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STUDIES ON THE USE OF SEPHAROSE-*N*-AMINOHEXANOYL-GLUCOSAMINE FOR PURIFICATION OF RABBIT RED BLOOD CELL HEXOKINASE

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N-acetyl glucosamine is a competitive inhibitor ($K_i = 0.7$ mM) of red blood cell hexokinase with respect to glucose. This property has been utilized for the purification of hexokinase by means of Sepharose-*N*-aminohexanoyl-glucosamine. Studies with this matrix have proved that ionic strength and pH play a very important role in the binding of hexokinase to the affinity column. Therefore their control is essential in order to minimize nonspecific binding and to maximize the purification. Methods for rejuvenation of columns, the effect of protein concentration, and the nature of the binding are also discussed in this paper.

INTRODUCTION

The most effective methods of enzyme purification are those that make use of interactions between the enzymes and their specific ligands, as in affinity chomatography (1). The successful application of affinity chromatography requires the consideration of several parameters, and the effective resolution depends on the optimization of all the experimental conditions.

In this paper we have examined systematically such parameters during the use of Sepharose-*N*-aminohexanoyl glucosamine for the purification of red blood cell hexokinase (EC 2.7.1.1). There are previous reports on homogeneous purifications of rat hexokinase by affinity chromatography (2–4) but these methods cannot be used in the case of the red blood cell enzyme, which represents only 0.0003% (w/w) of the total protein. Since the only results published on red cell hexokinase purification (5) are not completely satisfactory (specific activity, 14.3 U/mg; yield, 14%) we have investigated the use of Sepharose-*N*-aminohexanoyl-glucosamine as a rather simpler method to obtain homogeneous red blood cell hexokinase.

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MATERIAL AND METHODS

Materials

Chemicals. Coenzymes and substrates were obtained from Sigma Chemical Co. (St. Louis, Mo.). Activated CH-Sepharose 4B was purchased from Pharmacia (Uppsala, Sweden). Glucosamine hydrochloride was from Merck (Darmstadt, Germany). All other reagents used were of analytical grade.

Proteins. Glucose-6-phosphate dehydrogenase (D-glucose-6-phosphate: $NADP^+$ oxidoreductase, EC 1.1.1.49), from yeast, grade I, (350 U/mg) and 6-phospho-D-gluconate: $NADP^+$ oxidoreductase (decarboxylating), EC 1.1.1.44, from yeast, (12 U/mg), were purchased from Boehringer (Manheim, Germany). Pronase (type VIII) was from Sigma Chemical Co.

Methods

Preparation of Sepharose-N-Aminohexanoyl-Glucosamine. The activated CH-Sepharose 4B was swollen in 10^{-3} M HCl. The gel was washed with distilled water on a sintered glass filter using approx. 300 ml per g dry powder. Four mg D(+) glucosamine hydrochloride per mg dry powder was dissolved in the coupling solution (0.1 M $NaHCO_3$ containing 0.5 M NaCl), of which there was 5 ml per g dry powder, and mixed with the gel. Excess of ligand was removed by washing with the coupling solution and the remaining active groups were blocked with ethanolamine (1 M, pH 9). The product was washed with three cycles of alternating pH, consisting of a wash at pH 4 (0.1 M acetate buffer, 1 M NaCl) followed by a wash at pH 8 (0.1 M Tris-HCl, 1 M NaCl). The product was stored at +4°C in 5 mM sodium potassium phosphate buffer, pH 7.5, containing 3 mM KF and 3 mM 2-mercaptoethanol.

Enzyme Assay. Hexokinase activity was measured at 37°C spectrophotometrically in a system coupled with glucose-6-phosphate dehydrogenase (D-glucose-6-phosphate:$NADP^+$ oxidoreductase, EC 1.1.1.49) and 6-phosphogluconate dehydrogenase (6-phospho-D-gluconate:$NADP^+$ oxidoreductase, EC 1.1.1.44). The assay mixture contained, in a total volume of 1 ml, 0.135 M glycylglycine (pH 8.1), 5.0 mM glucose, 5.0 mM ATP-$MgCl_2$, 0.5 mM $NADP^+$, 5.0 mM $MgCl_2$, 0.05 IU glucose-6-phosphate dehydrogenase, and 0.05 IU 6-phosphogluconate dehydrogenase. Initial rate measurements were performed by following the reduction of $NADP^+$ at 340 nm with a Beckman spectrophotometer Model 25. One unit of hexokinase activity is defined as the amount of enzyme that catalyzes the formation of 1 μmol of glucose-6-phosphate per min at 37°C.

