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Isolation and Partial Characterization of Apiogalacturonans from the Cell Wall of *Lemna minor*

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1. A mild, reproducible extraction procedure, using 0.5% ammonium oxalate, was developed for the isolation of polysaccharides containing D-apiose from the cell wall of *Lemna minor*. On a dry-weight basis the polysaccharide fractions extracted with ammonium oxalate made up 14% of the material designated cell walls and contained 20% of the D-apiose originally present in the cell walls. The cell walls, as isolated, contained 83% of the D-apiose present in *L. minor*. 2. After extraction with ammonium oxalate, purified polysaccharides were obtained by DEAE-Sephadex column chromatography and by fractional precipitation with sodium chloride. With these procedures the material extracted at 22°C could be separated into at least five polysaccharides. On a dry-weight basis two of these polysaccharides made up more than 50% of the material extracted at 22°C. There was a direct relationship between the D-apiose content of the polysaccharides and their solubility in sodium chloride solutions; those of highest D-apiose content were most soluble. 3. All the polysaccharides isolated appeared to be of one general type, namely galacturonans to which were attached side chains containing D-apiose. The D-apiose content of the apiogalacturonans varied from 7.9 to 38.1%. The content of esterified D-galacturonic acid residues in all apiogalacturonans was low, being in the range 1.0–3.5%. Hydrolysis of a representative apiogalacturonan with dilute acid resulted in the complete removal of the D-apiose with little or no degradation of the galacturonan portion. 4. Treatment of polysaccharide fractions with pectinase established that those of high D-apiose content and soluble in M-sodium chloride were not degraded, whereas those of low D-apiose content and insoluble in M-sodium chloride were extensively degraded. When the D-apiose was removed from a typical pectinase-resistant polysaccharide, the remainder of the polysaccharide was readily degraded by this enzyme. 5. Periodate oxidation of representative polysaccharide fractions and apiogalacturonans and determination of the formaldehyde released showed that about 50% of the D-apiose molecules were substituted at either the 3- or the 3′-position.

The branched-chain aldopentose D-apiose (3-*C*-hydroxymethyl-*aldehydo*-D-*glycero*-tetrose) has been identified as a constituent of a large variety of plants. In some plants it exists as a component of a flavone, isoflavone or phenolic glycoside such as apiin (Vongerichten, 1901; Nakaoki, Morita, Motosune, Hiraki & Takeuchi, 1955; Wagner & Kirmayer, 1957; Rahman, 1958), lanceolarin (Malhotra, Murti & Seshadri, 1956) or furcatin (Hattori & Imaseki, 1959). However, in *Posidonia australis* (Bell, Isherwood & Hardwick, 1954), *Tilia* sp. (Bacon, 1963), *Zostera marina* (Bacon, 1963; Williams & Jones, 1964; Ovodova, Vaskovsky & Ovodov, 1968), *Lemna gibba* (Beck & Kandler, 1965), *Lemna minor* (Duff, 1965; Beck & Kandler, 1965; Mendicino & Picken, 1965; Beck, 1966, 1967), *Zostera nana* (Duff, 1965), *Zostera pacifica* and *Phyllospadix* (Ovodova *et al.* 1968) it appears to be primarily a component of polysaccharides.

In a study of *L. minor* (duckweed), one of the richest known sources of D-apiose, Duff (1965) showed that more than 90% of the D-apiose present was not extracted by organic solvents. Extraction of the insoluble material under a succession of alkaline conditions indicated that the bound D-apiose was continuously extracted and that no single fraction contained a majority of the D-apiose. From a study of the incorporation of $^{14}CO_2$ into the D-apiose of *L. minor* and *L. gibba* under various conditions, Beck & Kandler (1965) concluded that the D-apiose was not part of a storage material but rather a component of the cell

wall. While the present study was in progress, Beck (1966, 1967) reported the isolation of two apiogalacturonans from *L. minor* that contained 28% and 25% of D-apiose, one of which contained, in addition, D-xylose and D-galactose.

We are interested in the isolation of plant polysaccharides containing D-apiose in order to determine the structure of such unique polysaccharides and to study the mechanism and control of their biosynthesis. Attainment of these goals depended on the development of reproducible methods for their isolation and fractionation. These methods should result in minimal degradation of the polysaccharides. This precluded the use of alkaline extractions because of the degradative effect of such conditions on polysaccharide material (Whistler & BeMiller, 1958; Neukom & Deuel, 1958). It also precluded the use of acidic conditions because of the acid-lability of the glycosidic linkage involving D-apiose (Vongerichten, 1901). Further, even near neutrality, high temperatures must be used with caution, since pectins may be degraded by a β-elimination reaction (Albersheim, 1959; Albersheim, Neukom & Deuel, 1960).

We therefore examined procedures that have been used to extract pectic substances, since it is known that their extraction may be facilitated by relatively mild conditions. When such conditions were applied to *L. minor* cell walls, polysaccharides rich in D-apiose were solubilized. The present study describes the isolation and partial characterization of these polysaccharides.

MATERIALS AND METHODS

Materials. *L. minor* was collected from the Battle Creek River at Bellvue, Mich., U.S.A. The plants were washed extensively with water and either used immediately or stored at −20°C.

D-Apiose was obtained from once-recrystallized apiin. Crystalline apiin was isolated from *Petroselinum crispum* (parsley) seeds by the method of Gupta & Seshadri (1952) and hydrolysed with 0.05M-H_2SO_4 for 30min at 100°C (Vongerichten, 1901). Pure D-apiose was isolated by partition column chromatography on acid-washed Celite 535 by the procedure of Lemieux (1962).

