Journal of Chromatography, 138 (1977) 25–32

CHROM. 10,062

ISOELECTRIC FOCUSING IN CYLINDERS OF GRANULATED GEL

SAMUEL H. LOVE

Department of Microbiology and Immunology, The Bowman Gray School of Medicine of Wake Forest University, Winston-Salem, N.C. 27103 (U.S.A.)

(First received December 15th, 1976; revised manuscript received February 16th, 1977)

SUMMARY

Sephadex G-75 (superfine) served as supporting medium during isoelectric focusing (IEF) in electrophoresis tubes. Nylon nets, which retained the gel during preparation of the column and loading the sample, were replaced with dialysis membrane prior to IEF. Up to 0.8 ml of sample containing at least 1.2 mg protein could be focused as one zone in each tube. Most samples were focused at 170 V, 4°, for 8–18 h. The procedure offers many of the advantages of electro-focusing on layers of granulated gel but on a smaller scale.

INTRODUCTION

Isoelectric focusing (IEF) on flat beds of granulated gel such as Sephadex G-75 is considered to be one of the most promising preparative methods available for the purification of proteins[1,2]. Precipitates formed during the procedure do not interfere with resolution, and recovery of the desired protein involves removal and elution of gel sections. The filter paper print method helps locate the protein bands for selection and elution.

The resolution obtained by analytical electrofocusing on polyacrylamide gel[3] is an ideal adjunct to polyacrylamide gel electrophoresis for the analysis of protein purification steps. These procedures are limited by the difficulty in recovering biologically active proteins from the gels; therefore, it may be difficult to know which band is the one of interest. Adaptation of sucrose density gradient IEF[4] to microscale[5,6] yields biologically active proteins in quantities too low to serve our purposes.

Our work required a system which would permit comparison of different samples which tended to precipitate near their p*I*, one which would be suitable for small volumes, and which permitted recovery of activity rather than just detection by staining. Based on Radola's[1,2] work with layers of granulated gels, we developed a small-scale preparative procedure involving the electrofocusing of proteins in vertical cylinders of granulated gels. While this work was in progress, a procedure was described for the IEF of large samples (35 ml) on specially prepared columns of Sephadex G-15 (ref. 7).

MATERIALS AND METHODS

Materials

Spectra dialysis membranes of 6000–8000 or 12 000–14 000 MW cut-off were obtained from Fisher Scientific (Pittsburgh, Pa., U.S.A.). Sephadex G-75 (superfine) was purchased from Pharmacia (Uppsala, Sweden), and Ampholine solutions were obtained from LKB (Rockville, Md., U.S.A.). Myoglobin (horse heart), cytochrome *c* type VI (horse heart) and ferritin type I (horse spleen) were purchased from Sigma (St. Louis, Mo., U.S.A.). Hoefer (San Francisco, Calif., U.S.A.) generously supplied the optional lower chamber which allowed insertion of tubes up to 25 cm long.

Apparatus

A Hoefer model 150 electrophoresis apparatus was modified by making 6-mm wide slits radiating out from the bottom positioning holes of the tube alignment plate to facilitate loading and unloading the cylinders fitted with O-rings.

Preparation of gel

Sephadex G-75 (superfine) was washed and dehydrated according to instructions supplied by LKB[8] for their preparative flat-bed electrofocusing tray. Portions (3.75 g) of the dried Sephadex were suspended in distilled water (100 ml) and stored at 4° until required. Unless otherwise indicated, Ampholine solutions were diluted to 1% with the gel suspension prior to degassing by reduced pressure, and preparation of the columns. The 0.5 × 12.5 cm tubes supplied with the apparatus were adopted for routine use after comparison with 0.5 × 25 cm tubes which fit the larger lower chamber.

