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Polysaccharides in Pollen

II. The Xylogalacturonan from Mountain Pine (*Pinus mugo* Turra) Pollen

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The polysaccharides in Mountain pine pollen have been isolated by alkaline extraction combined with mild ultrasonic treatment. Fractionation of the extract afforded the previously reported xylogalacturonan in an almost pure state with reference to other polysaccharides. Using methylation analysis and enzymic and acidic hydrolysis, it has been shown to consist of chains of α-(1→4)-linked D-galacturonic acid residues, approximately every second being substituted in the 3-position by a β-D-xylopyranose residue. A few α-D-galacturonic acid residues might also be linked to the main chains through 3-positions.

A previous communication has reported [1] the extraction, using monoethanolamine (EA), and subsequent fractionation of the polysaccharide constituents of Mountain pine (*Pinus mugo* Turra) pollen intine (*i.e.* the easily swollen layer located between the outer, chemically very resistant exine and the cytoplasm). The following were obtained: an arabinogalactan containing a high proportion of L-arabinose and also small amounts of L-rhamnose and uronic acid, a polysaccharide containing D-xylose and D-galacturonic acid in the approximate ratio 1:2 and a glucan. The latter, being the main polysaccharide, was shown to be a linear (1→3)-β-glucan. The presence of starch and of a fucose-containing polysaccharide was also indicated.

The use of EA as an extracting agent proved effective but resulted in the xylogalacturonan appearing in the extract with the uronic acid residues almost completely transferred into the corresponding N-(β-hydroxyethyl) amides. This indicates that, in the native state, the uronic acid residues were esterified. The modified polymer was unsuitable for structural examination. The use of boiling water or alkali at room temperature did not result in the extraction of any appreciable amounts of polysaccharides indicating that the intine was protected by a membrane resistant to other than drastic conditions. Ultrasonic treatment in aqueous suspension at an intensity of about 10 W/cm^2,

Table 1. Extraction of Mountain pine pollen (300 g) in a 6 litre 500/125 W ultrasonic tank.

Extracting agent	Volume (l)	Time (min)	Water-soluble (g)	Water-insoluble (g)	Residue (g)
Acetone	6	10	—	—	288
50 % aq. ethanol	9	15	—	—	212
12 % KOH	6	12	3.57		181
»	4	60	5.05	22.2	158
12 % KOH + 1 % H_3BO_3	4	60	7.31	14.1	130
12 % KOH	3	60	5.30	2.7	118

Table 2. Fractionation of water-soluble pine pollen polysaccharides (15.8 g) on a DEAE cellulose column 6 × 40 cm.

Fract.	Eluant (M KOAc)	Yield (g)	Principal sugars
1:1	0.04	7.00	Glu, Arab, Xyl
1:2	0.12	2.25	Arab
1:3	0.50	3.97	Xyl
1:4	1	0.95	Arab, Xyl
1:5	0.5 M KOH	0.78	Arab, Xyl

using a stub transducer (MSE Ultrasonic Disintegrator Model 60W) caused some disintegration of the membrane within 30—60 min with accompanying extraction of polysaccharides but only 2—4 g batches could be treated at any one time.

Satisfactory extraction of the intine polysaccharides was obtained by ultrasonic treatment of the pollen in 12 % potassium hydroxide at 30—35°. The apparatus used was a Mullard Equipment Ltd 500/125 W Ultrasonic Generator L 364 combined with a 6 litre L 368 tank. This equipment offered an intensity of 1—2 W/cm^2 and high capacity. The extraction results are summarised in Table 1. The 50 % aqueous ethanol extract contained, besides low-molecular weight constituents, large amounts of protein-like material. The addition of boric acid to the alkali [2] only slightly improved the extraction. After the fourth alkali extraction, the intine had dissolved completely, as observed by microscopy.

The alkaline extracts were acidified with acetic acid and the precipitate (mainly β-glucan) was removed by centrifuging. After some purification the final yield of water-soluble material was 15.8 g and of crude β-glucan *ca.* 45 g.

The mixture of water-soluble polysaccharides was passed through a DEAE-cellulose column irrigated with aqueous potassium acetate of stepwise increasing concentration (Table 2). The first fraction contained, apart from small amounts of neutral polysaccharides, mainly unabsorbed material. Fractions 1:2 and 1:3 on hydrolysis gave, respectively, arabinose and xylose as the main sugars.

