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APPLICATIONS OF IMMOBILISED PHENYLBORONIC ACIDS AS SUPPORTS FOR GROUP-SPECIFIC LIGANDS IN THE AFFINITY CHROMATOGRAPHY OF ENZYMES

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SUMMARY

Several different aminophenylboronic acid (APBA)–agarose matrices have been compared.

Nucleotides are able to bind to these matrices in the presence of either NaCl or $MgCl_2$. NAD^+ and FAD are more tightly bound to these columns when compared with other nucleotides.

The properties of NAD^+ and $NADP^+$ complexes with immobilised phenylboronic acids have been compared using pure 6-phosphogluconate and alcohol dehydrogenases. The $NADP^+$-dependent enzyme bound to the $NADP^+$ complex. Conversely the NAD^+-dependent enzyme was only retarded by the NAD^+-immobilised APBA complex.

Glucose-6-phosphate dehydrogenase from yeast has been partially purified on $NADP^+$ complexed to immobilized boronic acid–agarose columns.

The desorption of bound enzymes to nucleotide–phenyl boronate columns can be effected by salt gradients, the addition of diols; monosaccharides, sorbitol, cofactor competition, by lowering the pH of the eluent or by specific retardation.

Purification factors of 14-fold were achieved for yeast glucose-6-phosphate dehydrogenase by choosing the appropriate concentration of presaturating $NADP^+$. The enzyme was selectively retarded and did not require specific elution.

Similar experiments enabled yeast hexokinase to be purified by presaturation of APBA-agarose with ATP. Elution of the enzyme was dependent upon the concentration of ATP used to presaturate the column. The enzyme was simply retarded by the column and required no (specific) eluent.

INTRODUCTION

The ability of borates to form complexes with alcohols and particularly vicinal diols has been known for some time[1,2]. Biologically useful applications of the latter property using immobilised phenylboronate to selectively interact with diols were developed by Gilham and co-workers[3,4]. Thus, tRNA and tRNA-isoacceptors have been purified from yeast[5]. Other separations of ribonucleotides and ribonucleotide (ADPR)–protein conjugates have also been described[6-8]. Smaller molecules, phenolates[9], catecholamines[10], and sugars[8] have been separated by a variety of phenylboronates immobilised to polyacrylamide, Sephadex and polystyrene.

Immobilised phenylboronates have also found interesting applications in the enzymic assays of adenylate cyclase[11], ribonucleotide triphosphate reductase[12] and a phosphodiesterase[13].

Other applications of immobilised phenylboronic acids utilise a different property of boronic acids, namely their ability to inhibit proteases. Thus, the purification of proteases from *Bacillus subtilis* has been reported[14].

Three variables are particularly important for the design of successful affinity chromatographic separations. These are matrix, spacer arm, and ligand.

(i) Matrix: in the application of immobilised boronates, unlike many affinity systems, the ligands have been mainly based on supports other than agarose. It could be argued that cellulose and other glucose-based polysaccharides would be intrinsically unsuitable because of potential interactions between the ligand (boronate) and the matrix. This feature of the latter polymers is likely to be less pronounced for agarose which has fewer available hydroxyl groups, none of which have the 1,2-*cis*-diol configuration.

(ii) Spacers: we have examined the properties of agarose-immobilised phenylboronates with hydrophobic arms because of the known importance of hydrophobic "spacer arms" to enhance some low affinity interactions[15].

(iii) Ligand: the ligand used in previous work has been phenylboronate (PBA), most frequently as the derivative 3-aminophenylboronate (Fig. 1a). One of the purposes of this investigation was to examine different methods of immobilising aminophenylboronic acid to agarose and to select from these the most suitable adsorbent for nucleotide-based affinity separations. Thus, we hoped to be able to overcome undesirable features of immobilised nucleotides which include (a) the need for complex synthesis of derivatives suitable for immobilisation; (b) the instability of many described covalently attached nucleotides and the resulting limited life of such columns; (c) the cost; (d) the need to carry out separate immobilisation reactions for each nucleotide derivative.

We present data which suggest that phenylboronate–agarose adsorbents can retard nucleotides sufficiently to permit chromatography of dehydrogenases and kinases from yeast extracts.

