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High-performance liquid chromatographic separation of oligogalacturonic acids on a cyclomaltoheptaose (β-cyclodextrin) bonded-phase column ☆

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Abstract

A cyclomaltoheptaose (β-cyclodextrin) bonded-phase HPLC column was used for the first time to separate acidic oligosaccharides. Oligogalacturonic acids up to a degree of polymerization (dp) of 7 were separated with a 50:50 acetonitrile–sodium phosphate-buffered (pH 5) mobile phase and up to dp 17 with an isocratic pH 5.0, sodium phosphate-buffered mobile phase. A sodium acetate gradient elution allowed for improved resolution of all oligogalacturonic acids, up to a dp value of at least 24. Although the stationary phase contained no cationic or readily ionizable groups, these separations appeared to be governed by a classical anion exchange-type mechanism. The β-cyclodextrin-bonded phase, which displayed exceptional stability over one year of use, is a useful alternative to silica gel- or organic polymer-based anion exchangers for HPLC of acidic carbohydrates.

Keywords: HPLC; Oligogalacturonic acids; Cyclomaltoheptaose (β-cyclodextrin); Polygalacturonic acid; Pectin

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1. Introduction

Oligogalacturonic acids [linear α-(1 → 4)-linked D-galactopyranuronosyl oligosaccharides] are formed by the partial depolymerization of polygalacturonic acid (PGA)-rich regions of plant pectins. Depolymerization catalyzed by acid or by hydrolytic enzymes results in "normal" oligogalacturonic acids, whereas that catalyzed by transeliminases, such as pectate lyase, results in "unsaturated" oligogalacturonic acids that contain 4-deoxy-β-L-*threo*-hex-4-enopyranosyluronic acid residues on the non-reducing ends. The degree of polymerization (dp) of poly- and oligo-galacturonic acids appears to control their important textural properties in fruit tissue [1], as well as their recently discovered physiological roles [2] in plants. In order to study these structure–function relationships and in order to monitor the depolymerization of pectin in model or biological systems, it is necessary to have convenient methods for separating and quantifying oligogalacturonic acids over the widest possible range of dp values. Currently, the most commonly used method for these analyses is high-performance liquid chromatography (HPLC) using ion-pair reversed-phase [3–5], anion-exchange [6–10], size-exclusion [11], or combined ion- and size-exclusion [12] modes. Of these, the one that allows separation of the widest range of oligogalacturonic acids, from dp 2 to 50, is high-performance anion-exchange chromatography combined with pulsed amperometric detection (HPAEC–PAD) [8]. While this is an extremely useful method, it requires the use of specialized equipment that is not routinely available in all laboratories.

Cyclodextrin-bonded silica gel stationary phases have recently [13–15] been used with acetonitrile–water mobile phases to separate neutral mono- and oligo-saccharides. These exceptionally durable stationary phases appear to separate sugars in a normal-phase mode [13,15], similar to those separations accomplished on polar, aminopropylsilica gels [16]. We recently reported [17] that this same β-cyclodextrin stationary phase readily separated aldonic acids from their corresponding lactones and parent neutral sugars when it was eluted with acetonitrile–phosphate buffer mobile phases. Here, we report on the use of a β-cyclodextrin stationary phase in conjunction with isocratic and gradient eluents to separate both normal and unsaturated oligogalacturonic acids. Under the conditions employed here, the β-cyclodextrin stationary phase unexpectedly functioned as a weak base anion exchanger. This is the first demonstration of an anion-exchange-type separation of acidic oligomers on this stationary phase.

2. Experimental

General.—Polygalacturonic acid, KOH, and NaOH were purchased from Sigma Chemical Company (St. Louis, MO). The β-cyclodextrin (Cyclobond I) bonded-phase column (250 mm × 4.6 mm) was purchased from Advanced Separations Technologies, Inc. (Whippany, NJ). All solvents were from Baxter Chemical Co. (Edison, NJ). Normal and unsaturated oligogalacturonic acids (see Fig. 3) were prepared as described in ref. [7].