Preparation of columns

Acid-cleaned tubes were covered on one end with nylon net held in place with an 0.07 O-ring (5/32 in I.D.) to support the gel during filling and packing steps.The O-ring applicator supplied with an oxygen analyzer (model 53; YSI, Yellow Springs, Ohio, U.S.A.) facilitated this operation. Short lengths (3 cm) of tygon tubing were placed over the other end to serve as reservoir. The gel–ampholyte mixture (4 ml) was transferred to tubes positioned vertically in flat-bottomed sample storage tubes (1.2 × 11.3 cm; Bio-Rad Lab., Richmond, Calif., U.S.A.). Excess liquid was allowed to drain near the top of the gels, then the tubes were centrifuged at 125 *g* for 2 min in a horizontal rotor to pack the gel uniformly. The gel heights were adjusted to 12 cm by the addition or removal of gel with a Pasteur pipette.

Sample addition

When necessary, the samples to be focused were dialyzed overnight against 1% glycine to remove salts, then they were adjusted to contain 1% ampholyte of the selected pH range. Up to 800 μl of sample could be applied to the columns, but in practice, from 5 to 200 μl were applied and allowed to drain into the gel.

The O-ring and nylon net were removed while the columns were held in a horizontal position, then pre-soaked dialysis membranes of single thickness were substituted for the net and held in place with the O-ring. It was important to use tightly fitting O-rings during the focusing procedure to prevent distortion of patterns

due to leakage around membranes. Tubes were returned to the vertical position, filled with the ampholyte–gel suspension, and positioned in the electrophoresis apparatus.

Focusing conditions

Phosphoric acid (0.2%, v/v) was used as the anode solution which usually was placed in the bottom reservoir (1100 ml). Ethanolamine (0.4%, v/v) was used as the cathode solution in the upper reservoir (200 ml). A Lauda cooling system (Brinkmann, Westbury, N.Y., U.S.A.) maintained the temperature in the water jacket at 4° during focusing. Most samples were focused at 170 V for 8–18 h. More recent experiments suggests that 100 V for 16 h, followed by 400 V for 30 min effected less distortion of the visible bands.

Recovery of samples

Electrofocused samples were left in the vertical position until processed to recover the fractions. A plunger from a disposable syringe (1 ml) was modified to fit in the focusing tube by slipping a small O-ring over its tip, and by extending the length of the stem. The columns were wiped dry on the outside, placed in a horizontal position, and marked with a felt pen into 0.5–1-cm sections from the top of the gel. The O-ring and membrane were removed from the column, then the plunger was used to extrude samples into Pasteur pipettes plugged with glass wool. The gels were washed into the pipettes during the gradual addition of elution fluid (0.2–0.6 ml of water or 0.01 *M* NaCl) while the pipettes were held up-right in 13 × 100 mm tubes.

The control columns were handled differently. The locations of visible bands of marker proteins were recorded, then 1-cm sections of the gel were extruded into screw-cap tubes (13 × 100 mm). One milliliter of saline (0.01 *M*) was added to resuspend the gels. The tubes were capped and chilled on crushed ice prior to obtaining pH measurements with a microcombination electrode (Fisher Scientific). The control tubes indicated the pH gradients obtained and the position of visible reference proteins.

Bottom loading

Columns were loaded from the bottom by placing the sample on top of the nylon net while the column was inverted. Excess ampholyte solution (20–40 μl) obtained during preparation of the column was used to wash the sample into the gel prior to replacing the net with dialysis membrane.

Low pH gradient

The pH 2.5–4 gradients were obtained by topping the gels with ampholyte of the same pH, extending the columns above the cathode solution in the upper reservoir, and connecting the columns and cathode solution with wicks made from Whatman No. 1 filter paper. The wicks were rolled and folded into a U-shape. The procedure prevented distortion of the low pH gradient by the cathode solution.

RESULTS

Split-image focusing of hemoglobulin

The migration of top- and bottom-loaded hemoglobin A (adult human Rbc

extract) in the columns of 12.5 cm or 25 cm length is illustrated in Fig. 1A. Migration distances of visible bands were measured with a ruler from the outside of the transparent chamber without disrupting the apparatus. The samples formed sharp bands within 15 min and migrated to a common position by 105 min in the 12.5-cm tubes. The steady continuous migration of the focused band represented the previously observed cathodic shift[9].

