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Note

Problems encountered during the extraction, purification, and chromatography of pectic fragments, and some solutions to them

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While working with pectins and fragments of pectins, we have noticed several anomalies in our extractions and purifications of them. These include aberrant behavior upon chromatography, retention of even small (di- and tri-) oligogalacturonides upon extended dialysis, and great difficulty in dialyzing chelating agents away from pectins. We attribute all of these behaviors to the strong interactions between oligomers and polymers of D-galacturonic acid and especially between them and calcium ions or other divalent cations.

The interaction between D-galacturonic acid residues and calcium ions has been investigated in detail because of the importance of these interactions in the gelation of pectate[1]. From experiments examining the activity of Ca^{2+} *vs.* Na^{+} in the presence of oligomers and polymers of D-galacturonic acid, it is apparent that the interaction between pectic fragments and Ca^{2+} is stronger than one would predict for solely ionic interactions[2,3]. Thus, hydroxyl groups must also be coordinating to the Ca^{2+}. When measured as a function of concentration of polypectate, the activity of Ca^{2+} decreased dramatically as the concentration rose between one and ten mequiv./L. Such an abrupt change was attributed to cooperative binding of calcium ions between polymer chains to form soluble aggregates[3] with the structures of the "egg box" model of Morris *et al.*[1] Lower than predicted activity of Ca^{2+} was seen in solutions of oligomers of D-galacturonic acid of three or more residues[3], and a marked decrease in the activity of Ca^{2+} was seen in solutions of oligomers of around 15 or more residues[2]. Oligomers of 14 residues are known from competition experiments to be able to prevent gelation of polypectate[4].

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Extraction of pectins from cell walls using chelating agents. — Pectins are very often extracted from plant tissues using the Ca^{2+} chelators ethylenediamine tetraacetate (EDTA) or *trans*-1,2-diacetamidocyclohexane (cyclohexane-*trans*-1,2 diamine tetraacetate, CDTA)[5]. The pectins are presumed to be solubilized by breakage of the calcium cross-bridges between adjacent pectate sequences within the pectins. These chelators are effective in solubilizing pectin from the cotton suspension culture cell walls that we are studying[6]. However, we find by ^{1}H-n.m.r. spectroscopy that even after extensive (1-wk) dialysis against distilled water, CDTA or EDTA remains associated with the pectin. Both CDTA and EDTA give rise to several signals, among which an intense signal at 3.75 p.p.m. obscures the signal for the methyl protons of methyl esterified D-galacturonic acid residues. The final product in the dialysis bag is at least 50% chelator.

A partial solution to the problem of retention of CDTA after dialysis is to dialyze the CDTA–pectin mixture against a buffer rather than water. After dialysis against 100mM NH_4OAc buffer at pH 5.2 for 12 h, 30–40% more of the CDTA was removed when compared with dialysis against distilled water. Redgwell and Selvendran[5] seemed to be successful in dialyzing away CDTA from extracts using extensive dialysis against running tap water, rather than distilled water.

We have found two ways around the problem of residual CDTA. One way is to use imidazole buffer instead of CDTA for extraction; the other is to add size-exclusion chromatography to the purification procedure. When the CDTA-containing pectin preparations were subjected to size-exclusion chromatography using a TSK 4000 SW column in 300mM NH_4OAc buffer, the CDTA was completely removed from the pectin as judged by ^{1}H-n.m.r. spectroscopy.

It has been reported that concentrated imidazole solution is a good solvent for pectate[7]. If cell walls are extracted with 500mM imidazole buffer, pH 7, for the same length of time as would be used for extraction with CDTA, an equivalent amount of pectin with the same sugar composition is extracted. But, in this case, the imidazole can be dialyzed away completely into distilled water, as judged by the lack of a signal around 7.5 p.p.m. in the ^{1}H-n.m.r. spectrum which imidazole would give. Table I shows the sugar compositions of pectins extracted from cotton suspension culture cell walls using 50mM CDTA or 500mM imidazole as extractant. From the weights of the residues, it appears that equal amounts were extracted by the two treatments.

