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PREPARATIVE CHROMATOGRAPHY OF OLIGOGALACTURONIC ACIDS

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SUMMARY

A mixture of oligogalacturonic acids was prepared by partial enzymatic hydrolysis of citrus pectin-derived α-D-polygalacturonic acid. Both a commercial fungal "pectinase" preparation and a purified *endo*-polygalacturonase isolated from this source by chromatography on carboxymethyl Sephadex C-50 were used to catalyze the hydrolysis. Different product distributions resulted, but even when using pectinase there was no evidence for the formation of unsaturated products from activity of polygalacturonic acid lyase. Individual oligogalacturonic acids were isolated by step-gradient elution (sodium formate, pH 4.7) from the macroporous strong base anion-exchange resin AG MP-1 in the formate form. Pure oligomers to heptagalacturonic acid were isolated in a single run, including gram quantities of tri-, tetra-, and pentagalacturonic acid. The individual oligogalacturonic acids were characterized by fast-atom bombardment mass spectrometry in positive and negative modes.

INTRODUCTION

The dominant structural feature of pectin in the cell walls of higher plants is a linear 1→4-α-linked D-galactopyranuronic acid chain (Fig. 1). In pectin, the carboxyl groups are esterified to various degrees with methoxyl groups. Fundamental studies in both plant biochemistry and pathology have required procedures for the production and isolation of pure oligogalacturonic acids from pectin. The availability of sufficient quantities of such standards with known degrees of polymerization (DP) allows examination of substrate specificities and kinetics of *exo*- and *endo*-polygalacturonases[1], and polygalacturonic acid lyases from various sources[2,3]. Also, it is known that oligogalacturonic acids which result from the action of such enzymes on plant cell walls elicit defense responses by the host plant against certain microorganisms[4–6]. Often the response is elicited by fragments with discrete DP values, so preparative methods for these compounds to use in bioassays are required.

Both polystyrene and Sephadex based strong anion-exchange packings have been used for the separation of oligogalacturonic acids. The polystyrene based resins have higher loading capacity and are especially useful for separation of lower DP oligomers. Dowex 1-X8 in the formate form was applied to the separation of DP-1

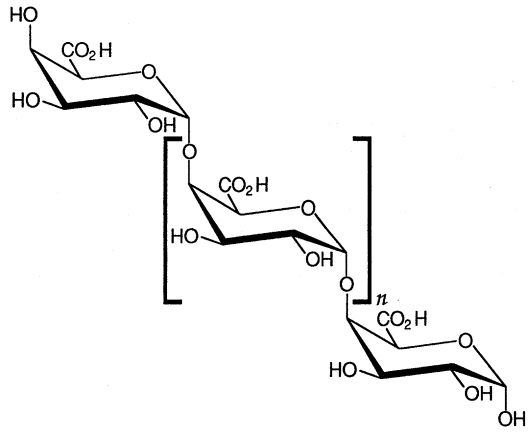

Fig. 1. Polygalactopyranuronic acid structure.

through DP-8, but re-chromatography of pooled fractions was required to obtain pure compounds[7]. Similar results were achieved using AG 1-X8, a refined form of Dowex 1-X8 (ref. 8). Separation on both resins utilized step gradient elutions with sodium formate (pH 4.7). Large elution volumes were used, and the gravity flow-rates were quite slow, so separations required several days. For baseline resolution of small quantities of oligogalacturonic acids to about DP 10, and some resolution of even higher oligomers, chromatography on DEAE-Sephadex A-25 is effective[5], but also very time-consuming. QAE-Sephadex has also been used to isolate milligram quantities of higher oligomers[9].

In this report, we describe the first application of the macroporous strong base anion-exchange resin AG MP-1 in the formate form to the isolation of oligogalacturonic acids to DP-7. To our knowledge, this resin has not previously applied to carbohydrate separations, but its versatility has been exemplified in separations of nucleotides[10] and proteins[11,12]. Preparative-scale separations of oligogalacturonic acids on AG MP-1 are more rapid, selective, and efficient than on other resins.

