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POLYACRYLAMIDE–BORONATE BEADS SATURATED WITH BIOMOLECULES: A NEW GENERAL SUPPORT FOR AFFINITY CHROMATOGRAPHY OF ENZYMES

RAMONCITA R. MAESTAS, JOE R. PRIETO, GLENN D. KUEHN and JAMES H. HAGEMAN

Department of Chemistry, Box 3C, New Mexico State University, Las Cruces, N.M. 88003 (U.S.A.)
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SUMMARY

Aminoethyl polyacrylamide beads (P-150) were quantitatively coupled to *m*-aminobenzeneboronic acid to yield a product containing 1.3 mmoles of boronic acid per gram (dry weight) of gel. The average pK_a of the insolubilized boronic acid was determined to be 9.2. At pH 8.45 a number of enzyme substrates and cofactors, including NAD^+, citric acid, pyridoxal, and epinephrine, were shown to bind to P-150–boronate beads packed in chromatography columns. At saturation, the P-150–boronate beads bound 2.5 to 80 μmoles of substrate or cofactor per ml of wet packed gel. A P-150–boronate column saturated with uridine 5′-triphosphate was used to achieve a 1000-fold purification of the enzyme uridine 5′-diphosphate glucose pyrophosphorylase (EC 2.7.7.9) from the slime mold, *Physarum polycephalum*.

INTRODUCTION

The usefulness of boronic acid derivatives attached to insoluble supports for the chromatography of sugars, nucleosides, nucleotides and nucleic acid polymers was first realized by Gilham and co-workers[1,2]. Subsequently, such boronic acid derivatives have been used for the purification of an ADP-ribosyl protein[3] and proteases from *Bacillus subtilis*[4], the assays of ribonucleotide reductase[5], adenylate cyclase[6] and cyclic nucleoside 3′,5′-monophosphate phosphodiesterase[7], the isolation of catecholamines from urine[8], and for the trapping and purification of nucleosides[9,10].

A review in the literature regarding boric acid reactions with molecules of biological interest[11] suggested to us that boronic acids attached to insoluble supports might bind a broader range of biomolecules than had previously been tested. In addition, we thought such column supports, when saturated with a specific biomolecule, might serve for the affinity chromatography of some enzymes[12]. Both of these suppositions are supported by the findings reported in this paper.

EXPERIMENTAL

Reagents

Aminoethyl P-2 and P-150, each 100–200 mesh, and 1-ethyl-3(3-dimethylaminopropyl)carbodiimide·hydrochloride (EDAC) were products of Bio-Rad Labs. (Richmond, Calif., U.S.A.). *m*-Aminobenzeneboronic acid, nicotinamide adenine dinucleotide oxidized (NAD^+) and reduced (NADH) forms, flavin adenine dinucleotide (FAD), pyridoxal, epinephrine, citric acid, lactic acid, uridine 5'-triphosphate (UTP) and Tris were products of Sigma (St. Louis, Mo., U.S.A.). N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) buffer was purchased from Calbiochem (Los Angelos, Calif., U.S.A.). [U-^{14}C]glucose ($1.14 \cdot 10^5$ cpm/μmole) was obtained from New England Nuclear (Boston, Mass, U.S.A.). All other chemicals were of reagent grade and were used without further purification.

Synthesis and analysis of boronate gel

The synthesis of the boronate gel was carried out as previously described[6]. The degree of coupling was determined by oxidative cleavage of the coupled boronate groups from the acrylamide beads with colorimetric quantitation or by spectrophotometric determination of the amount of uncoupled *m*-aminobenzeneboronic acid remaining in neutralized washes of the gel[6].

The titration of P-150–boronic acid gel with potassium hydroxide was accomplished using approximately 10 ml of washed, wet-packed gel suspended in 50 ml of deionized water. The titration was followed with a Metrohm-Brinkmann Model 104 pH meter fitted with a EA120 glass combination electrode. The potassium hydroxide solution was standardized by titrating against potassium acid phthalate (J. T. Baker Analyzed, primary standard; J. T. Baker, Phillipsburg, N.J., U.S.A.) with phenolphthalein indicator. The titration was corrected for the titration of an equal volume of 0.1 *N* sodium chloride solution which was performed in parallel.