For the inhibition studies glucose was removed by adding ATP-$MgCl_2$ to the enzyme solution at room temperature for 30 min, followed by passage through a Sephadex G-25 column (usually a 6 ml sample was applied to a 12×2 cm column operated in 5 mM phosphate buffer, pH 7.5, containing 3 mM KF, 3 mM 2-mercaptoethanol and 9% (v/v) glycerol), and the enzyme used immediately. The dissociation constants (K_i) were determined by plotting slope against inhibitor concentrations.

Protein Determination. Protein content was determined according to Lowry et al. (6) with bovine serum albumin as a standard, or spectrophotometrically at 280 nm.

Hemoglobin Estimation. Hemoglobin concentration was determined spectrophotometrically, at 540 nm, with Drabkin's solution as described by Beutler (7).

Operation of the Affinity Columns. Red blood cell hexokinase was obtained as previously described by Magnani et al. (8). All operations were carried out at +4°C. The columns were unpacked and washed by suction in a Buchner funnel with 0.5 M NaCl in 5 mM phosphate buffer, pH 7.5, containing 3 mM KF and 3 mM 2-mercaptoethanol. This operation was repeated each time after use. Pronase treatment, when required (usually after five or six operations), was carried out as described by Holroyde et al. (2). The gel (30 g) was first washed with KCl/urea solutions and then suspended in 5 mM phosphate buffer, pH 7.5, at +4°C. Two mg of pronase was added and the gel suspension mechanically stirred. Two further additions of 1 mg of Pronase were subsequently made, and the suspension was left stirring gently overnight. The treated gel was then washed carefully in a Buchner funnel with water (1 liter), KCl (5 liter), urea (5 liter), and water (5 liter), followed by the appropriate buffer (3 liter).

RESULTS

Studies on the inhibition of hexokinase activity have shown that glucosamine is a competitive inhibitor with respect to glucose. The K_i value determined at pH 7.5 in phosphate buffer at 37°C is 2.4 mM, but this value is too high for a successful application of this ligand in the affinity chromatography of hexokinase. Lower K_i values (0.7 mM) have been obtained for *N*-acetyl glucosamine. As described above, we have then synthesized an amide link between the amino group of glucosamine and the carboxyl group of 6-amino-hexanoic acid covalently bound to Sepharose 4B. The structure of this glucosamine derivate is much closer to acetyl glucosamine than to glucosamine. This is confirmed by the chemical data of Holroyde et al. (2) and by retention of hexokinase activity when applied to the Sepharose-*N*-aminohexanoyl-glucosamine column. The biospecific interaction between

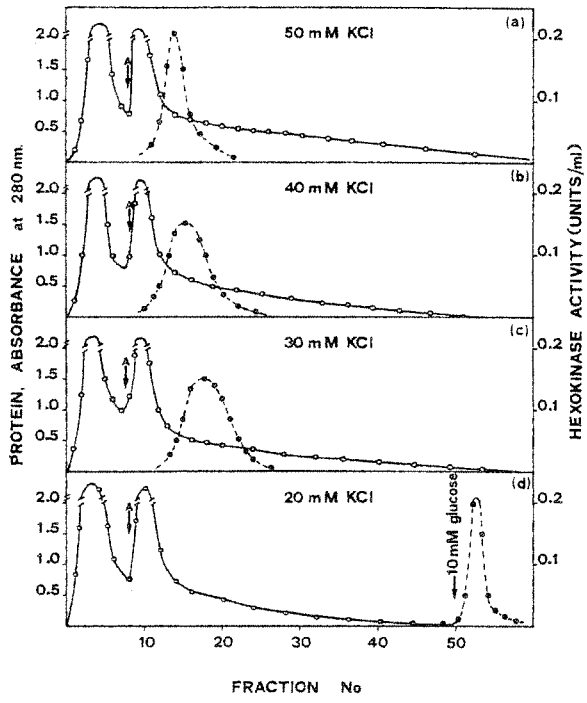

FIG. 1. Effect of ionic strength on affinity chromatography of rabbit red cell hexokinase. Each column (4 × 1 cm) was equilibrated in 5 mM sodium potassium phosphate buffer, pH 7.5, containing 3 mM 2-mercaptoethanol, 3 mM KF and 9% (v/v) glycerol and operated at 15–20 ml/h. 3.5 ml fractions were collected. 6.5 ml of sample (containing 2.0 U of hexokinase activity/ml) was applied to each column, followed by 25 ml of the equilibration buffer. At "A", included in the buffer was (a) 50 mM KCl, (b) 40 mM KCl, (c) 30 mM KCl, and (d) 20 mM KCl. The elution of hexokinase activity was obtained by adding 10 mM glucose to the buffer. (○—○), protein E_{280}; (●- -●), hexokinase activity.

hexokinase and Sepharose-*N*-aminohexanoyl-glucosamine was also demonstrated by the selective elution of the enzyme when glucose 10 mM was added.