Partial hydrolysis of polygalacturonic acid.—Polygalacturonic acid was partially hydrolyzed according to published procedures [8,18] by autoclaving a 1% polygalactur-

onic solution (in water, adjusted to pH 4.4 with NaOH) at 121°C for 40 min. The solution was then cooled, and any solid material that remained was removed by centrifugation (10,000 *g*, 10 min). The supernatant was collected, and the pH adjusted to 2 with dilute HCl. The resulting precipitate was removed by centrifugation as before, and the supernatant (pH 2-soluble fraction) was collected. The pH of this fraction was raised to 7 with dilute NaOH and lyophilized. Alternatively, prior to lyophilization, an equal volume of 22% ethanol (11% final concentration) was added to the pH 2-soluble fraction (pH 7), and this solution was refrigerated for 24 h. The ethanol precipitate of the pH 2-soluble fraction was collected by centrifugation, and the supernatant was discarded. This precipitate was solubilized in water prior to lyophilization.

Chromatography.—The HPLC system used was a Gilson Model 303 dual pump system equipped with a Model 811 Dynamic Mixer and a Rheodyne 7125 fixed-loop (20 μL) injector. Samples were detected with a Waters Model R-403 Differential Refractometer, and the data were recorded with a Rainin Dynamax Method Manager. A Dionex 4000i system, which included a quaternary gradient pump, eluant degas (He) module, postcolumn delivery system (pressurized reservoir) and pulsed amperometric detector (PAD II, with gold working electrode), was used, and the chromatograms were recorded with a Hewlett–Packard 3390A integrator. For optimal detector sensitivity, 500 mM potassium hydroxide was added postcolumn using a mixing tee. The triple-pulse sequence used for amperometric detection included the following potentials and durations: $E_1 = 0.15$ V ($t_1 = 480$ ms), $E_2 = 0.7$ V ($t_2 = 120$ ms) and $E_3 = -0.6$ V ($t_3 = 360$ ms). The integration time was set at 200 ms, and the response time at 1 s.

3. Results and discussion

Oligogalacturonic acids have recently been separated by anion-exchange HPLC using either aminopropylsilica gel [6,7] or organic polymer-based anion exchange resins [8–10]. In both of these systems, chromatographic separation is affected by the interaction between the analyte's negatively charged carboxylate groups and the station-

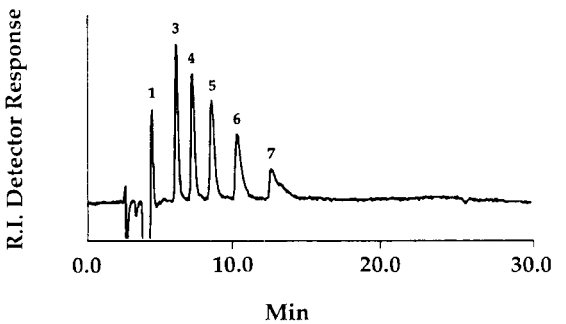

Fig. 1. The separation of oligogalacturonic acid standards on a Cyclobond I column using a 50:50 acetonitrile–sodium phosphate buffer (100 mM, pH 5.0) mobile phase at room temperature and at 0.75 mL/min using refractive index detection. The number above the peaks represent the dp value.

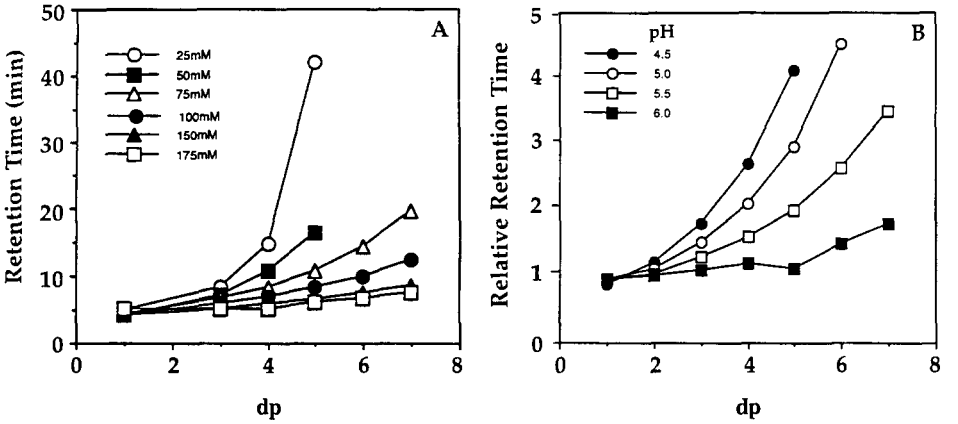

Fig. 2. Graphs of the effect of (A) buffer concentration and (B) pH on the retention times of oligogalacturonic acids on a Cyclobond I column. See Fig. 1 for chromatographic conditions.

