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SIMPLE METHOD FOR THE ISOLATION AND PURIFICATION OF HEMOGLOBIN COMPONENTS

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SUMMARY

A simple method for the isolation and purification of hemoglobin components from starch gel by electrophoresis is described. An inverted bottle with a cut off bottom is used. Pieces of gel containing the hemoglobin are embedded in potato starch, wetted with buffer, and placed in the neck of the inverted bottle, the mouth of which has been closed with filter paper and tape. Connection with the negative tank buffer is achieved by inserting a layer of starch gel between the potato starch and the negative tank buffer. By electrophoresis, the hemoglobin migrates and accumulates in a dialysing tube tied around the mouth of the bottle and hanging into the positive tank buffer.

INTRODUCTION

Separation of hemoglobin components can be achieved by various methods. Excellent results are obtained by starch gel electrophoresis, using Tris–EDTA–boric acid buffer; however, this method is normally not used for the isolation and purification of specific components because they are difficult to elute from the gel.

Several methods for the elution of hemoglobin or other proteins from starch gels have been reported[1–12]. However, these are all either technically cumbersome, not very effective since the recovery of the components is very incomplete, or require complex equipment to elute the hemoglobin or other proteins from pieces of gel.

A simple method for the purification and complete elution of hemoglobin using uncomplicated equipment is described in the present communication.

MATERIALS AND METHODS

Materials

Hemoglobin components are separated by the standard method of starch gel electrophoresis[9], with a suitable buffer such as Tris–EDTA–boric acid buffer (pH 8.6).

The equipment for elution consists of an ordinary inverted household bottle with a smoothly curved neck (such as a wine or syrup bottle), of which the bottom

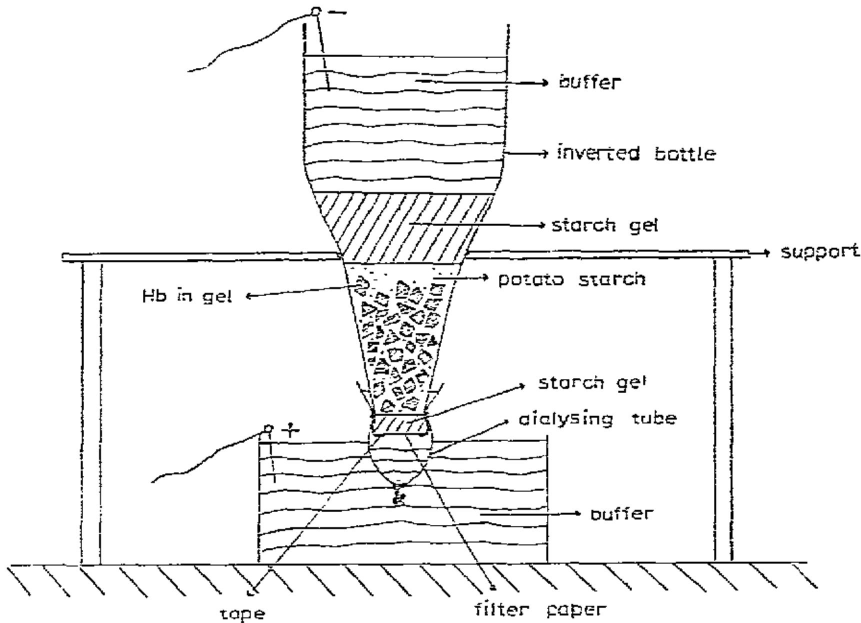

Fig. 1. Diagram of apparatus for elution of hemoglobin from starch gels making use of an inverted bottle.

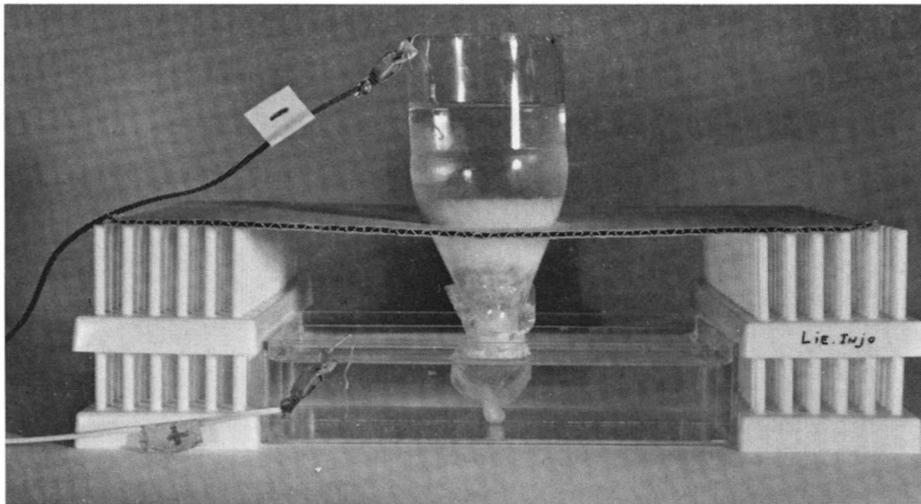

Fig. 2. Actual set-up of the method used for elution of hemoglobin from pieces of starch gel.

part has been cut off (Figs. 1 and 2) and other items commonly used for starch gel electrophoresis.

