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Note

Improved method of separating and quantitating hemoglobin compositions by isoelectric focusing

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Isoelectric focusing on polyacrylamide gels is a popular analytical tool for separating and identifying many different component mixtures[1–4]. The many advantages and some disadvantages of this technique have been described in detail elsewhere[1]. One of these disadvantages is that after completing the electrophoresis, the polyacrylamide gels must be removed from the gel tubes. The gels are either placed in special 10-cm cuvettes for scanning in a spectrophotometer or they may be fixed and stained prior to scanning. We have found that removing isoelectric focusing gels from the tubes is sometimes difficult, especially when using "soft" gels. Cutting gels to fit into the cuvette is also not very precise. Quantitation of the amount of materials in the polyacrylamide gels is determined by measuring the area under each peak of the resultant chromatogram.

In this communication, we describe a simple, rapid method of running long polyacrylamide isoelectric focusing gels and quantitating the amounts of materials present without removing the gels from the tubes. Another advantage of this method is that we have increased the width of the pH gradient over the gels and thus improved the separation of the hemoglobins.

EXPERIMENTAL

Equipment. The isoelectric focusing equipment used was the standard cylindrical unit for gel tubes. To accomodate the longer gel tubes used, new, lower buffer chambers were built. These chambers were made of plastic cylinders cemented to a square base with the appropriate polymer solvent. The cylinder was 7 3/4 in. × 5 in. O.D. × 4 1/2 in. I.D. The base was 6 in. × 6 in. × 1/2 in.

The power supplies used were either a Büchler Model 3-1014A or a Hewlett-Packard Model 6521A. Measurements of pH were made on a Radiometer Model 26 pH meter.

Chemicals. Carrier ampholytes were obtained from LKB (Stockholm, Sweden). We used Ampholine pH 6–8, batch numbers 25, 26, and 27. Electrophoresis-grade acrylamide and N,N′-methylene bisacrylamide were purchased from Eastman (Rochester, NY, U.S.A.). All other chemicals were analytical-reagent grade from various sources.

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Methods. Two electrophoresis gel tubes, 10 cm × 8 mm O.D. × 5 mm I.D., were connected by a 1.25-cm piece of 1/4 in. × 1/16 in. Tygon tubing. The bottom of the joined tubes were covered with parafilm and then inserted into the electrophoresis apparatus. A stock solution of 6.67% acrylamide containing 0.33% bisacrylamide was made and stored refrigerated for up to a month. A 45-ml volume of this stock solution was placed in a 50-ml graduated cylinder to which 2.5 ml of the ampholine, 25 μl of N,N,N′,N′-tetramethylethylenediamine (TEMED) and water were added to a total volume of 50 ml. A 25-mg amount of ammonium persulfate was placed in a 125-ml erlenmeyer flask to which the 50 ml of solution was added and swirled. This solution was rapidly transferred with a Pasteur pipette into the 20-cm gel tubes. Water was layered over the top and the gels were allowed to polymerize for 1 h. The upper reservoir buffer was 0.5% monoethanolamine and the lower reservoir buffer was 0.2% sulphuric acid. The gels were pre-electrophoresed at 200 V for 1 h and the buffers were discarded. Samples (50–100 μl in 15% sucrose) were layered onto the top of the gel. A 5% sucrose solution was layered over the top of the samples to fill the tubes. New buffers were put into the cathode and anode compartments. The electrophoresis was started at 200 V for 1/2 h, then 300 V for the next 1/2 h and finally set at 400 V for 17 h. The power supply was set on constant voltage. As the electrophoresis progressed, the current gradually decreased. All electrophoresis experiments were performed in the cold room at 4°C.

At the end of each run the pH gradient was determined on one of the gels. The gel was cut into 1-cm slices and soaked in 1 ml of deionized, distilled water. The 20-cm tubes which contained samples were cut with a razor blade at the junction between the two tubes. The tubing that connected the two tubes was completely removed and each of the two 10-cm electrophoresis tubes and gels was placed directly into the Gillford Model 240 gel scanner cuvette holder. The tubes were scanned at 2 cm/min at 540 nm and the absorbance was recorded on a strip chart recorder.

RESULTS

A representative sample of the pH distribution along the gels is shown in Fig. 1. By extending the length of the gel, we have expanded the pH gradient, especially in the pH 7–8 region which works well for hemoglobin separations. A typical hemo-

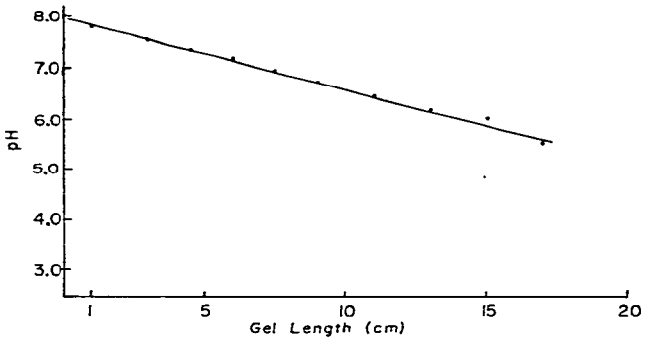

Fig. 1. pH Gradient using LKB ampholyte pH 6–8. Gel length is measured from the bottom of the meniscus.