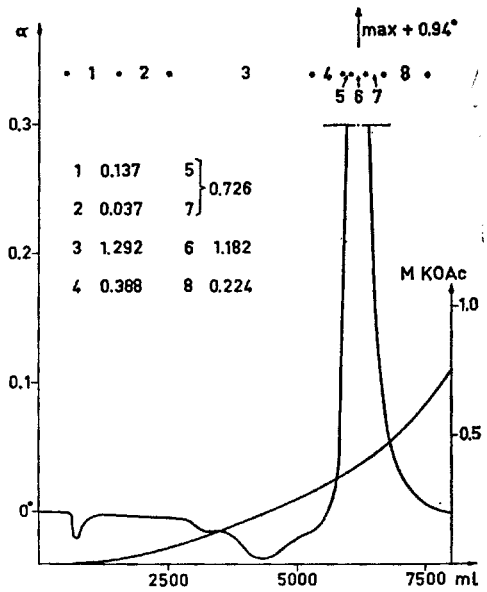


Fig. 1. Purification of pine pollen xylogalacturonan by chromatography on DEAE-cellulose (gradient elution).

Fraction 1:3 was refractionated on DEAE-cellulose using gradient elution (Fig. 1). The optical rotations of the fractions were measured at 578 nm on a Perkin-Elmer Polarimeter 141. Fractions in the main peak having rotations greater than + 0.5° were combined to give 1182 mg of ethanol-precipitable material (fract. 2:6); fractions with rotations of 0.1—0.5° gave fract. 2:5 + 7 (726 mg). These fractions, which were eluted by 0.35—0.45 M potassium acetate, gave on hydrolysis xylose and galacturonic acid plus traces of arabinose, and this corresponded to the previously reported xylogalacturonan.[1] An earlier fraction 2:3, as well as 1:2, was found to consist of almost pure acidic arabinogalactan. On refractionation of other xylose-rich samples, more than 80 % of the xylogalacturonan was eluted by 0.35—0.45 M KOAc, showing good reproducibility as to the concentration at which the polysaccharide was displaced from the column.

The potassium salt of the xylogalacturonan was soluble in water at moderate concentrations. The free acid was soluble in hot water but

Table 3. Properties of pine pollen xylogalacturonan fractions.

		2:6	2:5 + 7
Anhydro-D-xylose	%	22.4	19.7
	mmoles/10 mg	0.0169	0.0150
Anhydro-D-galacturonic acid	%	60.0	57.5
	mmoles/10 mg	0.0344	0.0327
Anhydro-L-arabinose	%	1.3	2.2
[α] (K-salt)	at 578 nm	+179.2°	
N	%	1.48	
Periodate consumed	mmoles/10 mg	0.0512	
Formic acid liberated	mmoles/10 mg	0.0193	

precipitated on cooling unless the concentration was below 0.5 %. As seen from Table 3, where some properties of the purest samples are summarised, xylose and galacturonic acid account for only 83 % of the sample. Acidic arabinogalactan, as deduced from the arabinose content,[3] is present in about 3 %. The nature of the remaining 14 % is not known but protein (N × 6.25 = 9.2 %) and moisture might constitute part of it.

By titration with sodium thiosulphate in the presence of iodide-iodate the polysaccharide was found to contain 0.0344 mmoles/10 mg of acidic groups. It consumed, as the potassium salt, 0.0452 mmoles/10 mg of periodate with the liberation of 0.0170 mmoles/10 mg of formic acid. After correcting for the potassium content, as calculated from the titrated acidity, the polysaccharide consumed 0.0512 mmoles/10 mg of periodate with the liberation of 0.0193 mmoles/10 mg of formic acid. Thus, besides residues giving rise to formic acid, there are present 0.0126 mmoles/10 mg of residues containing only two vicinal hydroxyl groups.

The polysaccharide was methylated first with methyl sulphate and potassium hydroxide and then with iodomethane and silver oxide in dimethyl formamide. Reduction with lithium hydridoaluminate and subsequent methylation to OCH_3 = 45.6 % gave an oily product which, on hydrolysis, yielded the methyl ethers listed in Table 4. 6-*O*-Methyl-D-galactose arises most probably from incomplete methylation. As can be seen from the table, the quantity of ethers corresponding to terminal, non-reducing residues, considerably exceeds those corresponding to branching points, indicating that severe degradation had taken place. From the physical properties of the intermediary products, this appears to have occurred in the first methylation using silver oxide. Therefore, in methylation of polysaccharides of the present type it seems advisable to esterify with diazomethane and reduce before methylating with iodomethane and silver oxide.