EXPERIMENTAL

Resin conversion

AG MP-1 anion-exchange resin (Cl^-, 200–400 mesh) was purchased from Bio-Rad Labs. (Richmond, CA, U.S.A.) and converted to the formate form as follows. The hydroxide form was generated by stirring the resin (500 ml) in 1.0 *M* sodium hydroxide (12 l) for 3 h. After settling the resin and decanting the alkali, it was rinsed with water (3 l) in a column mode, until the pH of the eluent was about 9. Then 1.0 *M* formic acid (2 l) was passed through the column, followed by water (2.4 l). The resin was removed to a beaker and twice stirred and decanted from water (2.0 l), under which it was stored. These last washings removed excess formic acid and resin fines, which affect chromatographic performance of the resin.

Monitoring column fractions

Oligogalacturonic acids were qualitatively determined by high-performance thin-layer chromatography (HPTLC)[13] on silica gel (4.5 μm particle size, 200 μm layer) HPTLC plates (10 × 10 cm), type HP-K (Whatman, Clifton, NJ, U.S.A.). Elution of the plates at 35°C with ethanol–25 m*M* acetic acid (42:58) allowed effective resolution of products to DP-9. Spots were detected by spraying plates with a solution[14] of aniline (4 ml), diphenylamine hydrochloride (4.0 g), *tert*.-butyl alcohol (120 ml), and ethanol (80 ml), and placing them on a hot plate for a few seconds. For quantitation of oligogalacturonic acids in column fractions, the colorimetric method based on reaction with *m*-hydroxybiphenyl[15] was used.

Isolation of endo-polygalacturonase from "pectinase"

The crude pectinase (50 ml, *Aspergillus niger* source, solution in 40% aqueous glycerol, Sigma, St. Louis, MO, U.S.A.) was dialyzed for 2 h against water, changing to new dialysis tubing each 20 min due to cellulase breakdown of the tubing. The pectinase was then dialyzed against 20 m*M* sodium acetate buffer (pH 4.4) for 2 h at 4°C, again with frequent changes of the dialysis tubing. The retentate (about 400 ml) was passed through a column of 300 ml carboxymethyl Sephadex C-50 (sodium exchanged), and eluted with 1 l acetate buffer (20 m*M*, pH 4.4). Proteins were fractionated by eluting with a linear gradient (1.2 l) of 0 to 0.3 *M* sodium chloride in 20 m*M* acetate buffer (pH 4.4), monitoring for protein at 280 nm. Three activities were separated, an *exo*-polygalacturonase followed by two *endo*-polygalacturonases. The final peak (*endo*-2, elution volume from 600 to 850 ml) possessed a specific activity of 1219 units/mg protein, while pectinase possesed an activity of just 9.1 units/mg protein. One unit liberates 1.0 μM reducing carbohydrate from polygalacturonic acid per min at pH 4.0 at 25°C. A total of 5320 ± 150 units *endo*-2 was isolated and was used for subsequent polygalacturonic acid (PGA) hydrolyses. Enzyme activity was determined by the 2-cyanoacetamide spectrophotometric assay for monitoring formation of reducing end groups from PGA[16].

Partial hydrolysis of PGA with endo-polygalacturonase

A solution of PGA (21.0 g, citrus origin, Sigma) in water (2.0 l) was adjusted to pH 4.4 with sodium hydroxide. Sodium chloride (11.7 g) was added to a concentration of 0.1 *M*. Some precipitated PGA was re-dissolved by heating to 30°C and readjusting the pH to 4.4. Purified *endo*-2 (300 units) was added, and the solution was maintained at 30°C and shaken at 150 rpm for 4 h. After adjusting to pH 7.0 with sodium hydroxide, barium acetate (13.8 g) was stirred into the solution, which was then cooled to 10°C. The insoluble precipitate which formed was removed by vacuum filtration through Celite. The oligogalacturonic acid mixture was then precipitated by stirring an equal volume of acetone into the filtrate. The precipitate was collected by centrifugation at 4000 *g*, and stirred into water (400 ml). Barium salts of the oligogalacturonic acids were converted to the free acid form by stirring for 3 h with 75 ml of Amberlite IR-120 (H^+) ion-exchange resin (Aldrich, Milwaukee, WI, U.S.A.). The resin was removed by filtration and washed with an additional 150 ml water. Combined filtrates were lyophilized to yield the oligogalacturonic acid mixture (16.6 g, 79% yield).