The same apparatus with a larger lower reservoir accepts 25-cm tubes, thus providing a greater distance for establishment of the gradient and separation of components. The 25-cm column increased the focusing time approximately four-fold, using hemoglobin as a guide. This procedure of split-image focusing was very useful for determining the time required to focus visible proteins such as myoglobin and hemoglobin but the rubber stoppers obscured the view of cytochrome *c* when the cathode solution was in the upper chamber.

Fig. 1B illustrates the pH gradients obtained by 6.5 h during the above procedure and the position of hemoglobin as determined spectrophotometrically on eluted samples (1 ml). The pH gradients were acceptable in both cases, and less steep in the 25-cm column, as expected. Two separate experiments indicated recovery of 85–98% of the hemoglobin in the region of the major band. We selected the 12.5-cm columns for routine use because the smaller columns were more economical, more easily handled, and less time-consuming to operate.

Electrofocusing of normal rabbit serum as an example of a complex mixture

Following the electrofocusing of normal rabbit serum (60 μl), samples of 3 μl were subjected to immunoelectrophoresis and detected with goat anti-rabbit whole serum to obtain a comprehensive impression of the method (Fig. 2). Albumin was recovered in fractions of pH 4.4–5.5 separated from most of the γ globulin. α-1 globulin was concentrated in the pH 4.4 fraction, α-2 globulin was detected in fractions

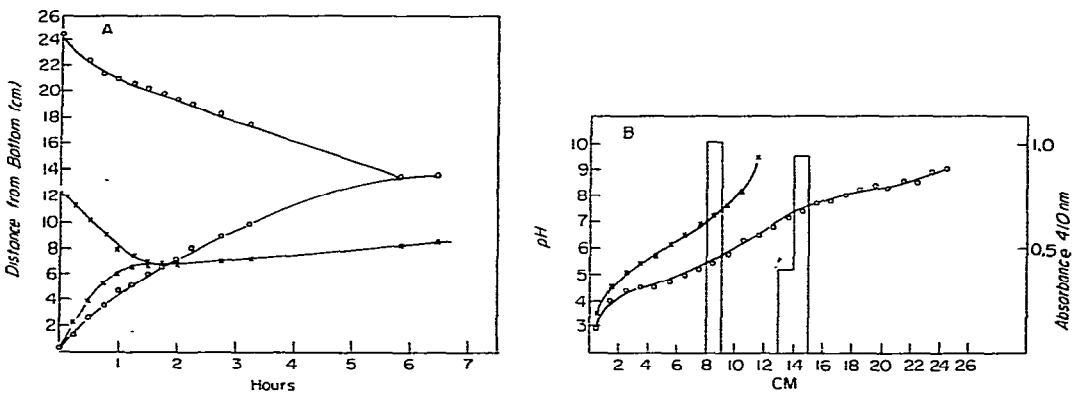

Fig. 1. Split-image electrofocusing of hemoglobin. A: samples (10 μl) of a human red blood cell extract were loaded on the top and bottom of cylinders (12.5 or 25 cm) of Sephadex G-75 (superfine); the migration distances were measured with a ruler by inspection through the clear chamber; the pH gradients were established with 1% Ampholine (pH 3.5–10) at 340–400 V and 4°. B: the gels were extruded from the tubes in 1-cm sections, suspended in 1.5 ml water plus 0.3 ml NaCl (0.85%), and placed in an ice bath prior to measuring the pH; the absorbance at 410 nm was determined in 1.5-ml cuvettes with a 1-cm light path.

of pH 5 and 5.5, and β-globulin focused in the pH 5.5 segment. Fractions of pH 6–6.6 appeared to contain the highest concentrations of γ-globulin free of other components. Based on these preliminary results, we expect the procedure to have wide application in the early and late phases of protein purification from small samples.

Comparisons of washed and unwashed Sephadex suspended in different concentrations of ampholyte

The optimal ampholyte concentration for electrofocusing on washed or unwashed gel was determined for the pH 3.5–10 gradient. Fig. 3 illustrates the appearance and locations of the marker proteins. Good separation occurred in each case but myoglobin was distorted in the unwashed gel containing 1% Ampholine. As the concentration of ampholyte was increased, the effective focusing limits approached the anode and cathode as evidenced by the locations of ferritin and cytochrome *c*, respectively. Cytochrome *c* migrated into the liquid portions above the gels containing 4% Ampholine and was removed when blotting the tubes prior to photography. The basic system found to be acceptable and most economical was the use of 1% Ampholine with washed gel.