D-[U-^{14}C]Apiose was prepared from UDP-D-[U-^{14}C]-glucuronic acid (New England Nuclear Corp., Boston, Mass., U.S.A.). The procedure of Gustine & Kindel (1969) was used to convert UDP-D-[U-^{14}C]glucuronic acid into Compound III, which was the name given by these workers to the D-apiose-containing compound obtained by their procedure. After paper chromatography in solvent *E*, Compound III was chromatographed in solvent *D*. For the preparation of free radioactive D-apiose, Compound III was hydrolysed with 0.1M-H_2SO_4 for 90min at 100°C and the hydrolysate was treated as described by Gustine & Kindel (1969). Before hydrolysis, sufficient non-radioactive D-apiose was added to give the specific radioactivity indicated below. The free D-[U-^{14}C]-apiose from the hydrolysis was chromatographed on paper in solvents *A*, *B* and *C*, in each of which it migrated as a single radioactive peak. The specific radioactivity was determined by measuring reducing sugar by Nelson's (1944) method with an appropriate elution blank, and radioactivity was measured by liquid-scintillation counting. The specific radioactivity of the D-[U-^{14}C]apiose was 68160d.p.m./μmol of D-apiose. The radioactive D-apiose was called D-[U-^{14}C]apiose since it was derived from the glucuronic acid portion of UDP-D-[U-^{14}C]glucuronic acid.

Fungal pectinase was purchased from Sigma Chemical Co., St Louis, Mo., U.S.A. The enzyme was purified 30-fold over the crude preparation by the manufacturer, who stated it still contained several other enzymes. The specific activity of this batch (lot 125B-0350) as measured by Sigma was 0.7 unit per mg of solid. A unit was defined as the amount of enzyme that liberated 1μmol of D-galacturonic acid per min at 25°C and at pH4.0 with de-esterified citrus pectin as the substrate.

General methods. Solutions were concentrated under reduced pressure by rotary evaporation at temperatures below 35°C. Polysaccharide material was desiccated to constant weight *in vacuo* and over P_2O_5. $CaCl_2$ and $AgNO_3$ were used to test for oxalate and Cl^- ions respectively. Polysaccharide fractions were centrifuged at 35000*g* for 15–20min at 4°C. Radioactivity on chromatograms was detected with a Packard radiochromatogram scanner, model 7201 (Packard Instrument Co., Downers Grove, Ill., U.S.A.). All other radioactivity measurements were made with a Packard Tri-Carb liquid-scintillation counter, model 3310, in Bray's (1960) solution. Optical rotations were determined with a Zeiss Photoelectric Precision Polarimeter 0.005° (Carl Zeiss, Oberkochen, Germany) at 22°C with a polarimeter tube having a 1cm light-path and light of 578nm (Hg) wavelength. Polysaccharides were dissolved in 67mM-KH_2PO_4–Na_2HPO_4 buffer, pH7.7, unless otherwise noted, and their concentrations were in the range 9.1–21mg/ml.

Paper chromatography. Descending paper chromatography was used and was carried out with Whatman no. 3MM paper prewashed with 0.1M-citric acid followed by water. The following solvents were employed: *A*, ethyl acetate–water–acetic acid–formic acid (18:4:3:1, by vol.); *B*, propan-2-ol–water (9:1, v/v); *C*, butan-1-ol–acetic acid–water (4:1:5, by vol., upper phase); *D*, propan-1-ol–ethyl acetate–water (7:1:2, by vol.); *E*, aq.95% (v/v) ethanol–M-ammonium acetate, pH7.5 (7:3, v/v); *F*, pyridine–ethyl acetate–acetic acid–water (5:5:1:3, by vol.). Sugars were detected on chromatograms by spraying with aniline hydrogen phthalate (Partridge, 1949) or by using the $AgNO_3$ dip method (Trevelyan, Procter & Harrison, 1950).

DEAE-Sephadex column chromatography. Column chromatography of the five polysaccharide fractions (see the fractionation experiment in the Results section) was carried out on DEAE-Sephadex A-25 (medium grade, capacity 3.5m-equiv./g, 100–270 mesh; Pharmacia Fine Chemicals, Uppsala, Sweden). Before use the DEAE-Sephadex was repeatedly suspended in 67mM-KH_2PO_4–Na_2HPO_4 buffer, pH7.7, and the fines were removed. Before a polysaccharide fraction was applied, the column was washed with 2–3 bed volumes of the phosphate buffer. The polysaccharide fractions were dissolved in either water or the same buffer and applied to the column at

approx. the same rate as the column was to be operated. After a washing with the phosphate buffer, the polysaccharides were eluted with a step gradient of 0.1–0.3M-NaCl in the phosphate buffer. The individual polysaccharides were obtained by combining the appropriate fractions from the columns and dialysing them against water until they were free of Cl^-. The non-diffusible material was then freeze-dried and desiccated.

Before characterization, some polysaccharides were converted into the H^+ form by passage through a column of Dowex 50W (X8; 50–100 mesh; H^+ form). The polysaccharide solutions were then freeze-dried and desiccated.

Analysis of DEAE-Sephadex column fractions for uronic acid and D-*apiose.* The column fractions were assayed for uronic acid by a modification of the method of Dische (1962). Samples (0.5ml) or appropriate samples diluted to 0.5ml with water in test tubes were cooled to 4°C. On top of each sample was layered 0.2ml of 0.1% (w/v) carbazole (recrystallized once from benzene) in ethanol. The tubes were cooled again to 4°C and then 6.0ml of 15.8M-H_2SO_4 was added. The solutions were thoroughly mixed with a Vortex Jr. mixer (Scientific Industries, Queens Village, N.Y., U.S.A.) at 22°C, heated at 100°C for 20min, cooled in water to 22°C and the E_{525} was determined immediately. With these conditions L-arabinose, D-xylose and D-apiose gave 3.4, 4.8 and 2.4% respectively of the colour obtained with D-galacturonic acid. In the above procedure, the samples were heated after the addition of carbazole. This is in contrast to the original Dische procedure and has two advantages; the sensitivity of the method is increased and the extinction readings can be made immediately after the 20min heating period (Gauthier & Kenyon, 1966).

