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Miniature Electrophoresis for Speed and Productivity

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ABSTRACT

Attempts to miniaturize electrophoresis (EP) to save time or enhance productivity and efficiency remains a challenge for science and industry. Other advantages of miniaturization include: increased sensitivity, saving of reagents, greater yield of data, and enabling studies where only small samples are available. Since electrophoresis that takes hours may be reduced to a matter of minutes, the limitations of miniaturization in clinical, industrial, and research applications are evaluated. Clinical electrophoresis (EP) on cellulose acetate media can be performed in 3.5 min instead of 20–45 min and on SDS polyacrylamide gels in 15–30 min compared with conventional 3–8 h.

Index Entries: Electrophoresis; miniaturization; microelectrophoresis; scanning densitometry; laser scanner.

INTRODUCTION

Past attempts to miniaturize electrophoresis (EP) started decades ago for the purpose of increased sensitivity for biological samples and were successfully pioneered by Edstrom (*1*). He was able to electrophorese nucleic acid bases from individual cells along a silk thread. Polyacrylamide fibers were used by Matioli and Niewisch (2) for electrophoresis of single erythrocytes to detect hemoglobin variants.

The various factors affecting the resolution of electrophoretic systems, such as passive diffusion, migration velocity, and interaction of proteins with supporting medium along with experimental means of controlling

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them by adjustments of buffers, voltage, current, and method of application of samples, has been discussed in detail for agar gel electrophoresis by Wieme (*3*) and cellulose acetate by Briere and Mull (*4*) and various media by Smith (*5*). The requirements for scaling down electrophoretic methods have been reported, including use of pH 8.6 barbital buffer with 2 m*M* calcium lactate in 1% agarose medium for high resolution agarose electrophoresis by Johansson (*6*), for polyacrylamide methods by Poehling and Neuhoff (*7*), and starch gel methods by Wright (*8*).

The present routine macro methods for polyacrylamide gel electrophoresis use either rod or slab gels. Rod gels are made in glass tubes 0.5 cm in diameter by 7 cm long and take 40–120 min to run. Slab gels vary from 10 to 20 cm wide by 20 cm long and 2–5 mm thick and take 8 h to run at fairly high voltages or overnight (12–18 hours) at lower voltages (about 3–5 volts/cm). Higher voltages are selected for speedier runs, so there is less time for diffusion to occur. However, since heating increases as the square of the voltage, there is a practical limit to the maximum running voltage owing to protein denaturation and gel distortion. Furthermore, diffusion increases with temperature. Temperature control is dependent upon the efficiency of the cooling system in use, the thickness of the gel, and thinness of the insulation. Proper fixing of these gels with TCA or other chemicals usually requires 1–3 h. Thinner gels permit faster diffusion of fixative and reduces diffusion of protein bands. Routine staining takes 0.5–2 h.

Destaining, to remove unbound stain, is usually done by flooding with agitation and frequent changes of destaining solution over a 24-h period. Destaining has no effect on smearing since the proteins are fixed, but excess destaining reduces sensitivity of detection of bands. Electrophoretic destaining is achieved by applying electric potential across the gel, resulting in rapid and complete removal of excess stain in 20–60 min.

Ultra-micro PAGE methods were developed by Grossbach (*9*) using 50–100 μm diameter x 25 mm glass capillaries (0.05–0.2 μL volumes) that require only 5–10 min for electrophoresis. Fixing and staining of capillary electrophoresis gels with the usual chemicals reduces the time of fixation, leading to lower smearing and improved retention of resolution. The only drawback to this method of capillary electrophoresis is that a high technical skill and micromanipulators are required to handle the small tubes.

The smallest scale for practical micro methods, developed by Neuhoff and colleagues, requires only more careful handling than with standard methods. Analytical and preparative methods using 5 μL capillary tubes have been developed for PAGE by Neuhoff, Schill, and Sternbach, (*10,11*), for molecular weight determinations in SDS-PAGE by Peter, Wolfrum, and Neuhoff (*12*) and analysis of enzyme kinetics by Cremer, Dames, and Neuhoff (*13*).

Miniaturization of electrophoretic separation has found other fields of application. Micro-PAGE followed by crossed immuno-electrophoresis

was developed by Dames, Maurer, and Neuhoff (*14*). Preparative methods using 50 μL capillaries were devised by Dames and Maurer (*15*). Micro-isoelectrifocusing was achieved by Bispink and Neuhoff (*16*). Miniaturized methods of Condeelis (*17*), using 100–250 μL capillaries requiring about the same technical skill as standard methods, were preferred for routine use.