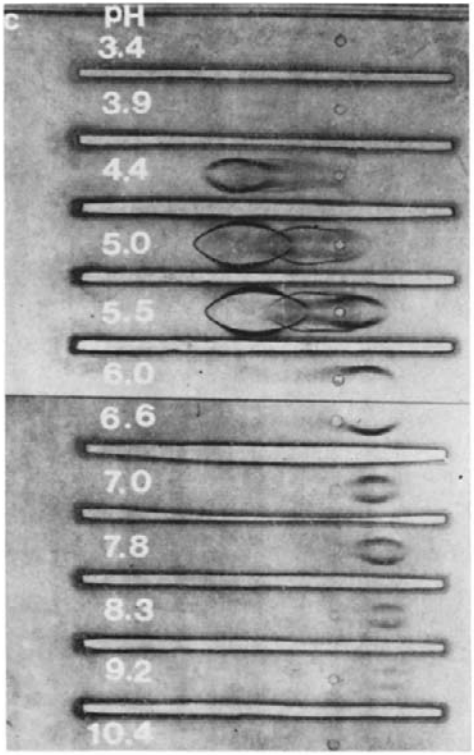

Fig. 2. Immunoelectrophoresis of electrofocused fractions of normal rabbit serum. Normal rabbit serum (60 μl) was subjected to electrofocusing in a 1% Ampholine mixture comprised of 4 parts pH 3.5–10 Ampholine and 6 parts pH 6–8 Ampholine. Focusing occurred at 170 V and 4°, for 17 h. The gel fractions (1 cm) were extruded into vials (4 ml) and suspended in 0.1 ml borate buffer (pH 8.6). Samples (2.5 μl) from each fraction were subjected to immunoelectrophoresis using goat anti-rabbit serum as antibody. The plates were stained with amido black.

Capacity

In an attempt to determine the capacity of the columns for focused protein, myoglobin was focused on a wide (steep) and a narrow (shallow) gradient, on separate occasions. Fig. 4A indicates that comparable separation was obtained with samples of 0.5–2 mg of myoglobin in the pH 3.5–10 gradient. Based on recovery experiments, 1.2 mg myoglobin derived from the 2-mg sample focused as the upper band and appeared to represent the capacity for one focused zone of a steep gradient.

The better separation provided by the shallow gradient suggested that greater quantities of protein could be accommodated (Fig. 4B), and the shallow gradient would facilitate recovery of isozymes. When sections were extruded for elution of the samples, 61% of the myoglobin was recovered in the upper band, 22% in the middle band, and 6% in the lower band. The p*I* values of the isozymes (6.2, 6.5 and 7.1) agreed with published values[10]. When a companion tube containing myoglobin was eluted dropwise, the three major bands were evident and the presence of a peak