EXPERIMENTAL

Materials

Enzymes, their substrates and nucleotides were purchased from Boehringer (Mannheim, G.F.R.). 3-Aminophenylboronic acid (APBA), 1,4-butanediol-digly-
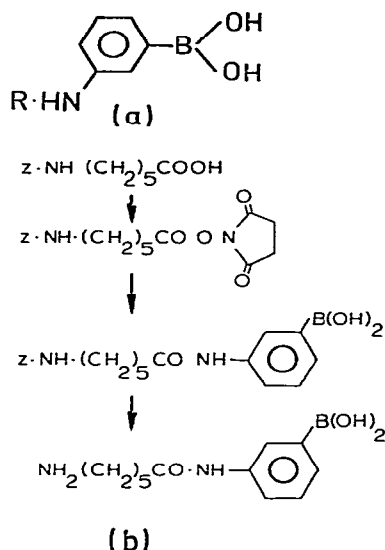
Fig. 1. The structure of phenylboronic acid (a) and synthesis of 6-aminocaproyl-3-aminophenylboronic acid (b).

cidyl ether, and diphenylcarbazone were obtained from Aldrich (Milwaukee, WI, U.S.A.). Cyanogen bromide and yeast enzyme concentrate were obtained from Sigma (St. Louis, MO, U.S.A.). Hydrogen bromide and 6-aminocaproic acid were purchased from BDH (Poole, Great Britain). Sepharose 6B was obtained from Pharmacia (Uppsala, Sweden). Matrex Gel phenylboronate was obtained from S. Fulton (Amicon, Lexington, MA, U.S.A.). (Ligand concentration was 41 μmoles/ml).

Methods

Thin-layer chromatography (TLC) was performed on Kieselgel GF 254-coated plates (Merck, Darmstadt, G.F.R.). The spots of boron-containing compounds were visualized by spraying with a 0.2% (w/v) solution of diphenylcarbazone in methanol[16]. A 0.2% (w/v) ninhydrin solution in acetone was used for detecting primary amino groups. Nuclear magnetic resonance (NMR) spectra were recorded at 220 MHz on a Perkin-Elmer R-34 instrument.

Measurements of retention volumes. (Least volume before nucleotide could be detected). Each nucleotide (1 mg) was dissolved in 50 mM Hepes buffer, pH 8.45 (0.2 ml) containing 0.1 M MgCl$_2$ and applied to a column of immobilized APBA (1 ml) attached to CNBr-activated Sepharose 6B (ligand concentration, 8 mg APBA/ml wet weight). The temperature of the column was 4°C throughout. Flow-rates were maintained at 1.0 ml/h using a peristaltic pump (LKB Varioperpex). Fractions (0.5 ml) were collected and monitored for O.D. at 260 nm.

Immobilisation methods. (a) Cyanogen bromide activation: Sepharose 6B (25 g moist weight) was activated with cyanogen bromide (2 g) according to the method of Axen *et al.*[17]. The gel was washed with ice cold 0.1 M NaHCO$_3$, pH 9.5 and added to a solution of APBA (500 mg) or aminocaproyl aminophenyl boronic acid (ACA-PBA, 500 mg) in 0.1 M NaHCO$_3$, pH 9.3 (20 ml), the suspension was rotated over-

night on a Coulter mixer at 4°C. The matrix was washed with 0.1 M NaHCO$_3$. The ligand concentration was estimated spectrophotometrically by subtracting the amount of unbound ligand from the total added initially.

(b) Epoxy activation: The method of Porath[18] was used to couple APBA to Sepharose 6B. The ligand concentration of the matrix was estimated spectrophotometrically by subtracting the amount of unbound APBA from the total amount added.

Synthesis of 6-aminocaproyl-3-aminophenylboronic acid (see Fig. 1b).

(a) Synthesis of benzyloxycarbonyl 6-aminocaproic acid (Z-ACA): 6-Amino-caproic acid (10 mmoles) was dissolved in 4 M NaOH (50 ml). Benzyloxycarbon-ylchloride (20 mmoles) was added with cooling and shaking over a period of 20 min. During the latter period, additional 4 M NaOH (50 ml) was added. The solution was extracted with diethyl ether (3 × 100 ml). The aqueous phase was acidified with HCl at 0°C, and extracted with ethyl acetate (3 × 100 ml). The ethyl acetate extracts were combined, dried (Na$_2$SO$_4$) and evaporated to dryness. The resulting solid was re-crystallised from chloroform m.p. 54–56°C (Lit.[19] m.p. 54°C) yield 85%. TLC showed one spot (R_F, 0.41, ethyl acetate; 0.66, ethanol). NMR [d$_6$-dimethyl sul-phoxide (DMSO)]: δ 1.38 (complex multiplet, 1.2 to 1.6, 3 × CH$_2$): 2.2 (t, J = 6 Hz, CH$_2$): 3.0 (q, J = 6 Hz, CH$_2$): 5.20 (s, ArCH$_2$): 7.23 (complex m, NH): 7.36 (s = 5ArH): 9.8 (s, CO$_2$H) ^{2}H$_2$O exchangeable.