A commercial pectinase hydrolysed the xylogalacturonan slowly, as compared to a citrus pectin, with splitting of galacturonosidic linkages only. The stable end-products were D-galacturonic acid and a disaccharide, with $[\alpha]$ + 36.4°, which was easily hydrolysed by acids to xylose and galacturonic acid. Methylation followed by esterification, reduction, a further methylation and hydrolysis gave 2,4,6-tri-*O*-methyl-D-galactose and 2,3,4-tri-*O*-methyl-D-xylose. From these results the disaccharide appears to be 3-*O*-β-D-xylopyranosyl-D-galacturonic acid (I). This pseudoaldobiouronic acid has previsously been isolated by Aspinall and Baillie [4] from tragacanthic acid.

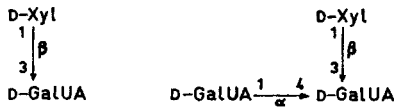
As intermediates of the enzymolysis there could be isolated a diuronic acid, indistinguishable by paper chromatography from 4-*O*-α-D-galacto-

Table 4. Methyl ethers from methylated, hydrolysed pine pollen xylogalacturonan.

		Mole %
D-Galactose,	2,6-Me_2 * **	33
»	2,3,6-Me_3	19
»	2,3,4,6-Me_4	7
D-Xylose,	2,3,4-Me_3	41

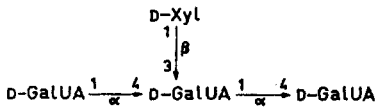
* 2,6-di-*O*-methyl-D-galactose etc. ** Incl. *ca.* 10 % 6-*O*-methyl-D-galactose.

pyranuronosyl-D-galacturonic acid and a tri- and a tetrasaccharide, both of which contained xylose. The results from enzymic and partial acid hydrolyses, from enzymic hydrolysis of reduced samples and from periodate oxidation followed by reduction and total hydrolysis [5] showed these to be 4-*O*-α-D-galactopyranuronosyl-(3-*O*-β-D-xylopyranosyl-)-D-galacturonic acid (II) and *O*-α-D-galactopyranuronosyl-(1→4)-*O*-(3-*O*-β-D-xylopyranosyl-)-α-D-galacturonosyl-(1→4)-D-galacturonic acid (III).



I

II



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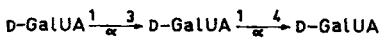
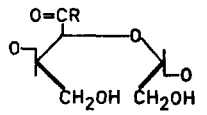


IV



V

The enzymic degradation was made stepwise with intermediate separation of lower oligosaccharides from the dextrins. The latter were then treated with fresh enzyme. Citrus pectin was converted to galacturonic acid within 16 h by the enzyme. The xylogalacturonan required 80 h in two steps for a 60 % conversion into tetrasaccharides or lower. The remaining part required more than 200 h, in several steps, before less than 2 % of the starting material remained as higher oligosaccharides. This indicates the accumulation of more resistant fragments at the later stages of the enzymolysis. Branching involving D-galacturonic acid residues combined through (1→3)-linkages to the main chain, as discussed below, might result in this resistance. Some support for this is provided by the release of comparable amounts of galacturonic acid and xylose on mild alkaline treatment of the residual dextrins, indicating that, besides xylose, galacturonic acid may also be linked to the 3-position of the reducing end-groups of the dextrins. However, further examination of this matter seems to be required.

There was available for partial hydrolysis only impure fractions containing 60—80 % of xylogalacturonan. Treatment of this material with 0.125 M

Table 5. Properties of oligosaccharides from acidic and enzymic hydrolysis of xylo-galacturonan.

Oligosaccharide	R_A *	[α]	Acid hydrolysis gave	Enzymic hydrolysis gave
A_2	0.46	+128°	A	
A_2'	0.56	+101°	A	
A_3	0.26	+149°	A,A_2	A
A_3'	0.32	—	A,A_2,A_2'	A,A_2'
XA	0.75	+ 36°	X,A	Stable
XA_2	0.31	+104°	X,A,A_2,(XA)	A,XA
XA_3	0.19	+133°	X,A,A_2,A_3(XA_2)	A,XA,XA_2

* Mobility in solvent B relative to galacturonic acid (= A).

