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SEPARATION OF PLANT POLYPHENOLICS BY CHROMATOGRAPHY ON A BORONATE RESIN

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SUMMARY

m-Aminophenylboronic acid attached to polyacrylamide beads by a short aliphatic chain strongly and selectively binds vicinal dihydroxyphenyl substituted compounds at pH 7–8. At lower pH these substances are released quantitatively thus affording a simple and rapid method for separation and purification of catecholic materials.

INTRODUCTION

Separation and identification of plant phenolics usually involves extractive workup of plant material followed by various chromatographic procedures. Chromatography columns employing silica gel[1], cellulose[2], polyamide (polycaprolactam[3] or polyvinylpyrrolidone[4]) and gel filtration media[5], as well as many other adsorbents, have all been used to carry out preparative isolations of these substances occurring as components of complex mixtures of free phenols and their glycosides. In an earlier report[6] we described a boronate affinity resin prepared by polymerizing *p*-vinylphenylboronic acid interstitially upon macroreticular polystyrene beads and illustrated its use by separating L-dihydroxyphenylalanine from L-tyrosine.

Such a resin, which acts by selective complexation of vicinal hydroxyl groups (Fig. 1a), should have general utility in separation of catechols. However, the capacity of that resin was limited; not all phenolic materials were satisfactorily desorbed; and the polymerization of *p*-vinylphenylboronic acid *in situ* lacked reproducibility. Other workers have described boronic acid derivatives of cellulose powder[7] as well as a copolymer of methacrylic acid possessing boronate groups[8,9]. Separations of sugars, nucleic acid components and catecholamines that utilized selective complexation with vicinal diols were reported. More recently, a boronate affinity gel based on polyacrylamide and having a capacity of *ca.* 1.0 mmol/g has become commercially available (Bio-Rad, Affi-Gel® 601), and it has found use in nucleoside determination in human plasma[10]. We have prepared a similar resin starting from polyacrylamide gel (Bio-Rad, Bio-Gel® P-2) and have used it to fractionate flavonoid mixtures from corn silk in which substances that are inhibitory toward the larval growth of the corn earworm (*Heliothis zea* Boddie) possess as an essential structural feature the *o*-dihy-

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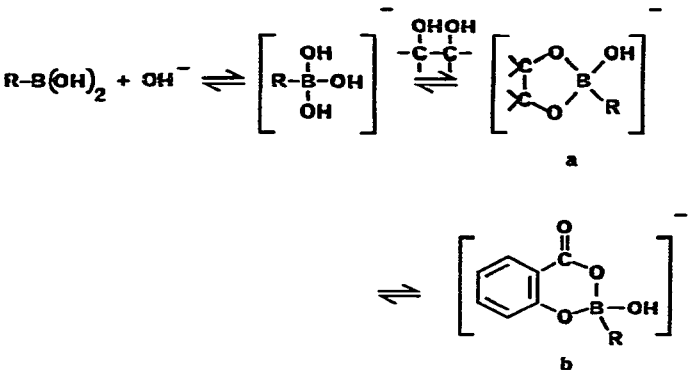

Fig. 1. Two types of boronate complex.

droxyphenyl moiety[11]. Other flavonoids not having the requisite substitution pattern for biological activity were separable only with difficulty from active material, which reduced yields upon the usual chromatography media. These interfering substances were removed easily by use of this boronate resin. We have extended the use of polyacrylamide bound boronic acid resin additionally to a wide variety of other plant phenolics and their glycosides.

