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Note

Rapid purification of glucokinase and glycerokinase from *Bacillus stearothermophilus* by hydrophobic interaction chromatography

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Glucokinase (ATP:α-D-glucose-6-phosphotransferase, E.C. 2.7.1.2) and glycerokinase (ATP:glycerol-3-phosphotransferase, E.C. 2.7.1.30) from *Bacillus stearothermophilus* have been previously purified by triazine dye affinity chromatography. Dye affinity chromatography is a rapid and inexpensive step giving far greater binding capacities than affinity chromatography with an immobilised ligand[1–3].

In the course of an investigation into the association and dissociation of glucokinase subunits by fluorescence polarisation spectroscopy it was observed that the enzyme fluoresced even in the absence of covalently bound fluorescent label. This fluorescence was found to be caused by breakdown-products of the dye, Procion Brown H-3R, used as a ligand when the enzyme was purified by dye affinity chromatography. Because this contamination could not be removed by any mild technique and its presence could interfere in any structural studies to be carried out on this enzyme, an alternative purification was developed.

This paper describes a purification method suitable for gram quantities of glucokinase. Triazine dyes and conventional affinity chromatography were avoided by using hydrophobic interaction chromatography. Glycerokinase was also found to bind to the hydrophobic matrix and could be completely separated from the glucokinase. The two enzymes previously proved difficult to separate by methods which did not include the use of triazine dyes and even then low level contamination (0.1%) was evident, reducing the value of the enzyme for diagnostic purposes. Pure glycerokinase and glucokinase were obtained by a procedure which included a total of three pseudo-affinity chromatography steps following partial separation by ion-exchange chromatography[2,3].

Glucokinase and glycerokinase of diagnostic grade were obtained free of each other and free of contaminating dyes by one step on Phenyl-Sepharose. The procedure is simple and involves commercially available chromatography matrices.

MATERIALS AND METHODS

N-2-Hydroxyethylpiperazine-N-2-ethanesulphonic acid (Hepes), bovine serum albumin, dithiothreitol and phenylmethanesulphonyl fluoride were obtained from Sigma (London). Phosphate buffers, deoxyribonuclease and 2-mercaptoethanol were

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obtained from BDH. All enzyme assay reagents were obtained from Boehringer. DEAE-Sepharose CL-6B and Phenyl-Sepharose CL-4B were obtained from Pharmacia. Ultrogel AcA 34 was obtained from LKB.

Assay methods

The assay mixture for glucokinase contained 92 m*M* triethanolamine–HCl (pH 7.6), 1.9 m*M*ATP, 5.4 m*M* magnesium chloride, 1.8 m*M* potassium chloride, 0.32 m*M* NADP, 2.55 m*M* α-D-glucose and 3.5 E.C. units of glucose-6-phosphate dehydrogenase (E.C. 1.1.1.49) in a final volume of 1 ml. The reaction was initiated with 1–10 μl of glucokinase solution.

Glycerokinase was assayed the method described by Garland and Randle[4]. The assay mixture contained 75 m*M* potassium chloride , 6.5 m*M* phosphoenolpyruvate, 0.3 m*M* $NADH_2$, 2.4 m*M* glycerol and 8 E.C. units of pyruvate kinase (E.C. 2.7.1.40)/lactate dehydrogenase (E.C. 1.1.1.27) in a final volume of 1 ml. The reaction was initiated with 1–10 μl of glycerokinase solution.

One E.C. unit is defined as the amount of enzyme which catalyses the conversion of 1 μmol substrate per min under these conditions at 30°C.

Protein was estimated by the Coomassie Brilliant Blue binding method of Bradford[5] with bovine serum albumin as standard. Column eluates were monitored by absorbance at 280 nm.

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate was carried out with 10% slab gels by the method of Laemmli[6].

ENZYME PURIFICATION

Enzyme purification was carried out at 4°C. All buffers contained 0.1 m*M* phenylmethanesulphonyl fluoride and 10 m*M* 2-mercaptoethanol.