Method

(a) Cut two or three round pieces of filter paper big enough to cover the

mouth of the bottle and place them over the mouth opening. Secure just the edges of the filter paper with a piece of strong tape, either by taping over the filter paper and cutting a hole in the middle of the tape or by taping the edges to the outer sides of the bottle opening. In either case, the goal is to expose a large amount of the filter paper, so that hemoglobin will not be trapped in the corners above the taped portions of the filter paper.

(b) Pour hot hydrolysed starch gel prepared in Tris–EDTA–boric acid buffer (pH 8.6) (degassing is not necessary) on the filter paper, forming a layer of gel about 1 cm thick. Let the gel cool and solidify.

(c) Tie a knot in one end of a dialysing tube which has been soaked in water and which has a diameter greater than that of the bottle mouth. Fill the tube with Tris–EDTA–boric acid buffer (pH 8.6). Invert the bottle and immerse its mouth in the buffer in the dialysing tube. Secure the dialysing tube tightly around the bottle neck with a rubber band, placing it below the buffer surface so that no air is trapped in the tube. This can be carried out more easily by two persons. Do not discard the buffer that remains in the dialysing tube above the rubber band because it ensures that no air will enter the tube.

(d) Mix potato starch powder with Tris–EDTA–boric acid buffer (pH 8.6) to make a paste. Neatly cut the specific hemoglobin component to be isolated from the starch gel or gels in which it has been run. Cut the gel into smaller pieces (crushing or smashing is not necessary). Mix the pieces of the gel with the potato paste and place them in the inverted bottle above the layer of gel already on the filter paper.

(e) After the potato starch has settled, some excess buffer will be seen on the surface of the potato starch. Add dry potato starch slowly until all gel pieces containing the hemoglobin are embedded. Add more potato starch powder until the surface looks damp but fluid is not visible. Because the pieces of starch gel containing the hemoglobin tend to rise to the surface of the potato starch, do not use too much starch paste because it will unnecessarily lengthen the path traveled by the hemoglobin molecules. If too much dry potato starch is accidentally poured on the surface, add drops of buffer until the surface is damp.

(f) Pour a layer of hot hydrolysed starch gel prepared in Tris–EDTA–boric acid buffer (pH 8.6) (degassing is not necessary) over the potato starch and let it cool and solidify. After the gel has solidified, carefully pour Tris–EDTA–boric acid buffer (pH 8.6) on it. Then place an electrode in this negative tank buffer and connect it to the negative pole of a power supply.

(g) Place the inverted bottle, with the dialysing tube hanging downward, on a support, putting the bottle neck through a round hole in the support (Figs. 1 and 2). Fill a tank with Tris–EDTA–boric acid buffer (pH 8.6); position the support over the tank so that the dialysing tube extends into the positive tank buffer. However, do not immerse the filter paper-covered bottle mouth in the buffer, since this will lead to incomplete elutions.

(h) Place an electrode in the positive tank buffer and connect it to the positive pole of the power supply. Start a potential gradient of 8–10 V/cm (voltage usually around 80–100 V) through the system, and keep it running overnight. The next day usually all hemoglobin will have accumulated in the buffer in the dialysing tube, leaving the gel and potato starch free of hemoglobin. The elution is now complete. Slow-moving components such as Hb A_2 take a longer time for elution.

Caution

Do not freeze the gel from which the hemoglobin is to be eluted. Elution from frozen pieces of gel is usually incomplete, as frozen gel has a fibrous consistency and tends to trap the hemoglobin.

RESULTS AND DISCUSSION

The method currently used for isolation and purification of hemoglobin components is usually performed by ion-exchange column chromatography, in which different types of ion exchangers are used. However, many laboratories have difficulty obtaining consistent results because success of the procedure depends upon the batch of ion exchanger used and proper packing of the column. Hemoglobin components eluted from the column can be contaminated with others, even if the procedure is performed correctly, so that it has to be repeated, preferably using a different type of ion exchanger. Starch gel electrophoresis is usually used for the final judgment of purity of the hemoglobin components. Therefore, the most reliable and effective method of isolating and purifying hemoglobin would be to elute the hemoglobin from the starch gel after electrophoretic separation.