Chromatographic separation of endo-polygalacturonase (endo-2) generated oligogalacturonic acids on AG MP-1 ($HCOO^-$)

AG MP-1 ($HCOO^-$) resin (520 ml) was slurried with water and packed into a glass column (60.0 × 3.8 cm I.D.) after inserting a glass wool plug into the column end. An aqueous solution of the oligogalacturonic acid mixture (7.0 g/350 ml), produced by hydrolysis of PGA by endo-2, was passed through the column. The resin was eluted with water (350 ml), followed by a step-gradient of sodium formate, pH 4.7, consisting of the following: 0.33 *M*, 200 ml; 0.36 *M*, 400 ml; 0.39 *M*, 1 l; 0.42 *M*, 1 l; 0.45 *M*, 800 ml; 0.55 *M*, 1 l; 0.65 *M*, 800 ml; and 0.75 *M*, 500 ml. The gravity flow-rate was 2.4 ml/min and fractions of 19 ml were collected. Each third tube was monitored both by HPTLC[13] (described above) and colorimetry[15], but either procedure allowed the pooling of tubes containing pure oligomers of like DP. Tubes were pooled as follows: DP-2 (51–71), DP-3 (75–84), DP-4 (96–198), DP-5 (213–270), and DP-6 (283–288). Galacturonic acid-containing tubes (28–36) were discarded.

The quantity of product in each fraction was determined from the colorimetric assay or estimated by HPTLC. A 50% molar excess of barium acetate was stirred into each pooled fraction, and the individual oligogalacturonic acids were precipitated as their barium salts by addition of acetone (3 volumes to DP-2 fraction, 2 volumes to DP-3 and DP-4, and 1 volume to DP-5 and DP-6). The precipitates were collected by centrifugation at 4000 *g*, and stirred into water (100 ml). Amberlite IR-120 (H^+) was then added, from 10 to 40 ml, in proportion to the quantity of barium acetate that had been added to each fraction. After stirring for 1 h, the resin was removed by filtration and washed with another 50 ml water. Filtrates were combined and lyophilized, yielding the oligogalacturonic acids.

Partial hydrolysis of PGA with pectinase

PGA (20.0 g) was stirred into 0.1 *M* sodium bicarbonate (800 ml) over the course of 1 h, during which time the pH of the solution dropped from 8.21 to 4.28. Pectinase (3.2 ml, 370 units) was added, and after stirring for 3 min, the reaction was quenched by heating to 80°C in a water bath. After cooling to about 50°C, the solution was clarified by filtration through celite. Oligogalacturonic acids were precipitated by addition of 95% aqueous ethanol (2 volumes), and collected by centrifugation at 4000 *g*. The centrifugate was twice washed with absolute ethanol, re-centrifuged, and dried *in vacuo* at 40°C to yield the oligogalacturonic acid mixture (17.1 g, 85.5%).

Chromatographic separation of pectinase generated oligogalacturonic acids on AG MP-1 ($HCOO^-$)

AG MP-1 ($HCOO^-$) resin (133 ml) was packed as a slurry with water into a glass column (40 × 2.2 cm). A water (100 ml) solution of the oligogalacturonic acid mixture (2.0 g) produced by partial pectinase hydrolysis of PGA was passed through the column. The resin was eluted with water (100 ml), followed by a step-gradient of sodium formate (pH 4.7), consisting of the following: 0.30 *M*, 100 ml; 0.37 *M*, 100 ml; 0.44 *M*, 150 ml; 0.50 *M*, 200 ml; 0.55 *M*, 200 ml; 0.60 *M*, 250 ml; and 0.65 *M*, 250 ml. The eluent was delivered with a high-performance liquid chromatography pump (Perkin-Elmer Series 3, Norwalk, CT, U.S.A.) at a flow-rate of 2.4 ml/min, and fractions of 6 ml were collected. Each tube was monitored by colorimetry[15] and each fourth tube by HPTLC[13]. Either procedure allows appropriate pooling of fractions.

Tubes were pooled as follows: DP-2 (37–50); DP-3 (64–72); DP-4 (84–100); DP-5 (112–136); DP-6 (140–166); and DP-7 (188–202). Galacturonic acid-containing tubes (16–31) were discarded. Pure oligomers were isolated after precipitation of their barium salts from acetone and converted to the acid forms as described above for separation of endo-polygalacturonase generated oligogalacturonic acids.