In other experiments on cotton suspension culture cell walls, we have found that liquid hydrogen fluoride (HF) at −23° does not cleave galacturonosyl linkages, but does cleave those of rhamnosyl residues[6,8]. After this treatment, the rhamnose-rich portion of the pectin (what has been called rhamnogalacturonan I, ref. 9) is cleaved into disaccharides, but the homogalacturonan stays polymeric. Highly methyl esterified galacturonan can be extracted with water from cell walls treated in this way, but the little-esterified galacturonan can only be solubilized by chelators[8] or by imidazole. Table I includes a comparison of the amounts and sugar compositions of the material solubilized from walls by CDTA and imidazole buffer following these pretreatments. Again, it is evident that imidazole is as effective as CDTA in solubilizing pectins that are insolubilized by Ca^{2+}.

TABLE I

Molar sugar compositions and amounts of cell wall extracts

Sample	*(mg)*	*Composition (mole %)*						
		Ara	*Rha*	*Fuc*	*Xyl*	*Gal*	*Glc*	*GalA*
Whole cell walls	500	20	11	2	17	11	7	33
Extracts of whole cell walls:[a]								
50mM CDTA extract	495[b]	7	7	1	6	4	5	71
500mM imidazole extract	35	6	6	1	5	4	2	77
Residue from CDTA extract	425	26	11	2	23	12	7	23
Residue from imidazole extract	435	25	14	2	22	13	7	17
Extracts following −23° HF treatment:[c]								
HF–ether extract	147	32	9	4	36	10	4	5
Water extract	73	8	21	1	10	17	15	30
50mM CDTA extract	211[b]	1	1	—	1	trace	2	96
500mM imidazole extract	94	1	1	—	1	trace	1	96
Residue from CDTA extract	180	4	2	—	5	4	55	30
Residue from imidazole extract	179	4	2	—	3	5	53	34

[a] Extractions from 500 mg of cell walls. [b] Much of this weight is CDTA. [c] Paired sequential extractions (HF–ether, water, 50mM CDTA or HF–ether, water, 500mM imidazole) from 500 mg of cell walls.

We assume that the imidazole is extracting the pectin *via* the same mechanism as the chelating agents. It has already been shown that the non-protonated imino nitrogen of the imidazole ring of histamine can coordinate to Ca^{2+} strongly enough to form crystallizable complexes[10]. We propose that even though the imidazole is mono-dentate, it is present in such high concentrations that it can compete with the carboxyl groups of the D-galacturonic acid in Ca^{2+} complex formation and thus disrupt the "egg box" structures. It appears that the Ca^{2+} imidazole complexes can readily diffuse through the dialysis membrane.

Dialysis of oligomers of galacturonic acid. — A second anomaly we have found is unexpected behavior of small oligomers upon dialysis. There is much interest in preparing oligomers of D-galacturonic acid because of their biological activity in plants. Dimers or longer oligomers of D-galacturonic acid cause the induction of protease inhibitors in members of the Solanaceae[11]. The trimer of D-galacturonic acid suppresses lignification induced by a fungal elicitor in wheat[12], oligomers ~7 residues long induce lignification in castor bean[13], and oligomers ~10–13 residues long induce phytoalexins[14].