EXPERIMENTAL*

Materials

m-Aminophenylboronic acid hydrochloride was prepared by catalytic hydrogenation of *m*-nitrophenylboronic acid[12] over 5% Pd/carbon in methanol containing a slight excess of HCl. The hydrochloride obtained upon filtration and evaporation of solvent *in vacuo* was used in the next step without further purification. Treatment of *m*-aminophenylboronic acid hydrochloride with succinic anhydride in pyridine gave N-(*m*-dihydroxyborylphenyl)succinamic acid, m.p. 185–186°C(H_2O), lit.[7] 173–174°C. Aminoethyl-polyacrylamide having a functional group density of *ca.* 1 mequiv./ml[13,14] was prepared from Bio-Gel P-2 (200–400 mesh) and ethylenediamine. Coupling of the aminoethyl resin with N-(*m*-dihydroxyborylphenyl)succinamic acid was carried out using the mixed anhydride derived from ethyl chloroformate similarly to the procedure described for the attachment of bromoacetamidocaproic acid to hydrazide resin[14] in buffered water–dimethylformamide solution. Excess reagents were removed by filtration, and any amino groups remaining unreacted were acetylated with acetic anhydride. After thorough washing, the boronate resin obtained was stored in 0.1 *M* sodium phosphate buffer, pH 8, containing 20% methanol.

Test compounds were obtained from commercial sources, prepared by standard synthetic methods, or isolated from natural sources[15–17].

Bio-Gel P-2 resin was obtained from Bio-Rad Labs, (Richmond, CA, U.S.A.).

* Reference to a company and/or product named by the Department is only for purposes of information and does not imply approval or recommendation of the product to the exclusion of others which may also be suitable.

Apparatus

Chromatography was carried out using a Chromatronix CMP-3 metering pump with LKB Uvicord-II® monitor at 280 nm and Ultrorac® fraction collector. Altex glass chromatographic columns (250 × 9 mm) with adjustable plungers were used for analytical procedures, and a Kontes column (250 × 25 mm) with O-ring fittings was used for preparative experiments. Nuclear magnetic resonance spectra were obtained on a Varian Associates EM-390 90-MHz spectrometer.

Standard procedures

Buffer consisting of 0.1 *M* dibasic sodium phosphate adjusted to the desired pH with phosphoric acid in water–methanol (80:20) was degassed by sonication and then passed through a boronic resin column (150 × 9 mm) until pH was constant and UV absorption was minimal. Test substances were applied in 0.5-ml volumes for analytical runs using sample loop injection of *ca.* 5 mg/ml concentrations of compound. Up to about 50 column volumes of buffer were pumped through the column at 30 ml/h to ascertain the degree of retention of test compounds. To complete the elution of strongly retained substances, the column was then purged with degassed 0.5 *M* acetic acid in water–methanol (80:20) until UV absorbance in the effluent was no longer observed. Re-equilibration of the column was then effected with starting buffer. For preparative experiments, a resin bed (250 × 25 mm) was prepared, and test solutions were added by hand. Elution rate was 120 ml/h for the larger column.

RESULTS AND DISCUSSION

Phenolic acids

Table I illustrates how the behavior of phenolic acids at pH 8 can be grouped into three categories. The non-retained substances do not possess vicinal hydroxyls. Of the acids showing an intermediate affinity for the resin, all but 3-O-ferulogylquinic acid possess a hydroxyl moiety *ortho* to the carboxyl group. Association of this grouping with arylboronate (Fig. 1b) is not unexpected since complexation of *o*-hydroxybenzoic acids with boric acid is well established[18]. Such chelation is not favored for larger ring sizes in the complex, and *o*-hydroxyphenylacetic acid (6) does not bind to the resin. It may be noted that 3-O-feruloylquinic acid (20) possesses *cis*-vicinal hydroxyls on its cyclohexane ring, giving rise to the usual complexation observed for sugars and other aliphatic polyols.