Separation of sugar acids on AG MP-1 ($HCOO^-$)

Galactonic acid, galacturonic acid, and glucuronic acid (40 mg each) in 2.0 ml water were applied to a column of 20 ml AG MP-1 ($HCOO^-$), packed as a slurry with water. The column was eluted at 2.9 ml/min with a formic acid step-gradient consisting of the following: 0.02 *M*, 50 ml; 0.05 *M*, 50 ml; 0.07, 100 ml; and 0.10 *M*, 50 ml. Column fractions (5.0 ml) were monitored for composition by HPTLC at 35°C as described above, except that ethanol–25 m*M* acetic acid (65:35) was used as irrigant. Galactonic acid, galacturonic acid, and glucuronic acid resided in fractions 21–23, 27–31, and 37–45, respectively.

Mass spectrometric analysis of oligogalacturonic acids

Fast-atom bombardment mass spectra were recorded at the Center for Advanced Food Technology, Rutgers University, New Brunswick, NJ, U.S.A. using a VG-Analytical 7070E mass spectrometer in the negative ($[M-H]^-$) and positive ($[M+Na]^+$) ionization modes. For analysis in the negative mode, samples were slurried in a small amount of aqueous methanol, then dissolved in dithiothreitol–dithioerythritol (3:1). Signals observed in the positive mode result from sodium attachment, possibly from ppm sodium contamination in the sample.

RESULTS AND DISCUSSION

Oligogalacturonic acid mixtures to be resolved by chromatography on AG MP-1 ($HCOO^-$) were generated by incubation of polygalacturonic acid (PGA) with commercial fungal pectinase and with *endo*-polygalacturonase (*endo*-PGA_{ase}) isolated from this crude preparation. The PGA substrate was earlier determined to have a number-average degree of polymerization ($\overline{DP}_n$) of about 35 (ref. 17). The plot of incubation time *versus* $\overline{DP}_n$ is shown in Fig. 2, which is based upon results of kinetic studies with PGA and purified oligogalacturonic acids of DP-3 through DP-7. The pectinase enzyme is useful for the rapid preparation of pure oligogalacturonic acids (DP 2–7) in lower yields.

Isolation from pectinase of two fractions with *endo*-PGA_{ase} activity on carboxymethyl Sephadex C-50 is shown in Fig. 3. The final *endo*-2 peak possessed a specific activity of 1219 units/mg protein, representing a 134-fold purification over *endo*-activity in the crude pectinase. The earlier eluting *endo*-1 peak possessed a much lower specific activity (Fig. 2). The HPTLC profiles with time of the *endo*-2 catalyzed hydrolysis of PGA are shown in Fig. 4 (lanes a–e), along with the final profile of the 3-min pectinase reaction (lane f) and standards of DP-1 through DP-7 (lanes g–m). Note the presence of high DP oligomers at the origin in lane f for the pectinase-catalyzed hydrolysis (oligomers of DP greater than 9 have no mobility in this HPTLC system), along with a significant level of DP-1 due to the presence of *exo*-PGA_{ase}.

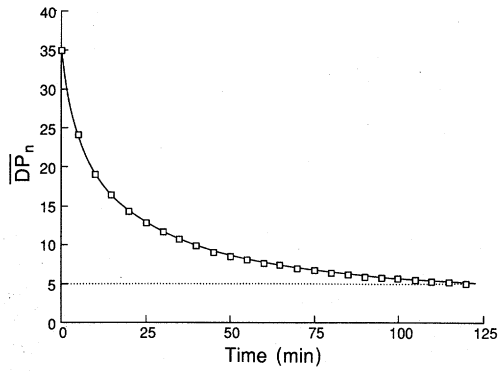

Fig. 2. Plot of the estimated number-average degree of polymerization ($\overline{DP}_n$) of polygalacturonic acid hydrolysis products *versus* incubation time with an *endo*-polygalacturonase (14 μmole min^{-1} per g of polygalacturonic acid). This estimation is based upon the observation that there is a 2% decrease in relative enzyme activity, $\frac{\partial R}{\partial t}$, for each unit decrease in substrate DP. The calculation was performed as follows:

$$(\overline{DP}_n)_i = \frac{M}{\left|(R)_{i-2} + \int_{t_{i-2}}^{t_{i-1}} \left(\frac{\partial R}{\partial t}\right)_{i-1} dt\right| N}$$

where M is the mass of the starting polymer solute, N is the molecular weight of the galacturonic acid monomer and $(R)_i$ is the number of moles of reducing end groups at various times, t_i.