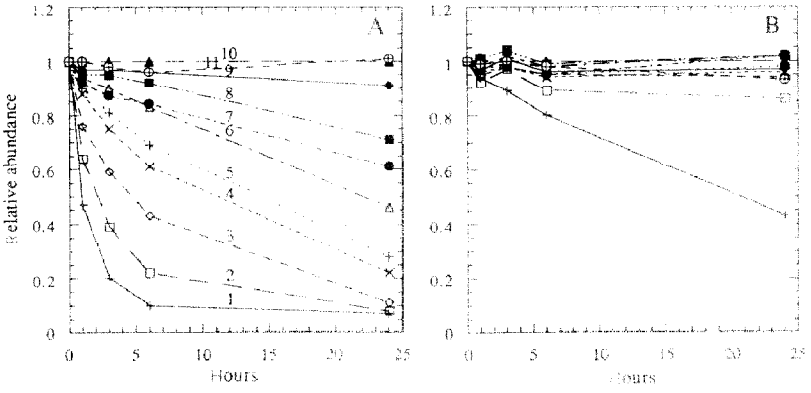

Fig. 1. (A) The abundances of oligomers of d.p. 1 +, 2□, 3 ◆, 4 X, 5 +, 6 △, 7 ●, 8 ■, 9 ◆, 10 ▲, and 11 +, after dialysis in 12–14 000 mol. wt. cutoff tubing against distilled water relative to their initial abundances in the pH 2 supernatant. To obtain the relative abundances, we normalized all the peak areas by dividing them by the area of the decamer at each time point, and then we divided this number by the normalized ones at time 0. The decamer was presumed to be retained by the dialysis tubing, since the relative abundances of larger oligomers did not increase over time with respect to its abundance. (B) The relative abundances of oligomers remaining in 1,000 mol. wt. cutoff dialysis tubing after various times of dialysis against distilled water. For details and symbols, see Fig. 1A.

Several methods are available for the generation, separation, and characterization of pure oligomers and mixtures of oligomers. We have used both partial enzyme hydrolysis with purified endopolygalacturonase[15] and autolysis of polygalacturonic acid in an autoclave[16] to produce a range of oligomers. We find, surprisingly, that if the partial hydrolysates are dialyzed extensively against distilled water, even very small oligomers do not pass through the dialysis membrane. When the acid-soluble portion of a mixture produced by autolysis was dialyzed, it gave the results presented in Fig. 1. Fig. 1 shows the relative proportions of the oligomers present before and after dialysis for periods up to 24 h against distilled water using 12–14 000 and 1,000 mol. wt. cutoff dialysis tubing. Oligomers of d.p. 10 or more were retained inside the 12–14 000 mol. wt. cutoff tubing, whereas oligomers as small as d.p. 2 passed only very slowly through the 1,000 mol. wt. cutoff tubing. When we added 0.5M imidazole to the sample and then dialyzed it in 12–14 000 mol. wt. cutoff tubing against 0.5M imidazole buffer for 12 h, followed by 12 h against distilled water, oligomers of d.p. < 18 passed through the dialysis tubing, oligomers of d.p. < 15 dialyzed from the tubing by more than 50%, and oligomers of d.p. < 6 dialyzed by more than 90%. In contrast, Figure 1A shows that, after 24 h of dialysis *vs.* distilled water, only oligomers of d.p. < 7 had dialyzed by more than 50%. D-Galacturonides understandably behave as molecules larger than dextrans of equivalent molecular weights because the electrostatic repulsion between adjacent D-galacturonic acid residues and restricted rotation around the α-(1→4) glycosidic linkages cause a rod-like structure[17,18]. The abnormal behavior is more pronounced, we think, due to aggregation of oligomers. We interpret the effect of imidazole as decreasing the formation of aggregates. (The high ionic strength of the imidazole would also

decrease electrostatic repulsion between adjacent D-galacturonic acid residues and thus, perhaps, allow more flexibility within the polymer chains.)

A similar aggregation effect is seen when mixtures of oligomers of D-galacturonic acid are precipitated with ethanol. When the pH 2 supernatant from the autoclave hydrolysate of pectic acid was made 70% in ethanol, all of the oligomers except the dimer, trimer, and tetramer were completely precipitated. A small proportion of the dimer, ~50% of the trimer, and most of the tetramer precipitated.

The lack of dialyzability of small (d.p. >9) oligomers of galacturonic acid using 12–14 000 mol. wt. cutoff tubing and of oligomers of d.p. >2 in the 1,000 mol. wt. cutoff tubing should allow one to avoid tedious or unwanted procedures for recovering desalted purified oligomers after their separation by ion-exchange chromatography. For example, Robertsen[16] purified large amounts of individual oligomers by ion-exchange chromatography, but then introduced barium chloride to allow precipitation of the oligomers with ethanol. For oligomers of over four residues prepared by ion-exchange chromatography using the same buffers and exchange medium as Jin and West[19], we have concentrated the pooled effluent from the column containing the oligomer of interest by lyophilization and then removed the salt by dialysis through 1,000 mol. wt. cutoff tubing.

