A RAPID METHOD FOR ISOELECTRIC FOCUSING IN POLYACRYLAMIDE GEL

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Received 1 May 1972

1. Introduction

During the last years different techniques for analytical gel electrofocusing have been developed and the results obtained indicate that this method gives a resolution superior to that obtained in sucrose gradients [1]. Gel electrofocusing also offers a considerable saving of time and materials [2–4]. Gel rods in an ordinary disc electrophoresis apparatus were mostly used [2–5] but recently a flat bed or thin layer technique has been developed [6–11]. This offers several advantages: (i) a very simple apparatus [11]; (ii) many samples can be run in parallel; (iii) it is simple to handle, e.g. for pH measurements; (iv) the thin gel slabs used permit efficient cooling. The effective removal of the heat evolved, was necessary to develop the ordinary gel slab electrofocusing to the rapid method described in this paper.

2. Materials and methods

Acrylamide and *N,N'*-methylene bisacrylamide were purchased from British Drug House (Poole, England), Coomassie Brilliant Blue R (formerly R 250) from ICI (Manchester, England) and Ampholine from LKB-produkter (Bromma, Sweden). The following proteins were used: ovalbumin (Sigma, St. Louis, Mo., USA) and sperm whale myoglobin (Schwartz-Mann, New York, USA) All chemicals used were of analytical grade.

The gel was prepared between two glass plates measuring 200 × 110 × 0.8 mm assembled with a gasket of 1.5 mm soft polyvinylchloride 5 mm wide, as recently described [11]. The gel slab will measure about 190 × 95 × 1.5 mm. The following recipe was used to prepare one gel plate: 1.4 ml Ampholine (Amph) 3–10, 0.10 ml Amph 4–6, 0.10 ml Amph 5–7, 0.20 ml Amph 8–10, 0.4 g glycine, 0.40 ml riboflavin (from a stock solution of 4 mg/100 ml) and 25 ml distilled water. A stock solution of 30% w/v acrylamide and 1.5% w/v methylene bisacrylamide was prepared and 6.0 ml of this solution was added to the mixture described above. The final concentration of Ampholine in the gel was 2.1% w/v and acrylamide T = 5.8% w/v and C = 4.8% w/w [12]. Several plates were prepared and kept at +4° overnight or for longer periods. It is possible to dispense with the siliconization of one glass plate [11] if one is working carefully.

The apparatus, constructed as a cooling-plate, was made in principle as described earlier [11] with a 2 mm thick surface plate. The plate was cooled from a refrigerated thermostat bath (Kälte-Kleinthermostate K4R Electronic, Messgerätewerk Lauda, Tauber, Germany) to 2–4°. The gel, on its 0.8 mm thick glass plate was put on the cooling plate with some distilled water evenly spread on the surface to ensure good thermal contact. Care was taken to remove all air bubbles between the plates and in the apparatus. The gel was allowed to cool for about 10 min and the carbon electrodes (230 × 8 × 4 mm; Le Carbonne-Lorraine, Paris, France), which were first soaked in 1 M phosphoric acid (anode) and 1 M sodium hydroxide (cathode) for several hours, were then applied [11]. These rectangular electrodes gave a better surface contant with the gel than the round ones used previously [11] and in preliminary experiments. Protein samples were applied on filter papers (10 × 10 mm, Whatmann 3 MM) which were put on the gel with a forceps at different distances between the electrodes. The samples had to be applied immediately after the

papers were put on the gel: 2–200 μg of protein was generally applied in 50 μl. A glass plate (250 X 140 X 6 mm) was then put on top of the electrodes to ensure a good contact between the gel and the electrodes. The electrodes were connected with a power supply (Buchler Model No 3-1014 A, Fort Lee, N.J., USA) used in the constant voltage mode and manually regulated to constant wattage, i.e. the product of voltage and current was kept constant.

After the experiment was stopped, the gel was immediately put in a bath containing 2% w/v sulphosalicylic acid, 11% w/v trichloroacetic acid and 27% v/v methanol in distilled water, which solution was preheated to 65° [13]. The gels were kept in this solution for 20 min and then washed twice with the destaining solution (see below) at 20° before staining at 65°. The rest of the staining and destaining procedures were carried out in principle as described by Vesterberg [11,13] with a few minor modifications. The stain was dissolved in 8.5% v/v acetic acid and 27% v/v ethanol in distilled water, i.e. the same solution as used for destaining [13]. This modification of the original procedure had the same sensitivity, but was easier to work with in our hands.

3. Results

Fig. 1 shows the time relationship for the current and voltage in a typical experiment. A plateau was reached after about 30 min at 50 W, when the voltage was 1 000 V corresponding to 130 V/cm for the effective separation distance of 75 mm. Comparison of the proteins, applied at various distances between the electrodes, showed that the proteins migrated to the same positions during this short time, and thus reached equilibrium. This was also confirmed by measuring the pH on the gel with a surface electrode. The power supply limited the carrying out of complete experiments at 100 W and 250 W. However, local overheating probably caused blurring of some of the zones of myoglobin, which separated in a few minutes, while the ovalbumin zones were still sharp. No cracking of the gel was observed as described at a much lower final voltage [14]. Much sharper zones were also found after staining, in comparison with an experiment run at a lower final voltage (33 V/cm) for a longer period of time (fig. 2). A slow migration towards the cathode,

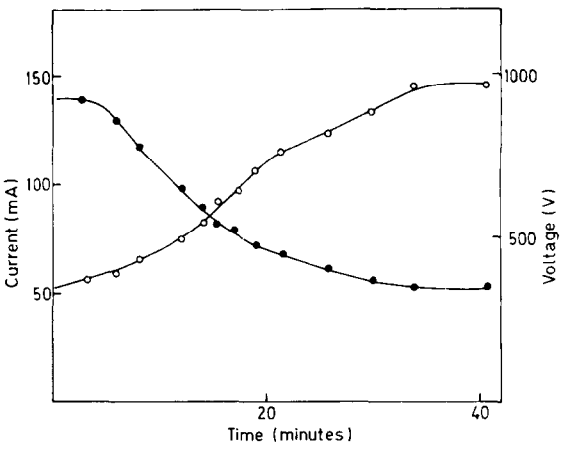

Fig. 1. Time relationship for current (●——●——●) and voltage (○——○——○) in a 50 W experiment.

due to drift of the pH gradient as observed with the gel rod technique, was not observed in experiments run for about 30 min ([2,5,14], T. Wadström, unpublished observations).

