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Note

Chromatographic fractionation of multiple forms of red blood cell hexokinase

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It is well established that four hexokinase (E.C. 2.7.1.1) isozymes (named I, II, III and IV in order of increasing electrophoretic mobility towards the anode) are present in mammalian tissues[1,2]. Conflicting results have been reported for the hexokinase pattern in the erythrocytes[3–14]. The discrepancies are mainly due to the presence of sub-types or multiple forms of hexokinase I and to the difficulties related to their separation. Electrophoresis on starch gel, agarose gel, cellulose acetate membrane, polyacrylamide gel, and isoelectric focusing or ion-exchange chromatography have been employed as separation techniques of the red blood cells isozymic pattern. Unfortunately starch gel, agarose gel and cellulose acetate have been found unsuitable as media for hexokinase isozymes separation owing to the lack of resolution. Polyacrylamide disc gel electrophoresis or electrofocusing, on the other hand, inactivate some isozymes.

In this paper we propose the use of small DE-52 ion-exchange columns for the resolution and the complete recovery of the hexokinase isozymic pattern in red blood cells. This approach has been used successfully in studies of the hexokinase isozymic patterns of red cells of different ages[15] and of different mammalian species[16,17]

MATERIALS AND METHODS

Materials

Coenzymes, enzymes, substrates and dithiothreitol were obtained from Sigma (St. Louis, MO, U.S.A.). DE-52 was purchased from Whatman (Maidstone, Kent, Great Britain). All other reagents were of analytical grade.

Methods

Rabbit and human blood samples were collected using EDTA as anticoagulant. Red blood cells were washed and haemolysed as previously described[15]. Rabbit reticulocytes were obtained as in ref. 15. DE-52 column chromatography (24 × 0.35 cm I.D. unless otherwise indicated) was performed at 4°C in 5 m*M* sodium potassium phosphate buffer (pH 7.5) containing 1 m*M* glucose, 3 m*M* KF, 3 m*M* 2-mercaptoethanol and 5 m*M* dithiothreitol. Flow-rates were maintained at 5.0 ml/h using a peristaltic pump (Gilson minipuls 2). Fractions (0.7 ml) were collected in a fraction collector (LKB Ultrorac II) and assayed for hexokinase activity; the absorbance was

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monitored at 280 nm. Columns were developed with 280-ml linear gradients by an automatic gradient former (Gilson Mixograd) from 0 to 0.4 *M* KCl in the same sodium potassium phosphate buffer.

Enzyme assay

Hexokinase activity was measured spectrophotometrically at 37°C in a system coupled with glucose-6-phosphate dehydrogenase (EC 1.1.1.49) and 6-phosphogluconate dehydrogenase (EC 1.1.1.44) as previously described[16]. One unit of enzymatic activity is defined as the amount of enzyme that catalyses the formation of 1 μmol of glucose-6-phosphate per minute at 37°C.

RESULTS AND DISCUSSION

As part of the study of the regulatory and biochemical properties of the multiple forms of hexokinase in the erythrocytes, we have developed a method that permits the separation and recovery of hexokinase isozymes starting from haemolysates.

After some preliminary experiments with polyacrylamide disc gel electrophoresis, isoelectric focusing and agarose gel electrophoresis, we decided to approach this study by utilising the DE-52 ion-exchange chromatography method. However, the successful application of this method requires consideration of several parameters and depends on the optimization of all the experimental conditions.

Influence of column dimensions

Fig. 1 shows the experiments carried out to determine the influence of column dimensions on hexokinase resolution. When the ratio (*R*) of the height to the diameter of the column was varied from 20 to 70 the column resolution increased. At higher *R* values the two hexokinase isoenzymes are separated with loss of resolution (Fig. 1D). These results were obtained by loading 1-ml samples; larger volumes of haemolysates significantly affect the separation. Furthermore, correct column packing plays a significant role in separation of the hexokinases. In order to obtain reproducible results the ion-exchanger was pre-equilibrated in the elution buffer degassed by a water pump, loaded into the column at a flow-rate of 5 ml/h and packed for at least 20–30 h.

Influence of gradients

Two different gradients have been tested for separation of the hexokinase isozymes. The first was a pH gradient from pH 8.0 to 6.5. Under these conditions two main problems affect the separation: (a) the bulk of the haemoglobin is strongly bound to the exchanger at the basic pH values with consequent blockage of the column; (b) the enzyme recovery is greatly affected by the pH of the elution buffer (Fig. 2). For these reasons further experiments have been done with salt gradients at a constant pH of 7.5.

Fig. 3 shows the results of experiments at different gradient slope. Peak width decreases with increasing ionic concentration from 0 to 0.5 *M* KCl. The best results were obtained with 280-ml linear gradient 0 to 0.4 *M* KCl in the equilibrating buffer at pH 7.5.