Anion-exchange chromatography. — We frequently find that chromatography of mixtures containing oligomers of D-galacturonic acid seems to be influenced by their history, as well as by the conditions of the chromatography. This is perhaps because of very slow disaggregation even after the conditions which induced aggregation are removed. A striking example is shown in Fig. 2, which is a chromatogram of a mixture of oligogalacturonides separated on a TSK DEAE-2 SW column using an NH_4OAc gradient. The series of peaks labeled 2, 3, 4 etc. were found by rechromatography on a PA-1 column in NaOH, to be individual oligomers of galacturonic acid residues. The

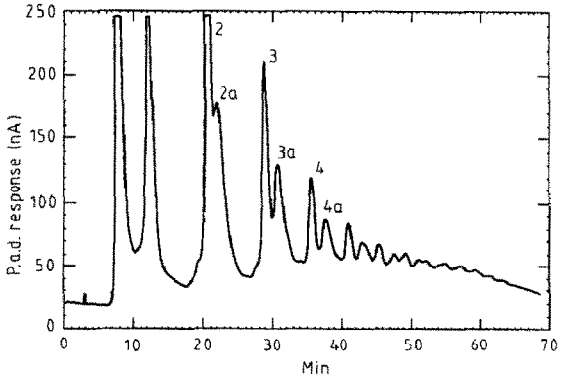

Fig. 2. Anion-exchange chromatography of a mixture of D-galacturonic acid oligomers obtained by Ba^{2+}-precipitation of a subfraction of the pH 2 supernatant. Two mg of this fraction were dissolved in distilled water (500 μL) and applied to a TSK DEAE-2 SW column. Elution was by a linear gradient of 50–200mM NH_4OAc buffer pH 5.2 at 1 mL/min. A fraction (5%) of the eluate was passed through an anion micro-membrane suppressor to remove ammonium ions prior to addition of 1 mL/min of N NaOH and detection by a pulsed amperometric detector (p.a.d.).

peaks labeled 2a, 3a, and 4a etc. were found to be mixtures of a range of oligomers including shorter and longer oligomers than the preceding peak. Similar results have been observed with the same TSK column using NaOAc gradients and with DEAE Sephadex chromatography using an NH_4OAc gradient. The proportion of the secondary peaks is variable. It may be especially high following exposure to divalent cations, *e.g.*, from Ba^{2+} added to aid precipitation, but we have seen the effect in an endopolygalacturonase digest of wheat cell walls and in other samples to which divalent cations had not deliberately been added.

In our experience with separating mixtures of oligogalacturonides on a PA-1 column using a gradient of NaOAc in NaOH, we have not observed this doubling of peaks. Inferring that the alkali dissociated aggregates (which were causing the secondary peaks), we made samples alkaline by additions of at least 1 mol. equiv. of NaOH per mol. equiv. of D-galacturonic acid residue for 15 min at room temperature. The samples were then neutralized with 10% acetic acid. Upon chromatography of samples treated in this manner on the TSK DEAE column described above, only single peaks were obtained at the expected elution time of each oligomer. We were not able to obtain the same disaggregating effect by treating the samples with 500mM imidazole buffer.

Size-exclusion chromatography. There has been a considerable amount of discussion of size-exclusion chromatography of large (> 5,000 mol. wt.) pectins, and it is well known that they exhibit large increases in their radii of gyration as the ionic strength of the elution buffer falls below 0.1 (ref. 17). Even at ionic strengths of 0.1 and above, pectins behave as segmented rods and consequently elute much earlier from size-exclusion columns than would pullulans or dextrans of equivalent mol. wt.

The behavior of short oligomers of D-galacturonic acid (less than 10 residues) was investigated by Thibault[20]. He found that the elution position for D-galacturonic acid chromatographed on Bio-Gel P-2 approached the void volume as the ionic strength of the elution medium approached 0, but reached a constant value more reflective of its size at ionic strengths greater than 0.05. He ascribed the anomalous chromatographic behavior to ionic exclusion; that is, exclusion of the negatively charged D-galacturonic acid from the pores of the gel by the small number of fixed negative charges on the gel.

