Journal of Chromatography, 346 (1985) 265–270
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 17 978

ADAPTATION OF THE EQUIPMENT FOR HIGH-PERFORMANCE ELECTROPHORESIS TO ISOELECTRIC FOCUSING

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(Received June 25th, 1985)

SUMMARY

Fast and reproducible isoelectric focusing experiments have been performed in glass capillaries of 0.2 mm I.D., wall thickness 0.1 mm, and length 120 mm. These narrow, thin-walled tubes permit rapid removal of the Joule heat to allow high voltages (3000 V) and short run times. The experiments were carried out in equipment designed for high-performance electrophoresis. The protein zones were detected by on- or off-tube measurements of their UV absorption. This detection technique requires that the proteins can be mobilized following the isoelectric focusing step. This mobilization has been achieved either by pumping the zones past the stationary UV monitor (with the voltage applied to avoid zonal broadening during the elution) or by electrophoretic elution, accomplished by replacing the acid at the anode by a base (or the base at the cathode by an acid), which caused the pH gradient —and thereby the proteins— to move in the tube. The electrophoretic mobilization procedure is more universal because it is also applicable when the focusing is performed in a gel.

INTRODUCTION

Two types of apparatus for high-performance electrophoresis (HPE) have been described, one for analytical[1,2] and one for micropreparative as well as analytical experiments[3–5]. In the former version we use an on-tube and in the latter an off-tube detection technique. The HPE equipment can be employed not only for zone electrophoresis[1–5] but also for displacement electrophoresis[6]. In this paper we show that the application range can be extended to include isoelectric focusing. Since the HPE apparatus can detect only migrating solute zones we had to develop methods to mobilize the train of focused zones, preferably without impairment of the resolution obtained in the focusing step. In this paper two such mobilization methods will be described.

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0021-9673/85/$03.30

MATERIALS AND EQUIPMENT

Human transferrin was a gift from KABI/Vitrum, Stockholm, Sweden. Hemoglobin was prepared from outdated human erythrocytes. The carrier ampholytes (Pharmalyte™, pH 3–10) were bought from Pharmacia, Uppsala, Sweden. The protein zones in the electrophoresis tube were recorded either on-tube as they migrated electrophoretically past a detector consisting of a monochromator giving an 0.2–0.3-mm wide UV beam, which passed through the electrophoresis tube onto a photomultiplier connected to a recorder[1,2], or off-tube as they left electrophoretically the capillary tube and were transferred to a high-performance liquid chromatographic (HPLC) detector by an HPLC pump[4].

EXPERIMENTAL AND RESULTS

Isoelectric focusing and subsequent mobilization of the protein zones by hydrodynamic flow (Method A)

The electrophoresis tube had the dimensions 0.2 I.D. × 0.4 O.D. × 120 mm. Its inside wall was coated with methylcellulose (as described on p. 163 in ref. 7) to eliminate zone distortion caused by electroendosmosis and possible adsorption of the solutes (proteins) to the tube wall (an equation showing the favourable effect of methylcellulose is given on pp. 171 and 172 in the same reference). The electrophoresis tube was then filled with a 1% solution of Pharmalyte (pH 3–10) containing hemoglobin and transferrin. Phosphoric acid (0.02 *M*) served as anolyte and sodium hydroxide (0.02 *M*) as catholyte. The focusing was conducted at 1000 V for 7 min (the current decreased from 40 to 15 μA) and then at 3000 V for 13 min (visual inspection of the hemoglobin zones showed that a steady state had been attained after this time). A pump connected to the cathodic end of the electrophoresis tube via a T-tube was then started to deliver 0.02 *M* phosphoric acid into the electrophoresis tube at a rate of *ca.* 0.05 μl/min. The free end of the T-tube, closed with a dialysis membrane to prevent non-controllable liquid flow in the electrophoresis tube, was immersed in the cathodic vessel during both the focusing and the mobilization steps. The protein zones were monitored at 280 nm as they were pumped past the stationary UV detector (Fig. 1). The voltage (3000 V) was maintained during the pumping in order to eliminate distortion of the zones.

Isoelectric focusing and subsequent electrophoretic mobilization of the protein zones (Method B)

The methylcellulose-coated electrophoresis tube had the same dimensions as the tube employed in the above experiment, and the same sample, Pharmalyte, anolyte and catholyte were used. An agarose gel plug (*ca.* 3 mm long) at the cathodic end of the electrophoresis tube eliminated the risk of hydrodynamic flow in the tube. At a voltage of 1200 V the proteins became focused within 20 min. The phosphoric acid in the anode vessel was then replaced by 0.02 *M* sodium hydroxide and the voltage was increased to 3000 V. The current was initially 14 μA, increased gradually during the mobilization, was *ca.* 30 μA when the last peak had passed the detector and then continued to increase thereafter. All protein zones had passed the detector (280 nm) after electrophoresis for *ca.* 15 min. The electropherogram is shown in Fig. 2a.