ary phase's positively charged ammonium functionalities. The Cyclobond I column is packed with silica gel, modified with a β-cyclodextrin-bonded phase. No amine functionality [13] or other cationic group is present on this phase. Despite this fact, we recently reported [17] that this phase functioned like an anion exchanger: when eluted with acetonitrile–water mobile phase, aldonic acids were very tightly bound. When the aqueous portion of the mobile phase was buffered to pH 5 with sodium dihydrogen phosphate, the sugar acids were eluted from the column. In fact, as expected for anion-exchange separations, retention times of the sugar acids were affected both by pH and ionic strength.

We have now found that these unusual properties of the Cyclobond I column can be used for performing separations of acidic oligosaccharides. Oligogalacturonic acid standards [7], up to dp 7, were readily separated on the Cyclobond I column with a mobile phase of 50:50 acetonitrile–sodium phosphate buffer (100 mM, pH 5.0) (Fig. 1). As shown earlier for aldonic acids [17], oligogalacturonic acid separations were affected both by ionic strength and mobile-phase pH. As the concentration of the buffer increased, the retention time of the oligogalacturonic acids decreased (Fig. 2A). An increase in mobile-phase pH also caused a decrease in retention times (Fig. 2B). While the conditions shown in Fig. 1 were appropriate for separation of dp 2–7 oligogalacturonic acids, higher oligomers could not be separated, due to their insolubility in the (acetonitrile-containing) mobile phase. Hotchkiss et al. [7], however, were able to separate oligogalacturonic acids up to dp 12 on aminopropylsilica gel columns using a purely aqueous (no organic modifier), phosphate-buffered mobile phase. Using this approach, the β-cyclodextrin bonded-phase column separated oligogalacturonic acids up to dp 10 when a 100 mM, pH 5.0 sodium phosphate buffer was used as a mobile phase (Fig. 3A). This separation was also affected by ionic strength. Increasing the buffer concentration from 100 to 150 mM caused all oligomers to elute earlier (compare Figs. 3A to 3B and 3C), permitting analyses of oligomers up to dp 17. The selectivity of this phase also allowed the differentiation of unsaturated versus normal oligogalacturonic