Frozen cell paste (1 kg) was suspended in 2 l of 100 m*M* potassium phosphate buffer, pH 8.0, containing 0.5 μg/ml deoxyribonuclease to reduce viscosity caused by nucleic acids[7]. The thawed cell suspension was disrupted with a 15M-8BA Type Manton-Gaulin homogeniser operated at 550 kg/cm^2. The homogenate was centrifuged at 1000 *g* for 1 h and the supernatant filtered through glass wool to remove cell debris and lipid. The supernatant was mixed with an approximately equal volume of 100 m*M* potassium dihydrogen phosphate and the pH adjusted to 5.6 with 3.2 *M* orthophosphoric acid. The raised ionic strength prevented adsorption of the enzyme on to the precipitate. The precipitate was removed by centrifugation at 1000 *g* for 1 h. The pH of the supernatant was raised to 8.0 with 10 *M* potassium hydroxide.

Ion-exchange chromatography on DEAE-Sepharose CL-6B

The supernatant was loaded onto a 3.5-l DEAE-Sepharose CL-6B column (22 cm × 14 cm I.D.) equilibrated with 100 m*M* potassium phosphate buffer, pH 8.0. Enzyme was eluted at 600 ml/h with a 20-l linear gradient from 100 m*M* to 400 m*M* potassium phosphate at pH 8.0, collected in fractions of 500 ml.

Hydrophobic interaction chromatography on Phenyl-Sepharose CL-4B

Active glucokinase and glycerokinase fractions were pooled and concentrated to 500 ml with an Amicon CH4 ultrafiltration unit fitted with an H10P10 hollow-

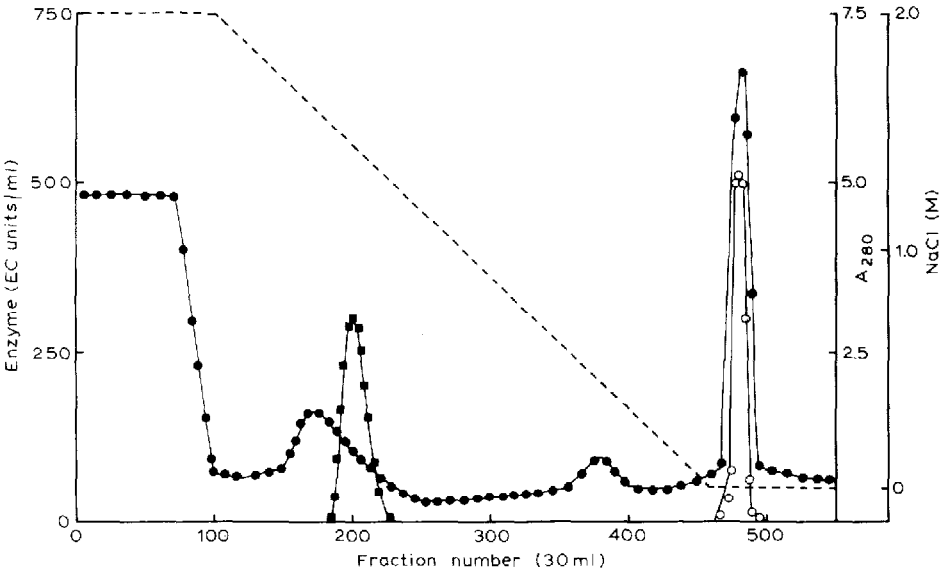

Fig. 1. Chromatography of glucokinase (■) and glycerokinase (○) on Phenyl-Sepharose. Conditions are given in the text. The broken line indicates the sodium chloride content of the elution buffer. Absorbance at 280 nm (●).

fibre cartridge. The enzyme pool was made 2 *M* in sodium chloride and chromatographed on a 1-l Phenyl-Sepharose column (16 cm × 9 cm I.D.) equilibrated with 10 m*M* Hepes, pH 7.0, containing 2 *M* sodium chloride. It was not necessary to change the buffer component of the enzyme solution prior to loading the column. The column was washed with 3 l of equilibration buffer and enzyme eluted with an 11-l linear gradient from 2 *M* to 0 *M* sodium chloride in 10 m*M* Hepes, pH 7.0, at 1500 ml/h, collected in fractions of 30 ml.