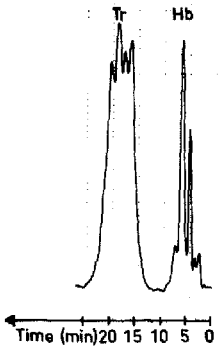

Fig. 1. Elution of focused protein zones by hydrodynamic flow and monitoring by on-tube detection. The sample consisted of 10 μg of human hemoglobin (Hb) and 15 μg of human transferrin (Tr). The experiment was carried out in the HPE equipment[1,2]. The protein zones were recorded as they passed a stationary UV detector monitoring at 280 nm.

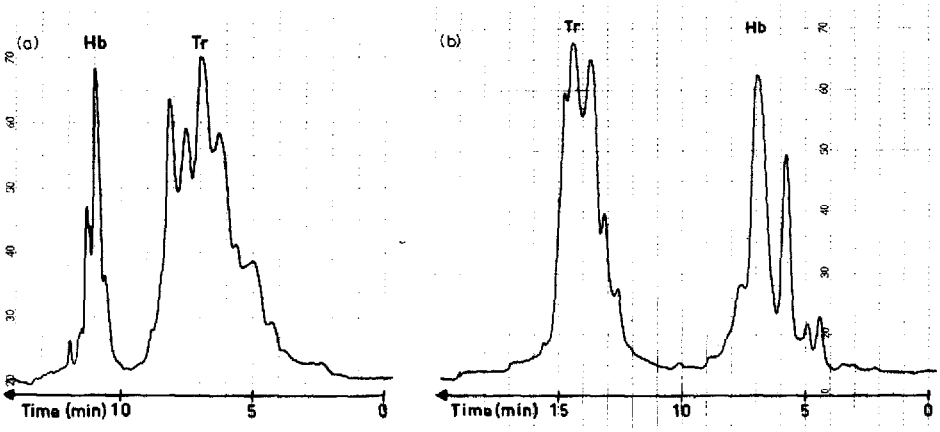

Fig. 2. Electrophoretic elution of focused protein zones and their monitoring by on-tube detection. The same sample and equipment were used as in the experiment shown in Fig. 1. The elution was achieved by replacing the acid at the anode with a base (a), or the base at the cathode with an acid (b).

To determine whether alterations in the protein pattern and in the pH gradient occur during the mobilization of the protein zones, the above experiment was repeated in the free-zone electrophoresis apparatus, where stabilization against convection is achieved by slow rotation (40 rpm) of the horizontal quartz electrophoresis tube (380 × 3 I.D. × 8 mm O.D.) around its long axis[7]. The latter apparatus is very suitable for this type of measurement since it permits scanning of the protein pattern at any time during both the focusing and the mobilization and also allows one to withdraw zones from the tube after completion of a run, for instance, for pH measurements. The result of such an analysis is shown in Fig. 3. As curve I indicates, the pH gradient in the electrophoresis tube extended from 7.0 to 9.6 at the moment when the transferrin zone had just migrated out of the tube. This experiment was carried out overnight at a constant voltage of 500 V.

The experiment shown in Fig. 2a was repeated, with the difference that the

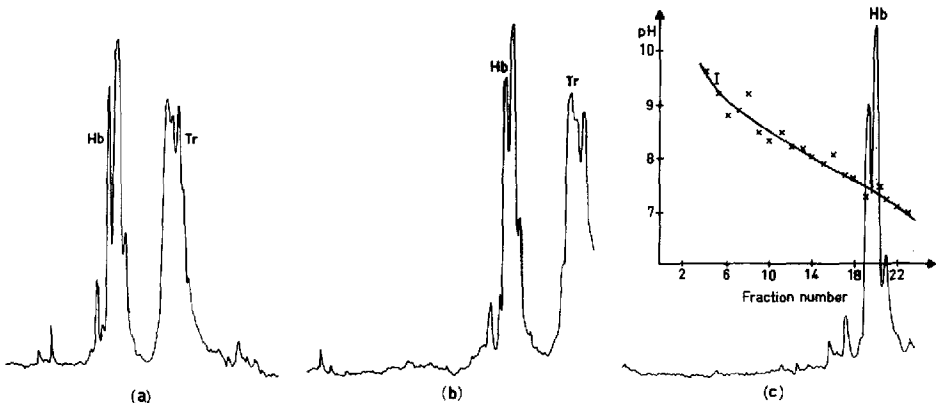

Fig. 3. Isoelectric focusing (a) of the same proteins as were used in the experiment shown in Fig. 2 and subsequent elution of the protein zones by replacing the acid at the anode by a base (b, c). The experiment was carried out in the free-zone electrophoresis apparatus[7]. After completion of the run, 1-cm fractions were withdrawn from the revolving electrophoresis tube for pH measurements (curve I).

mobilization step was performed with 0.02 *M* phosphoric acid in both the electrode vessels (Fig. 2b). When this experiment was performed in the above free-zone electrophoresis apparatus, the pH gradient extended from 3.0 to 7.5 at the moment when the hemoglobin left the electrophoresis tube.

Electrophoretic elution of focused zones is applicable both in free solution (Figs. 2a and 2b) and in gel media. The example shown in Fig. 4 refers to an experiment performed under the same conditions as that shown in Fig. 2a, with the difference that the focusing was done in a polyacrylamide gel of the composition $T = 3\%$; $C = 3\%$ (for definition of these parameters, see ref. 8), rather than in free solution.

