Journal of Chromatography, 366 (1986) 79–89
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 18 779

USE OF TRIAZINE DYES AS LIGANDS FOR THE LARGE-SCALE AFFINITY CHROMATOGRAPHY OF A THERMOSTABLE GLYCEROKINASE

PETER M. HAMMOND*, TONY ATKINSON and MICHAEL D. SCAWEN
Microbial Technology Laboratory, PHLS Centre for Applied Microbiology & Research, Porton Down, Salisbury, Wiltshire SP4 0JG (U.K.)
(Received April 18th, 1986)

SUMMARY

Glycerokinase from the thermophilic bacterium *Bacillus stearothermophilus* was found to have an affinity for several reactive, triazine dyes. The enzyme was found to have a strong affinity for several dyes when the dyes were immobilised on an agarose support matrix. Matrices based on cellulose, or polymers of acrylic acid, were less suitable. The study of the effect of various physicochemical properties is described, including ligand concentration, pH, ionic strength, column dimensions and flow-rate. Glycerokinase could be bound to Procion blue MX-3G–Sepharose at low ionic strength (pH 7.5) and was most readily recovered by substrate elution. This matrix was used to purify to homogeneity the enzyme derived from 1 kg of bacteria, in three steps, with an overall recovery of 44%.

INTRODUCTION

Glycerokinase (EC 2.7.1.30) is produced by a wide range of mesophillic microorganisms[1–3] and by the thermophile *Bacillus stearothermophilus*[4,5]. The *B. stearothermophilus* enzyme has been purified by a number of conventional techniques[5], but the procedure involves a large number of purification steps and results in poor recoveries.

Preliminary studies have shown that a four to five-fold purification, with good recovery, can be achieved using affinity chromatography on AMP or ATP substituted agarose[5,6]. However, such nucleotides are costly, and the amount required to purify several grams of glycerokinase would be prohibitively expensive. A more cost effective alternative is the use of the reactive dyes as affinity ligands. These dyes, which are sulphonic acid-substituted azo, phthalocyanine or anthroquinone compounds contain a mono- or dichlorotriazinyl group. Thus, they may be readily attached to agarose, or other support matrices through the reactive triazine ring. Many proteins have been shown to interact with these dyes and they have been used in the purification of numerous enzymes[7–9]. Glycerokinase has previously been shown to bind to two immobilized Procion dyes, Yellow MX-6G and Yellow MX-R under mildly acidic conditions. Non-specific elution gave a four or five-fold purification with a recovery in excess of 65%[6].

0021-9673/86/$03.50

This paper reports the use of other Procion dyes as affinity ligands and investigates the optimum conditions for the bio-specific application of these dyes to the purification of glycerokinase.

EXPERIMENTAL

Materials

Phenylmethylsulphonyl fluoride, NAD and ATP were obtained from Sigma (London) Chemical Co., Poole, Dorset, U.K. Glycerol-3-phosphate dehydrogenase was obtained from Biozyme Laboratories, Blaenavon, South Wales, U.K. Bovine deoxyribonuclease and all other chemicals were obtained from BDH, Poole, Dorset, U.K. DEAE-Sephadex A50, DEAE-Sepharose CL-6B, Sepharose 4B, Sepharose 6B, Sepharose CL-4B, Sephacryl S-200, Sephadex G-150 and Blue Sepharose CL-6B were obtained from Pharmacia (G.B.); Ultrogel AcA 34 was obtained from LKB; cellulose powder CF11 was obtained from Whatman Chemical Separation. 6-Aminohexyl cellulose was prepared by treating CNBr activated cellulose with 1,6-diaminohexane[10]. Procion dyes were a kind gift from Dr. C. V. Stead, ICI, Blackley, Manchester, U.K. Cibacron Blue F-3GA was obtained from Ciba-Geigy, Manchester, U.K.

Culture methods

B. stearothermophilus NCIB 11270 was grown according to the method of Sargeant *et al.*[11] as modified by Comer *et al.*[5].