Determination of biomolecule saturation

To 1 ml of wet-packed gel in a plastic column (6×1 cm I.D., Quick Sep columns from IsoLab, Akron, Ohio, U.S.A.; Cat. No. QS-P) was added 0.1–0.5 ml of a stock solution containing 5–20 m*M* of biomolecule. After a 10-min incubation, the column was eluted with 5 ml of 0.05 *M* HEPES–potassium hydroxide, pH 8.45, which contained 0.10 *M* magnesium chloride·6 H_2O. This procedure was repeated until the boronate gel column was fully saturated with bound ligand; that is, until all of the biomolecule applied in the small aliquot eluted through the column, reflecting saturation of boronate binding groups. To discharge the bound biomolecules, each column was washed with 0.05 *M* sodium borate, pH 10.2, which contained 0.3 *M* sodium chloride. Elution of the column was continued until no further biomolecule could be detected in the column effluent.

The determination of concentrations of biomolecules was conducted spectrally in most cases. The extinction coefficients used, from standard references, were: NAD^+, $\varepsilon_{340} = 1.78 \cdot 10^4\ M^{-1}\ cm^{-1}$; NADH, $\varepsilon_{340} = 6.29 \cdot 10^3\ M^{-1}\ cm^{-1}$; FAD, $\varepsilon_{375} = 9.30 \cdot 10^3\ M^{-1}\ cm^{-1}$; pyridoxal, $\varepsilon_{318} = 8.20 \cdot 10^3\ M^{-1}\ cm^{-1}$; and epinephrine, $\varepsilon_{278.5} = 3.47 \cdot 10^3\ M^{-1}\ cm^{-1}$. Colorimetric methods were used to estimate citric acid[13] and lactic acid[14] concentrations.

Affinity chromatography of uridine diphosphoglucose (UDPG) pyrophosphorylase

A glass column (20 × 1 cm I.D.) was filled with the P-150–boronate gel described above. The gel was equilibrated in 50 m*M* Tris·HCl containing 50 m*M* magnesium acetate, pH 8.4, at 5°. A solution of 20 m*M* UTP in the same buffer was passed through the column until 4.7 μmoles of UTP per ml of gel was bound. A crude, clarified, cell-free extract of *P. polycephalum* was prepared from vegetative microplasmodia as previously described[15]. The enzymatic activity of UTP: α-D-glucose 1-phosphate uridyltransferase (EC 2.7.7.9) (UDPG pyrophosphorylase), was assayed by following the incorporation of [U-^{14}C]glucose into UDPG[15]. The enzyme catalyzes the reaction: glucose-1-P + UTP $\rightleftarrows$ UDPG + inorganic pyrophosphate. Protein was estimated by the method of Lowry[16]. Enzyme units are expressed as μmoles of UDPG formed/min. Specific activity is defined as units/mg of protein.

RESULTS AND DISCUSSION

Table I shows that it has been possible to obtain quantitative coupling of *m*-aminobenzeneboronic acid to aminoethyl polyacrylamide beads. Although it was possible to couple more boronate to the P-2 gel than the P-150 gel, the former has not been studied extensively. It was felt that the lower degree of cross-linking in P-150 would make these derivatives more useful for enzyme purification. The P-2 derivatives, however, would be expected to have a greater capacity to trap small molecules and might be more useful than the P-150 beads in such applications.

TABLE I

COUPLING OF *m*-AMINOBENZENEBORONIC ACID TO AMINOETHYL POLYACRYLAMIDE BEADS

Preparation	*Gel type*	*Milliequivalents of original amino group per g dry weight**	*Millimoles of bound boronate per g dry weight*	*Percent coupling*
1	P-150	1.2	1.08	90
2	P-150	1.2	1.35	113
3	P-150	1.2	1.38	115
4	P-150	1.2	1.36	113
5	P-2	2.0	2.46	123

* Reported by Bio-Rad.

Uziel *et al.*[10] had shown earlier that the binding capacity of P-2–azidoboronate gels for adenosine decreased 4.5-fold when the buffer pH was decreased from 10 to 8. Since many enzymes are quite unstable at alkaline pH, it was important to know the pK_a of the boronic acid group when attached to the P-150 beads. Fig. 1 shows a corrected titration curve for boronate-derivatized P-150 beads. The average pK_a from three titrations was calculated to be 9.2. This is in good agreement with the value of 8.86 reported for underivatized benzeneboronic acid[17]. The amino substituent at the *meta*- position on the benzene ring would be expected to slightly increase the pK_a of *m*-aminobenzeneboronic acid, compared to that of benzeneboronic acid, due to its electron-donating capacity[18]. The observed pK_a was indeed slightly higher than that of benzeneboronic acid.