In all the above experiments the protein zones were monitored by an on-tube detection method. However, a pattern similar to that shown in Fig. 2a was obtained when the experiment was repeated with off-tube detection[3]. The pump delivered 0.02 *M* sodium hydroxide to the flow cuvette of the HPLC detector at a rate of 0.15 ml/min.

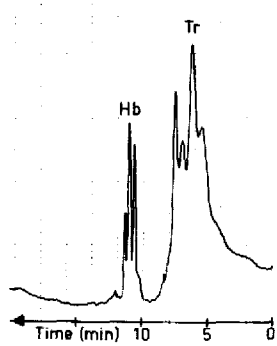

Fig. 4. An isoelectric focusing experiment similar to that shown in Fig. 2a, with the main difference that it was performed in a polyacrylamide gel instead of in free solution.

DISCUSSION

In 1976 McCormick *et al.*[9] and in 1978 McCormick *et al.*[10] showed that, following isoelectric focusing, "selective zone recovery could be achieved by transposition of the gels into either isoelectric ampholytes or charged buffers". We believe that this paper is the first to describe successful one-step mobilization of all the isoelectrically focused protein zones by changing the composition of the electrode solutions. Although our studies on mobilization of the train of focused proteins by changing the composition of the anolyte or catholyte originated from attempts to use the HPE electrophoresis equipment for isoelectric focusing, the mobilization technique described can be adapted to runs in other types of column as well.

For a discussion of the mobilization of the protein zones following the focusing step we refer to the experiment presented in Fig. 2a. When the acid at the anode is replaced by sodium hydroxide, the hydroxyl ions begin to migrate through the electrophoresis tube toward the anode and the sodium ions at the anode begin to move toward the cathode. The migrating sodium ions will cause a continuous increase in the conductivity of the solution in the electrophoresis tube, and the migrating hydroxyl ions an increase in both conductivity and pH. The change in pH will not be abrupt because the carrier ampholytes have a good buffering capacity. (Since the hydrogen ions in the sodium hydroxide solution are present at much lower concentration than the hydroxyl ions, they will contribute much less than the hydroxyl ions to a change in pH and conductivity in the electrophoresis tube, and can therefore be neglected in this discussion.) When the ampholytes and the proteins are titrated by the migrating hydroxyl ions they become negatively charged and will move towards the anode in agreement with Fig. 3. The above-mentioned increase in conductivity caused by the sodium and hydroxyl ions, and strengthened by the charged ampholytes, is manifested as an increase in the current (at constant voltage) during the mobilization process.

It is premature to discuss whether elution by hydrodynamic flow (Fig. 1) or by electrophoresis (Figs. 2 and 4) gives the higher resolution. However, a comparison of these figures does not reveal any large differences in the separation pattern.

In Fig. 2a the resolution of the transferrin components is better than in Fig. 2b, whereas the hemoglobin components are resolved better in Fig. 2b. If this reflects a general phenomenon one should perform the mobilization of the proteins toward both the positive and the negative pole to see which procedure gives the higher resolution.

To judge from Fig. 3 the resolution is not affected much during the mobilization of the protein zones. This is in agreement with the observation that hemoglobin is positioned in the moving pH gradient at a pH close to its isoelectric point, which is *ca.* 7.0; (see curve I in Fig. 3c).

Elution of separated protein zones by hydrodynamic flow (Method A above) is of course, not applicable if the capillary tube contains a gel. In such cases one can use the alternative technique and perform the mobilization by changing the composition of the catholyte or anolyte (Method B above). We have used the latter technique when the isoelectric focusing was carried out in a polyacrylamide gel (Fig. 4).

In agreement with observations made by other authors[11–14], both transferrin

and hemoglobin appeared in multiple forms upon isoelectric focusing (Figs. 1–4). Since we have not taken any precautions to prevent release of iron from the transferrin, some of the transferrin peaks obtained may correspond to molecules with reduced iron content. Since the focusing experiments (including the mobilization step) performed in the free-zone electrophoresis equipment took much longer (run overnight) than those carried out in the HPE equipment (*ca.* 30 min), the differences between the protein patterns obtained with these different types of apparatus (Fig. 3 and Figs. 1, 2 and 4, respectively) can possibly be ascribed to transferrin molecules with different numbers of iron atoms.

Perhaps the HPE apparatus with the off-tube detection system gives electropherograms with somewhat lower resolution than the apparatus with on-tube monitoring. On the other hand, the former apparatus has the advantage that it permits recovery of the separated proteins and can thus be used for micropreparative runs.

In this paper we have shown the potentialities of a hydrodynamic and an electrophoretic method to mobilize the pH gradient and the focused proteins. Both methods permit rapid detection of the protein zones without time-consuming staining. Therefore we feel it is important to publish the results at this early stage in the development of the methods, even if much remains to be investigated.

ACKNOWLEDGEMENTS

The authors are much indebted to Mrs. Karin Elenbring for very skilful technical assistance. The work was supported by the Swedish Natural Science Research Council.

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