Early in this work it was discovered that the 22°C and 70°C NaCl-soluble and -insoluble polysaccharide fractions quantitatively released their D-apiose on mild acid hydrolysis without the release of significant amounts of other reducing material. This property was used to develop the following assay for D-apiose in the column fractions. A 1.0ml sample or an appropriate sample diluted to 1.0ml with water was mixed with 0.1ml of M-HCl and heated at 100°C for 30min. The samples were cooled and the liberated reducing material was determined by the method of Nelson (1944). E_{540} was measured in all cases.

Quantitative determination of sugars in polysaccharide material. The D-galacturonic acid content was determined after saponification (McComb & McCready, 1952), by the modification of the H_2SO_4–carbazole method described above.

D-Apiose was determined by isotope dilution. For each determination approx. 5–10mg of polysaccharide material was suspended in 1.0ml of D-[U-^{14}C]apiose solution containing 9474d.p.m. and 0.139μmol of D-apiose and 1.0ml of 0.5M-H_2SO_4 was added. The solution was heated at 100°C for 1h, cooled to 22°C and neutralized to pH6–7 with NaOH and then 9vol. of ethanol was added. The resulting precipitate was removed by centrifugation. The supernatant was decanted, concentrated and streaked on paper, and the chromatogram was developed in solvent *B*. A strip of the chromatogram was treated by the $AgNO_3$ dip method. The material that co-chromatographed with the radioactive D-apiose was eluted and restreaked on paper and the chromatogram developed in solvent *C*. After elution, the specific radioactivity of the radioactive D-apiose was determined by measuring reducing sugar and radioactivity as described above. The amount of D-apiose released from the polysaccharide material was calculated from the percentage of the total radioactive D-[U-^{14}C]apiose recovered and the specific radioactivity of the isolated and added D-[U-^{14}C]apiose.

Determination of the content of esterified D-*galacturonic acid residues in polysaccharide fractions.* The methoxyl content of polysaccharide fractions was determined by the method of Schultz (1965). Polysaccharide fractions (20–50mg) were dissolved in CO_2-free water, titrated to a phenolphthalein end point with carbonate-free 5mM-NaOH, and then 5–10ml of carbonate-free 0.1M-NaOH was added. The solutions were kept at 22°C for 30min and then an equal volume of 0.1M-HCl was added. The solutions were titrated to a phenolphthalein end point under N_2 with the 5mM-NaOH. With this method a value of 8.3% was obtained for the methoxyl content of citrus pectin (grade II; Sigma Chemical Co.).

The proportion of esterified D-galacturonic acid residues in the polysaccharide fractions was calculated from the D-galacturonic acid content, obtained from the H_2SO_4–carbazole test, and the methoxyl content obtained as described above.

Preparation of partially hydrolysed polysaccharide. D-Apiose was removed from the 22°C NaCl-soluble fraction by hydrolysis with 0.1M-HCl for 30min at 100°C. The solution was cooled, neutralized with NaOH to pH7 and then dialysed against water until the diffusate was negative for Cl^-. The non-diffusible material was freeze-dried and stored over P_2O_5 *in vacuo*. This material was used in the pectinase experiment depicted in Fig. 4.

Pectinase hydrolysis of polysaccharide fractions. Polysaccharide fractions were incubated with pectinase at 37°C in 25mM-sodium acetate buffer, pH4.5. The enzyme was dissolved in 50mM-sodium acetate buffer, pH4.5, and the polysaccharide fractions were dissolved in water. The reaction was followed by measuring the increase in reducing material with time.

Periodate oxidation of polysaccharide materials and release of formaldehyde. Polysaccharide material (approx. 5.0mg) was dissolved in 1.0ml of 50mM-sodium acetate buffer, pH5.0, and then 1.0ml of 0.1M-$NaIO_4$ was added. The solution was kept at 22°C for 2h in the dark. Preliminary experiments showed that the release of formaldehyde was virtually complete after 1h. Formaldehyde was determined by using chromotropic acid (Speck, 1962). The chromotropic acid was recrystallized once from aq.50% (v/v) ethanol.

RESULTS

Extraction and fractionation of L. minor. *L. minor* plants were initially extracted so that cell walls were isolated and these were then fractionated as in the typical experiment described below.

Fresh *L. minor* (350g, 24.15g dry wt.) was homogenized for 1min periods in a Waring Blendor successively with M-sodium chloride (three times), water (once), 0.1M-sodium chloride (twice) and water (three times), 1 litre quantities being used for each homogenization. The suspension was filtered

through six layers of cheesecloth after each homogenization. The final residue was washed with water until the washings were free of Cl^-, dried by solvent exchange with aq. 95% (v/v) ethanol, ethanol, ethanol–diethyl ether and diethyl ether and desiccated to yield 7.97g of white material, designated cell walls.

Cell walls (7.50g) were suspended in 1 litre of water and stirred at 60–70°C for 8h. The suspension was filtered by suction and the process was repeated twice. The combined filtrates were concentrated, freeze-dried and desiccated to yield 280.6mg of brown material. This was called the 70°C water fraction.

The residue was suspended in 1litre of 0.5% (w/v) ammonium oxalate, pH 6.2, and stirred at 22°C for 3h. The suspension was filtered and the process was repeated. The residue was then extracted with 1 litre of water at 22°C for 3h and the suspension was filtered. The extraction of the residue with water was repeated once and the four filtrates were combined and concentrated to approx. 400ml. The solution was dialysed against water until the diffusate was negative for oxalate ion. The solution of non-diffusible material was transferred to a beaker and 10% (w/v) calcium chloride was added slowly to the stirred solution until no further precipitation occurred. After cooling to 4°C, the suspension was centrifuged and the supernatant was decanted and discarded. With stirring, the precipitate was redissolved in approx. 400ml of 0.5% (w/v) ammonium oxalate at 22°C. After cooling to 4°C, the insoluble calcium oxalate was removed by centrifugation and discarded. After decanting, the supernatant was dialysed against water until the diffusate was negative for oxalate ion. The solution of non-diffusible material was concentrated to 300ml and an equal volume of 2.0M-sodium chloride was added dropwise to the constantly stirred solution. After cooling to 4°C, the suspension was centrifuged. The supernatant was concentrated to about 250ml and then dialysed against distilled water until the diffusate was negative for Cl^-. No further precipitation of polysaccharides occurred during this decrease in volume even though the concentration of the sodium chloride in the supernatant increased to at least 2M. The non-diffusible material was freeze-dried and desiccated to yield 371mg of white material. This material was designated the 22°C sodium chloride-soluble fraction. The material precipitated by the M-sodium chloride was resuspended in water and dialysed against water until the diffusate was negative for Cl^-. The solution of non-diffusible material was freeze-dried and desiccated to yield 321mg of white material. This material was designated the 22°C sodium chloride-insoluble fraction.