In our experience with size-exclusion chromatography of oligomers containing D-galacturonic acid, we have also seen what we propose are the effects of persistent aggregate formation. When a pectolyase digest of wheat cell walls[21] was chromatographed on a variety of size-exclusion media in a range of buffers, elution profiles were variable between runs under seemingly identical conditions. After learning that strong imidazole buffer is a very good solubilizing agent for pectins, and knowing from the results of Thibault[20], *i.e.*, that for proper chromatography, a suitable ionic strength buffer must be used, we have developed the following protocol for preparing samples for size-exclusion chromatography:

(*i*) Add 1 mol. equiv. of dry imidazole per mol. equiv. of D-galacturonic acid to the dry sample. (*ii*) Add an appropriate volume of distilled water to dissolve the sample. (*iii*) Add buffer to a final concentration of 0.3M NaOAc or NH_4OAc at pH 5.2. The samples are then eluted through the column in 0.3M NaOAc or NH_4OAc buffer at pH 5.2. Using

this exact protocol, we were able to consistently obtain the same elution profile. Mixtures of oligomers of D-galacturonic acid fractionated, as expected, on Toyopearl HW 40S and Bio-Gel P-2 columns when treated in this manner.

Advantage can be taken of the anomalous behavior of oligomers of D-galacturonic acid for desalting them by size-exclusion chromatography. If samples containing salt, *e.g.*, 10 mg $GalA_2$ or $GalA_3$ containing 20 mg NH_4Cl are passed through a 10 × 500 mm column of TSK Toyopearl HW 40S and eluted with water, the D-galacturonides elute in the void volume and the salt in the included volume. Fishman *et al.*[17] have also reported the elution of apparently low molecular weight pectin fragments in the excluded volume of a large-pore, size-exclusion column using water as eluant.

CONCLUSIONS

Several anomalies are encountered while working with pectins and pectic fragments; however, there are means of circumventing them: (*i*) Chelators do not dialyze away readily from pectins. However, 0.5M imidazole buffer can substitute for the chelators, CDTA and EDTA, for solubilizing pectins, and it does readily dialyze away. (*ii*) Oligomers of D-galacturonic acid with as low a degree of polymerization as 3, in 1,000 mol. wt. cutoff dialysis tubing, or 10, in 12–14 000 mol. wt. cutoff tubing, are retained upon dialysis against distilled water. (*iii*) There is a tendency for oligomers of D-galacturonic acid to remain aggregated during ion-exchange chromatographic separations. This leads to coelution of more than one size of oligomer under conditions which separate non-aggregated oligomers. A short treatment with alkali disaggregates the oligomers and allows subsequent, predictable chromatography. (*iv*) D-Galacturonic acid-containing oligomers behave upon size-exclusion chromatography as much larger molecules than one would expect, unless aggregates are disrupted before the chromatography and the ionic strength of the elution buffer is at least 0.1.

EXPERIMENTAL

Materials. — Pectic acid (Aldrich Chemical Co. or Sigma Chemical Co.) was used to produce a wide range of lengths of oligomers of D-galacturonic acid by the autoclave hydrolysis procedure of Robertsen[16]. A 1% solution of pectic acid in distilled water was adjusted to pH 4.2 with N NaOH. The solution was then heated in an autoclave for 20 min at 121°. After cooling, the pH was adjusted to pH 2 with N HCl, large oligomers were allowed to precipitate overnight at 6°, and the material was then centrifuged at 4000*g* for 20 min. The supernatant (designated pH 2 supernatant) was adjusted to pH 5.2 with N NaOH and freeze-dried. Cell walls from suspension cultures of cotton (cultivar Acala 44) were prepared as previously described[6]. Preparation and digestion with Pectolyase (Sigma) of wheat leaf cell walls has been previously described[21].

Chromatographic separation of oligogalacturonides. — The separation of 2-aminopyridine labeled samples on a TSK DEAE-2 SW column has been previously described in detail[15].