sulphuric acid at 100° for 28 h gave an ethanol-precipitable fraction which was made up almost exclusively of D-galacturonic acid residues. Examination of the low-molecular weight fraction showed, besides neutral sugars, a mixture of acids apparently originating from the acidic arabinogalactan present in the starting material. Some 4-*O*-α-D-galactopyranuronosyl-D-galacturonic acid was also shown to be present by chromatography. The high-molecular weight fraction was further hydrolysed as described in the experimental section, to give, after fractionation, two di- and two trisaccharides designated A_2, A_2', A_3, and A_3'. Their only product of hydrolysis was galacturonic acid. Owing to the extensive degradation of galacturonic acid residues during the hydrolysis,[6] the yields of the acidic fragments were low, making it possible to carry out only superficial characterisation using partial acidic and enzymic hydrolyses, the relative rates of alkaline degradation and the examination of products from periodate oxidation. A_2 and A_3, being the main products, were chromatographically identical to the di- and trigalacturonic acids, isolated from a citrus pectin hydrolysate. They were both rapidly hydrolysed by pectinase. A_3 gave only A_2 and galacturonic acid on partial hydrolysis. A_2 was degraded by alkali with similar rate to the digalacturonic acid from pectin. A_2 and A_3 therefore are composed of D-galactopyranosyluronic acid residues combined through α-(1→4)-linkages, as would be expected when the results from methylation analysis and enzymic degradation are considered. A_2' was only very slowly degraded by the pectinase. It was degraded 3—4 times faster by alkali than A_2. Therefore A_2' most probably possesses a 3-*O*-α-D-galactopyranuronosyl-D-galacturonic acid structure. A_3' was degraded by the pectinase considerably faster than A_2'. The products were comparable amounts of galacturonic acid and A_2' indicating the presence of one linkage that was more rapidly split than the other. Partial hydrolysis gave, besides A_2', a component with the same chromatographic mobility as A_2. Partial hydrolysis of a reduced sample gave virtually only A_2'. Finally, periodate oxidation followed by reduction and total hydrolysis [5] showed the presence of unoxidised galacturonic acid. From this A_3' appears to have an *O*-α-D-galactopyranuronosyl-(1→3)-*O*-α-D-galactopyranuronosyl-(1→4)-D-galacturonic acid structure (IV).

Owing to the fact that impure fractions were used in the partial hydrolysis, there is some ambiguity as to whether the (1→3)-linked galacturonic acid residues originate from the xylogalacturonan or not. However, unless galacturonic acid residues are considerably more stable towards degradation by acids when combined through (1→3)- than through (1→4)-linkages, a separate polysaccharide containing α-(1→3)-linked galacturonisidic residues would be present in quantities that would not be easily overlooked. For a satisfactory explanation of the absence in the enzymic digest of any lower oligosaccharides containing (1→3)-linkages, information is required concerning the relative stabilities to the enzyme of the (1→3)- and (1→4)-linkages in the presence of each other.

In the previous communication [1] conclusions, now known to be false, as to the structure of the xylogalacturonan were based mainly on the results of Smith degradation [7] and on the lability of the xylose residues towards alkali. The present results show that the normal conditions (0.5 N acid at room temperature) in the Smith degradation are not sufficient for splitting off oxidised galacturonosyl residues. When the xylogalacturonan [1] was subjected to Smith degradation, it therefore retained its polymeric nature owing to the relatively acid-stable elements of the structure V combined to galacturonic acid residues which had been protected towards periodate oxidation by xylosyl substituents in the 3-positions. The lability of the xylose residues towards alkali would indicate these to be attached to the β-position of the carboxylic groups in the galacturonic acid residues. This is found not to be correct; the lability of the xylose residues is not understood.

The present results show that, in the pine pollen xylogalacturonan, all xylose units occur as terminal, non-reducing residues, β-linked to the 3-positions of D-galacturonic acid residues, the majority of which are combined through α-(1→4)-linkages to form the main chain. Some units in this chain appear to be substituted in the 3-position by α-D-galacturonosidic residues; from such elements oligosaccharides A_2' and A_3' would arise. Using the analytical figures, of 100 units there would be approximately 62 D-galacturonosyl units which are resistant towards periodate or oxidised without liberating formic acid. Of these, 33 are substituted in the 3-positions by β-D-xylose units (calculated from xylose content) and 5 by α-D-galacturonic acid units (from formic acid liberation — xylose content). The xylose units are not regularly distributed along the chain, as indicated by the formation of A_2 in the enzymic hydrolysis of the polysaccharide. The presence of branches involving galacturonic acid residues is supported by the methylation data, and to some extent by the results from partial hydrolysis, but requires further confirmation. These branches would be single units rather than chains of several units when the strong film-forming properties of the polysaccharide are considered.