The residue from the 22°C extraction procedure was further extracted with 0.5% (w/v) ammonium oxalate and water at 70°C in the same way as it was extracted at 22°C. The material solubilized by the 70°C extraction procedure was taken through the same steps as that solubilized by the 22°C extraction procedure. The material designated the 70°C sodium chloride-soluble fraction weighed 211mg and was yellow, whereas that designated the 70°C sodium chloride-insoluble fraction weighed 105mg and was white.

The residue from the 70°C extraction was dried by solvent exchange as described above for the cell walls. It was then desiccated to yield 5.15g of material. The results of this experiment are summarized in Table 1.

In a separate experiment the efficiency of the

Table 1. *Fractionation of* L. minor

The weights of the six fractions obtained from the cell walls were calculated on the basis that 7.97g of cell-wall material was fractionated.

Fraction	Amount obtained		D-Apiose content	D-Apiose recovered in each fraction	
	(g)	(%)	(%)	(%)*	(%)†
Dry plants	24.15	—	5.8	100	—
Cell walls	7.97	100	14.6	83.1	100
70°C Water	0.298	3.7	3.1	0.7	0.8
22°C Sodium chloride-soluble	0.395	5.0	33.1	9.3	11.2
22°C Sodium chloride-insoluble	0.341	4.3	10.1	2.5	3.0
70°C Sodium chloride-soluble	0.224	2.8	21.6	3.5	4.2
70°C Sodium chloride-insoluble	0.112	1.4	14.0	1.1	1.3
Residue	5.47	68.6	16.2	63.3	76.2
		85.8			96.7

* Values calculated on the basis of the D-apiose content of the dried plants.

† Values calculated on the basis of the D-apiose content of the dried cell walls.

individual ammonium oxalate and subsequent water extractions was examined. *L. minor* cell walls were prepared and extracted as described above. The values given are based on cell wall material (3.8g) that had been extracted with water at 70°C and dried. The first and second 0.5% ammonium oxalate extracts at 22°C yielded 8.8 and 0.6% of the starting material and the first and second at 70°C yielded 2.4 and 0.7%. The corresponding aqueous extracts yielded 0.5 and 0.3%, and 0.7 and 0.1% of the starting material.

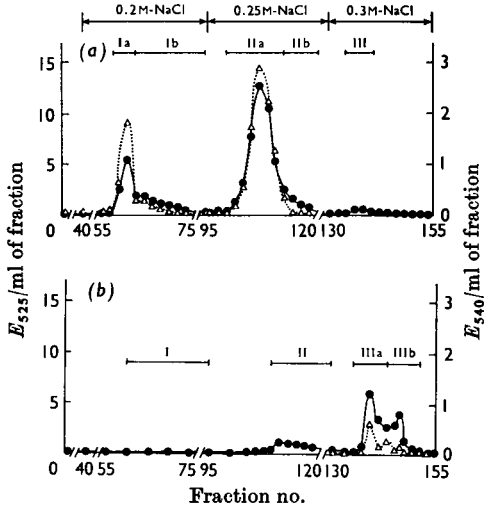

Fig. 1. Column chromatograms of 22°C sodium chloride-soluble (*a*) and -insoluble (*b*) fractions. The columns (both 2.8cm internal diam. × 25cm) were of DEAE-Sephadex and were developed identically. The columns were treated with 67mM-KH_2PO_4–Na_2HPO_4 buffer, pH7.7, for fractions 1–16 and with 0.1M-NaCl in the same buffer for fractions 17–39. From both columns fractions (15ml) were collected at a rate of 0.3ml/min. The fractions were assayed for uronic acid (●; E_{525}) and D-apiose (△; E_{540}) as described in the text.

Column chromatography of polysaccharide fractions on DEAE-Sephadex. The five fractions obtained from the fractionation experiment described in the preceding section were subjected to column chromatography on DEAE-Sephadex. The procedure described in the Materials and Methods section was followed.

The 70°C water fraction (110mg) was suspended in 35ml of water but did not dissolve completely. The material that did dissolve (96.8mg) was bound irreversibly to the column under the conditions used.

The 22°C sodium chloride-soluble fraction (335.2mg) was applied to the column in 20ml of the phosphate buffer. The elution profile is given in Fig. 1(*a*). The indicated fractions were combined and the polysaccharides were isolated as described in the Materials and Methods section. The analytical results are summarized in Table 2.

The 22°C sodium chloride-insoluble fraction (285.5mg) was applied to the column in 40ml of the phosphate buffer. Further washing of the column with 0.5M-sodium hydroxide eluted no more polysaccharide material. The elution profile is given in Fig. 1(*b*). The indicated fractions were combined and the polysaccharides were isolated. The analytical results are summarized in Table 2.

Table 2. *DEAE-Sephadex chromatography of 22°C sodium chloride-soluble and -insoluble fractions*

The polysaccharides from the column were weighed as their sodium salts. They were converted into the H^+ form before characterization as described in the text. The sugar values are percentages of the dried polysaccharides in their H^+ form expressed as glycosyl residues.