General methods

Buffers. Potassium phosphate buffers were prepared by mixing 1.0 *M* solutions of dipotassium hydrogen phosphate and potassium dihydrogen phosphate in the correct proportions to give the desired pH, followed by dilution to the required concentration. Imidazole buffer for cell breakage contained 20 m*M* imidazole, 1 m*M* EDTA and 1 m*M* glycerol, adjusted to pH 8.0 with 3.2 *M* phosphoric acid. All buffers contained 10 m*M* 2-mercaptoethanol and 0.1 m*M* phenylmethylsulphonyl fluoride. All column dimensions are given as bed height × internal diameter.

Enzyme assay. Glycerokinase assays were based on the method described by Bublitz and Weiland[12]. A semi-micro cuvette (10 mm light path) contained, in a final volume of 1 ml, 0.2 *M* glycine, 1.0 *M* hydrazine hydrate adjusted to pH 9.8 with 2 *M* potassium hydroxide, 2 m*M* magnesium chloride, 2 m*M* ATP, 0.5 m*M* NAD, 0.5 U glycerol-3-phosphate dehydrogenase and 0.8–2.4 U glycerokinase sample. The reaction was initiated by adding 25 μl of 0.1 *M* glycerol. All assays were carried out at 30°C; one unit of activity was defined as that amount of enzyme which converted 1 μmol of substrate per minute under the given conditions.

Protein estimation. Protein was estimated by the method of Lowry *et al.*[13] using bovine serum albumin (Sigma, U.K.) as standard. Column eluates were monitored at 280 nm.

Preparation of cell extract. A 100-g sample of frozen cell paste was suspended in 300 ml of 20 m*M* imidazole buffer, pH 8.0. The suspension was sonicated at 20 kHz for six 30-s intervals, with cooling between each exposure, using a Dawe Soniprobe (Dawe Instruments). The homogenate was centrifuged at 13 700 *g*, at 4°C for 30 min. The supernatant was adjusted to pH 5.1 with 3.2 *M* phosphoric acid and

centrifuged as above. The supernatant was then adjusted to pH 8.0 with 5 *M* potassium hydroxide and the conductivity adjusted to that of 100 m*M* potassium phosphate (pH 8.0) using 1 *M* potassium phosphate, pH 8.0. The supernatant was applied to a 200-ml column (13 × 4.4 cm I.D.) of DEAE Sephadex A50, equilibrated in 100 m*M* potassium phosphate, pH 8.0. The column was eluted with a 1-l linear gradient between 100 m*M* and 400 m*M* potassium phosphate (pH 8.0). Fractions of 10 ml were collected. Fractions were pooled for peak glycerokinase activity and concentrated to an activity of 300 U/ml by ultrafiltration using Amicon PM10 membranes, prior to storage at 4°C. The yield was *ca.* 10^4 U glycerokinase, with a specific activity of 20–25 U/mg protein. Before use in subsequent studies samples of enzyme were dialysed exhaustively against the required buffer.

Coupling of dyes. Triazine dyes were coupled to Sepharose 4B by using the methods described by Baird *et al.*[14], but using 0.1 *M* sodium hydroxide in place of sodium carbonate[15]. A total of 37 dye–Sepharose 4B conjugates were prepared. Procion Blue MX-3G was also coupled to various other matrices by using the same procedure. Differing levels of substitution of Blue MX-3G on Sepharose 4B were obtained by withdrawing samples from the reaction mixture at intervals over the time period from 5 min to 3 h. The degree of substitution was estimated by hydrolysing samples in 7.8 *M* acetic acid at 85°C for 15 min, followed by spectrophotometric measurement of the dye present. The usual level of substitution was between 1.5 and 3.0 mg dye/g wet weight Sepharose 4B.

Screening of dye–Sepharose conjugates. Columns of 1-ml volume (1.6 × 0.9 cm I.D.) of each conjugate were prepared and equilibrated in 10 m*M* potassium phosphate at pH 5.5 or 7.5, at a flow-rate of *ca.* 0.2 ml/min. Then 100 U of glycerokinase, prepared as described above, were applied to each column. The columns were washed with 4 ml of equilibration buffer and eluted as follows:

(i) Columns equilibrated at pH 5.5; 100 m*M* potassium phosphate buffer (pH 7.5) followed by the same buffer containing 1 *M* potassium chloride.