As early as 1950 Gordon *et al.*[3] attempted to elute protein components from agar gel slabs by electrophoresis. By their method, an agar gel slab containing the protein to be eluted is placed on a cellophane membrane, isolating a pool of buffer at one end of the slab. This is followed by a period of electrophoretic elution into the pool thus formed. Since the agar slab and the buffer containing the collected protein are in the same compartment (in our method they are in separate compartments) it is always difficult to keep the buffer from migrating to the opposite side. Moreover, the buffer level must continuously be adjusted to compensate for electro-endosmosis and to prevent the slab from becoming dry. In addition, a particular protein component cannot be cut out from different gels, pooled and placed in a small space in their method. This can easily be done in our method. The whole set-up of their method is also more complicated than the inverted bottle method, and not as flexible.

In other publications, hemoglobin was reportedly isolated by crushing or smashing the pieces of gel containing the hemoglobin component and eluting it with buffer or water. However, the isolated hemoglobin is always found to be accompanied by starch gel in colloidal solution, which cannot be separated by filtering. Repeated freezing and thawing is somewhat beneficial. In our earlier attempts, we tried to get rid of starch gel in colloidal solution by freezing and thawing the hemoglobin solution and running and eluting it through a short column of DEAE-cellulose with 0.05 *M* Tris–HCl buffer (pH 6.5). Small amounts of material can be handled in this way, but large amounts will cause blockage of the column because accumulated gel remains on top of the column.

In the method of Tsuyuki[12] and in all its modifications reported to date, the gel containing the hemoglobin is cut in small pieces or is mashed and suspended in buffer. It is then placed in a separate compartment, which has to be connected to the negative and positive tank buffers. This poses a problem. In order to achieve these connections, special equipment had to be devised; modifications of Tsuyuki's method vary only in the equipment devised. Another problem with Tsuyuki's method and its modifications is that the hemoglobin in the buffer, being free, tends to circulate back

to the cathode part of the compartment instead of progressing to the anode side. The result is that recovery is never complete, no matter how long the electrophoresis is carried out.

In our method, the gel pieces containing the hemoglobin are embedded in potato starch; therefore, hemoglobin migrating to the anode side cannot circulate back to the cathode side. Further, the connection between the medium carrying the hemoglobin and the tank buffer is easily made by placing a gel layer on top of the potato starch and placing the buffer on top of the gel layer. Another advantage of our method is that all dimensions are flexible —for large amounts big bottles can be used, while for minute quantities very small bottles (even short stem funnels or plastic bottles) can be employed. Furthermore, the layers of potato starch and starch gel can be varied considerably according to the amount to be eluted. A small layer of starch gel between the filter paper and potato starch is important for freeing the hemoglobin from colloidal gel, which would go through filter paper, but not through starch gel.

The method is also suitable for the isolation of proteins other than hemoglobin. It is especially useful for the separation of hemoglobin components that migrate very near to each other (such as Hb A and Hb F, or Hb Bart's and Hb H) and therefore are difficult to separate by other methods. We usually obtain pure components with the first attempt (Fig. 3). Our method has served in the laboratory for some time with great satisfaction.

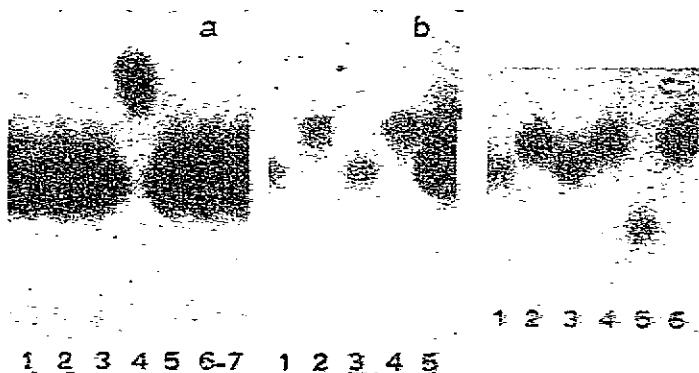

Fig. 3. Starch gel electrophoretic patterns of purified hemoglobin components, pH 8.6. (a) Slot 4, purified Hb Bart's obtained from newborn with 5.6% Hb Bart's; slots 1, 2, 3, 5, 6 and 7, normal hemolysates with Hb A and Hb A_2. (b) Slots 1 and 3, purified Hb F; slots 2 and 4, purified Hb A; slot 5, normal cord blood hemolysate. (c) Slots 1 and 3, purified Hb F components; slot 5, purified Hb A_2; slots 2, 4 and 6, normal Hb A; results obtained at first attempt of purification.

ACKNOWLEDGEMENTS

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