Polysaccharide	Amount recovered from column (mg)	(%)	D-Apiose (%)	D-Galacturonic acid (%)	$[\alpha]_{578}^{22}$
(*a*) 22°C sodium chloride-soluble fraction					
Ia	49.9	14.9	36.1	46.3	+91.8°
Ib	69.8	20.8	33.7	45.1	+99.5
IIa	169.9	50.7	38.1	52.8	+106.9
IIb	18.4	5.5	—	—	—
III	10.4	3.1	—	—	—
		95.0			
(*b*) 22°C sodium chloride-insoluble fraction					
I	10.0	3.5	—	—	
II	38.0	13.3	20.2	56.1	
IIIa	29.4	10.3	16.6	65.4	
IIIb	14.0	4.9	11.8	65.0	
		32.0			

The 70°C sodium chloride-soluble fraction (190mg) was applied to the column in 30ml of the phosphate buffer. Further washing of the column with 0.5M-sodium hydroxide eluted no more polysaccharide material. The elution profile was essentially the same as that for the 22°C sodium chloride-soluble fraction (Fig. 1*a*). The appropriate fractions were combined and the polysaccharides were isolated. On a weight basis, the amount of material recovered as polysaccharides Ia, Ib, IIa, IIb and III was 9.8, 6.4, 28.8, 15.3 and 5.1% respectively of that applied to the column. Only polysaccharide IIa was analysed. In the H^+ form it contained 34.1% of D-apiose and 52.3% of D-galacturonic acid and had $[\alpha]_{578}^{22}$ +107.1°.

The 70°C sodium chloride-insoluble fraction (91.0mg) was suspended in 40ml of water and stirred for several hours. Since not all the material dissolved, the suspension was centrifuged. The supernatant was decanted and the precipitate was dried with acetone and weighed (9.9mg). The material in the supernatant (81.1mg) was chromatographed. Further washing of the column with 0.5M-sodium hydroxide eluted no more polysaccharide material. The elution profile was essentially the same as that for the 22°C sodium chloride-insoluble fraction (Fig. 1*b*). The appropriate fractions were combined and the polysaccharides were isolated. On a weight basis, the amount of material recovered as polysaccharides II, IIIa and IIIb was 20.6, 17.3 and 33.8% respectively of that applied to the column. No further work was done with these polysaccharides.

Fractionation of sodium chloride-insoluble fractions with sodium chloride. The 22°C sodium chloride-insoluble fraction from a different preparation (500mg) was dissolved in 100ml of water and 2.0M-sodium chloride was added dropwise slowly to the constantly stirred solution until the desired concentrations were reached. Precipitated polysaccharides were removed at 0.27M-, 0.41M- and 1.0M-sodium chloride by centrifugation. Increasing the sodium chloride concentration to 2.0M by concentrating the supernatant from the final centrifugation did not result in further precipitation. The precipitated polysaccharides were suspended in water and these suspensions and the supernatant were dialysed until negative for Cl^- and then freeze-dried. The polysaccharides were characterized after desiccation. The results are summarized in Table 3.

The 70°C sodium chloride-insoluble fraction (200mg) was dissolved in 40ml of water and 2.0M-sodium chloride was added as above. Precipitated polysaccharides were removed at 0.41M- and 1.0M-sodium chloride by centrifugation. Increasing the sodium chloride concentration to 2.0M as described above did not result in further precipitation. The polysaccharides from the precipitated fractions and the supernatant were isolated as described above and characterized. The results are summarized in Table 3.

Identification of polysaccharide components. The procedure used to determine the D-apiose content of the polysaccharide fractions in Table 1 also served to identify D-apiose as a component of these fractions. Solvents *B* and *C* distinguish between D-apiose, L-rhamnose, and D-fucose.

The identification of D-galacturonic acid as a component of the sodium chloride-soluble and

Table 3. *Fractionation of 22°C and 70°C sodium chloride-insoluble fractions with sodium chloride*

The polysaccharides were weighed and characterized as their sodium salts. The sugar values are percentages of the dried polysaccharides in the Na^+ form expressed as glycosyl residues. The polysaccharides were dissolved in water for the optical-rotation measurements.

Polysaccharide	Concn. of NaCl (M)	Type of precipitate	Amount recovered (mg)	Amount recovered (%)	D-Apiose (%)	D-Galacturonic acid (%)	$[\alpha]_{578}^{22}$
(*a*) 22°C sodium chloride-insoluble fraction							
A	0.19–0.27	Flocculent	29.6	5.9	7.9	77.5	+210.5°
B	0.27–0.41	Gel	335.0	67.0	9.2	80.4	+223.6
C	0.73–1.00	Flocculent	59.2	11.8	17.2	62.3	+136.9
D	Supernatant	—	39.5	7.9	24.7	59.2	+111.9
				92.6			
(*b*) 70°C sodium chloride-insoluble fraction							
B	0.27–0.41	Gel	147.1	73.6	7.9	74.1	+192.1
C	0.73–1.00	Flocculent	35.3	17.7	11.3	60.8	+178.8
D	Supernatant	—	15.2	7.6	25.5	41.0	—
				98.9			

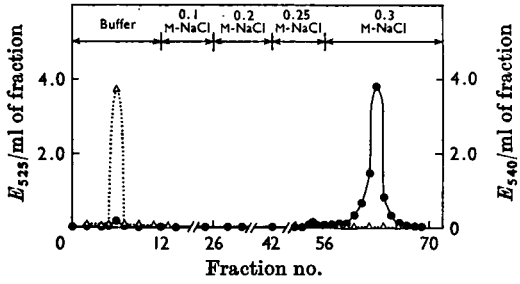

Fig. 2. Column chromatogram of partially hydrolysed polysaccharide IIa. The 22°C NaCl-soluble polysaccharide IIa (25mg) was hydrolysed in 0.1M-HCl for 30min at 100°C. The solution was neutralized with NaOH and applied to a DEAE-Sephadex column (1.4cm internal diam. × 20cm). The column was eluted first with 67mM-KH_2PO_4–Na_2HPO_4 buffer, pH7.7, and then with a step gradient of 0.1–0.3M-NaCl in the same buffer. Fractions (6.0ml) were collected at a rate of 0.12ml/min and were assayed for uronic acid (●; E_{525}) and D-apiose (△; E_{540}) as described in the text.