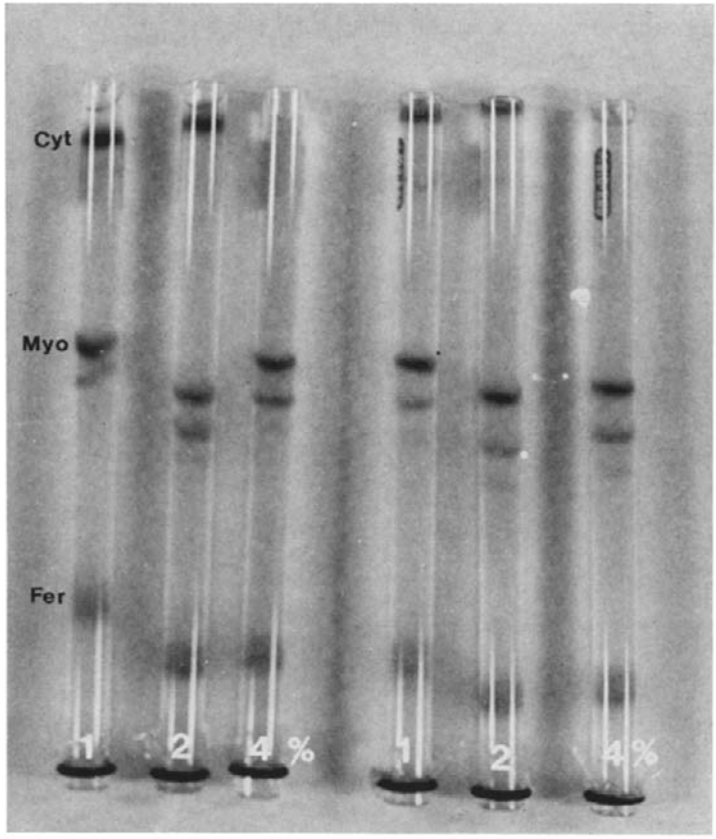

Fig. 3. Washed and unwashed Sephadex with different concentrations of ampholyte. Columns were prepared to contain washed or unwashed Sephadex G-75 (superfine) suspended in 1, 2, 3 or 4% Ampholine, pH 3.5–10. Each column received 150 μl of a mixture prepared with equal volumes 1% cytochrome *c*, 1% myglobin, and 1% ferritin; Ampholine was diluted in the sample mixtures to match the concentration contained in the recipient columns. The samples were focused at 100 V for 15 h and 400 V for 0.5 h, at 4°.

intermediate between bands 1 and 2 was suggested. Columns packed by centrifugation flowed slowly and discouraged recovery of samples by the drop method. Most of our experience involved the extrusion method for recovery of proteins, but the direct elution from gravity-packed columns may be advantageous when comparing closely related proteins by co-focusing.

DISCUSSION

This new procedure of electrofocusing in cylinders of granulated gels is easily managed and very convenient for comparative and small-scale preparative purposes. Columns of the desired pH can be prepared in advance and stored in screw-capped jars at 4°. We have not determined the maximal storage time but it would be limited primarily by the stability of the ampholyte employed. When stored, it is necessary to replace the weakened O-ring after adding the samples and installing the membranes.