Recent developments include the use of immobilized pH gradients (IPG) in 50×43×0.5 mm slab gels for isoelectricfocusing in the first dimension of 2-D microelectrophoresis by Gorg et al. (*18*). Since very small amounts of protein are involved in these micro methods, there is a significant problem of losing it through adsorption to equipment and materials. This problem can be avoided by the use of a soy bean trypsin inhibitor as a protein carrier, as shown Hinson et al. (*19*).

Scanning densitometry with fluorescence chemistry of microgels by Zimmer and Neuhoff (*20*) demonstrated that exacting quantitative analysis was possible for micro separation techniques. Increased resolution compatible with miniaturization was accomplished using laser scanning densitometry by Nijm and Zeineh (*21*).

Another field where miniaturization was applied is micro slab technology developed by Maurer and Dati (*22*), Matsudaira and Burgess (*23*) and for isoelectricfocusing by Gorg, Postel, and Westermeier (*24*). Gels were poured into molds made with standard equipment or microscope slides. Samples as small as 0.1–2.0 μg could be loaded as vol of 0.5–5.0 μL. Runs and processing times were reduced to 1.5–2 h with sharper bands owing to more efficient cooling. Staining and destaining times were also shortened.

Especially important in enabling miniaturization are improvements in staining technology. The early standard stain used was Amido Black 10B. Coomassie Brilliant Blue R 250 is about 5 times more sensitive, and a good method is described by Chrambach et al. (*25*). The high resolution and sensitivity of autoradiographic methods was closely rivaled by the silver staining technique of Switzer, Merril, and Shifrin (*26*) and Oakley, Kirsch, and Morris (*27*), which is about 110 times more sensitive than Coomassie Brilliant Blue R250. Silver staining, taking about 4–5 h, is faster than other stains and is more sensitive, thereby contributing to miniaturization. To reduce the loss of reduction owing to diffusion in gels, Manabe et al. (*28*) transferred proteins on to nitrocellulose. They then used concanavalin A-peroxidase staining to obtain glycoprotein specificity showing complexity within the more than 20 protein moieties observed.

In general, the high resolution of electrophoresis is manifested by electrophoregrams coming close to an ideal with thin, well-separated, straight, adequately-stained bands with low background stain. Deviations from this ideal separation may result from any number of the following factors. Optimal sample size is necessary to detect proteins in low concentration, requiring a large enough sample to detect them and yet a small enough sam-

ple so not to overload with the predominant proteins. Smearing of bands may also be a result of particulate cellular material remaining in poorly clarified samples. Incomplete or disturbed gel polymerization can result in band irregularities, such as curves and S-shapes. The method of application should ideally place the sample in the thinnest possible starting line for electrophoresis. This is done with stacking gels in PAGE, thin applications onto cellulose acetate membranes, or with thin paper inserts in starch gels. For isoelectricfocusing, the width of the applied sample does not matter because the method itself separates and sharpens the protein bands at their respective isoelectric points.

All micro methods reduced the time for processing and the amount of sample and reagents used. Clearly, the results showed that micro gel methods significantly saved time at all steps of experimentation and in the quantities of reagents and sample used, as reviewed by Poehling and Neuhoff (*7*). In recent methods, IEF was accomplished in 45–65 min and EF in 30–60 min (*18,19*).

The focus of this report is the development of routine clinical and industrial electrophoresis when many runs are performed frequently and steadily. Most reports to date are oriented towards R & D applications. At the present, there is room for improvement regarding yield, productivity, savings of material and technical time, and speeding up the turn-around time. Present attempts in miniaturization are coupled with automation and multiple runs, such as achieved by the AMBIS (San Diego, CA) automated electrophoresis system and the PHAST system of PHARMACIA (Uppsala, Sweden).

Although separation technologies achieved high resolution at the 50 μm level in the past, this was lost, however, by using incompatible, conventional, white light scanning densitometers of 200–400 microns resolution. The observation was that well-resolved stacked bands observed by the naked eye appeared as overlapping peaks in densitometry tracings. Recently, a special customized scanning microdensitometer was assembled by Tarczynski and Outlaw (*29*) using a Leitz Diavert MPV Microphotometer and a computer controlled scanning stage. Optical density measurements were converted from analog to digital signals and entered into computer files for further mathematical and graphical processing. The method has a scanning step size of 1 μm capable of resolving bands 10 μm apart. This result shows that the technology exists for analyzing electrophoresed proteins since they occur in wider bands and spacings. With 2000 detectible steps in optical density, the system can measure a wide quantitative range of proteins detecting differences as small as 0.3 ng.