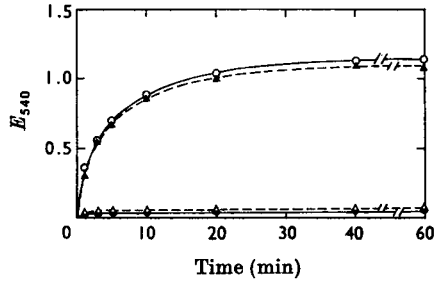

Fig. 3. Pectinase hydrolysis of polysaccharide fractions. Each point represents one assay mixture of 1.0ml final volume. Each assay mixture contained 500μg of the sodium salt of the polysaccharide fraction and 200μg of pectinase. Reactions were terminated by addition of the Nelson alkaline copper reagent and then immediately heating at 100°C. In the zero-time incubations the order of addition of materials to the incubation tubes was the same as in the sample tubes except that the Nelson reagent was added before the pectinase solution. Incubations were started such that all finished together. Reducing material was determined by the Nelson (1944) method. The polysaccharide fractions hydrolysed were: △, 22°C NaCl-soluble fraction; ▲, 22°C NaCl-insoluble fraction; ●, 70°C NaCl-soluble fraction; ○, 70°C NaCl-insoluble fraction.

-insoluble fractions was based on the following. All four fractions reacted positively in the sulphuric acid–carbazole test. The insoluble fractions, and the soluble fractions after mild acid hydrolysis, were degraded in the presence of pectinase. Paper chromatography of the enzymic hydrolysates of the insoluble fractions in solvent *A* revealed that D-galacturonic acid was the predominant sugar present. Finally, paper chromatography of representative polysaccharide fractions in solvents *A* and *F* after acid hydrolysis under reflux for 15h showed that D-galacturonic acid was the major sugar present. These solvents distinguish between D-galacturonic and D-glucuronic acids. The D-galacturonic acid was further distinguished from D-glucuronic acid by its inability to form a lactone.

In the experiments for determining the D-apiose content of polysaccharide fractions we normally found a single spot, corresponding to D-apiose, on the chromatograms after chromatography with solvent B. Occasionally one and rarely two faint spots were also present. These were not identified. These results suggest that either other sugars are not present at all or only in small amounts in these polysaccharide fractions or that hydrolysis was incomplete.

Content of esterified D-galacturonic acid residues in polysaccharide fractions. The content of esterified D-galacturonic acid residues in the 22°C and 70°C sodium chloride-soluble and -insoluble fractions was determined. The values for all four fractions were similar, being in the range 1.0–3.5% with most values between 2.5 and 3.5%.

Partial acid hydrolysis of polysaccharide IIa. The results of the partial acid hydrolysis and rechromatography on DEAE-Sephadex of 22°C sodium chloride-soluble polysaccharide IIa (Fig. 1*a*) are shown in Fig. 2. Paper chromatography in solvents *A* and *B* of samples of the column fractions containing reducing material revealed that D-apiose was the only sugar present. No reducing material was associated with the uronic acid-positive material. The uronic acid-positive material that was eluted corresponded to about 30% of the theoretical value and was characterized as a galacturonan by its lack of reactivity in the Nelson test and by its conversion into D-galacturonic acid on treatment with pectinase. No additional D-apiose was released on further acid hydrolysis of the eluted galacturonan.

Pectinase hydrolysis of polysaccharide fractions. The results of the pectinase hydrolysis of the sodium chloride-soluble and -insoluble fractions are shown in Figs. 3 and 4. Fig. 3 shows that only the sodium chloride-insoluble fractions were extensively degraded by the pectinase treatment. Paper chromatography of the pectinase hydrolysates of these two fractions in solvent *A* revealed that the reducing material was predominantly D-galacturonic acid. The maximum reducing values attained by the insoluble fractions correspond to a D-galacturonic acid release of about 80% of the theoretical value. The sodium chloride-soluble fractions were resistant to pectinase-catalysed

hydrolysis. However, incubation of such a polysaccharide fraction with pectinase after removal of the D-apiose by dilute acid hydrolysis resulted in extensive degradation, as shown in Fig. 4. The maximum reducing value attained with the hydrolysed 22°C sodium chloride-soluble fraction corresponds to a D-galacturonic acid release of about 60% of the theoretical value.

Release of formaldehyde on periodate oxidation of polysaccharide material. The 22°C sodium chloride-soluble and -insoluble fractions and certain of the polysaccharides obtained after separation of the 22°C sodium chloride-soluble fraction on DEAE-Sephadex were oxidized with sodium metaperiodate and the amount of formaldehyde released was measured. The 22°C sodium chloride-soluble polysaccharide IIa (Fig. 1*a*) was treated with sodium metaperiodate both before and after it was subjected to a hydrolysis procedure that liberated only D-apiose. These results are summarized in Table 4.

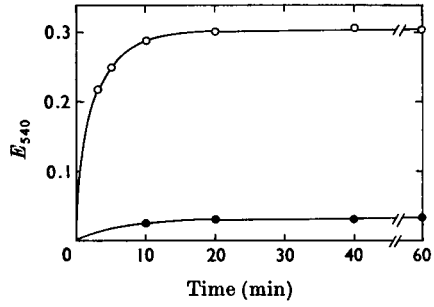

Fig. 4. Pectinase hydrolysis of the 22°C NaCl-soluble fraction before (●) and after (○) removal of D-apiose. Each point represents one assay mixture of 1.0 ml final volume. Each assay mixture contained either 200 μg of unhydrolysed or 120 μg of hydrolysed polysaccharide fraction, both as the sodium salts, and 200 μg of pectinase. The experimental procedure described in the legend of Fig. 3 was followed. Reducing material was determined by the Nelson (1944) method.