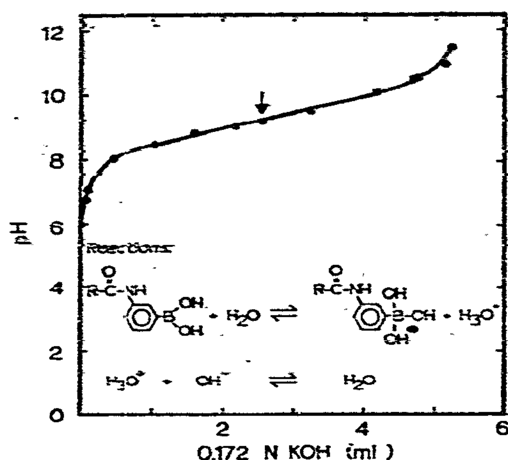


Fig. 1. Titration of P-150–boronate gel. P-150–boronate gel was prepared[6] and washed exhaustively with distilled water to remove residual electrolytes carried over from its synthesis. A sample of 10 ml of wet-packed P-150–boronate acrylamide containing 0.93 g dry-weight of derivatized gel, was suspended in 50 ml of distilled water. This suspension was stirred and titrated with 0.172 N potassium hydroxide. A 50-ml sample of 0.1 N sodium chloride was similarly titrated and the results were used to construct the corrected titration curve shown above for the P-150–boronate gel. From this titration curve it was calculated that 0.961 mequiv. of boronate groups was bound per g dry weight of derivatized gel. The average pK_a from three titration trials was calculated to be 9.2 (arrow).

Lorand and Edwards[19] have emphasized that only the boronate ion binds significantly to *cis*-diols; thus, small molecules which might serve as affinity ligands for enzyme chromatography bind best at a pH above 9, an alkalinity at which many enzymes are unstable. We therefore chose to examine the binding of several biomolecules at pH 8.4–8.5, a range in which the P-150–boronic acid derivative would be about 15% ionized.

The maximum binding capacities of P-150–boronic acid beads for a variety of molecules of biochemical interest were determined. The binding results and the capacity of borate to displace these biomolecules are shown in Table II. It may be noted

TABLE II

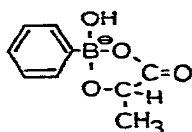
MAXIMUM BINDING CAPACITIES OF P-150–BORONIC ACID BEADS FOR SEVERAL BIOMOLECULES

All numbers are averages of duplicate runs.

Biomolecule	*Amount bound (μmoles/ml wet gel)*	*Estimated percent saturation of boronate groups**	*Amount recovered in borate wash (μmoles/ml wet gel)*
NAD$^+$	52	39	37
NADH	23	17	20
FAD	7	5	6
Pyridoxal	2.5	2	0.76
Epinephrine	16	12	16
Citric acid	21	16	1.6
Lactic acid	83	61	0

* The percent was calculated assuming 0.135 mequiv. boronate/ml wet-packed gel.

that even the most weakly bound molecule, pyridoxal, bound sufficiently well that a column of P-150–boronate thus derivatized might, in principle, serve for enzyme affinity chromatography; enzyme affinity chromatography supports that are commercially available typically contain 1–5 μmoles of biomolecule per ml of wet gel. One of the potential advantages of boronate beads is that the free boronate group can be regenerated. As Table II shows, four of the biomolecules tested (those with adjacent diols) can be displaced quite efficiently by simply washing the columns with borate buffer. The reason that borate failed to displace citric acid or lactic acid is unknown. However, it was shown that borate did not merely interfere in the standard assays for these ligands. Benzeneboronic acid was reported to form only a single type of complex with lactic acid[20]. The complex has a single negative charge:



By assuming a water regain or swelling factor of 10, it could be calculated from the data in Tables I and II that at saturation nearly 40% of the boronate groups were complexed with NAD^+. The observation that NAD^+ bound better than NADH is consistent, in order but not in magnitude, with the binding constants for borate–NAD^+ and borate–NADH reported by Smith and Johnson[21]. These authors attributed the differences in binding constants to the electrostatic attraction possible between the positive charge on the nicotinamide ring of NAD^+ and the negative charge on the cyclic borate diester formed at the ribose *cis*-diols. Both NAD^+ and NADH, of course, have two diol sites, each capable of reacting with benzeneboronate; it may be that the negative matrix of the boronate beads accentuates the electrostatic attraction.