(ii) Columns equilibrated at pH 7.5; 10 m*M* potassium phosphate buffer (pH 7.5) containing 5 m*M* neutralised ATP and 5 m*M* magnesium chloride.

All eluates were assayed for glycerokinase.

Subsequent experiments were carried out using 10-ml columns (5 × 1.6 cm I.D.) loaded with 3500 U of glycerokinase.

RESULTS AND DISCUSSION

Screen of dye–Sepharose conjugates

At pH 5.5, more than 80% of glycerokinase activity bound to 19 of the 37 dye Sepharose conjugates examined. However, it could be eluted from only six of these by a change in pH or ionic strength. It was eluted from Procion Red HE-7B, Procion Yellow MX-R and Procion Green HE-4BD by 100 m*M* potassium phosphate buffer (pH 7.5). It was eluted from Procion Blue H-B and Procion Blue MX-3G by 100 m*M* potassium phosphate containing 1.0 *M* potassium chloride. With Procion Blue HE-RD *ca.* 50% of the bound activity was eluted by 100 m*M* buffer and a further 30% was eluted by 100 m*M* buffer containing 1.0 *M* potassium chloride.

At pH 7.5, glycerokinase bound to only four dye–Sepharose conjugates: Procion Blue H-B, Procion Blue MX-3G, Procion Blue MX-R and Cibacron Blue F-

3GA. In each case it could be eluted by 5 m*M* ATP, 5 m*M* magnesium chloride at pH 7.5 in 10 m*M* potassium phosphate buffer.

The capacities of these dye–Sepharose conjugates for binding glycerokinase were similar in each case at *ca.* 300 units (2.5 mg) per ml settled matrix, with the exception of Procion Blue MX-R–Sepharose, which bound only 180 units/ml. This matrix continually leached enzyme on washing with equilibration buffer.

Of the dyes to which glycerokinase bound, five were selected for further study: Procion Red HE-7B, Procion Yellow MX-R and Procion Green HE-4BD, which bound the enzyme at pH 5.5 and from which it was eluted by 100 m*M* buffer (pH 7.5); Procion Blue MX-3G, which bound the enzyme at both pH 5.5 and pH 7.5 from which it was eluted by 1.0 *M* potassium chloride at pH 7.5 or by 5 m*M* ATP at pH 7.5; and Cibacron Blue F-3GA, which bound the enzyme at pH 7.5, and from which it was eluted with 5 m*M* ATP. Although this latter dye bound the enzyme at pH 5.5 it could not be eluted with 1.0 *M* potassium chloride. Procion Blue H-B was not included as it is equivalent to Cibacron Blue F-3GA.

The behaviour of glycerokinase on the above dye–Sepharose matrices was investigated in greater detail using 10-ml columns. From the results shown in Table I it is apparent that substrate elution at pH 7.5 was superior, in terms of both enzyme recovery and purification achieved. Of the two dyes, Procion Blue MX-3G and Cibacron Blue F-3GA, Procion Blue MX-3G gave higher recovery and slightly better purification, and so was selected for all subsequent work.

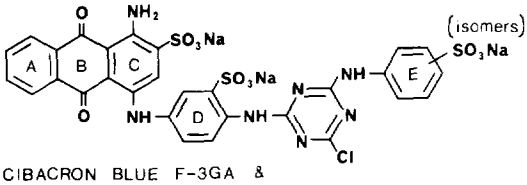
A comparison of the structures of Procion Blue MX-3G, Procion Blue H-B, Procion Blue MX-R and Cibacron Blue F-3GA (Fig. 1)[9,16] is revealing. Cibacron Blue and Procion Blue H-B are structural isomers about the sulphonic acid residue on ring E. Removal of this amino benzene sulphonic acid group gives Procion Blue MX-3G. These three dyes all had a similar affinity for *B. stearothermophilus* glycer-