(b) Synthesis of benzyloxycarbonyl 6-aminocaproyl-N-hydroxysuccinimide ester (Z-ACA-NHS): Dicyclohexylcarbodiimide (5.5 mmoles) was added to a so-lution of Z-ACA (5 mmoles) plus N-hydroxysuccinimide (5.5 mmoles) in dioxan (25 ml) with cooling. The reaction was left for 10 h at 12°C. The white precipitate which formed (dicyclohexylurea) was filtered off and the remaining solution evaporated to give an oil. The oil was washed with water followed by 1 M HCl and was used for the next step without further purification.

(c) Synthesis of benzyloxycarbonyl-6-aminocaproyl-3-aminophenylboronic acid (Z-ACA-APBA). To a solution of Z-ACA-NHS (5 mmoles) in ethanol (10 ml) under nitrogen was added APBA (5 mmoles) in water (10 ml) containing NaHCO$_3$ (10 mmoles). The mixture was left for 10 h at room temperature. The precipitate which formed was filtered off, washed with water and recrystallized from ethyl acetate to give Z-ACA-APBA as plates m.p. 172–174°C (yield, 20–40%).

TLC revealed a single UV-absorbing spot (R_F, 0.32, (ethyl acetate); 0.76 (eth-anol); 0.84 (ethanol–water, 1:1) (APBA gave R_F 0.26 (ethyl acetate)). NMR (d$_6$-DMSO): δ 1.4 (complex multiplet, 1.2 to 1.7, 3 × CH$_2$): 2.25 (t, J = 6 Hz, CH$_2$): 2.97 (q, J = 6 Hz, CH$_2$): 4.97 (s, ArCH$_2$): 7.3 (s, 5ArH): 7.17–7.93 (complex multiplet, H). APBA gave (^{2}H$_2$O): 7.07 (complex multiplet): 7.28 (d, J = 2 Hz): 7.34 (d, J = 2 Hz) all aromatic H atoms. λ_{max} (dioxan) = 253, 284 (shoulder).

(d) Synthesis of 6-aminocaproyl-3-aminophenylboronic acid (ACA-APBA): Benzyloxycarbonyl-6-aminocaproyl-3-aminophenylboronic acid (150 mg) was added to 45% (v/v) HBr in acetic acid (4 ml) and acetic acid (4 ml). The mixture was left at room temperature for 40 min. Diethyl ether (100 ml) was added to precipitate the HBr salt of ACA-APBA as a white hygroscopic powder. The precipitate was further washed with diethyl ether (500 ml) and dried *in vacuo*. The product moved as a single spot on TLC (SiO$_2$) R_F, 0.18 (ethanol); (cellulose) 0.68, (ethanol), m.p. > 250°C (d). Yield, 80%. NMR (d$_6$-DMSO): significant peaks were δ 1.13 (t, J = 6 Hz, CH$_2$); 1.4 (complex multiplet, CH$_2$); 1.65 (complex multiplet, CH$_2$); 2.38 (broad t, J = 6 Hz,

CH_2); 2.85 (t, J = 6 Hz, CH_2); 7.62 (complex multiplet, 4 ArH); 7.92 (d, J = 6 Hz) and 9.88 (s, $B(OH)_2$) in the presence of ^2H_2O the peaks at δ 7.92 and 9.88 were removed by exchange. $\lambda_{max.}$ (0.1 M sodium phosphate buffer, pH 7.0), 242 nm.

RESULTS AND DISCUSSION

Of the eleven nucleotides tested for retardation by CNBr-immobilized APBA, only NAD^+, FAD and NADH seemed to bind tightly (Table I). The effect of varying the pH and salt concentration on the retention of nucleotides was examined (Table II). It can be seen from Table II that Hepes buffer, pH 8.45 improved the binding of FAD when compared with phosphate buffer (pH 7.0) but that either 1 M NaCl or 0.1 M $MgCl_2$ produced a further 6–10-fold increase in retention volume.

TABLE I

RELATIVE RETARDATION OF NUCLEOTIDES BY PBA-AGAROSE COLUMNS

Nucleotides (1 mg) were separately applied in 50 mM Hepes–NaOH buffer, pH 8.45 containing 0.1 M $MgCl_2$ to columns (1 ml) of PBA–Sepharose 6B (CNBr-activated); ligand concentration, 58 μmoles/ml (wet weight) at 0°C at a flow-rate of 1.0 ml/h. Retention volumes were measured as in Fig. 2.