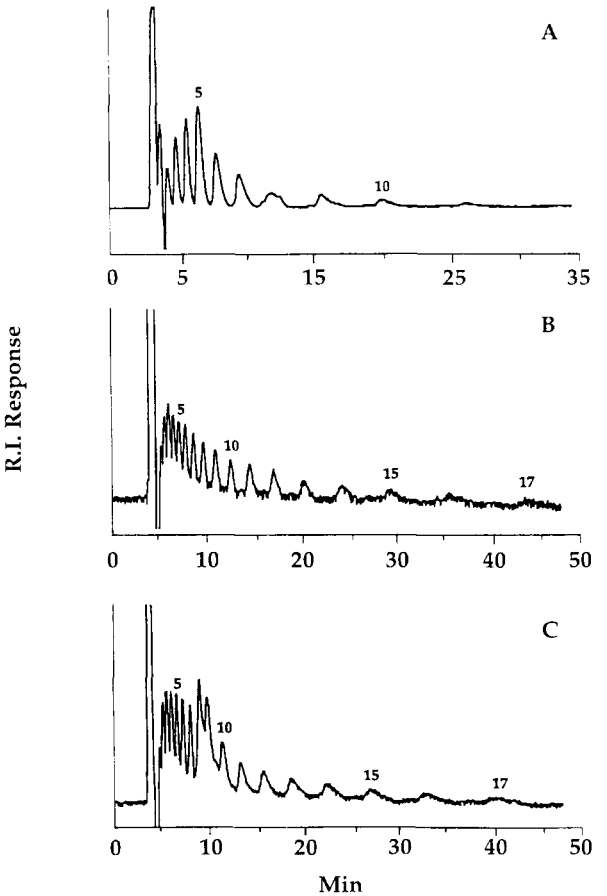

Fig. 3. Isocratic HPLC separations of (A) normal oligogalacturonic acids, (B) unsaturated oligogalacturonic acids, and (C) partial hydrolysis of polygalacturonic acid produced by autoclaving a polygalacturonic acid sample as described in the Experimental section. Conditions: Cyclobond I column eluted at room temperature and at 0.75 mL/min. Monitored with RI detection. Mobile phase was pH 5, sodium phosphate buffer at (A) 100 mM and (B and C) 150 mM.

acids. Unsaturated oligogalacturonic acids (Fig. 3B) consistently eluted later than the analogous normal oligogalacturonic acid (Fig. 3C). This elution order was also seen when the same compounds were chromatographed on strong base HPAEC systems [9,10]. Retention times of oligomers were, as before, also dependent on the pH (Fig. 4) of the mobile phase.

In order to extend separations to even higher dp values, the Cyclobond I column was eluted with the sodium acetate gradient shown in Table 1. Since the gradient elution prevented use of an RI detector, a PAD was used. The samples used in these analyses were obtained as described in the Experimental section. Two fractions were obtained: a pH 2-soluble fraction that was relatively enriched in oligomers dp < 20 and a pH 2-soluble, ethanol-precipitated fraction relatively enriched in oligomers dp 10–20. When

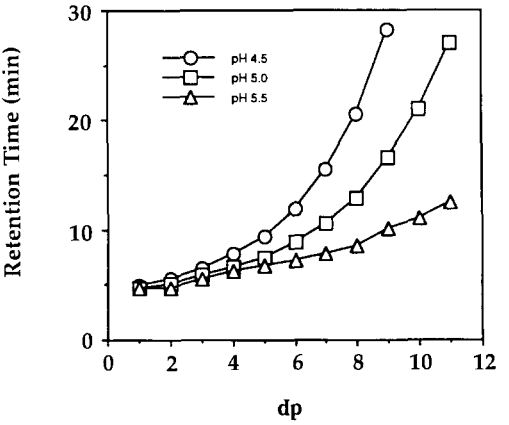

Fig. 4. Effect of buffer pH on the retention times of oligogalacturonic acids. See Fig. 3B and C for chromatographic conditions.

the former sample was analyzed, oligosaccharides up to dp 21 were resolved using the sodium acetate gradient elution (Fig. 5A). Analysis of the pH 2-soluble, ethanol-precipitated fraction revealed, as expected, greater amounts of oligomers above dp 10, and peaks corresponding to at least dp 24 were evident. Using conditions that reflect its relatively low ion-exchange capacity, chromatographic results obtained were almost as good as those we reported earlier [8] for separation of these same oligomers on a CarboPac PA-1, commercial, high performance anion-exchange chromatography stationary phase.

It is well known that cyclodextrin-bonded phases can be utilized in normal and reversed-phase modes. In addition, the ability of the bound cyclodextrin to form inclusion complexes with small solutes results in exceptional selectivity in the HPLC of diasteromeric and enantiomeric isomers. Our earlier results [17], and those presented here, now establish yet another useful chromatographic mode for cyclodextrin-bonded

Table 1
Conditions used for gradient separation of oligogalacturonic acids on Cyclobond I

Time (min)	% A [a]	% B [b]
0–1	5	95
8	9	91
15	10	90
25	11	89
35	11	89
45	13	87
55	14	86
65	15	85
70	20	80

[a] A = M CH_3COONa, pH 5.
[b] B = H_2O.