TABLE I

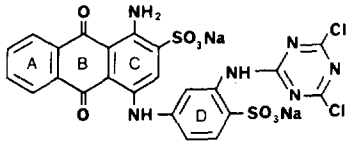
CHROMATOGRAPHY OF GLYCEROKINASE ON VARIOUS TRIAZINE DYE–SEPHAROSE COLUMNS

The columns were 10 ml (5.0 × 1.6 cm I.D.) and equilibrated as shown in either 10 m*M* potassium phosphate buffer (pH 5.5) or in 10 m*M* potassium phosphate buffer (pH 7.5). A total of 3500 units of enzyme, with a specific activity of 20 U/mg, and equilibrated in the relevant buffer was applied to each column. The columns were washed with 30 ml of equilibration buffer and eluted as shown with: (A) 100 m*M* potassium phosphate buffer (pH 7.5); (B) 100 m*M* potassium phosphate buffer (pH 7.5) containing 1 *M* potassium chloride; (C) 10 m*M* potassium phosphate buffer (pH 7.5) containing 5 m*M* neutralised ATP and 5 m*M* magnesium chloride.

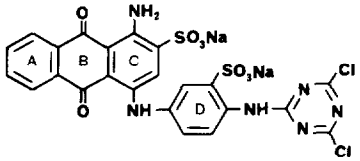
	Columns equilibrated at pH 5.5			*Columns equilibrated at pH 7.5*		
	Procion Red HE-7B	*Procion Yellow MX-R*	*Procion Green HE-4BD*	*Procion Blue MX-3G*	*Procion Blue MX-3G*	*Cibacron Blue F-3GA*
Enzyme bound (U)	2700	2620	3000	3140	2820	2950
Elution procedure	A	A	A	A, B	C	C
Enzyme eluted	1450	260	1300	980	2030	1790
Specific activity (U/mg)	98	9	37	57	120	116
Recovery (% of enzyme bound)	59	11	48	35	79	67



CIBACRON BLUE F-3GA &
PROCION BLUE H-B



PROCION BLUE MX-R



PROCION BLUE MX-3G

Fig. 1. Structures of Cibacron Blue F-3GA, Procion Blue H-B, Procion Blue MX-R and Procion Blue MX-3G.

okinase, although the recovery of eluted material was slightly better for Procion Blue MX-3G. Inversion of the two substituents around ring D gives Procion Blue MX-R, which had a much lower affinity for the enzyme, although it showed no marked differences in behaviour from Cibacron Blue in the binding and elution of pig heart lactate dehydrogenase (unpublished data).

Space-filling models of these dyes have been constructed and suggest that the major difference between Procion Blue MX-R and the other three dyes lies in the orientation of the sulphonic acid group on ring D. In Procion Blue MX-R this group is in a different orientation with respect to the rest of the molecule, and it is likely that this difference accounts for the lower affinity of glycerokinase for Procion Blue MX-R–Sepharose.

Effect of ligand concentration

Both adsorption and desorption of glycerokinase on Procion Blue MX-3G–Sepharose were affected by ligand concentration, as shown in Fig. 2.

The decreased capacity of the matrix at high levels of substitution may be due to the steric effect of a large number of dye molecules. However, the enzyme that is

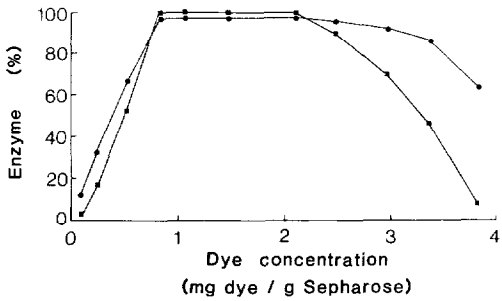


Fig. 2. Effect of ligand concentration on the binding and elution of glycerokinase on Procion Blue MX-3G–Sepharose. Columns of 1 ml (1.6 × 0.9 cm I.D.) were prepared containing Sepharose 4B substituted with various amounts of Procion Blue MX-3G and equilibrated in 10 m*M* potassium phosphate buffer (pH 7.5). The columns were loaded with 300 units of glycerokinase, washed with 4 ml of equilibration buffer and eluted with 4 ml of calibration buffer containing 5 m*M* ATP and 5 m*M* magnesium chloride. Circles, enzyme bound as percentage of that loaded; squares, enzyme eluted as percentage of that bound.

bound under these condition is not readily eluted, perhaps because the enzyme is bound at several sites. Glycerokinase exhibits somewhat unusual behaviour in that the enzyme which binds at low levels of substitution of the matrix is also not readily eluted. At low dye concentrations, therefore, enzyme binding may occur at different sites to those used at high dye concentrations.