DISCUSSION

The procedure developed for the isolation of polysaccharides containing D-apiose from the cell wall of *L. minor* is simple, mild and reproducible. The procedure is reasonably quantitative, since 86% of the starting cell-wall material and 97% of the D-apiose of the cell wall could be accounted for in the various fractions. With this procedure the major apiogalacturonans can be obtained in sufficient quantity to permit structure determination. We established that the polysaccharides isolated are components of the cell wall by isolating cell walls before any fractionations were performed. An extensive series of extractions was used in the preparation of the cell walls to minimize possible contamination by other cellular components. Although this resulted in the solubilization of two-thirds of the plant material, 83% of the D-apiose was still present in the final product, designated cell walls. This is a minimum value since some of the cell-wall material may have been solubilized by the shearing action of the blender and some may have been lost in the cheesecloth during the filtrations. The cell walls were not further charac-

Table 4. *Formaldehyde released on periodate oxidation of polysaccharides extracted at 22°C*

Periodate oxidations were performed as described in the text. The 22°C NaCl-soluble Ia, Ib and IIa samples refer to the polysaccharides obtained from DEAE-Sephadex column chromatography of the 22°C NaCl-soluble fraction (Fig. 1*a*). Hydrolysed 22°C NaCl-soluble polysaccharide IIa was prepared by heating this polysaccharide for 30 min at 100°C in 0.5 ml of 0.1M-HCl; after cooling an equal volume of 0.1M-NaOH was added. Then 1.0 ml of 50 mM-sodium acetate buffer, pH 5.0, was added and the periodate oxidation was performed. The unhydrolysed polysaccharide was dissolved in 1.0 ml of 0.1M-NaCl and an equal volume of 50 mM-sodium acetate buffer, pH 5.0, was added. The theoretical yield of formaldehyde was calculated assuming that one D-apiose residue yielded one molecule of formaldehyde.

Sample	D-Apiose	Formaldehyde found	Yield
	(μmol/mg of polysaccharide)	(μmol/mg of polysaccharide)	(% of theoretical)
22°C NaCl-insoluble	0.844	0.532	63.0
22°C NaCl-soluble	2.590	1.428	55.1
Ia	2.735	1.571	57.5
Ib	2.550	1.301	51.2
IIa	2.880	1.518	52.8
22°C NaCl-soluble IIa			
Unhydrolysed	2.880	1.621	56.3
Hydrolysed	2.880	2.845	98.8

terized to determine purity. The results clearly show that, at least in *L. minor*, most of the D-apiose present in this plant is in cell-wall components. Its function there is unknown.

The polysaccharides isolated with our procedure belong to the pectic acid group of plant cell-wall polysaccharides. The degree of esterification of the D-galacturonic acid in all polysaccharide fractions was very low. Similar results have been reported by Beck (1967). This appears to be their natural condition since the isolation procedure does not contain any steps that would lead to de-esterification. Highly esterified polysaccharides, if present in these cell walls, were probably lost during the preparation of the cell walls. Water-soluble polysaccharides, in general, would be lost during the isolation of cell walls by our procedure. Because of this, the weight value for the 70°C water fraction in Table 1 is a minimum value.

The results in Table 1 show that, on a dry-weight basis, the four polysaccharide fractions extracted with ammonium oxalate made up 14% of the cell walls and contained 20% of the D-apiose originally present in these cell walls. They were presumably non-covalently bound in the cell wall, possibly as calcium salts, since they were readily extracted with ammonium oxalate. A portion of the apiogalacturonans could only be extracted by ammonium oxalate at 70°C. All of our experiments showed this material to be similar to that extracted at 22°C but it may be partially degraded due to the temperature.

At M-sodium chloride, the material extracted by ammonium oxalate at 22°C was separated into two fractions with strikingly different D-apiose contents, 33.1% against 10.1%. Similar, though not so striking, results were obtained with the material extracted at 70°C. The 22°C sodium chloride-soluble fraction could be separated on DEAE-Sephadex into two or possibly three polysaccharides, although the differences between these polysaccharides were slight and may represent natural variations within one type of polysaccharide or possibly some degradation of one type of polysaccharide. The DEAE-Sephadex chromatography of the 22°C sodium chloride-insoluble fraction was not entirely successful. The yields were low and the resolution was incomplete. However, sufficient information was obtained to show that it was a mixture of partially separable components. The chemical and physical properties of the polysaccharides obtained from the 22°C sodium chloride-insoluble fraction are different from those of the 22°C sodium chloride-soluble fraction. The elution profiles of these two fractions show that polysaccharide II of each fraction is eluted at about the same position. Since the results in Table 2 show that these two polysaccharides are different, it is clear that, in order to obtain pure 22°C sodium chloride-soluble polysaccharide II, the M-sodium chloride fraction step is essential.

The 22°C and 70°C sodium chloride-insoluble fractions were resolved into four and three components respectively by fractionation with sodium chloride. In both cases the recovery of material was virtually quantitative, in contrast with the recoveries from the DEAE-Sephadex columns. The polysaccharides of both insoluble fractions were precipitated at similar and definite sodium chloride molarities. The three polysaccharides of the 70°C insoluble fraction have properties similar to polysaccharides B, C and D of the 22°C insoluble fraction, again showing that the material extracted at each temperature is very similar. These experiments and those discussed above show that sodium chloride can be used successfully to fractionate the apiogalacturonans. They confirm that the two insoluble fractions are mixtures of polysaccharides. Fractionation of pectic substances from plant material with inorganic univalent cations has been used successfully by Bhattacharjee & Timell (1965) and Zitko & Bishop (1965).