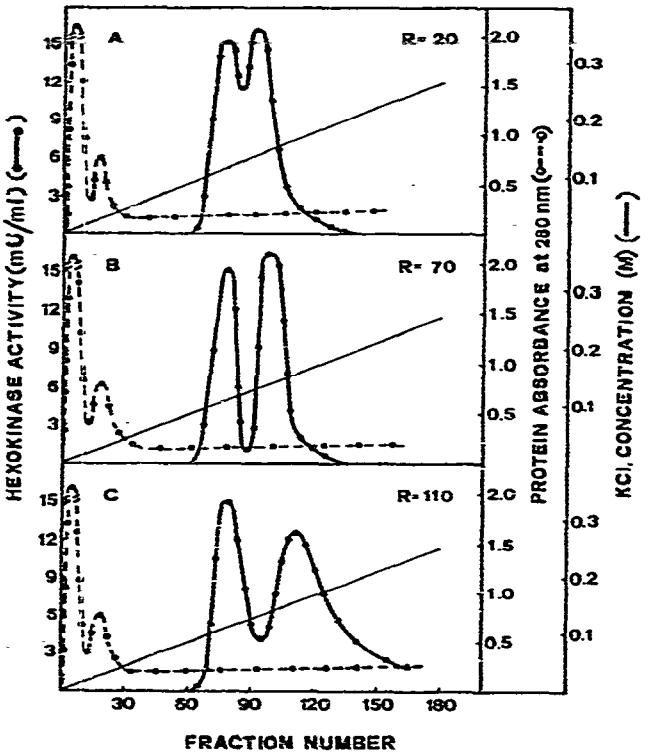

Fig. 1. Influence of column dimensions on resolution of rabbit reticulocytes hexokinase pattern. Haemolysate (1 ml) was applied to three different columns with height/diameter ratios of 20, 70 and 110, respectively. Elution was obtained by a linear gradient of 280 ml from 0 to 0.4 *M* KCl in 5 m*M* sodium potassium phosphate buffer (pH 7.5) contraining 1 m*M* glucose, 3 m*M* KF, 3 m*M* 2-mercaptoethanol and 5 m*M* dithiothreitol. Column packing and equilibration were performed in the same buffer system at a flow-rate of 5 ml/h. Fractions of 0.7 ml were collected and assayed for enzyme activity (●—●) and protein absorbance at 280 nm (○---○).

Enzyme stability

As discussed above, the column chromatography buffer greatly affects hexokinase recovery. Optimal enzyme stability was obtained between pH 7.5 and 8.0. Four different buffer systems have been tested. Complete hexokinase recovery was obtained in sodium potassium phosphate buffer, while with glycylglycine, Tris-HCl and triethanolamine the recovery range was 20–70% of the activity introduced on the columns. Furthermore, 1 m*M* glucose and 5 m*M* dithiothreitol were essential for good enzyme stability in any buffer system.

CONCLUSION

DE-52 column chromatography, under the conditions described, provides the most efficient method so far reported for the study of the hexokinase isozymic pattern in mammalian red blood cells. Fig. 4 shows that it is possible to resolve as complex a

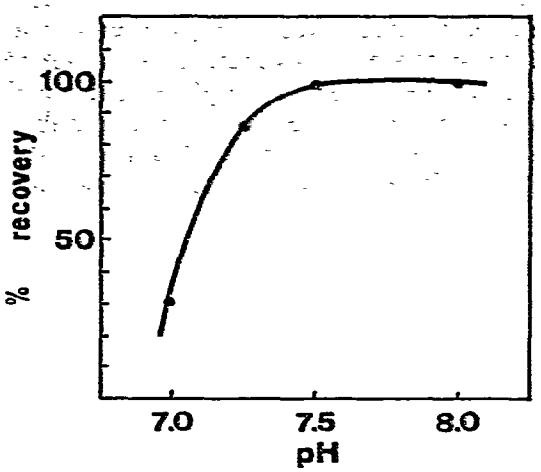

Fig. 2. Hexokinase activity recovery after ion-exchange chromatography. Experiments were carried out as in Fig. 1B except that the pH was varied as reported.