The soft laser scanner, also having a resolution in the 10 micron range, was introduced more than a decade ago by Zeineh et al. (*19*) and is available to other laboratories using miniaturized methods. The possibility of any limitation owing to scanning densitometry in miniaturization is evaluated in this report.

METHODS

Electrophoresis of dog serum proteins was miniaturized to separate along a length of 1.6 cm on cellulose acetate by using the BECKMAN Microzone Electrophoresis System. The BECKMAN applicator, having two parallel wires separated by 0.5 mm, was adapted by inserting a 5–8 mm short rectangular piece of office paper (nonglossy) snugly in to the applicator loop protruding downward by 1.5–2 mm. The applicator was loaded by immersing it in serum sample for about 30 s, wiping off excess serum, resting the applicator on top of the internal cover of the electrophoresis chamber, and releasing the wire loop-paper to contact the equilibrated cellulose gel under moderate and even pressure from the loop's own weight for 30 s. The gel was run at a constant 45 volts DC for 3.5 min, instead of the macro method at 100 volts for 45 min. The gel membrane was removed, fixed, stained, and destained as per manufacturer's instructions. Buffer conditions at 0.085*M* Tris/HCl at pH 8.4 were optimal.

In another miniaturization study, EP gels separating wheat gliadin proteins were obtained from Ian Batey of the Commonwealth Scientific Industrial Research Organization (CSIRO) Wheat Research Unit of Australia. The routine electrophoretic separation was performed on 10×10× 0.4 cm polyacrylamide gels, run for 6 h, stained with Coomassie Brilliant Blue R 250, and washed over 18 h. A step toward miniaturization used gels of 10×3×0.2 cm, runs for 30 min, followed by staining and washing for 6–12 h.

RESULTS

Miniaturization of the cellulose acetate electrophoresis of dog serum proteins was achieved (Figs. 1 a–c). Scanning the electrophoregram (Fig. 1d) revealed over 90% correlation between macro and micro methods for the amounts of the different globulins and 85% for the albumins.

A gel representing the original standard method of PAGE of the wheat gliadins is shown in Fig. 2a and a stage in the progress toward miniaturization in Fig. 2b. Further refinements on the technique of miniaturization need to be developed in order to obtain similar patterns as the standard method. An estimate of whether the laser scanning densitometry should be able to detect and present the results suitable for mathematical analysis and graphic representation when miniaturization becomes successful was carried out. Laser scans were made of photographic negatives of the original size gel and 1/2, 1/4, and 1/8 size reductions for six samples. A photographic composite of the 1/2 and 1/8 reductions of one sample is shown in Fig. 3. The scans for the different reductions were compared by electronically adjusting them with the Flexible Superimposition Program of BIOMED INSTRUMENTS and then combining them in a

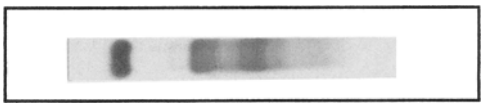

FIG. 1a

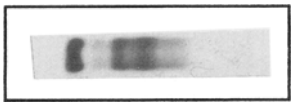

FIG. 1b

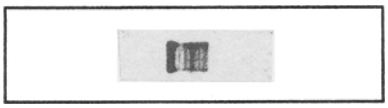

FIG. 1c

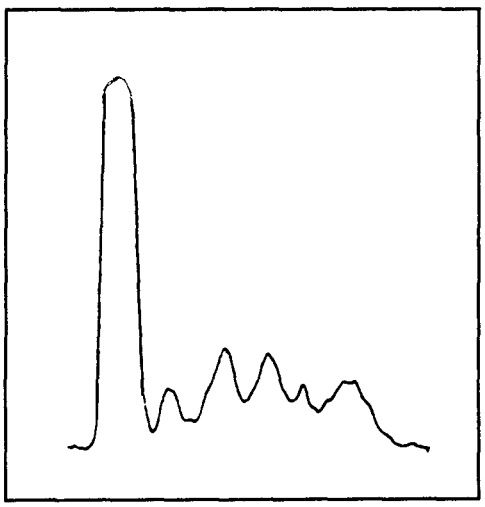

FIG. 1d

Fig. 1. Laser scanning of dog serum proteins using microzone electrophoresis: (a) standard electrophoresis on cellulose acetate run; (b) partial miniaturized run; (c) miniaturized run; and (d) laser scanning densitometer tracing of miniaturized run in (c).