The results in Table 3 show that there is a direct relationship between the D-apiose content of a polysaccharide and its solubility in sodium chloride solutions; the higher the D-apiose content, the greater the solubility. The apiogalacturonans, which contained the greatest percentage of D-apiose, were soluble even in 2M-sodium chloride. However, they were readily precipitated by low concentrations of the bivalent Ca^{2+} ion. In view of the very low D-galacturonic acid methyl ester content of all of the apiogalacturonans isolated, the sodium chloride fractionations reported here are probably based on the ability of D-apiose to interfere with the formation of ionic bonds between the negative charges of the apiogalacturonan and the positive Na^+ ions. If precipitation occurred by the formation of ionic bonds between polysaccharide molecules via Na^+ and Cl^- and if the D-apiose sterically shielded the negative charges on the polysaccharide molecules, those polysaccharides of highest D-apiose content would have the greatest difficulty in forming such bonds and thus in aggregating. Consequently, these polysaccharides would be the most soluble in salt solutions.

The results of several of the experiments described show that the polysaccharides isolated consist of a galacturonan to which are attached side chains containing D-apiose. The pectinase hydrolysis results (Fig. 3) suggest that in the sodium chloride-soluble polysaccharides the D-apiose is attached to the galacturonan throughout its length, since these polysaccharides are resistant to pectinase-catalysed hydrolysis. This is substantiated by the results in Fig. 4, which show that these polysaccharides are readily hydrolysed by

treatment with pectinase if their D-apiose is removed. Since there is very little methyl esterification, the inhibition must have been due to the D-apiose side chains. If the D-apiose was concentrated at a few specific points in these polysaccharides, a large proportion of the D-galacturonic acid should have been released by pectinase treatment before acid hydrolysis. Since this was not the case, such a structure can be excluded. With the sodium chloride-insoluble polysaccharides, their relatively small D-apiose content dictates that large portions of the galacturonan backbone must be free of D-apiose side chains, thus making them susceptible to pectinase. In these polysaccharides two types of structure are possible. Either the D-apiose is attached throughout the length of the galacturonan with sufficient space in between the D-apiose molecules to allow the pectinase to act, or all the D-apiose molecules are concentrated in one portion of the galacturonan, leaving the remainder susceptible to pectinase. The available data do not allow us to make a choice. Because the apiogalacturonans from *L. minor* were degraded by the pectinase, we have assumed that they consist of α-(1→4)-linked D-galacturonic acid residues. The two apiogalacturonans isolated by Beck (1967) were both extensively degraded by pectinase.

The ability of D-apiose to confer resistance to pectinase on apiogalacturonans of high D-apiose content has not been observed before and could be physiologically significant. Recent studies have shown that an early consequence of pathogen infection in plants is the degradation of cell-wall polysaccharides (Bateman, Van Etten, English, Nevins & Albersheim, 1969; English & Albersheim, 1969). Thus one function of D-apiose in *L. minor* might be to protect the pectic substances from degradation by infecting pathogens. If D-apiose functions in this manner in *L. minor*, it probably functions similarly in other plants that contain D-apiose. Pertinent to this is the isolation by Ovodova *et al.* (1968) of a polysaccharide containing D-apiose from three species of the Zosteraceae family that also contained D-galacturonic acid. Polysaccharides containing D-apiose have only been isolated from *L. minor* and those three species of the Zosteraceae family. In an early study on D-apiose Bell *et al.* (1954) suggested that the resistance of *P. australis* and *Z. marina* fibres to natural decomposition may be due to the presence of a D-apiose derivative.

Polysaccharides similar to those described here have been isolated from plant material by Aspinall & Baillie (1963) and by Bouveng (1965). However, in these polysaccharides the side chains consist of sugars other than D-apiose. This type of polysaccharide where sugars are attached as side chains to a galacturonan backbone may be widely distributed in plants.

The acid hydrolysis and autohydrolysis results obtained by Beck (1967) and his inability to detect disaccharides or higher homologues of D-apiose led him to suggest that the D-apiose was attached to the galacturonans as monomeric side chains. However, our formaldehyde values indicated that the D-apiose was partially substituted, since only about 50% of the theoretical quantity of formaldehyde was obtained on periodate oxidation. Only D-apiose will yield formaldehyde in these polysaccharides, and it would have to be substituted at position 3 or 3′ to block formaldehyde production during periodate oxidation. The theoretical amount of formaldehyde (i.e. 1mol/mol of D-apiose) was obtained if the apiogalacturonan was first hydrolysed under conditions that resulted in the release of only free D-apiose. The most plausible explanation of the findings is to postulate the existence of acid-labile disaccharide side chains of D-apiose attached to a galacturonan. Recently we have isolated a disaccharide of D-apiose from several apiogalacturonans by heating an aqueous 0.3% (w/v) solution of the apiogalacturonan at 100°C for 3h at pH 4.5 (D. A. Hart & P. K. Kindel, unpublished work).

The results in Table 1 show that, on a dry-weight basis, 76% of the D-apiose of the cell wall of *L. minor* was not solubilized by our extraction procedure. The structure of polysaccharides containing D-apiose remaining in the residue remains to be determined. If D-apiose is functioning to protect the pectic substances, the D-apiose remaining in the residue may be functioning similarly but protecting different polysaccharides, in which case different polysaccharides containing D-apiose would be present. The isolation by Williams & Jones (1964) of a crude polysaccharide fraction containing D-xylose and D-apiose in the ratio of approx. 1:1 from the marine plant, *Z. marina*, showed that other types of polysaccharides containing D-apiose are present in Nature. Alternatively, the D-apiose could be present in polysaccharides that are the same as those reported here but for unknown reasons are not extracted. A major obstacle to further extraction of the *L. minor* cell wall is in developing sufficiently mild procedures for solubilizing the polysaccharides containing D-apiose still in the residue. One possible approach would be to use a purified enzyme to degrade selectively one type of polysaccharide (e.g. cellulose) and to determine if the remaining polysaccharides were then more readily extractable.

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University in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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