine clinical chemistry but also perhaps replace both standard electrophoresis and IEF of immunoglobulins.

By decreasing the size of the IEF and SDS-PAGE gels to one-third, analysis of a sample should be completed in a day. Of course, the maximum sample load would have to be decreased, but this perhaps could be compensated for by increasing the staining sensitivity.

Of the several published "micro" 2-DGE procedures (13–20), all have notable disadvantages: the slab gels must be prepared by the users, the staining procedure is not automated, and the resolution of plasma proteins is not well comparable with that achieved for large 2-D gels.

Here we describe an automated method for running and staining mini 2-DGE, from sample load to picture analysis, in <6 h on "ready-to-use" slab gels. We show high-resolution mini 2-DGE pictures of plasma, and their computer analyses, as well as the image for a comparable large gel.

Materials and Methods

Apparatus

We used a PhastSystem® electrophoresis apparatus (Pharmacia, Uppsala, Sweden) and an IEF chamber, Model 155 (BioRad, Richmond, CA). A 5000-V power supply (Camag, Muttenz, Switzerland) was connected to the IEF chamber. The gel scanner was a high-precision scanner, built at The Swiss Federal Institute of Technology in Zurich, with a cooled (−10 °C) charged coupled device array of 2048 elements moving with a precision of <1 µm. The scanner has been built as the back box of a 500C/M camera (Hasselblad, Goteborg, Sweden) with macro lenses optics (Zeiss, Oberknen, F.R.G.). The scanner device was connected to a 3/160 Sun graphic workstation (Sun Co., Santa Clara, CA), which has 4 megabytes of central memory and 400 megabytes hard disk. The home-made light box contained 14 high-frequency (20-kHz) neon tubes.

Reagents

We used reagents from the following sources: acrylamide, N,N'-methylenebisacrylamide, N,N,N',N'-tetramethylethylenediamine (TEMED; BioRad, Richmond, Switzerland); ammonium persulfate, glycine, SDS (Fluka, Buchs, Switzerland); "Ampholine" carrier ampholytes (LKB, Bromma, Sweden); Servalyte carrier ampholytes (Serva, Heidelberg, F.R.G.); 3,5-(3-cholamidopropyl)dimethylamino)-2-hydroxy-1-propanesulfonate (CHAPS); Nonidet P-40 detergent, TriS (Sigma Chemical Co., St. Louis, MO); dithioerythritol, urea (Merck, Darmstadt, F.R.G.); silver nitrate, ethanol,
acetic acid, glutaraldehyde, formaldehyde, phosphoric acid, sodium hydroxide, and ammonium hydroxide (Lotti, Geneva, Switzerland).

Procedures

Preparation of IEF microcapillary gels. We prepared the following stock solutions: acrylamide/N,N'-methylenebisacrylamide solution (respectively 30 and 0.8 g dissolved in 100 mL of distilled water); Nonidet P-40/CHAPS (50 µL of Nonidet P-40, 150 µg of CHAPS, and 450 µL of distilled water). We also dissolved 5 g of urea in 3.25 mL of distilled water in a small beaker and added 1.5 mL of acrylamide solution, 500 µL of Nonidet P-40/CHAPS solution, 10 µL of TEMED, 100 µL of 5–7 Ampholines, 200 µL of 3.5–10 Ampholines, and 200 µL of 3–10 Servalyt. The final solution was mixed, degassed, then flushed with nitrogen. To initiate polymerization, we added 20 µL of 10 g/L ammonium persulfate solution. After washing glass capillaries (55 × 0.3 mm i.d.; Becton and Dickinson Co, Rutherford, NJ) with distilled water, we filled them with the gel reagents by capillary action to a height of 45 mm.