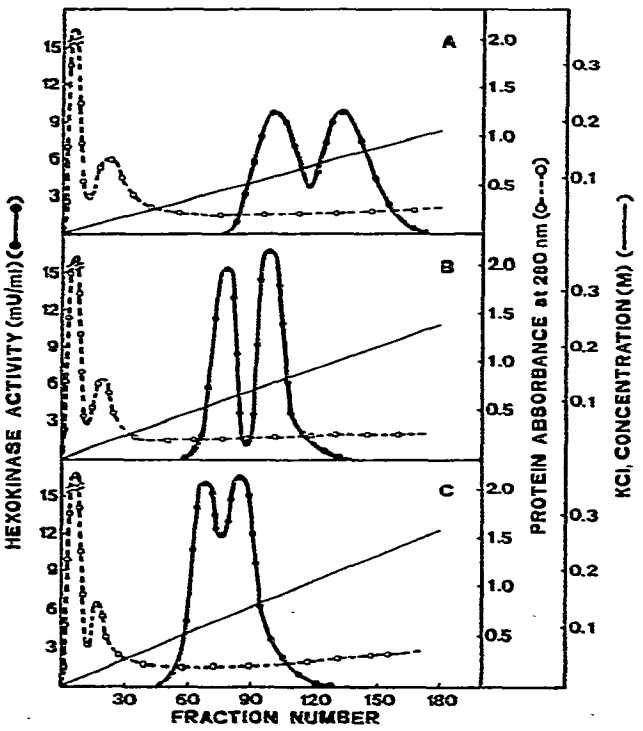

Fig. 3. Effect of salt gradient on the chromatographic fractionation of hexokinase isozymes. Three different gradients, 0 to 0.3 *M* KCl (A), 0–0.4 *M* KCl (B) and 0–0.5 *M* KCl (C), were tested. The column used had an *R* value of 70 (24.5 × 0.35 cm I.D.). Other experimental conditions were as given in Fig. 1.

pattern as that of the hexokinase in human erythrocytes and to weight each of the molecular forms present. This is very important because of the different decay rate of each isozyme during cell ageing. Furthermore, other systems that employ the removal of the bulk of haemoglobin before the study of the red cell hexokinase pattern, inevitably cause a modification of the isozymic pattern, as clearly shown in Fig. 4C. The method described permits the use of the haemolysate as starting material because of the non-retention of haemoglobin.

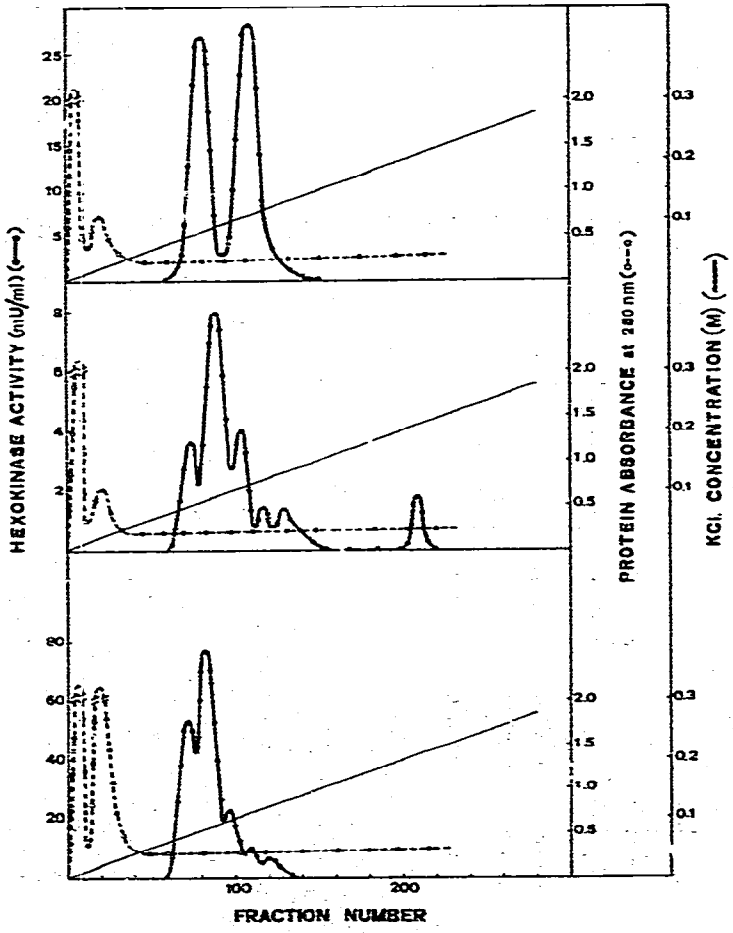

Fig. 4. DE-52 ion-exchange chromatography of hexokinase activity from rabbit reticulocytes (A), human red blood cells (B) and ammonium sulphate fraction, 35–75%, from human red cells (C), prepared as in ref. 18. All the experimental conditions were as in Fig. 1B.

Furthermore this approach could be useful not only in the study of the red cell hexokinase system but also in the investigation of the pattern of many other erythrocyte enzymes and their genetic variants.

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