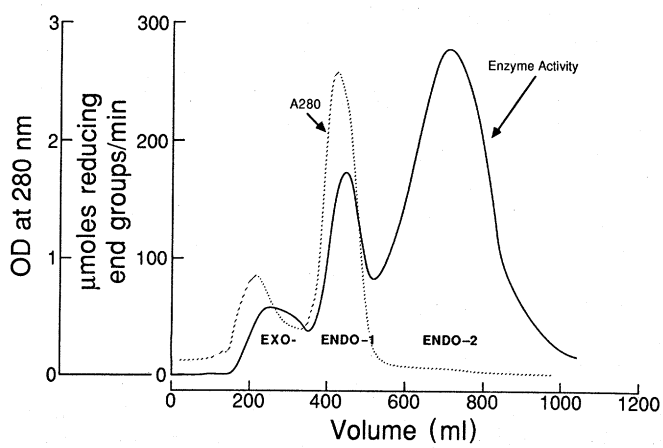

Fig. 3. Chromatographic profile of enzymes in crude pectinase on a carboxymethyl Sephadex C-50 column. Mobile phase: linear gradient of 0–0.3 M sodium chloride in 20 mM sodium acetate buffer, pH 4.4.

Fig. 4. HPTLC on silica gel. Lanes a–e, time course of oligogalacturonic acid formation resulting from partial hydrolysis of polygalacturonic acid by purified *endo*-polygalacturonase (0.5, 1, 2, 3 and 4 h, respectively); lane f, oligogalacturonic acid profile resulting from partial hydrolysis of polygalacturonic acid by pectinase; lanes g–m, galacturonic acid, and pure oligogalacturonic acids DP-2 through DP-7, respectively.

Incubation of PGA with *endo*-2 resulted in increasing proportions of oligomers from DP-3 through DP-5 with time (lanes a–e), no high DP oligomers after 1 h, and minimal DP-1. Since oligogalacturonic acid profiles in mixtures generated by pectinase and *endo*-2 catalyzed hydrolysis of PGA were quite different, optimal step-gradient programs for separation of oligomers on AG MP-1 ($HCOO^-$) resin also varied.

The resolution of DP-1 through DP-6 oligomers, produced by action of endo-2 on PGA, is shown in Fig. 5. From the 7.0 g applied to the column, a total of 5.672 g (81.0% total yield of oligogalacturonic acids DP-2 through DP-6) was obtained. Gram quantities of pure DP-3 through DP-5 were obtained, more rapidly than previously described[7,8], and without the need for re-chromatography in order to obtain pure oligomers. The only column fractions that contained more than one oligomer were tubes 85–95, apparent from HPTLC analysis. These tubes were discarded and appropriate tubes of like DP were combined and processed to yield the pure oligogalacturonic acids.

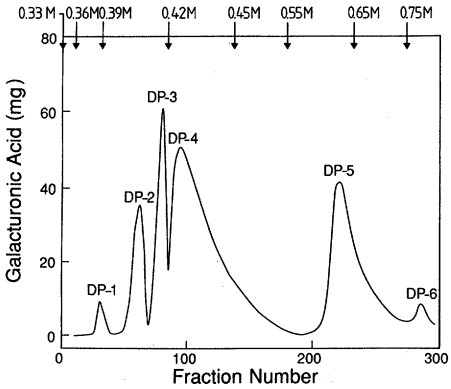

Fig. 5. Separation on AG MP-1 ($HCOO^-$) of oligogalacturonic acids (7.0 g, DP-1 through DP-6) resulting from partial hydrolysis by purified *endo*-polygalacturonase (*endo*-2) of polygalacturonic acid. Chromatographic conditions: mobile phase, sodium formate (pH 4.7); flow-rate, 2.4 ml/min; fraction volume, 19 ml; detection, *m*-hydroxybiphenyl assay[15].

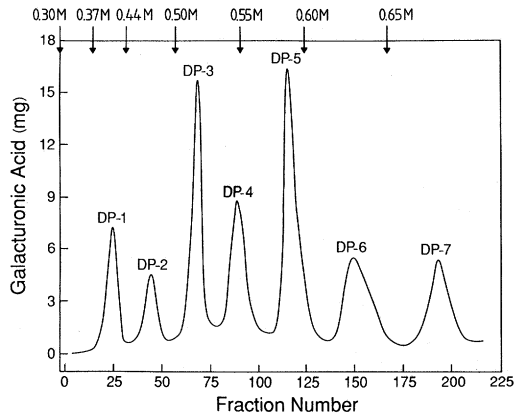

Fig. 6. Separation on AG MP-1 ($HCOO^-$) of oligogalacturonic acids (2.0 g, DP-1 through DP-7) resulting from partial hydrolysis by pectinase of polygalacturonic acid. Chromatographic conditions: mobile phase, sodium formate (pH 4.7); flow-rate, 2.4 ml/min; fraction volume, 6 ml; detection, *m*-hydroxybiphenyl assay[15].