Elution was continued with 10 m*M* Hepes, pH 7.0, after completion of the gradient. Glucokinase eluted at about 1.5 *M* sodium chloride while glycerokinase eluted only in the absence of sodium chloride (Fig. 1).

Gel filtration chromatography on Ultrogel AcA34

Active glucokinase fractions were concentrated to 10 ml with Amicon PM10 membranes. The concentrate was adjusted to pH 7.6 and loaded on to a 1.75-l column of Ultrogel AcA34 (90 cm × 5 cm I.D.), equilibrated with 50 m*M* potassium phosphate at pH 7.6. Fractions of 10 ml were collected at 60 ml/h. Active glycerokinase fractions were concentrated to 15 ml and chromatographed as described for glucokinase. Enzyme fractions were pooled on the basis of activity and electrophoretic homogeneity.

Glucokinase and glycerokinase pools were concentrated to a protein concentration of 100 mg/ml using Amicon PM10 membranes and preserved by addition of 0.1 m*M* dithiothreitol and 0.02% sodium azide. The results of this purification are summarised in Tables I and II. Both enzymes were shown to give single protein bands by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate.

TABLE I

PURIFICATION OF GLUCOKINASE FROM 1 kg *B. STEAROTHERMOPHILUS*

Purification step	*Volume (ml)*	*Amount of protein (g)*	*Enzyme activity (E.C. units)*	*Specific activity (E.C. units/mg protein)*	*Activity recovered (%)*
Homogenate	2000	60	81 600	1.4	100
pH 5.6 supernatant	3600	61	75 200	1.2	92
DEAE-Sepharose	4000	5.6	55 600	9.9	68
Phenyl-Sepharose	330	0.27	50 400	187.0	62
Ultrogel AcA 34	100	0.12	40 800	340.0	50

TABLE II

PURIFICATION OF GLYCEROKINASE FROM 1 kg *B. STEAROTHERMOPHILUS*

Purification step	*Volume (ml)*	*Amount of protein (g)*	*Enzyme activity (E.C. units)*	*Specific activity (E.C. units/mg protein)*	*Activity recovered (%)*
Homogenate	2000	60	95 500	1.6	100
pH 5.6 supernatant	3600	61	86 400	1.4	90
DEAE-Sepharose	4000	5.6	74 400	13.3	78
Phenyl-Sepharose	180	0.7	64 800	92.8	68
Ultrogel AcA 34	140	0.5	58 400	117.0	61

DISCUSSION

Hydrophobic interaction chromatography can be applied to the large-scale purification of glucokinse and glycerokinase as an alternative to pseudo-affinity chromatography with triazine dyes. This paper describes purification of the enzymes from 1 kg of bacterial cells but the procedure has been applied to extraction from 25 kg of cells[8]. Advantages of hydrophobic interaction chromatography are high flow-rates, excellent recovery of diagnostic-grade enzyme from a commercially available matrix, enzyme free of contamination from triazine dye and low cost relative to conventional affinity chromatography. Phenyl-Sepharose also has a high binding capacity; more than 6 mg of glucokinase and 3 mg of glycerokinase have been shown to bind to 1 ml of matrix. Triazine dye affinity matrices used in the purification of both enzymes bind about 3 mg of glucokinase and 1.5 mg of glycerokinase per ml of column[2,3]. It should be noted, however, that gel filtration after pseudo-affinity chromatography is unnecessary for glycerokinase. The hydrophobic interaction chromatography profile shows that complete separation of glucokinase from glycerokinase is possible from a single elution.

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