Those acids not eluted from the boronate column at pH 8, but which come off at pH 3, all have the *o*-dihydroxyphenyl substitution pattern. Apparently at the higher pH the equilibrium established favors complexation with the catechol system much more strongly than is the case for the purely aliphatic polyols or with the *o*-hydroxyaromatic acids. An exception, though, exists in the intermediate affinity of 2,3-dihydroxybenzoic acid (14) for the resin. Strong hydrogen bonding of carboxylate to the *ortho*-phenolic hydrogen accounts for the lack of complexation between the vicinal hydroxyls and boronate. If 2,3-dihydroxyphenylacetic acid (24) is substituted for 14 then unfavorable geometry prevents internal hydrogen bonding between carboxylate and *o*-phenol, and strong affinity toward the resin is again observed. It might be expected that the overall polarity of individual substances will influence binding to boronate resin giving rise to decreased affinity with increased polarity. If this effect is

TABLE I

ELUTION BEHAVIOR OF PHENOLIC ACIDS AT pH 8

Acid (trivial name)	*Elution volume* (V_e/V_0)
Non-retained	
(1) 3-Hydroxybenzoic	1.4–3.0
(2) 4-Hydroxybenzoic	2.6–4.0
(3) 3,5-Dihydroxybenzoic	1.4–3.6
(4) 4-Hydroxy-3-methoxybenzoic (vanillic)	1.2–3.4
(5) 3,5-Dimethoxy-4-hydroxybenzoic (syringic)	1.2–4.0
(6) 2-Hydroxyphenylacetic	1.2–2.6
(7) 2-Hydroxycinnamic (*o*-coumaric)	1.2–4.0
(8) 3-Hydroxycinnamic (*m*-coumaric)	1.0–3.6
(9) 4-Hydroxycinnamic (*p*-coumaric)	1.0–3.6
(10) 2,4-Dihydroxycinnamic	1.0–2.8
(11) 3-Methoxy-4-hydroxycinnamic (ferulic)	1.2–3.4
(12) 3,5-Dimethoxy-4-hydroxycinnamic (sinapic)	1.0–3.8
Retarded	
(13) 2-Hydroxybenzoic (salicylic)	7–20
(14) 2,3-Dihydroxybenzoic	11–26
(15) 2,4-Dihydroxybenzoic	13–44
(16) 2,5-Dihydroxybenzoic (gentisic)	10–23
(17) 2,6-Dihydroxybenzoic	7–18
(18) 2-Hydroxy-3-methoxybenzoic	6–17
(19) 2,4,6-Trihydroxybenzoic	12–38
(20) 3-O-Feruloylquinic	12–40
Strongly bound (released at pH 3)	
(21) 3,4-Dihydroxybenzoic	
(22) 2,3,4-Trihydroxybenzoic	
(23) 3,4,5-Trihydroxybenzoic	
(24) 2,3-Dihydroxyphenylacetic	
(25) 3,4-Dihydroxyphenylacetic	
(26) 3,4-Dihydroxycinnamic (caffeic)	
(27) 3,4-Dihydroxyphenylalanine (DOPA)	
(28) 3-O-Caffeylquinic (cholorogenic)	
(29) Caffeylglucaric	

present, it is overshadowed by the complexiметric behavior since we have not noticed a lessening of affinity even with caffeylglucaric acid (29) which does not adsorb readily to macroreticular polystyrene[16] as a consequence of its high polarity. Polarity factors are of importance for other types of boronate resins. For example other workers[9] have reported that 3,4-dihydroxyphenylalanine (DOPA) (27) was not adsorbed by a boric acid gel column and could be separated from urinary catecholamines which were strongly bound. The properties of their resin may differ from those of the acrylamide based material described herein, however, since the linkage of phenylboronate in their example is much closer to the backbone of the polymer.