Nucleotide	Retention volume (× V_0)	Capacity ($\mu mol/ml$)
NAD^+	>15	5–7
NADH	>15	5–7
$NADP^+$	9–10	5–7
FAD	>15	NT*
Adenosine	8	NT
AMP	6	NT
ADP	6.5	NT
ATP	5.5	4
GMP	5.5	NT
GDP	6	NT
GTP	5	NT

* NT = not tested.

TABLE II

THE EFFECT OF VARYING THE pH AND SALT CONCENTRATION ON THE INTERACTION OF FAD WITH PBA–AGAROSE COLUMNS

Columns containing PBA–Sepharose 6B (CNBr-activated, 1 ml) were equilibrated with the appropriate buffer before application of a pulse of 1 mM FAD (0.2 ml) at 4. Flow-rate 0.7 ml/h. Retention volumes were measured as in Fig. 2.

	Retention volume (× V_0)
0.1 M potassium phosphate, pH 7.0	2.5
0.1 M potassium phosphate + 1 M NaCl	4.6
50 mM Hepes, pH 8.4	4.0
50 mM Hepes + 1 M NaCi	26
50 mM Hepes + 0.1 M $MgCl_2$	25

These results indicate that nucleotides could be used as affinity ligands when complexed to immobilized phenylboronic acid–agarose provided that enzyme and enzyme-nucleotide interactions are stable to either 1 M NaCl or 0.1 M MgCl$_2$ and to pH values in excess of 8.0 and preferably 8.4.

The increased stability of the PBA–nucleotide interaction in the presence of 1 M NaCl or 0.1 M MgCl$_2$ has useful chromatographic applications. Thus, enzyme samples can be applied to a nucleotide–PBA–agarose complex at low salt concentrations (the complex being stabilised with MgCl$_2$). The bound enzyme may be desorbed using either 1 M NaCl or nucleotide (see below).

The interaction of NADP$^+$ with immobilised PBA was determined to some extent by the chemistry of the linkage with the column. Fig. 2 shows the differing abilities of PBA derivatives to bind NADP$^+$ at various applied NADP$^+$ concentrations. The retention volume was also affected by the amount of nucleotide applied to columns of immobilized phenylboronic acid (Fig. 2), decreasing in response to an increase in the amount of NADP$^+$ applied. This change of retention volume was clearly related to the capacity of the boronate ligand to bind the nucleotides. Thus the retention volumes of both epoxide and CNBr-immobilized PBA fell below ten column volumes when more than 2–3 mg of NADP$^+$ were applied. Columns with retention volumes in excess of ten column volumes were effective as affinity supports. Thus, we conclude from our experiments with CNBr-coupled aminocaproyl-3-aminophenylboronic acid that, at the ligand concentration used, this derivative of phenylboronic acid with NADP$^+$ cannot easily be used for affinity experiments.
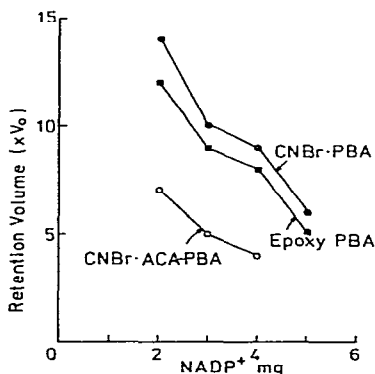

Fig. 2. The variation of retention volume with the concentration of applied NADP$^+$ for three types of immobilised PBA. Buffer was continuously applied until the applied nucleotide emerged. The retention volume is defined as the lowest volume after which the nucleotide was detected and is expressed as a multiple of the column volume. The ligands were prepared as described in Experimental. The ligand concentrations were 58 μmoles/ml (CNBr-PBA); 146 μmoles/ml (epoxy-PBA) and 34 μmoles/ml (CNBr-ACA-PBA). Temperature, buffers and flow-rate used were as quoted in Table II. NADP$^+$ was applied in 0.2 ml to each column (1 ml).