Chromatography of the oligogalacturonic acid mixture (2.0 g) produced by pectinase catalyzed hydrolysis is shown in Fig. 6. The sodium formate (pH 4.7) step-gradient used above for the *endo*-2 produced mixture was modified, since the poduct profile (Fig. 4) as well as the sample and column size had changed. Pure oligomers through DP-7 were obtained, and the separation required less than 9 h. A total of 0.839 g (42% total yield of oligogalacturonic acids DP-2 through DP-7) was obtained. So nearly double the yield of useful products was obtained using purified *endo*-2. This was because significant levels of both galacturonic acid (DP-1) from *exo*-PGA_{ase} activity and higher-DP oligomers result from partial hydrolysis of PGA with pectinase. Yields are summarized in Table I.

TABLE I

OLIGOGALACTURONIC ACIDS ISOLATED AFTER PARTIAL HYDROLYSIS OF POLYGALACTURONIC ACID WITH *endo*-POLYGALACTURONASE AND PECTINASE

Product	*endo-Polygalacturonase**		*Pectinase***	
	Yield (g)	*Yield (%)*	*Yield (g)*	*Yield (%)*
Digalacturonic acid	0.480	6.8	0.031	1.5
Trigalacturonic acid	1.500	21.4	0.114	5.7
Tetragalacturonic acid	2.561	36.6	0.155	7.7
Pentagalacturonic acid	1.080	15.4	0.237	11.8
Hexagalacturonic acid	0.051	0.7	0.200	10.0
Heptagalacturonic acid	–	–	0.102	5.1
Total	5.672	81.0	0.839	41.9

* 7.0 g polygalacturonic acid substrate.
** 2.0 g polygalacturonic acid substrate.

Separations as efficient as those above can be achieved at flow-rates higher than 2.4 ml/min, provided that proportions of column input sample to column bed volume are reduced to 1 g/100 ml. For example, by applying a column head pressure of 2.5 p.s.i. nitrogen, flow-rates were increased to 4.0 ml/min, and a 1.0-g sample (produced by pectinase) yielded pure oligogalacturonic acids in less than 5 h. Yields from DP-2 through DP-7 were 10, 60, 80, 130, 90 and 100 mg, respectively. Product mixtures contained high levels of galacturonic acid (resulting from *exo*-PGA_{ase} activity), much of which remained in the supernatant when desired mixtures were precipitated by addition of 2 volumes of ethanol.

For separations of oligogalacturonic acids on strong base anion-exchange resins such as AG MP-1, formate would appear to be the most suitable resin counter ion and column eluent. In an earlier attempt to separate oligogalacturonic acids on Dowex 1-X8, the acetate form rather than formate form of the resin was used along with acetate as eluent[18]. The resin displayed a lower relative selectivity for acetate than for formate and higher oligogalacturonic acids were strongly retained on the column. Also, organic acid counter ions and eluents are preferable to inorganic anions such as orthophosphate because their barium salts are highly soluble in acetone–water