All subsequent experiments were carried out at a ligand concentration of *ca.* 2 mg dye/g Sepharose.

Effect of pH

As shown in Fig. 3 the adsorption and elution of glycerokinase on Procion Blue MX-3G Sepharose were independent of pH over the range pH 6–8. Outside of this range both the capacity and ease of elution decreased rapidly.

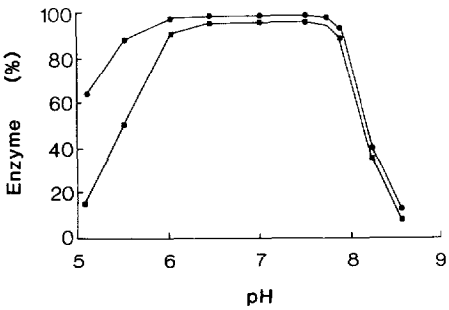


Fig. 3. Effect of pH on the adsorption and elution of glycerokinase on Procion Blue MX-3G–Sepharose. Columns of 1 ml (1.6 × 0.9 cm I.D.) were prepared containing Sepharose 4B substituted with 2.0 mg of Procion Blue MX-3G/g. The columns were equilibrated in 10 m*M* potassium phosphate buffer over the pH range 5.0–8.6. The columns were loaded with 300 units of glycerokinase equilibrated in the appropriate buffer, washed with 4 ml of equilibration buffer and eluted with 4 ml of the appropriate buffer containing 5 m*M* ATP and 5 m*M* magnesium chloride. Circles, enzyme bound as percentage of that applied; squares, enzyme eluted as percentage of that bound.

Effect of ionic strength

The binding capacity of Procion Blue MX-3G–Sepharose for glycerokinase decreased rapidly at buffer concentrations greater than 50 m*M*, although the bound enzyme was eluted with increasing yield. This indicates that hydrophilic, as opposed to hydrophobic sites are involved in binding of the enzyme to the dye. These results are shown in Table II.

TABLE II

EFFECT OF IONIC STRENGTH ON THE BINDING OF GLYCEROKINASE TO PROCION BLUE MX-3G–SEPHAROSE 4B AT pH 7.5

Experimental details are given in the text.

Potassium phosphate buffer (mM)	*Enzyme bound (U/ml column)*	*Enzyme eluted (U)*	*% eluted*
10	300	220	73
25	300	220	73
50	280	220	78
100	190	165	87
150	35	33	95
200	0	–	–

Effect of matrix

Although agarose is generally regarded as the optimum matrix for the preparation of affinity chromatography adsorbents[9,17], the possible use of other supports was investigated. Table III shows the results obtained when glycerokinase was applied to various matrices substituted with Procion Blue MX-3G. These data confirm that agarose, in the form of Sepharose or cross-linked Sepharose, is superior to other potential supports, in terms of both binding capacity and recovery on elution. Both Sephacryl and Ultrogel, which are partly composed of polyacrylamide, proved to be unsuitable supports because they required high concentrations of salt for enzyme elution and recoveries were low. Cellulose was found to be an excellent matrix for dye attachment but exhibited poor enzyme binding properties. This was presumably due to its poor interaction with water. Thus, the enzyme can only gain access to a binding site on the dye when a spacer arm is introduced, for example as in 6-aminohexyl cellulose. However, this latter matrix is not particularly stable owing to hydrolysis of the aminohexyl-cellulose bond.

Effect of column dimensions

The dimensions of chromatographic columns are an important consideration in the scale-up of an enzyme purification procedure, with short columns of large diameter being preferred because of their improved flow properties. However, the dimensions of the column can also critically affect the behaviour of the enzyme, particularly with regard to elution. As shown in Table IV, the capacity of Procion Blue MX-3G–Sepharose for glycerokinase decreased slightly when a column of

TABLE III

EFFECT OF MATRIX ON THE BINDING OF GLYCEROKINASE TO PROCION BLUE MX-3G CONJUGATES AT pH 7.5

Columns were 1 ml (1.6 × 0.9 cm I.D.) and were packed with the dye-substituted matrix shown. The columns were equilibrated in 10 m*M* potassium phosphate buffer (pH 7.5). They were loaded with 300 units of glycerokinase, washed with 4 ml of equilibration buffer and eluted with 4 ml of equilibration buffer containing 5 m*M* ATP and 5 m*M* magnesium chloride, followed by 4 ml of equilibration buffer containing 2 *M* potassium chloride.