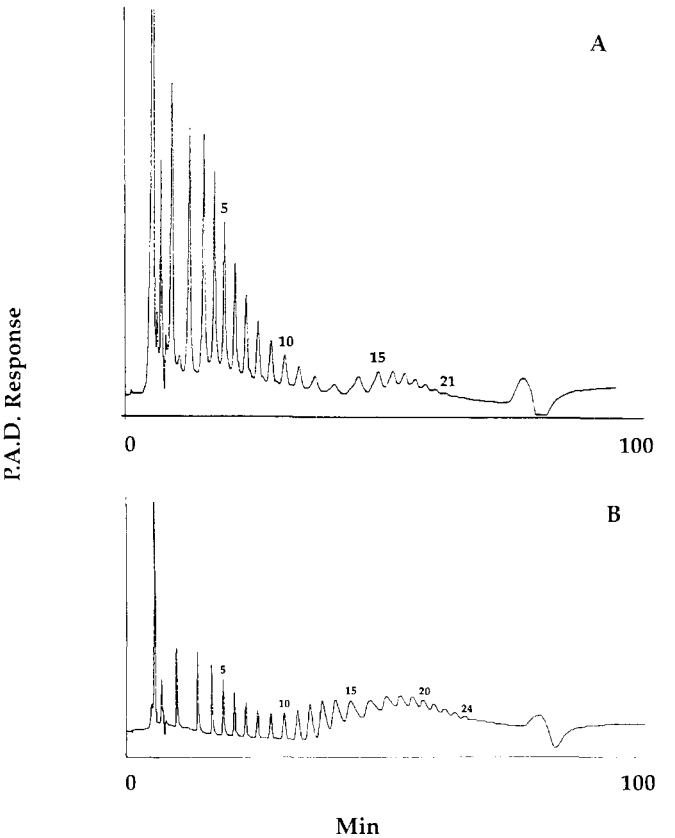

Fig. 5. Gradient separation of oligogalacturonic acids on Cyclobond I column using gradient described in Table 1. Flow rate was 0.75 mL/min. Sample monitored by PAD. (A) pH 2.0-soluble oligogalacturonic acids, (B) pH 2.0-soluble, ethanol-precipitated oligogalacturonic acids. See Experimental section for sample preparation and PAD pulse sequence.

phases: anion exchange. Although this is the first report of anion exchange-type separations on a cyclodextrin-bonded phase in conjunction with a fully aqueous (buffered) mobile phase, there have been reports of ion exchange-type behavior in other applications. Beeson and Vigh [19] separated 2-arylpropionic acid (profen) enantiomers on cyclodextrin-bonded phases eluted with acetonitrile–citrate buffer. In that reversed-phase system the authors noted that the pH of the buffer had a great effect on separations and that anionic forms of analytes bound more strongly to the stationary phase than did their free acid forms. Similarly, Wilder et al. [20] observed a similar effect while separating sulfonated aromatics on a cyclodextrin-bonded phase eluted with methanol–ammonium acetate buffer. Recognizing that the cyclodextrin stationary phase contained no ionizable functionalities, Wilder et al. [20] postulated that a cationic component from the mobile phase, such as ammonium ion, might have become included in the cyclodextrin cavity, forming a dynamic anion-exchange site. The fact that cyclic oligosaccharides of several

types have now been reported [21–24] to form inclusion complexes with cations such as Cu^{2+}, Na^{+}, K^{+}, and Ba^{2+}, adds additional support for this hypothesis.

Although this work primarily deals with separation phenomena, a few items regarding quantification of separated analytes should also be noted. Low dp oligogalacturonic acids separated on the Cyclobond I column can be readily quantified when the refractive index detector is used, since homo-oligosaccharides with values ≤ 8–10 are known [25] to give reasonably equivalent chromatographic peak areas per mg of oligosaccharide injected. Hence, one can usually quantify the amount of each oligosaccharide in a mixture by using the detector response factor calculated from injection of only one pure dp standard of that series. Quantification of eluted oligogalacturonic acids by PAD is more problematic, however. Oligogalacturonic acids with different dp values do not give the same molar or weight concentration PAD response factors [8]. Therefore, PAD is only quantitative if exact standards for the analytes of interest are available. Despite this limitation, the ability of PAD to monitor gradient separations and to profile oligogalacturonic acid mixtures from dp 2 to at least 50 [8], makes it a useful detection system for these acidic oligosaccharides.

Unlike aminopropylsilica gel columns used extensively in our laboratory for anion-exchange separations, the β-cyclodextrin-bonded stationary phase column showed little decrease in capacity or efficiency during extended (one year) use. This enhanced column stability, which has been reported previously [13–15,17], along with its ability to function in multiple chromatographic modes, makes this phase quite useful for these and other separations.

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