Flavonoid compounds

The elution behavior of flavonoids (Fig. 2) bearing various substituents is

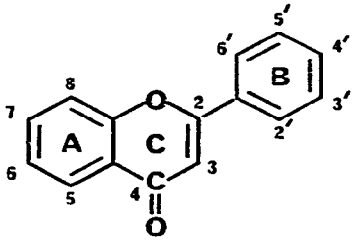

Fig. 2. The flavone system.

presented in Table II. Generally, these compounds have a non-specific affinity for polyacrylamide and therefore are eluted more slowly and with considerable tailing. For this reason, we have included methanol in our buffer systems which does not impair the use of the resin at concentrations up to 20% and which minimizes the non-specific interactions so that symmetrical peaks are obtained in reasonable elution volumes. Again, as in the case of the phenolic acids, two categories of behavior are observed: only those substances having the *o*-dihydroxyphenyl grouping are strongly

TABLE II

ELUTION BEHAVIOR OF FLAVONOIDS AT pH 8

rha = Rhamnose; glc = glucose; ketofuc = ketofucose.

Compound	*Hydroxylation*	*Other substituents*	*Elution volume* (V_e/V_0)
(30) Liquiritin	7	2,3-Dihydro 4'-O-glc	1.4–6.0
(31) Liquiritigenin	7,4'	2,3-Dihydro	2.0–6.4
(32) Naringin	5,4'	2,3-Dihydro 7-O-(rha-glc)	4.0–16
(33) Naringenin	5,7,4'	2,3-Dihydro	2.4–7.0
(34) Neohesperidin	5,3'	2,3-Dihydro 4'-OMe, 7-O-(rha-glc)	4.0–18
(35) Hesperetin	5,7,3'	2,3-Dihydro 4'-OMe	2.4–8.0
(36) 3'-O-Methylmaysin	5,7,4'	6-C-(rha-4-ketofuc) 3'-OMe	1.0–14
(37) Isovitexin	5,7,4'	6-C-glc	7.0–20
(38) Kaempferol	3,5,7,4'		6.0–16
(39) Morin	3,5,7,2',4'		32–56
Compounds not eluted at pH 8 (released at pH 3)			
(40) Astilbin	5,7,3',4'	2,3-Dihydro 3-O-rha	
(41) Dihydroquercetin	3,5,7,3',4'	2,3-Dihydro	
(42) Eriodictyol	5,7,3',4'	2,3-Dihydro	
(43) Catechin	3,5,7,3',4'	2,3-Dihydro, 4-deoxo	
(44) Iso-orientin	5,7,3',4'	6-C-glc	
(45) Maysin	5,7,4',4'	6-C-(rha-4-ketofuc)	
(46) Rutin	5,7,3',4'	3-O-(rha-glc)	
(47) Quercitrin	5,7,3',4'	3-O-rha	
(48) Myricitrin	5,7,3',4',5'	3-O-rha	

bound to the resin at pH 8. Also, lowering of the pH to 3 is sufficient to release those compounds. We noticed that those materials that did emerge from the column at pH 8 differed among themselves in elution volume depending upon substitution. These minor affinity effects may also serve to effect purification of such substances.

It was desirable to determine the relative contribution of boronate groups *per se* to this effect compared to the backbone of the polyacrylamide structure as well as the functionalities used to attach the affinity ligand. We therefore constructed an analogous polyacrylamide resin having all the structural features of the active resin, but bearing carboxylate groups instead of boronate. This synthesis was performed as described, but *m*-aminobenzoic was used instead of *m*-aminophenylboronic acid. In subsequent tests, it was found that neither the hydroxy acids nor the flavonoids were significantly retained. It appears, therefore, that not only the strong binding of *ortho*-hydroxyls but also the smaller differences in elution that are dependent on glycosylation and hydroxylation result from association with boronate groups as opposed to general adsorption to acrylamide and attached frunctionalities.