Matrix	*Dye concentration (mg/g)*	*Enzyme bound (units)*	*Enzyme eluted (units)*	
			ATP	*Potassium chloride*
Cellulose	1.5	15	0	5
	6.4	25	0	10
6-Aminohexyl cellulose Sepharose 4B	1.4	75	25	30
	1.2	300	270	–
	3.2	265	135	–
Sepharose 6B	0.7	220	160	–
	1.3	300	265	–
Sepharose CL-4B	0.7	210	155	–
	1.6	300	265	–
Sephacryl S-200	0.4	40	0	20
	1.4	185	45	90
Ultrogel AcA 34	0.5	35	10	10
	1.1	160	60	60
Sephadex G-150	1.3	220	175	–

height:diameter ratio of 0.45 was used. However, the elution volume of the adsorbed enzyme increased dramatically. In a production scale purification this increase in elution volume could perhaps be tolerated but a column of height:diameter ratio of 1.25 would be preferred and would still have acceptable flow properties.

TABLE IV

EFFECT OF COLUMN DIMENSIONS ON THE ADSORPTION AND ELUTION OF GLYCEROKINASE ON PROCION BLUE MX-3G–SEPHAROSE

Columns of various dimensions containing 4 ml of adsorbent were packed and equilibrated in 10 m*M* potassium phosphate buffer (pH 7.5) at a flow-rate of 1.8 ml/h. The columns were loaded with 1400 units of enzyme and washed with 15 ml of buffer. The columns were eluted with 5 m*M* ATP and 5 m*M* magnesium chloride in 10 m*M* potassium phosphate buffer (pH 7.5). Elution volume is the volume required to pool 70–75% of the eluted enzyme.

Column dimensions (cm)	*Height:diameter*	*Enzyme bound (%)*	*Elution volume (ml)*
6 × 0.9	6.67	100	1.1
3.6 × 1.2	3.0	100	1.1
2.0 × 1.6	1.25	100	1.4
1.0 × 2.2	0.45	88	2.4

Effect of flow-rate

For the application of a purification protocol to large-scale working, due consideration must be given to the flow-rate of the chromatography column. In order to achieve maximum throughput, flow-rates need to be as high as possible within the restriction imposed by the need to maintain adequate resolution. In the case of affinity chromatography sufficient time must be allowed for the protein in solution to interact with the immobilized ligand. If insufficient contact time is allowed the apparent capacity of the column will decrease. As shown in Table V, this effect was observed with glycerokinase on Procion Blue MX-3G–Sepharose at flow-rates in excess of one column volume per hour. A similar effect was observed on elution. Generally, the binding of glycerokinase to Procion Blue MX-3G at high flow-rates was good.

TABLE V

EFFECT OF FLOW-RATE ON ADSORPTION CAPACITY AND ELUTION OF GLYCEROKINASE ON PROCION BLUE MX-3G–SEPHAROSE

A column of volume 1.6 ml (2.5 × 0.9 cm I.D.) was equilibrated with 10 m*M* potassium phosphate buffer (pH 7.5). The column was loaded to saturation with enzyme at 290 units/ml and washed with 10 ml of buffer. It was eluted with 5 m*M* ATP and 5 m*M* magnesium chloride in 10 m*M* potassium phosphate buffer (pH 7.5). Loading, washing and elution were all carried out at the same flow-rate.

Flow-rate		*Enzyme bound (unit/ml adsorbent)*	*Enzyme eluted (% of that bound)*
ml/h	*Column volume/h*		
0.29	0.2	380	79
0.75	0.5	395	76
1.4	1.0	375	81
2.6	1.5	325	71
5.3	3.0	255	55
8.4	5.3	160	43
13.3	8.3	85	32

Large-scale purification

In order to demonstrate the validity of dye affinity chromatography to the large-scale purification of glycerokinase the enzyme from 1000 g of bacterial cell paste (250 g dry wt.) was purified to homogeneity by a process involving only three steps.