We have chromatographed purified enzymes on both NAD$^+$ and NADP$^+$-presaturated phenylboronic acid–agarose columns (Fig. 3). Neither yeast alcohol dehydrogenase (ADH) nor 6-phosphogluconate dehydrogenase (6-PGDH) were able to bind tightly to the NAD$^+$-phenylboronic acid complexes, whereas the NADP$^+$-specific yeast 6-PGDH bound tightly to the NADP$^+$–PBA columns.
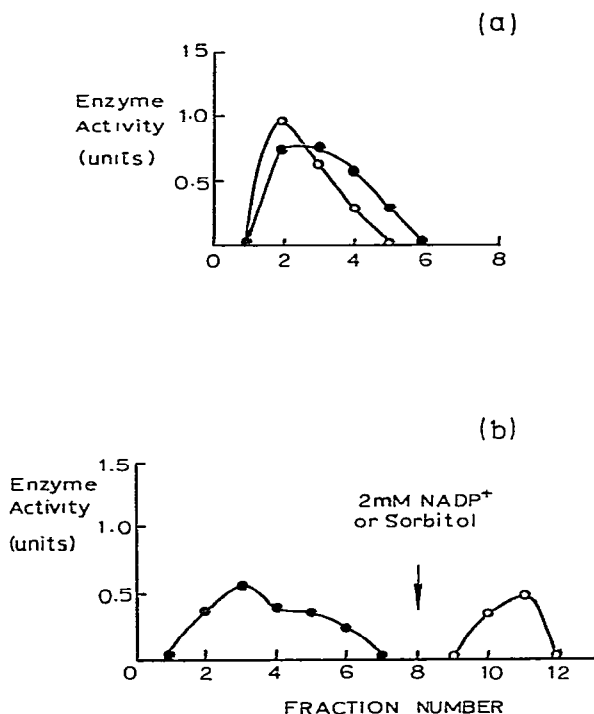
(a)

Enzyme Activity (units)

(b)

Enzyme Activity (units)

2mM NADP⁺ or Sorbitol

FRACTION NUMBER

Fig. 3. The chromatographic behaviour of purified yeast ADH and yeast 6-PGDH on NAD(P)$^+$-PBA-Sepharose. NAD$^+$ (a) or NADP$^+$ (b) (2 mg) was applied to APBA–Sepharose 6B (1 ml) (CNBr-activated) and the resulting adsorbent washed with 0.05 M Hepes–NaOH buffer, pH 8.45 containing 0.1 M MgCl$_2$ (1.0 ml). Columns were then loaded with a mixture of ADH (3 U) and 6-PGDH (2 U) (in 0.2 ml) and washed with the same buffer (7 ml). Elution buffers contained 2 mM NAD(P)$^+$ or 20 mM sorbitol. ●, ADH; ○, 6-PGDH (× 5).

Yeast protein mixtures were applied to NADP$^+$–PBA columns and the results (Fig. 4) confirm those obtained with purified enzymes. Thus, ADH appeared in the early fractions of the column whereas a linear gradient of KCl eluted glucose-6-phosphate dehydrogenase (G-6-PDH), which was separated from the main peaks of protein.

We do not understand why NAD$^+$-presaturated columns did not retard NAD$^+$-dependent dehydrogenases. Similar differences in behaviour have been observed for ribose-immobilised NAD$^+$ and NADP$^+$. Several well documented examples of successful application of such NADP$^+$-affinity columns exist[20,21] but few for NAD$^+$ systems. The reasons may be (i) that a large percentage (50%) of non-productive species are formed by attachment via the adenine ribose of the dinucleotide; or (ii) that NAD$^+$-dependent enzymes use both ribose moieties for successful binding of the cofactor[22]. The apparent inconsistency between free solution studies on periodate-oxidized (ribose) nucleotides (to which dehydrogenases do not bind) and ribose-immobilised NADP$^+$-affinity columns (which do bind NADP$^+$-dependent dehydrogenases) can be explained by the fact that the ribose ring in the latter has been reformed into a morpholine derivative and may well retain the molecular configuration of the original nucleotide.
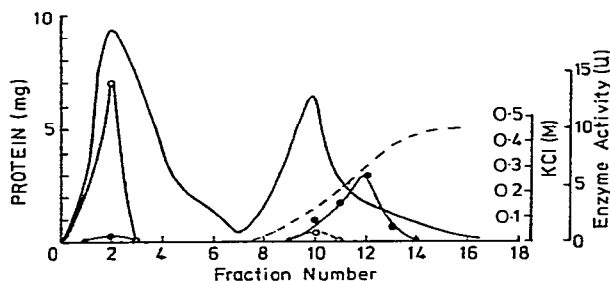
Fig. 4. The chromatography of yeast proteins on NAD(P)$^+$–PBA–Sepharose. Yeast enzyme concentrate (52 mg) in Hepes–NaOH buffer (1 ml), pH 8.45 (plus 0.1 M MgCl$_2$) containing G-6-PDH (5.5 U) and ADH (16 U) applied to a column of PBA–Sepharose 6B (CNBr-activated) (3 ml) presaturated with 6 mg NADP$^+$ (prepared as described in Fig. 3). Elution was carried out with a gradient of 0 to 0.5 M KCl (– · –) in the same Hepes buffer (20 ml total volume). Fractions of 2 ml were collected. Samples were assayed for G-6-PDH (●), ADH (○) and protein (——).