Effect of Ionic Strength and pH

As shown in Fig. 1, ionic strength plays a very important role in red blood cell hexokinase purification on Sepharose-*N*-aminohexanoyl-glucosamine. At KCl concentrations higher than 20 mM, hexokinase was

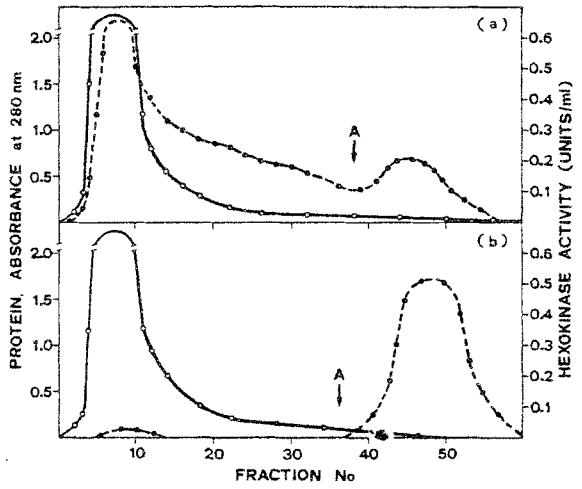

FIG. 2. Effect of pH on the release of hexokinase from Sepharose-*N*-aminohexanoyl glucosamine. To the two columns (4 × 1 cm) 7.0 ml of sample (containing 3.5 U of hexokinase activity/ml) was applied. The columns were operated in 5 mM phosphate buffer, containing 3 mM KF, 3 mM 2-mercaptoethanol and 9% (v/v) glycerol at 20 ml/h; 3.0 ml fractions were collected at (a) pH 7.2 and (b) pH 7.5. At arrow "A," 10 mM glucose was added to the buffer. (○—○), protein, absorbance at 280 nm. (●- -●), hexokinase activity.

only retarded as Rijksen and Staal (5) also reported for the human enzyme, while at 20 mM KCl, hexokinase can be eluted specifically with 10 mM glucose. This fact does not allow the column to be washed thoroughly with KCl at a high concentration to remove undesirable proteins; therefore the eluted enzyme contains some contaminants. The pH value also plays an important role in the binding of hexokinase to the affinity matrix (Fig. 2), and for this reason, good results can be obtained only at pH 7.5.

Effect of Protein Concentration

Since hexokinase is present only as a minor component of our starting material, several different interactions must occur between the applied proteins and the gel matrix. When applied to the column, samples of very high protein concentration severely limit the binding of hexokinase (Fig. 3), while a low enzyme concentration severely affects the recovery. It is therefore necessary to use concentrations of about 4–5 U per ml in order to obtain maximum purification.

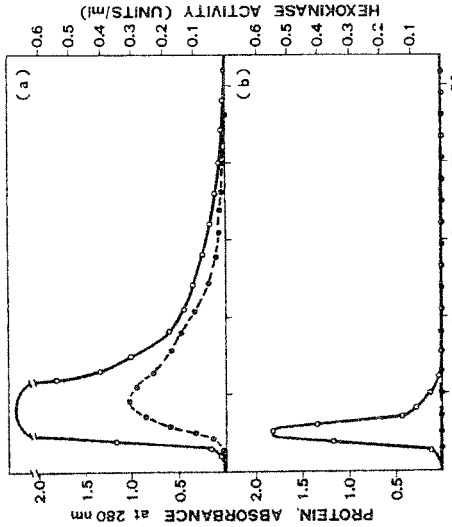

FIG. 3. Effect of protein concentration on the binding of hexokinase to Sepharose-*N*-aminohexanoyl glucosamine. Each column (4 × 1 cm) was equilibrated in the buffer as described in Fig. 2(b) and operated at 15–20 ml/h. (a) The sample (6.5 ml) contained 3 U/ml of hexokinase activity and 130–150 mg/ml of protein. (b) The sample (6.5 ml) contained 3 U/ml of hexokinase activity and 0.4–0.5 mg/ml of protein, (○—○), protein, absorbance at 280 nm. (●- -●), hexokinase activity.