For separation and collection of non-derivatized samples on the same column, the buffer was switched to NH_4OAc from NaOAc to allow easy sample recovery by lyophilization or by vacuum concentration (by a Savant Speed-Vac concentrator). The oligomers were detected using a pulsed amperometric detector (p.a.d., Dionex). The eluate from the column was split using a Kel-F tee, 95% to a fraction collector, 5% to the detector. Since ammonium ions interfere with the electrochemical detection, they were removed from the eluate using an anion micro-membrane suppressor (Dionex). As the suppressor has a limited capacity, it was only used to de-cationize the 5% of the eluate going to the detector. To make the eluate alkaline for the electrochemical detector, M NaOH was added at ~1 mL/min by way of another Kel-F tee.

Pure oligomers of known degrees of polymerization (as determined by liquid secondary-ion mass spectrometry on a ZAB 2SE mass spectrometer) were used to determine the elution times of the various oligogalacturonides. The relative amounts of oligogalacturonides were determined by a p.a.d. after separation on a CarboPac PA-1 (quaternary amine) column using a Dionex Bio-LC Carbohydrate System. A gradient of NaOAc in 0.1M NaOH was used with the following program: Inject at 0.1M NaOH, after 3 min a linear increase in NaOAc to 0.5M (0.1M NaOH) over 20 min, followed by a linear increase up to 0.75M NaOAc (0.1M NaOH) over 27 min. Post-column addition of M NaOH at ~0.6 mL/min was used to stabilize the baseline. The response of the detector to each oligomer was assumed to be linear to its concentration in the detector[22]. However, the relative molar response factors from one size oligomer to another were not determined. Hotchkiss and Hicks[23] have reported that responses are affected by the degree of polymerization and eluant composition.

Dialysis of oligogalacturonides. — Aliquots of a solution of the pH 2 supernatant (7 mg/mL) were dispensed into standard dialysis tubing (1 cm wide 12–14 000 mol. wt. cutoff) and into 1,000 mol. wt. cutoff tubing (Spectrum) (1.2 cm wide). Each sample was dialyzed against distilled water (1,500 mL) at 4° with constant stirring, and 200-μL aliquots were withdrawn from the bags at the indicated time intervals.

To test the effect of imidazole on the dialysis, a sample of the pH 2 supernatant was dissolved in 0.5M imidazole buffer (pH 7.0) and then dialyzed in 12–14 000 mol. wt. cutoff tubing against the same buffer for 12 h. The sample was then dialyzed against distilled water to remove the imidazole.

Extraction of pectins from cell walls. — Cell walls (500 mg) were suspended in either 50mM CDTA (50 mL), pH 6.5, or in 0.5M imidazole-acetate, pH 7.0, at room temperature. Sodium azide (0.05%) was added to prevent microbial contamination. After stirring for 12 h, they were filtered on a 0.45-μm pore size Nylon-66 filter (Alltech) and then washed four times with water (25 mL). The residue was re-extracted with extractant for 3 h and washed again in the same manner. The combined filtrates were dialyzed in 12–14 000 mol. wt. cutoff tubing extensively against distilled water (12 changes of water over 7 days). After dialysis, the samples were freeze-dried and weighed. The residues were also freeze-dried and weighed.

Cell walls were treated with liquid HF at −23° as previously described[6]. The reaction was stopped by addition of a ten-fold volume of dry-ice-cooled ether. The

suspension was filtered, and the HF–ether mixture was evaporated to give what was designated "HF–ether extract". The residue was extracted with water to give the "water extract". This residue was then extracted with either 50mM CDTA or 500mM imidazole buffer.

Carbohydrate analysis. — Aliquots of samples (50–100 μg) were pretreated with endopolygalacturonase, methanolized, and trimethylsilylated as previously described[23]. Carbohydrate compositions of samples were obtained by g.l.c. using a DB1 (0.25 mm × 30 m) capillary column with per-*O*-(trimethylsilyl)-*myo*-inositol as the internal standard[6]. The degree of methyl esterification of samples was determined by selective reduction of methyl esterified D-galacturonic acid residues to D-galactose[23]. ^{1}H-N.m.r. spectroscopy was conducted using a Varian XL-400 spectrometer with 3-(trimethylsilyl)propionic acid, sodium salt (TSP) as internal reference (0.0 p.p.m.).

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