Other phenols and related compounds

Table III includes miscellaneous substances that were investigated in our studies. Among the glycosides examined in this group no particularly strong adsorptive interaction occurred except for the two dihydrochalcones (55 and 56) and for adenosine (59). The latter compound has, of course, *cis*-hydroxylation on the furanose ring; and complexation with boronate is expected. It is especially instructive to compare 2′-deoxyadenosine (58) which is unretained. It was also observed that no unusual affinity toward the resin was evidenced by either of the two *o*-hydroxy aldehydes (52 and 53) or by *o*-nitrophenol (54). This is in contrast to the intermediate binding shown by the *o*-hydroxy acids. Neither did a hydrogen bonding group *ortho*- to the catechol system in 60 or 61 influence the strong affinity of the *o*-dihydroxy-

TABLE III

ELUTION BEHAVIOR OF MISCELLANEOUS PHENOLS AND OTHER GLYCOSIDES AT pH 8

Compound	*Elution volume* (V_e/V_0)
(49) Dhurrin	1.0–5.0
(50) Amygdalin	1.6–3.0
(51) Esculin	1.2–4.6
(52) Salicylaldehyde	1.8–4.8
(53) *o*-Vanillin	1.2–6.0
(54) *o*-Nitrophenol	1.0–3.0
(55) Naringin dihydrochalcone	3.0–14
(56) Phlorizin	3.4–15
(57) 2,4-Dihydroxy-7-methoxy-1,4-benzoxazin-3-one-glucoside	1.0–4.0
(58) 2′-Deoxyadenosine	1.6–4.0
(59) Adenosine	26–62
Compounds not eluted at pH 8	
(60) 2,3-Dihydroxybenzaldehyde	
(61) 3-Nitrocatechol	

phenyl moiety for the resin. Apparently, the perturbing effect of *o*-carboxylate discussed earlier is a unique phenomenon.

Also examined was the unusual hydroxamate glucoside (57) which is considered to be a resistance factor against the European cornborer[17]. No association toward the resin was observed even though hydroxamate chelates well with other systems.

Effect of pH

Below pH 8 the hydroxy acids have lowered affinity for the resin. At pH 7, 3,4-dihydroxybenzoic acid appeared as a broad peak in the column effluent with V_e/V_0 = 16–60 (not eluted at pH 8). Flavonoids still did not come off the column at this pH. At pH 6 a slow bleed of adsorbed material took place with the flavonoids, but no real elution maximum was seen. From a practical standpoint pH 7 represents the lower limit.

Effect of glycosylation

The strong affinity of *ortho*-phenolic hydroxyls for boronate completely exceeds that of sugar or glycoside hydroxyls. Where the *o*-dihydroxyphenyl grouping is absent, then effects associated with aliphatic hydroxyls are observed. Differences in glycosylation pattern and in the sugars involved cause a number of the glycosides examined to be significantly separated from one another although it is difficult to predict relative elution values for these compounds.

Separation of a naturally occurring mixture of flavonoids

A larger (250 × 25 mm) boronate resin column was charged with a mixture of phenolics obtained from "Zapolote Chico" corn silk, a variety noted for resistance to the corn earworm. Preliminary steps consisted only of methanol extraction and adsorption of the phenols onto macroreticular polystyrene (XAD-2). Fig. 3 shows the resulting elution pattern. Note that considerable separation among components has

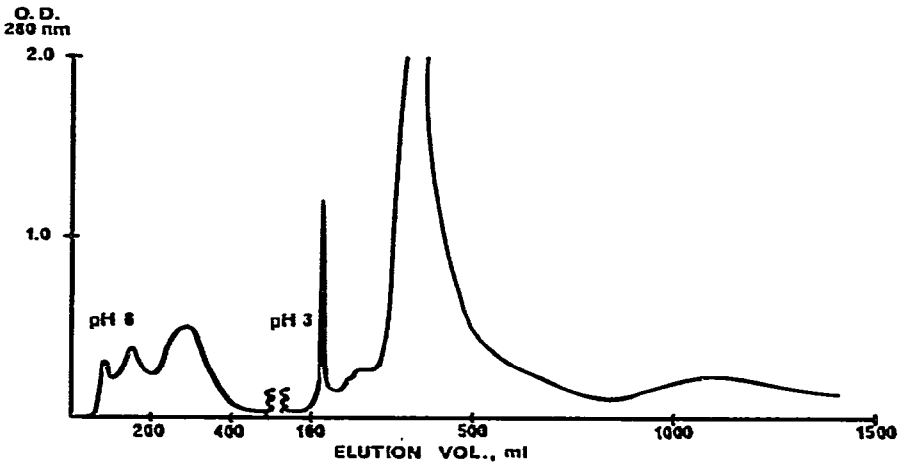

Fig. 3. Elution profile for corn silk phenolics on boronate resin.