The 1000 g of frozen bacterial cell paste was suspended in 1000 ml of 20 m*M* imidazole buffer (pH 8.0). The cells were lysed by a single passage through a Manton–Gaulin homogeniser operated at 55 MPa. The homogenate was clarified by centrifugation at 5500 *g* for 45 min at 4°C. The supernatant was adjusted to pH 8.0 with 5 *M* potassium hydroxide and the conductivity adjusted to that of 100 m*M* potassium phosphate buffer, pH 8.0, using 1.0 *M* potassium phosphate buffer at pH 8.0. The extract was then applied to a 2000-ml column (31 × 9 cm I.D.) of DEAE-Sepharose CL-6B equilibrated in 100 m*M* potassium phosphate buffer (pH 8.0). The column was eluted with a 10-l linear gradient between 100 m*M* and 400 m*M* potassium phosphate (pH 8.0) collected in *ca.* 250-ml fractions (Fig. 4). The fractions were

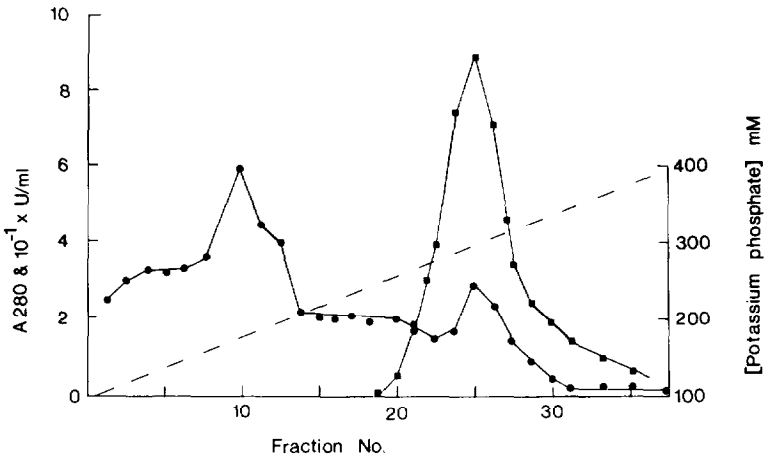


Fig. 4. Chromatography of glycerokinase on DEAE-Sepharose CL-6B. The enzyme from 1 kg of bacterial cell paste was chromatographed on a 2-l column of DEAE-Sepharose CL-6B equilibrated in 100 m*M* potassium phosphate buffer (pH 8.0). The column was eluted with a 10-l linear gradient of 100 m*M* to 400 m*M* buffer, collected in fractions of *ca.* 250 ml. Fractions 21–29 were pooled. Circles, A280; squares, glycerokinase activity, U/ml.

pooled for glycerokinase activity and concentrated to *ca.* 250 units/ml using Amicon H1P10 hollow fibre cartridges in an Amicon Model CH4 concentrator. The concentrated enzyme was adjusted to pH 7.5 with 3.2 *M* phosphoric acid and dialysed against 10 m*M* potassium phosphate buffer (pH 7.5). The dialysed enzyme was applied to a 600-ml column (16 × 7 cm I.D.) of Procion Blue MX-3G–Sepharose equilibrated in 10 m*M* potassium phosphate buffer (pH 7.5) at a flow-rate of 300 ml/h. The column was washed with 2500 ml of buffer to remove unbound proteins.

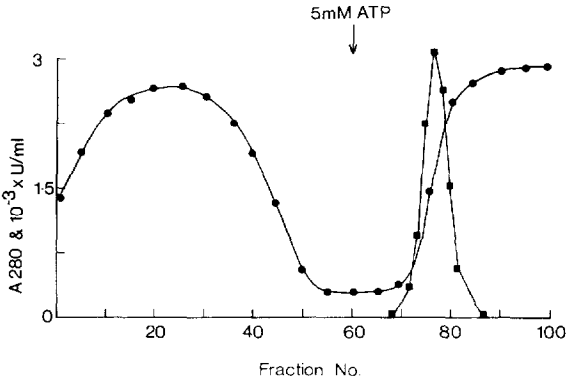


Fig. 5. Chromatography of glycerokinase on Procion Blue MX-3G–Sepharose 4B. The pooled enzyme from the DEAE–Sepharose column was loaded onto a 600-ml column of Procion Blue MX-3G–Sepharose 4B, equilibrated in 10 m*M* potassium phosphate buffer (pH 7.5). The column was washed with 2500 ml of buffer and eluted with 1200 ml of 5 m*M* ATP and 5 m*M* magnesium chloride in 10 m*M* phosphate buffer (pH 7.5). The column eluate was collected in *ca.* 15-ml fractions. Circles, A280; squares, glycerokinase activity, U/ml.