The next step in the present study was to determine if a P-150–boronate column, saturated with an enzyme substrate or cofactor, might be used to purify an enzyme by selective binding. It has indeed been possible to purify UDPG pyrophosphorylase from the slime mold *P. polycephalum* by use of a UTP-saturated boronate column. Fig. 2 shows that this enzyme, and few other proteins present in the cell-free extract, bound to the column. The enzyme was not eluted until the UTP was displaced by washing the column at pH 7.0 with a glucose-containing phosphate buffer. The specific activity of the crude extract applied to the column was 0.019 units/mg while the pooled fractions which eluted from the column had a specific activity of 20.2 units/mg; hence, a purification of 1060-fold was achieved on the basis of enzymatic assay. However, UDPG pyrophosphorylase from *P. polycephalum* demonstrates a marked activation after partial purification on a variety of chromatographic media[22]. Thus, the large purification factor reflected, in part, this phenomenon. An alternative demonstration that the enzyme was indeed retarded by the UTP–boronate column is seen in a comparison of the protein originally applied (4.85 mg) to the column and that recovered (0.52 mg) under the enzyme activity peak (Fig. 2). On the basis of protein recovered, a 9.3-fold purification was realized. UDPG pyrophosphorylase was not retarded on a P-150–boronate gel column that was not saturated with UTP.

It should be noted that this success was dependent on the fact that UDPG

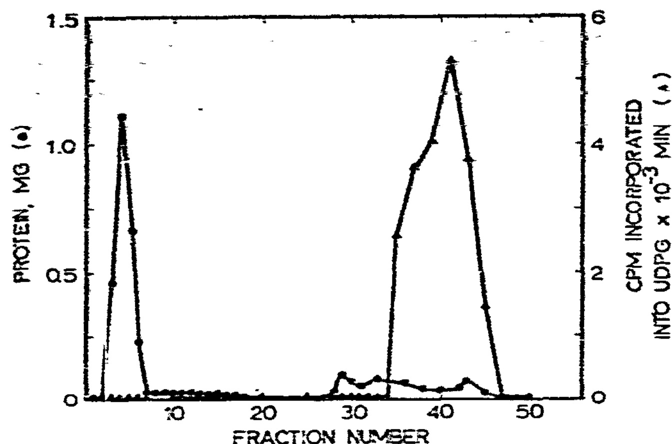


Fig. 2. Affinity chromatography of UDPG pyrophosphorylase from the slime mold *Physarum polycephalum* on a P-150–boronate acrylamide column saturated with UTP. A P-150–boronate acrylamide column (20 × 1 cm I.D.) was equilibrated with 50 m*M* TRIS·HCl, 50 m*M* magnesium acetate, pH 8.4, at 5°. UTP (74 μmoles) was bound to the P-150–boronate acrylamide as described in Experimental. A 1-ml volume of crude soluble extract protein (4.85 mg) prepared from vegetative microplasmodia of *P. polycephalum* was applied to the column. Fractions of 3 ml were collected. The Tris–magnesium acetate buffer was applied for the first 15 fractions. The buffer was then changed at fraction number 16 to 0.1 *M* potassium phosphate, pH 7.0, containing 0.1 *M* glucose. UDPG pyrophosphorylase activity (▲) was determined in 67-μl samples from each fraction by the rate of incorporation of [U-^{14}C]glucose (1.14·10^{8} cpm/μmole) into UDPG. Protein was determined by the method of Lowry (●).

pyrophosphorylase was stable at pH 8.4. When 3700 units of lactic dehydrogenase (Sigma) was applied to a 10 × 1 cm I.D. column of P-150–boronate equilibrated in 0.05 *M* HEPES–0.05 *M* magnesium acetate (pH 8.5) buffer and bearing 50 μmoles/ml of NAD^+, over 86% of the enzyme units bound to the column. Upon elution with NAD^+, very few units were recovered. This was shown to be due to the instability of lactic dehydrogenase in the pH 8.5 buffer. This pH problem, however, could probably be overcome by synthesizing boronic acid derivatives which have lower p*K* values. This work is underway in our laboratory.

Finally, the list of biomolecules which can, in principle, bind to these boronic acid supports is surprisingly large. Table III is a list of some types of compounds, and

TABLE III

BIOMOLECULES EXPECTED TO BIND TO P-150–BORONIC ACID COLUMNS

Class	*Examples*
Polyhydroxy compounds	Mannitol, fructose-6-phosphate, CDP-ethanolamine, glycoproteins
Aromatic *o*-dihydroxy compounds	Catechols, tannins, epinephrine
α-Hydroxy acids	Lactic acid, 6-phosphogluconate
Aromatic *o*-hydroxy acids and amides	Salicylic acid, salicylamide
1,3-Dihydroxy compounds	Tris, pyridoxine
Diketo and triketo compounds	Dehydroascorbic acid, benzil, alloxan
Other dihydroxy compounds	Steroids, prostaglandins

examples of each type, which are expected to have at least some affinity for boronic acid columns. We believe the main advantages of insolublized boronate derivatives over other types of column supports used for enzyme affinity chromatography are their extreme versatility and ease of coupling labile ligands under mild conditions. One may quite easily prepare mixed supports that contain more than one ligand type on the same column. Even these columns could be readily regenerated at the end of an experiment.

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