4. Discussion

All methods described up to now on gel electrofocusing have been performed at a final voltage of about 20–30 V/cm and run for 4–24 hr. No systematic study, except one recently published by Finlayson and Crambach [15], has been published. These authors found that runs for 8 hr at 33 V/cm at 5° provided optimal separation in large pore gels under "non-sieving conditions", while Wrigley earlier claimed that equilibrium is reached in 1.5–3 hr under similar conditions [16]. Measurements of pH with a surface electrode, after application of the samples at different distances between the electrodes, showed that equilibrium for each protein zone was reached after 30–40 min when experiments were carried out at 50 W (fig. 1). This was also compared with the pH values of each component after conventional separation: 33 V/cm; 1.5 mA; 4 hr.

The recipe given in this paper provides an even distribution of ampholytes in the gel which prevents local overheating and probably also visible side effects, such as local bulging at certain positions [14]. Separation in a similar apparatus built in acrylic plastic or an

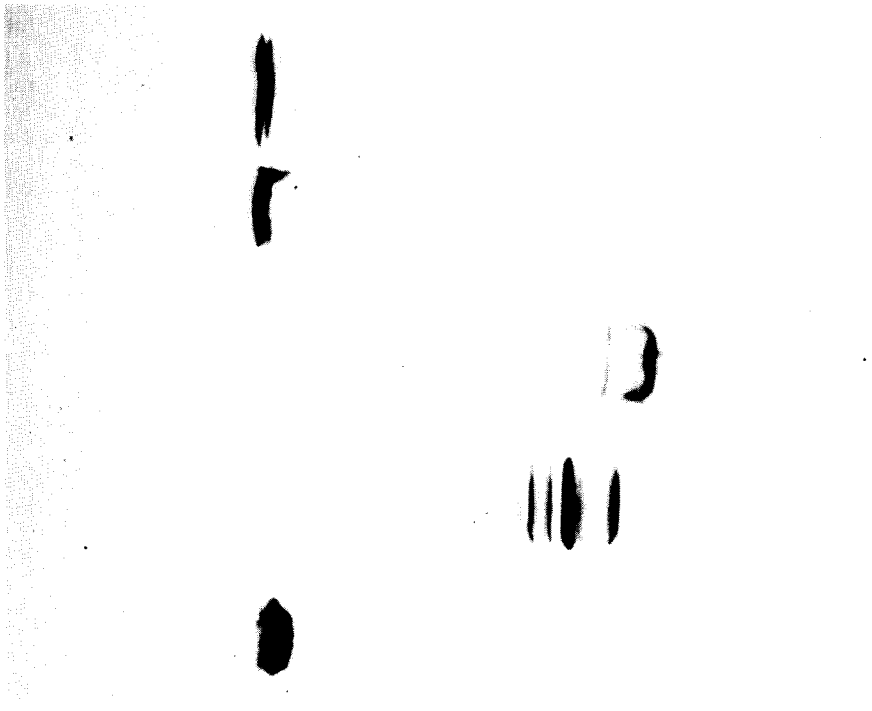

Fig. 2. Isoelectric focusing (from left to right) of human serum albumin (50 μg); sperm whale myoglobin (50 μg) applied between the middle of the plate and the cathode and the same protein (50 μg) applied between the middle and the anode; ovalbumin (50 μg) applied between the middle and the anode and the same protein (50 μg) applied between the middle and the cathode. Note that myoglobin in the second position was adsorbed to the filter paper and did not resolve into different components as in position one.

apparatus of the same material constructed for Laurell electrophoresis [17] gave much poorer results at 50 W, due to less efficient cooling capacity. For this reason it is very important that the equipment should be constructed in glass as recently pointed out by C.J.O.R. Morris (The British Biophysical Society meeting on "Separation Methods in Cell and Molecular Biology", 14–16 December 1971, University of Surrey, Guildford, Surrey, England, in general discussion) and Vesterberg [11].

The gel slab technique described in this paper has the following advantages over methods earlier described: (i) it permits a much faster separation and a complete analysis of the separated proteins in one day. (ii) it gives sharper bands and a higher resolution compared to separation at a lower final voltage for a longer period of time.

The staining procedure recently described by Vesterberg [11,13] was modified. In our opinion this modified method is easier to use.

Adsorption of protein on the filter paper, especially at lower protein concentrations (50–500 μg/ml) of albumin and several other proteins was observed, and must be taken into account when determining the protein detection limit by different staining methods. Recently protein samples applied in glass basins (10 × 10 mm) put on the gel surface solved this problem and will permit accurate comparisons.

Work is now in progress to develop a rapid and convenient method for analysing the separated protein zones by scanning the gel in a densitometer (J. Söderholm, O. Vesterberg and T. Wadström, in preparation).

Acknowledgements

One of us (J.S.) is thankful to J.S. Fawcett for stimulating discussions at the symposium in Guildford (see above) which initiated this study. Olof Vesterberg is gratefully acknowledged for stimulating criticism and Per Ingvar Kring for skilful technical assistance.

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