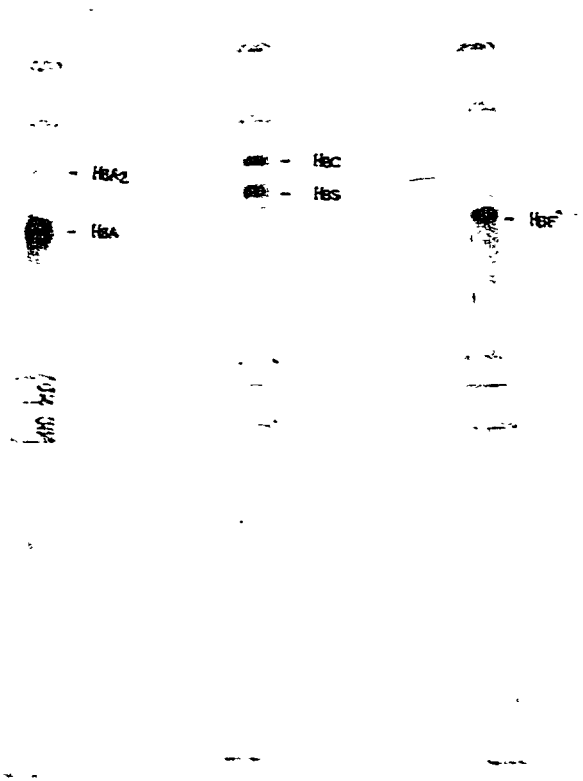

Fig. 2. The separation of human hemoglobins is shown. Note that two 10-cm gel tubes are connected by a short piece of tubing. The two tubes are removed from the Tygon tubing for quantitation of the amounts of material present.

globin separation is shown in Fig. 2. Here we have shown the separation of human hemoglobin into its major and minor components. In Fig. 3 we show the scan of the separation through the gel tube. The hemoglobin zones are separated readily and quantitated by calculating the area under each peak of the chromatogram.

DISCUSSION

We have modified the widely used technique of analytical isoelectric focusing on polyacrylamide gels to make quantitation of the separate zones more rapid and convenient. This has been accomplished by joining two 10-cm gel tubes together. Each tube exactly fits into the cuvette holder of the Gilford spectrophotometer scanning attachment. We have found that by lengthening the tube, we get a better pH gradient and hence a better separation of the hemoglobin components. Connecting more 10-cm tubes to give a 30- or 40-cm tube would be practical and feasable. By building a taller anode compartment, even longer gel tubes could be used. The advantage to connecting two or more 10-cm tubes together by tubing is that each of the pieces fit directly into the gel scanner, thus eliminating the need for removal of the gel

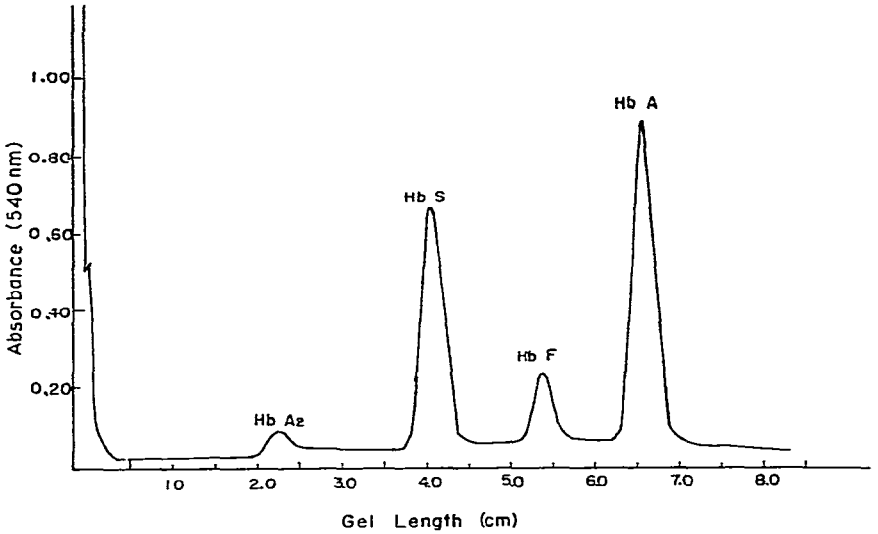

Fig. 3. Isoelectric fractionation of human hemoglobins. The wavelength and scanning conditions are described in the *Methods* section.

from the tube for quantitation. This technique should also work on non-colored proteins in the UV region if special tubes were purchased.

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