Sample preparation and loading. After treating 5 µL of plasma or serum samples with 10 µL of a dissociating buffer (1 g of SDS, 232 mg of dithioerythritol in 10 mL of distilled water) by heating at 95 °C for 5 min, we let the samples cool, then added 480 µL of a second buffer (100 µg of dithioerythritol, 400 µg of CHAPS, 5.4 g of urea, and 500 µL of Ampholines 3.5–10, plus 6 mL of distilled water) and 500 µL of distilled water, for a final 200-fold dilution of the original plasma or serum sample. Generally, we loaded 5 µL of the final diluted sample onto the gel-containing capillary glass tubes, using a 10-µL syringe (Hamilton, Bonaduz, Switzerland). The samples were then overlayed with the cathodic solution (see below).

IEF conditions. The cathodic solution was sodium hydroxide, 40 mmol/L, the anodic solution phosphoric acid, 10 mmol/L. The samples were loaded on the basic side of the capillary tubes as described above. We applied 100 V for 1 h, 200 V for 2 h, then 1000 V for 30 min (no bubbles should be left in the capillary tubes, otherwise orange sparks will appear at high voltage).

Preparation of slab gels and transfer. The "ready-to-use" 8–20% Phastgel® gradient slab gels (Pharmacia, Uppsala, Sweden) were first washed for at least 30 min with distilled water, then equilibrated for at least 60 min with a solution containing 1 g of SDS, 6 g of Tris, and 28.6 g of glycine in 1 L of distilled water. A groove 0.5 mm wide was cut in the stacking gel area, parallel to the interface between the porosity gradient gel and the stacking gel, and approximately 1 mm away from that line. Once ready, the gels were placed on the PhastSystem® apparatus as recommended by the manufacturer and cooled to 10 °C. After the first-dimension separation, we did not equilibrate the IEF gels. We used a 2-mL plastic syringe to extrude them from the capillary tubes onto a piece of Parafilm® (American Can Co, Atlanta, GA), wet the gels with a few drops of a transfer solution (8 mL of a 0.5 g/L bromphenol blue solution, 40 mL of 100 g/L SDS solution, 20 mL of 0.5 mol/L Tris buffer, pH 6.8, and 71 mL of distilled water), and immediately, using thin tweezers, layered these thin gels in the tiny groove cut in the stacking gel (Figure 1). No agarose was used to seal the capillary gels in the groove.

Second dimension separation assay conditions. To increase resolution and to abolish the background in silver staining, we used home-made SDS gels prepared with an agarose solution (2 g of agarose, 0.3 g of SDS, 1.8 g of Tris, 8.64 g of glycine, 0.02 g of sodium azide in a final volume of 100 mL of distilled water). We heated this solution to dissolve the agarose, then solidified it at room temperature in Pharmacia strip molds, so that the strip shape would fit the apparatus. (Such strips may become commercially available in the future.) We positioned the prepared strips on both sides on the gels under the electrodes. The running conditions were 4 mA per gel, 4 V · h, and then 6 mA per gel, approximately 95 V · h, until the migration front reached the positive electrode strips. The voltage increased to 150 V at the end of the run. The gels were removed and promptly placed in the staining chamber of the equipment.

Automatic staining procedure. With the PhastSystem apparatus, one can have 10 different bottles simultaneously connected to the staining device. We used eight outlets, in the following order: 1, waste liquid; 2, ethanol/acetic acid/distilled water (40/10/50 by vol); 3, ethanol/acetic acid/distilled water (5/5/50 by vol); 4, glacial acetic acid, 5 mL/L; 5, silver nitrate solution (6 g of silver nitrate, 1.5 mL of sodium hydroxide, 10 mol/L, 10 mL of 85% ammonium hydroxide solution, diluted to a final volume of 750 mL with distilled water); and 6, distilled water. Outlet 7 could be used for developing solution (1 mL of formaldehyde and 0.1 mg of citric acid in 1 L of distilled water) if desired, and outlet 8 for fixative solution (12.5 g of sodium bisulfite and 125 g of sodium thiosulfate per liter). The equipment was programmed to fix the gels with the first solution for 5 min, with the second for 10 min, to wash with distilled water for 5 min, and to fix again with the glacial acetic acid solution for 8 min. The gels were then washed five times for 3 min and three times for 5 min each, stained with the staining solution for 5 min, then washed again for 5 min with water. The gels were removed from the equipment and treated manually for 5 to 8 min with the developing solution so we could observe the appearance of the spots, then finally fixed with the fixative solution. This step can be automated, which ensures the best reproducibility for the technique. We then washed the gels with 100 mL/L solutions of glycerol and ethanol and dried them at 50 °C for 4 to 6 h. The dried stained gels could be stored for weeks or months without observable changes. This staining method was adapted from Oakley et al. (5).