Application of similar protein mixtures from yeast extracts to NADP$^+$– presaturated aminocaproyl aminophenylboronate columns (Fig. 5) resulted in retardation of the NADP$^+$-dependent G-6-PDH dehydrogenase. A specific elution step was not used and a 14-fold purification factor was achieved with 66% yield. This gel did not perform particularly well in NADP$^+$-saturation studies (Fig. 1) (partly because of its low ligand concentration, 34 μmoles/ml), which might explain why the enzyme did not require a separate elution step to be recovered from the column. Indeed boronate columns appear to be especially useful because of the manner in which enzymes may be selectively retarded[23].

Matrex gel phenylboronate (ligand concentration, 41 μmoles/ml) columns were also able to retard G-6-PDH from yeast protein when presaturated with 2 mg of NADP$^+$ per 1 ml column (Fig. 6). In this experiment, a purification factor of 12-fold and yield of 69% were recorded. Elution in the latter instance was effected by 2 mM NADP$^+$.
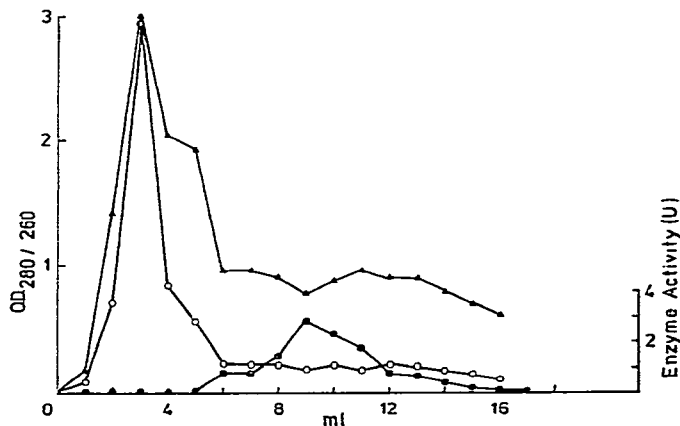

Fig. 5. Chromatographic retardation of yeast G-6-PDH by NADP$^+$-presaturated ACA-PBA–Sepharose. Yeast enzyme concentrate (7.1 mg in 0.5 ml) in 0.05 M Hepes–NaOH buffer (1 ml), pH 8.5 (plus 0.1 M MgCl$_2$) was applied to a column of ACA-PBA–Sepharose (1 ml) which had been preequilibrated with the above buffer (5 ml) followed by a solution containing NADP$^+$ (2 mg) in the same buffer (0.5 ml) followed by buffer (1 ml). The concentrate contained 18 U of G-6-PDH. Fractions of 1 ml were collected at a flow-rate of 1 ml/h. Fractions were assayed for enzyme activity (●), O.D. 260 (▲) and O.D. 280 (○).
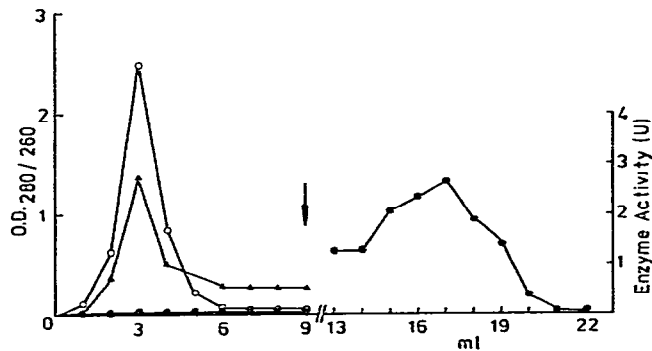
Fig. 6. Chromatography of yeast G-6-PDH by NADP$^+$–presaturated Matrex gel–PBA. Yeast enzyme concentrate (7.5 mg in 0.5 ml) in buffer (ligand concentration, 41 μmoles/ml) (see Fig. 5) was applied to a column of Matrex gel PBA (1 ml) preequilibrated as described in Fig. 5. The concentrate contained 19.4 U of G-6-PDH. Fractions (1 ml) were collected and assayed for enzyme activity (●), O.D. 260 (▲) and O.D. 280 (○). Elution was effected with 2 mM NADP$^+$ (arrow).