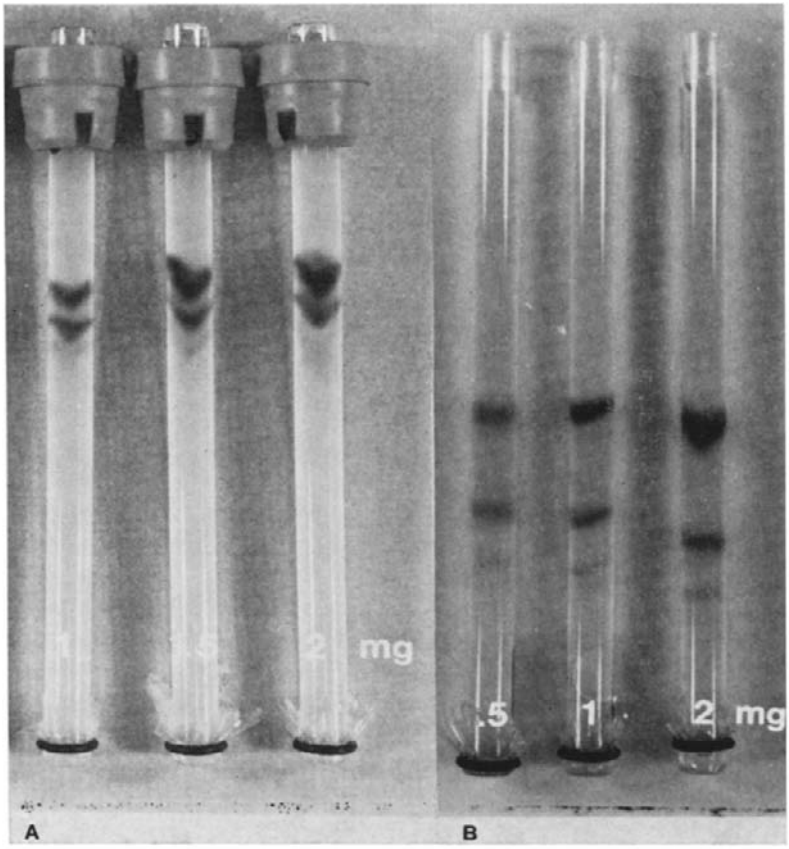

Fig. 4. Protein capacity of the focused zones. A: Ampholine (pH 3.5–10) was diluted 1:40 in 1% myoglobin. Samples of the mixture (50, 100, 150 or 200 μl) were applied to columns containing 1% Ampholine (pH 3.5–10) and focused at 170 V for 19 h and 400 V for 1 h, at 4°. B: in a separate experiment, the shallow gradient was established with Ampholine of pH 7–9; other conditions remained the same.

The capacity of the columns for electrofocused myoglobin was at least 1.2 mg; it occupied a 0.6-cm zone or 1/20 of the 12 cm bed volume. The apparatus employed accepts 12 tubes thus providing for the isolation of at least 14 mg of protein in one run. During preparative runs, comparable segments from parallel columns are collected in syringes for elution of samples[8].

While comparing closely related toxins from *Escherichia coli* and from *Vibrio cholerae*[11], 0.5-cm segments were extruded into wells of microtest plates for elution, and for quantitation by the single radial diffusion method. This method of electrofocusing facilitates the recovery of activity from small samples and is especially convenient in combination with the collection and storage of fractions in the microtest plates.

Drawbacks in the procedure include the inability to obtain direct filter-paper prints of the focused proteins for analytical purposes, and the remerging of close but distinctly separated bands during the extrusion process. Also, we need to establish the conditions required to obtain better shallow gradients in the neutral and alkaline ranges. In spite of these limitations, the system is ideally suited for obtaining the shallow gradient of pH 2.5–4, and the steep gradient of pH 3.5–10 which provides approximate determination of the p*I* values of proteins, and for use as an early or late semi-preparative procedure. The procedure is not precisely analytical nor optimal for preparative purposes; it appears to be intermediate between analytical electrofocusing in polyacrylamide gel[3] and preparative electrofocusing in large cylinders[7] or on flat beds of granulated gels[1,2]. Electrofocusing on sucrose gradients in 1-ml syringes[5,6] probably would be more suitable for the recovery of nanogram quantities of protein.

ACKNOWLEDGEMENTS

I am deeply grateful for the photographic assistance of Mrs. Lily K. Fainter and the technical assistance of Miss Devyani Desai. This project was supported by a National Heart, Lung, and Blood Institute Grant HL 16769, and the North Carolina Lung Association.

REFERENCES

1 B. J. Radola, *Biochim. Biophys. Acta*, 295 (1973) 412.
2 B. J. Radola, *Biochim. Biophys. Acta.*, 386 (1974) 181.
3 C. W. Wrigley, *Methods Enzymol.*, 22 (1971) 559.
4 O. Vesterberg, *Methods Enzymol.*, 22 (1971) 389.
5 J. Press and N. Klinman, *Immunochemistry*, 10 (1973) 621.
6 R. J. Gearhart, N. H. Sigal and N. R. Klinman, *Proc. Nat. Acad. Sci. U.S.*, 72 (1975) 1707.
7 T. J. O'Brien, H. H. Liebke, H. S. Cheung and L. K. Johnson, *Anal. Biochem.*, 72 (1976) 38.
8 A. Winter, H. Perlmutter and H. Davies, *LKB-Produkter AB Application Note* 198 (1975).
9 P. G. Righetti and J. W. Drysdale, *J. Chromatogr.*, 98 (1974) 271.
10 P. G. Righetti and T. Caravaggio, *J. Chromatogr.*, 127 (1976) 1.
11 S. H. Richardson, W. F. Osborne, D. E. Lockwood and S. H. Love, *Proc. 12th Joint Cholera Conf., 1976*, U.S.–Japan Cooperative Medical Science Program, National Institute of Health, Sapporo, Japan, 1976.