TABLE VI

PURIFICATION OF GLYCEROKINASE BY DYE-AFFINITY CHROMATOGRAPHY

	Enzyme (unit)	*Protein (mg)*	*Specific activity (U/mg)*	*Purification factor*	*Recovery (%)*
Crude extract	198 000	123 000	1.60		100
pH 5.1 supernatant	156 200	22 200	7.04	4.4	79
DEAE-Sephadex	122 400	4920	24.9	3.5	62
Procion Blue MX-3G–Sepharose	87 000	720	121	4.9	44

Glycerokinase was eluted with 1200 ml of 5 m*M* ATP, 5 m*M* magnesium chloride, in 10 m*M* potassium phosphate (pH 7.5), collected in *ca.* 15-ml fractions (Fig. 5).

The results of this purification are shown in Table VI. The pooled enzyme from the dye–Sepharose column was shown to be homogeneous by electrophoresis in the presence of sodium dodecyl sulphate. The final dye-affinity step replaced three steps of chromatography in the conventional purification[5], and accounts for the overall yield of 44% that was achieved, thus demonstrating the usefulness of dye-affinity chromatography for the purification of this enzyme. Because the interaction between enzyme and dye is relatively non-specific, biospecific elution gave a greater degree of purification, as well as a higher overall recovery than did non-specific elution with salt. In this respect ATP is an ideal ligand because of its low cost compared with other nucleotide cofactors.

REFERENCES

1 E. C. C. Lin, A. P. Levin and B. Magasanik, *J. Biol. Chem.*, 235 (1960) 1824–1829.
2 J. P. Koch, S. Hayashi and E. C. C. Lin, *J. Biol. Chem.*, 239 (1964) 3106–3108.
3 H. U. Bergmeyer, G. Holz, E. M. Kauder, H. Mollering and O. Wieland, *Biochem. Z.*, 333 (1961) 471–478.
4 A. Atkinson, C. G. T. Evans and R. G. Yeo, *J. Appl. Bact.*, 38 (1975) 301–304.
5 M. J. Comer, C. J. Bruton and A. Atkinson, *J. Appl. Biochem.*, 1 (1979) 259–270.
6 M. J. Comer, *Thesis*, Imperial College, London, 1976.
7 P. D. G. Dean and D. H. Watson, *J. Chromatogr.*, 165 (1979) 301–319.
8 C. R. Lowe, D. A. P. Small and T. Atkinson, *Int. J. Biochem.*, 13 (1981) 33–40.
9 C. R. Lowe and J. C. Pearson, *Methods Enzymol.*, 104 (1984) 97–113.
10 R. Axen, J. Porath and S. Earnback, *Nature*, 214 (1967) 1302–1304.
11 K. Sargeant, D. N. East, A. R. Whitaker and R. Ellsworth, *J. Gen. Microbiol.*, 65 (1971) iii.
12 C. Bublitz and O. Wieland, *Methods Enzymol.*, 5 (1962) 355.
13 O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, 193 (1951) 265–275.
14 J. K. Baird, R. F. Sherwood, R. J. G. Carr and A. Atkinson, *FEBS Lett.*, 70 (1976) 61–66.
15 A. Atkinson and M. J. Harvey, *U.K. Pat. Appl.*, (1978) 3505/78.
16 K. Venkataraman, (Editor), *The Chemistry of Synthetic Dyes*, Vol. 6, Academic Press, New York, 1972.
17 W. H. Scouten, *Affinity Chromatography: Bioselective Absorption on Inert Matrices*, Wiley, New York, 1980, Ch. 2, pp. 20–41.