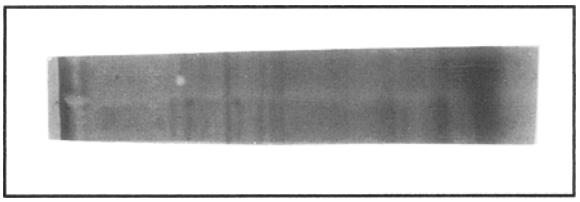

FIG. 2a

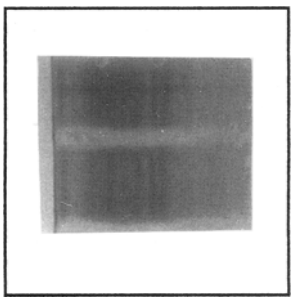

FIG. 2b

Fig. 2. Polyacrylamide gel electrophoresis of wheat gliadin proteins: (a) standard size gel and (b) present stage of gel miniaturization.

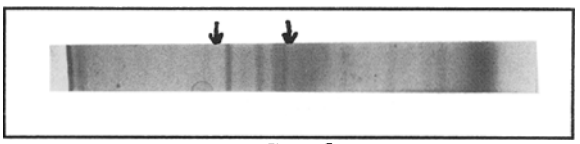

FIG. 3a

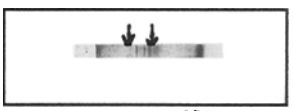

FIG. 3b

Fig. 3. Polyacrylamide gel electrophoresis of wheat gliadin proteins: (a) photographic reduction to 1/2 size of standard gel; and (b) photographic reduction to 1/8 size of standard gel.

single graph with the Superimposition subroutine of the Laser Scanning Program. Such a graph for the sample in lane 4 is given in Fig. 4. As far as it is possible to photographically miniaturize the gel pattern to as small as a 1/8 reduction, the laser scanner is able to provide suitable scans that can be analyzed and presented graphically.

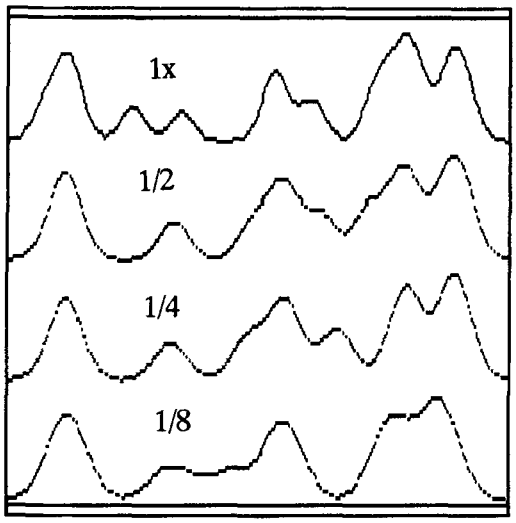

Fig. 4. Flexible superimposition of the segment indicated by arrows in Fig. 3 of the original 1× and 1/2, 1/4, and 1/8 photographic reductions from the top to bottom, respectively.

DISCUSSION

Miniaturization of EP on cellulose acetate was found to be feasible for electrophoresis of dog serum proteins. The limiting factor to achieving good resolution was the width of the applied sample and diffusion, which could be kept to a minimum.

When performing SDS-EP in polyacrylamide gels, the thinness of the application band can be very fine and is made very fine by use of the stacking gel technique. Therefore, sample width is less likely to be the limiting factor, whereas diffusion is more likely to be limiting. With IEP, the width of the application is not relevant because the method itself produces sharp thin bands. In the wheat gliadin experiment, the limiting factors at this technical development stage appear to be the amount of sample applied, the thickness of the gel, diffusion, and the time for staining and destaining. Although band widths and spaces between bands as small as 40 microns have been reported for SDS-EP (*30*), the wheat gliadin miniature gel did not obtain such a fine resolution. When it is attained by technical improvements, the soft laser scanner can detect, quantify, and compare the gel patterns.

SUMMARY

The previous limitation on utilizing the results of miniaturization of high resolution electrophoresis owing to incompatible white light scanning

densitometry is overcome by the use of soft laser scanning densitometry. The positive analysis of laser scannings of miniaturization reductions to as low as 1/8 times gives encouragement for further efforts for miniaturization of polyacrylamide gel electrophoresis without infringing on quality and accuracy. Miniaturization also reduced cellulose acetate electrophoresis to a quick micro method that could also be analyzed with laser scanning densitometry. Miniaturization of electrophoresis benefiting from the advantages of laser scanning densitometry can add a significant advantage and high value to routine clinical and industrial testing, quality control, and production procedures.

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