Thus, various elution procedures can be adopted which depend to some extent on the nature of the boronate ligand, its concentration and the agarose used as a matrix. Cross-linking of the matrix may have some effect and may explain the difference between Matrex gel® and Sepharose® since Matrex gel is cross-linked whereas Sepharose is not.

The specificity of "ligand mediated" separations using boronate columns can be changed by replacing NADP$^+$ with other nucleotides. Thus, we have used Matrex gel phenylboronate–ATP complexes to retard hexokinase. Control columns containing no ATP retard little or no hexokinase. Small amounts of material are bound however, which are released from the column with 100 mM sorbitol (Fig. 7).

On the other hand, when ATP was included in the irrigation buffer prior to the application of yeast extract, hexokinase was retarded (Fig. 8). Furthermore, the elu-
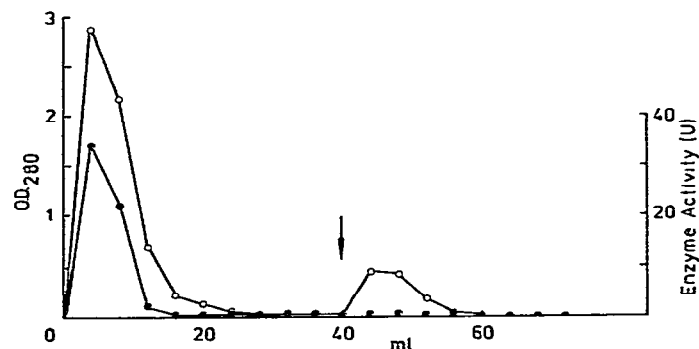
Fig. 7. Chromatography of yeast hexokinase on Matrex gel–PBA in the absence of presaturating ligand. Yeast enzyme concentrate (1 ml) in buffer (see Fig. 5) was applied to a column of Matrex gel–PBA (1 ml) (ligand concentration, 41 μmoles/ml) preequilibrated with 50 mM Hepes–NaOH buffer (containing 0.1 M MgCl$_2$), pH 8.45. The concentrate contained 62 U of hexokinase. Fractions (4 ml) were collected and assayed for hexokinase activity (●) and O.D. 280 (○). Elution was effected with 100 mM sorbitol in buffer (arrow).
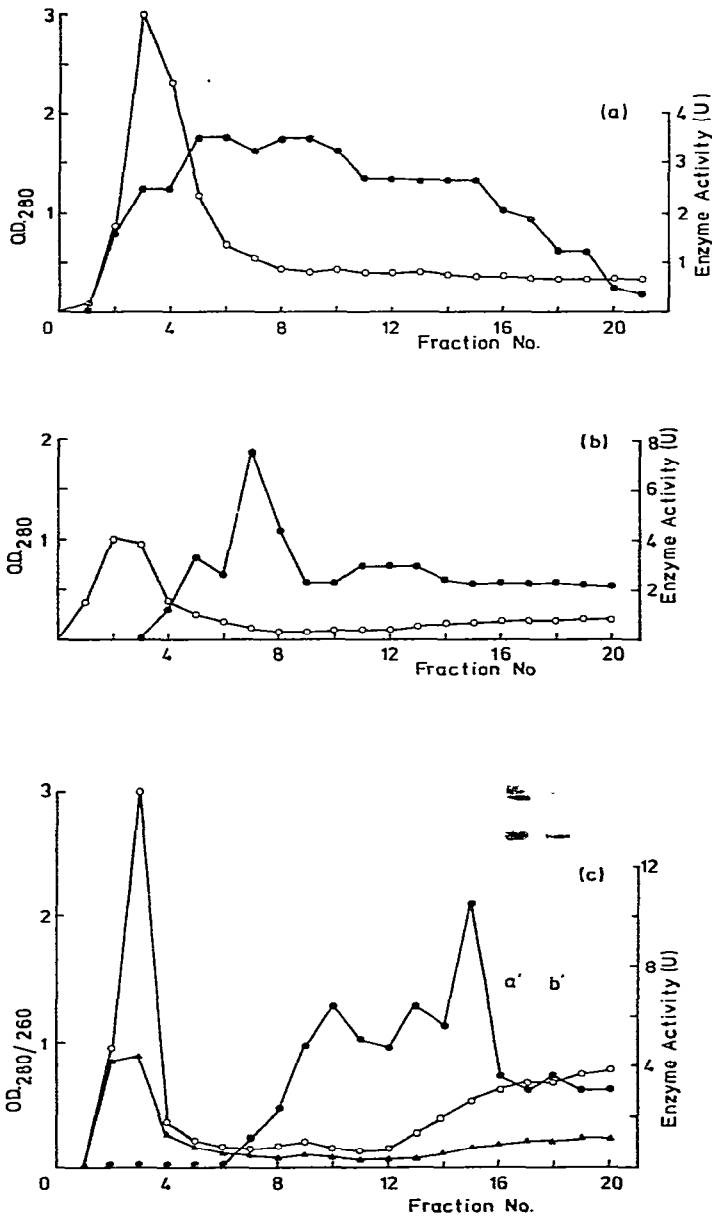
Fig. 8. Chromatographic profiles of hexokinase retarded by Matrex gel PBA presaturated with three different concentrations of ATP. Yeast enzyme concentrate containing hexokinase [60 (c), 66 (b) and 80 (a) U, respectively] in buffer (see Fig. 5) was applied to a column of Matrex gel PBA (4 ml, ligand concentration, 41 μmoles/ml) preequilibrated with ATP [8 (c), 12 (b) and 22 (a) mg in 0.5, 0.5 and 1 ml, respectively] in 50 mM Hepes–NaOH buffer (containing 0.1 M MgCl$_2$), pH 8.45 followed by the same buffer (4 ml). Fractions (4 ml) were collected and assayed for hexokinase activity (●), O.D. 280 (○) and 260 (▲). Insert Fig. 8c: Fractions were pooled (6 to 26) and analysed by sodium dodecyl sulphate–polyacryl-amide gel electrophoresis (gel a′). A commercial preparation of pure yeast hexokinase (Boehringer) is compared (gel b′).