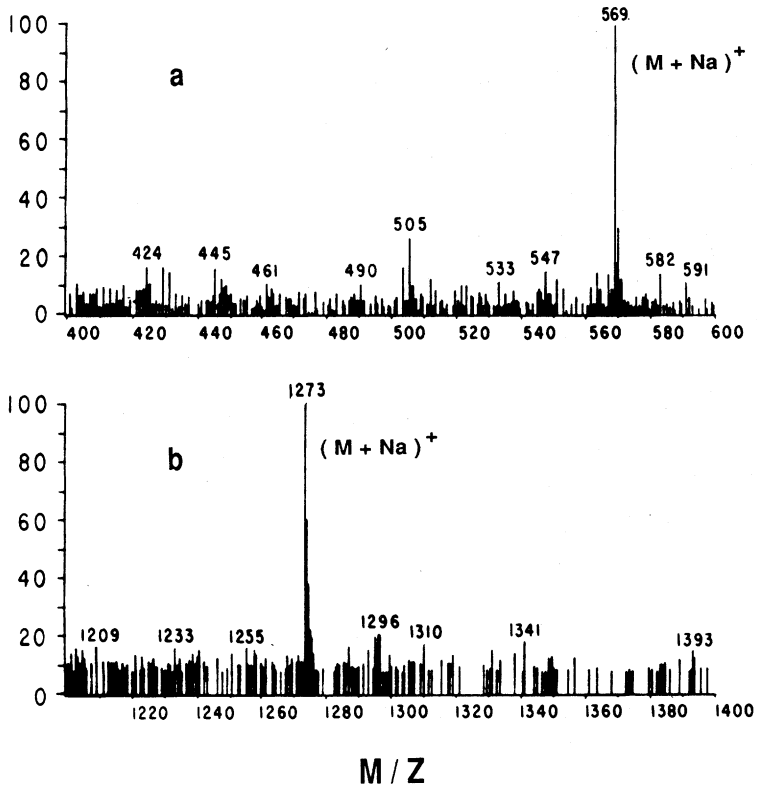

Fig. 7. Fast-atom bombardment mass spectrum in positive mode ($[M+Na]^+$) of DP-3 and DP-7.

solutions, which facilitates the precipitation of individual oligogalacturonic acids in pooled column fraction as their barium salts.

Characterization of the isolated products as pure oligogalacturonic acids was accomplished by UV spectroscopy and mass spectrometry. The absence of UV absorption at 235 nm confirmed the absence of terminal 4,5-unsaturated galacturonic acid residues, which would have resulted from polygalacturonic acid lyase-catalyzed hydrolysis of PGA. Such products, differing by a single carbon–carbon double bond from the desired oligomers, potentially could have co-chromatographed with the desired oligogalacturonic acids. Fast-atom bombardment mass spectral analysis, in the negative ($[M-H]^-$) and positive ($[M+Na]^+$) ionization modes confirmed both the structures and purity of the products. Expected molecular ions (m/z = 176DP + 18 + 23) resulted in the positive mode for oligomers from DP-2 to DP-7, as shown in Fig. 7 for DP-3 and DP-7. In the negative mode, spectra revealed the expected molecular ions (m/z = 176DP + 18 − 1) and ions resulting from loss of successive galacturonic acid residues. For example, DP-7 gave the $[M-H]^-$ ion at m/z 1249 as well as peaks at m/z 1073 (DP-6), 897 (DP-5) and 721 (DP-4).

The separation of individual sugar acids on AG MP-1 ($HCOO^-$) was tested, using a step-gradient eluent of formic acid (0.02–0.10 *M*). Effectively resolved in 1.5 h on a column of 20 ml resin were 40 mg each of galactonic acid (pK_a 3.60), galacturonic acid (pK_a 3.42), and glucuronic acid (pK_a 3.20). As expected on an anion-exchange column, these acids were eluted in order of decreasing pK_a. The separation of these compounds was more efficient than previously achieved by gravity flow chromatography. It is likely that this procedure can be adapted to the simple preparative separations of other sugar acids.

CONCLUSION

A commercial fungal pectinase preparation and an *endo*-polygalacturonase with high specificity isolated from pectinase have been used to catalyze the partial hydrolysis of citrus pectin-derived polygalacturonic acid. Column chromatography on the macroporous anion-exchange resin AG MP-1 (formate), using step-gradient elution with sodium formate (pH 4.7) allowed the isolation of pure oligogalacturonic acids, from DP-2 through DP-7. Column fractions were monitored by HPTLC and by colorimetric reaction with *m*-hydroxybiphenyl. Yields of desired oligomers were doubled by using purified endo-polygalacturonase rather than pectinase to catalyze PGA hydrolysis. Separations were superior in terms of speed and efficiency compared to those previously reported on the gel-type AG 1-X8 and Dowex 1-X8 anion-exchange resins. The identity and purity of isolated oligogalacturonic acids was confirmed by fast-atom bombardment mass spectrometry. By employing more limited hydrolysis with the endo-enzyme, and extending the gradient beyond 0.75 *M* sodium formate, it should be possible to isolate oligogalacturonic acids beyond DP-7. Preliminary experiments demonstrated that closely related acidic monosaccharides can also be efficiently resolved on AG MP-1.

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