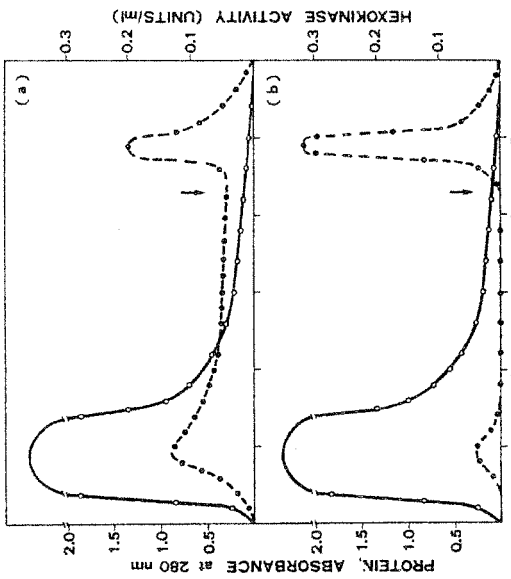

FIG. 4. Effect of Pronase treatment of the glucosamine derivative affinity columns. Sepharose-*N*-aminohexanoyl glucosamine used at least six times in previous experiments before (a) and after Pronase treatment (b). In each case hexokinase (2 U/ml), obtained from AS65 precipitation, was applied to the column (4 × 1 cm) operated in the buffer described in Fig. 2b. The arrows indicate where 10 mM glucose was included in the developing buffer. The columns were operated at 15–20 ml/h and 2 ml fractions were collected. (○—○), protein, absorbance at 280 nm. (●- -●), hexokinase activity.

Several proteins are also retained by these columns, even after 6 M urea, 0.5 M KCl treatement, and the Sepharose-glucosamine derivate loses much of its capacity to bind the enzyme after some operations. After six or seven cycles, the matrix had a pale yellow color, in contrast with the white appearance of an unused gel. The original white color of the matrix and all the capacity of the column were fully recovered when the used matrices were treated overnight with pronase, a nonspecific proteinase preparation (Fig. 4). Finally, the urea-KCl wash was routinely performed after each usage, and pronase treatment was applied every sixth time the column was used (see above under Material and Methods).

DISCUSSION

The results reported in this paper indicate the necessity in purification by affinity chromatography of a careful control of several parameters. This is very important in cases like ours when the enzyme concentration is particularly low. In fact, in our experiments hexokinase represents only 0.0003% (w/w) of the total protein in hemolysates and becomes 0.015% (w/w) after removing hemoglobin and ammonium sulphate fractionation. At this level the enzyme concentration is still very low, and therefore the control of all the parameters studied is particularly important. As suggested by the above experiments, we operated at 20 mM KCl and pH 7.5. Under these conditions all the hexokinase activity applied was retained by the Sepharose-*N*-aminohexanoyl glucosamine column. As for the binding specificity of hexokinase, we have shown that extensive column washing eliminates several contaminating proteins bound to the matrix, but the release of hexokinase by the introduction of glucose is still followed by other contaminants.

In fact, at this stage the yield of hexokinase is higher than 80%, with a 9.000–10.000-fold purification. However, this high recovery can be obtained only if the hexokinase activity is 4 U/ml. A second identical step allows us to eliminate most of the contaminant protein present in the first eluate and to obtain a 150.000-fold purification. Therefore the selective elution of hexokinase from Sepharose-*N*-aminohexanoyl-glucosamine and the kinetic data suggest that the interaction of the enzyme with the affinity column is essentially of a specific type. In the Rijksen and Staal (5) studies, human red blood hexokinase is only retarded when applied to an affinity matrix similar to the one we have used. These authors, however, include 0.5 M NaCl in their buffer, but as we show in Fig. 1, the ionic strength greatly affects the hexokinase purification.

Wright et al. (4) also reported that the ligand concentration strongly affects this chromatography step, and that at a ligand concentration of 12 μmol/g of Sepharose rat hexokinase type I is partially retained, while at 4 μmol/g of Sepharose this isoenzyme passes through unretained. In our experiments the ligand concentration used was about 10 μmol/g of gel, and in these conditions the 20–30 U of hexokinase applied were fully retained.

Our purpose was to determine the best operating conditions for the affinity chromatography step in red blood cell hexokinase purification. We are now able to obtain for the first time high yields of rabbit red blood cell hexokinase with a very high purification factor. Moreover, the results obtained can be useful in other enzyme purifications, particularly when the enzyme studied represents a minor component in the starting material.

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