taken place at pH 8. A total of 1500 ml of buffer was allowed to pass through the column, and flow was then switched to 0.5 *N* acetic acid containing 20% methanol. Strongly bound materials then appeared in the effluent. Examination of the early (pH 8) eluting material by NMR revealed that mainly two substances were present: 3'-O-methylmaysin (36) and the analog bearing only a 4'-hydroxyl on the B-ring. Neither of these compounds is significantly of importance in insect resistance. The major component that was eluted at pH 3 was almost 100% pure maysin (45), the substance responsible for insect resistance of the silk, and the amount represented *ca.* 1.5% of the original dry weight of the plant material. Previously[15], much more elaborate chromatographic procedures were necessary, and yields were lower primarily because of difficulty in removal of the maysin analogs which always co-occurred in the plant.

Potential application

We regard the boronate polyacrylamide resin as useful in separating a wide variety of plant phenolics. Solubility considerations dictate that mainly glycosidic or ionic compounds will be subjected to the resin; however, just these classes of substances are found to be important plant allelochemicals. Using 20% methanol as co-solvent in buffer mixtures broadens the range of solubilities and also reduces non-specific binding to the resin.

REFERENCES

1 M. B. Thomas and T. J. Mabry, *J. Org. Chem.*, 32 (1967) 3254.
2 J. W. Clark-Lewis and L. J. Porter, *Aust. J. Chem.*, 25 (1972) 1943.
3 T. J. Mabry, K. R. Markham and M. B. Thomas, *The Systematic Identification of Flavonoids*, Springer, New York, 1970, p. 18.
4 C. G. Van Teeling, P. E. Cansfield and R. A. Gallop, *J. Chromatogr. Sci.*, 9 (1971) 505.
5 K. M. Johnston, D. J. Stern and A. C. Waiss, Jr., *J. Chromatogr.*, 33 (1968) 539.
6 C. A. Elliger, B. G. Chan and W. L. Stanley, *J. Chromatogr.*, 104 (1975) 57.
7 H. L. Weith, J. L. Wiebers and P. T. Gilham, *Biochemistry*, 9 (1970) 4396.
8 H. Schott, *Angew. Chem., Int. Ed. Engl.*, 11 (1972) 824.
9 S. Higa, T. Suzuki, A. Hayashi, I. Tsuge and Y. Yamamura, *Anal. Biochem.*, 77 (1977) 18.
10 E. H. Pfadenhauer and S.-de Tong, *J. Chromatogr.*, 162 (1979) 585.
11 C. A. Elliger, B. G. Chan and A. C. Waiss, Jr., *Naturwissenschaften*, 67 (1980) 358.
12 W. Seaman and J. R. Johnson, *J. Amer. Chem. Soc.*, 53 (1931) 711.
13 J. K. Inman and H. M. Dintzis, *Biochemistry*, 8 (1969) 4074.
14 J. K. Inman, *Methods Enzymol.*, 34B (1974) 30–58.
15 C. A. Elliger, B. G. Chan, A. C. Waiss, Jr., R. E. Lundin and W. F. Haddon, *Phytochemistry*, 19 (1980) 293.
16 C. A. Elliger, R. E. Lundin and W. F. Haddon, *Phytochemistry*, 20 (1981) 1133.
17 O. Wahlroos and A. I. Virtanen, *Acta Chem. Scand.*, 13 (1959) 1906.
18 J. Boeseken, *Advan. Carbohyd. Chem.*, 4 (1949) 189–210.