Gel scanning and computer analysis. We placed the gels on the home-made light box and scanned them with our camera set for maximum magnification. The resolution of the 3 × 4 cm gel picture was 1024 × 1024 pixels. The pixel width was then about 40 µm. The picture were analyzed by our computer system MELANIE, developed at Geneva University from the Miller ELISE system (17).
Results and Discussion

We could analyze 25-nL samples of plasma, from sample load to computer analysis, in half a working day, with resolution comparable with that of large gels (Figure 2). More than 200 spots were detected per gel (Figures 2 and 3). Comparison with pictures published by Anderson et al. (21) or Tracy and Young (3) allowed us easily to identify different plasma proteins. The computed molecular masses and isoelectric points of the known proteins were also similar to those published (3, 21). The reproducibility of the method was adequate for computer-automated matching of the spots. The smaller gel size did not seem to compromise the accuracy of quantitative measurements, but further experiments are required to verify this.

Different IEF separation conditions have been tested. Capillary gels gave better results for the IEF separation and transfer than do IEF strips. A small Plexiglass chamber was built to run the IEF separation in capillary gels with the PhastSystem apparatus, but it was more convenient to run the IEF separation in the Model 155 electrophoresis chamber from Bio-Rad.

Many transfer conditions have been tested. Overlaying the stacking gel with the IEF capillary gels or IEF strips gave poor results. Good penetration of the samples into the SDS-PAGE gels was ensured only by cutting a groove in the stacking gel approximately 1 mm from the porosity gradient. The groove had to be ≤1 mm wide. Agarose sealing of the IEF capillary gels in the groove did not improve the picture and even decreased the reproducibility of the technique.

We tested also Pharmacia SDS electrode strips, but they produced a gray background with the silver-staining method used (5) and a yellowish background with Coomassie Blue staining; the resolution of the spots was also somewhat less than on the home-made strips. We investigated various electrolyte concentrations and used different equilibration times between first- and second-dimension separations. Figure 4 illustrates results obtained with strips with low electrolyte concentration (same as in the gel buffer) and an equilibration time of 10 min. Many protein spots disappeared, and the migration front showed severe distortion from electroendosmosis—and the strips started to burn. We determined that the electrolyte concentration of the strips had to be at least three times the concentration of the electrolytes in the gel (data not shown).

In our experience, by comparison with published illustra-
routinely has an internal diameter of 1.5 mm. In our laboratory, we routinely use capillary gels 165 × 1.4 mm (i.d.) for IEF and slab gels 200 × 180 × 1.5 mm.

The extreme sensitivity of the method was demonstrated by assays of 25 nL of plasma (roughly 1.5 µg of protein), where spots representing less than nanogram quantities of proteins were detected (Figure 5). Two hundred spots were detected by the computer. Because new low-molecular-mass spots were visible on the microgels but not on the large gels (Figures 2 and 5) despite an eightfold greater protein load on the large gels, we repeated the assay with fresh samples and fresh electrolyte solutions, the equipment and the capillaries having been carefully washed. The reproducibility of these spots ruled out the possibility that these were artefacts. Their detection was probably attributable to a better separation with very thin capillaries, a faster separation according to molecular mass, and a rapid staining procedure. However, good reproducibility depended on the expertise of the technician, notably when cutting the groove in the stacking gel. The resolution should be even improved if "ready-to-use" slab gels are manufactured with a groove for 2-DGE.

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