tion profile of hexokinase changed on increasing the amount of presaturating ATP from 8 to 22 mg. The purification factor was least (ten-fold) when the ATP concentration was highest. The precise ATP concentration required for maximizing the emergent specific activity is expected to be different for each batch of yeast protein. From the data presented a value between 8–12 mg ATP per (4-ml) column gave the best results.

Previously reported examples of immobilised nucleotides have been described where the ligand was attached covalently and irreversibly to support matrices. In the latter instances complex chemical syntheses are required prior to coupling the ligand which can incur considerable expense especially when large scale applications are considered.

We believe, despite the problem of preparing suitable immobilised phenylboronic acids that this ligand, when used as a complex with nucleotides, may overcome many of the criticisms of immobilised cofactors as group-specific ligands.

At present, two problems remain to be resolved.

(i) The pK of the immobilised PBA (8.9, see ref. 20 and Fig. 9) used in this study is by no means ideal, since the chromatography requires pH values in excess of 8.0. Work is in progress to synthesise more suitable boronic acids.
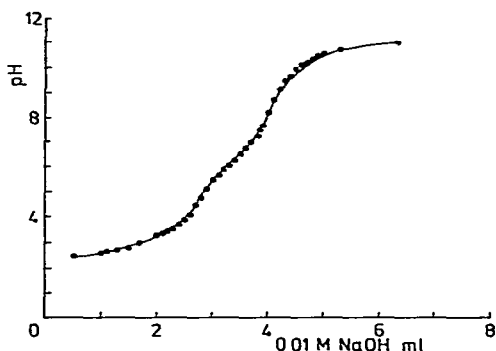

Fig. 9. Titration curve obtained with APBA–Sepharose (CNBr-activated). Samples of gel were washed with distilled water and the pH adjusted to a value of 2.5 with dilute HCl. The suspension was then titrated with 0.01 M NaOH with constant stirring under nitrogen. The titration was carried out at room temperature. Ligand concentration 8 mg APBA per ml gel (wet weight).

(ii) The ligand concentrations used in this work appear to limit the enzyme capacity of these gels. Therefore, a device is needed to augment the ligand concentration[24]. However, the ubiquitous nature of PBA–coenzyme interactions enables a very wide range of immobilised cofactors to be prepared with the same column. The column is not sensitive to enzymic degradation, and the ligand which is complexed to the boronic acid is readily replaced (or exchanged) simply by stripping the PBA matrix with low pH buffers or sorbitol (followed by urea and NaOH to remove any adsorbed protein, for cross-linked gels only).

Other applications of immobilised PBA are under investigation in this laboratory.

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