The analytical values are considered to be accurate enough to allow the above discussion of the structure of the polysaccharide. Considering the titrated acidic groups and the types of substituted residues known to be present from the methylation data, the results of periodate oxidation are able to account for 0.0512 mmoles/10 mg. The galacturonic acid (by titration) and xylose contents account for 0.0513 mmoles/10 mg. The ratio terminal non-reducing

galacturonic acid to terminal non-reducing xylose is by xylose content and formic acid liberation 1:6.6 and by methylation data 1:5.9.

The pine pollen polysaccharide bears structural resemblance to the tragacanthic acid fraction from gum tragacanth, investigated by Aspinall and Baillie.[4] This also contains a main backbone of D-galacturonic acid residues but the content of D-xylose (also combined to galacturonic acid residues through 3-positions) was almost equivalent to the galacturonic acid content. The side-chains consist not only of single D-xylosyl residues but also of 2-*O*-α-L-fucopyranosyl-D-xylosyl and 2-*O*-β-D-galactopyranosyl-D-xylosyl residues.

EXPERIMENTAL

Paper chromatograms were run on Whatman No. 1 and (for quantitative separations) Grycksbo No. 333 filter paper, in the following solvents:

A. Ethyl acetate, pyridine, water 8:2:1 (neutral sugars)
B. Ethyl acetate, acetic acid, water 3:1:1 (acidic sugars)
C. Butanol, ethanol, water 10:3:5 (methylated sugars)

Paper electrophoresis was carried out on Whatman No. 3 papers in 0.1 M borate buffer at pH 10. Anisidine hydrochloride was used as spray reagent. Optical rotations were measured in water, unless otherwise stated, at 578 nm. Enzyme stability tests on oligosaccharides were all run for 40 h and the relative differences estimated visually from the chromatograms.

Extraction and separation of polysaccharides

Extraction. The pollen (300 g) was suspended in the proper extractive agent in the ultrasonic tank and the series of extractions shown in Table 1 carried out. Acetone and 50 % aqueous ethanol extractions at room temperature removed lipids and low-molecular weight carbohydrates together with proteinaceous material. The temperature of the alkali extractions was allowed to rise to 30–35° and then kept there by the intermittent addition of ice. Any clots formed during the extraction were broken up by occasional stirring with a glass rod. Owing to the intense swelling of the pollen in alkali, it was extracted in three separate batches in the first steps, then in two.

After each alkali extraction, the pollen suspension was poured onto a sintered glass filter, washed with water, dilute acetic acid, ethanol and then air-dried. The filtrates and washings were combined and acidified and the precipitate formed, constituting the "water-insoluble" in Table 1, was removed by centrifuging. The centrifugate was concentrated and the soluble polysaccharides were precipitated with ethanol. The combined precipitates gave, after redissolving, removal of insolubles and reprecipitation 15.8 g of polysaccharides.

Fractionation of polysaccharides. The polysaccharide mixture was fractionated on a column of Whatman DE 50 DEAE-cellulose using aqueous potassium acetate of stepwise increasing concentration as shown in Table 2. Fraction 1:1 contained mainly incompletely absorbed material and fraction 1:2 mainly acidic arabinogalactan. Fraction 1:3, which gave a high proportion of xylose on hydrolysis, was refractionated on a column (5 × 55 cm) using gradients of 0 → 0.12, 0.12 → 0.36, and 0.36 → 1.0 M potassium acetate for elution. The gradients were of type

$$c = (c_1 - c_2)\left(1 - \frac{V}{V_1 + V_2}\right)^{V_1/V_2}$$

with $V_1/V_2 = 5/6$.[8] The fractions were examined by means of their optical rotations. The results are summarised in Fig. 1. Fraction 2:3 (1.29 g) contained almost pure acidic arabinogalactan and fraction 2:5 + 7 (0.73 g) and 2:6 (1.18 g) almost pure xylogalacturonan. Refractionations of different fractions, in a similar manner, gave a total of 3.50 g of xylose-free and 3.13 g of 80–90 % pure arabinogalactan. In addition, 600 mg of about 90 % pure xylogalacturonan was obtained. Apart from fractions containing mixtures of these polysaccharides, there was obtained 0.48 g of neutral polysaccharides

giving, on hydrolysis, besides glucose (from starch and/or β-glucan) comparable amounts of galactose, xylose, arabinose, and fucose.

Examination of the xylogalacturonan

The xylose content was estimated using an internal standard (glucose) by the Somogyi method [9] after hydrolysis and chromatographic separation. The periodate consumption was estimated by the arsenite method and the formic acid release with sodium thiosulphate after adding ethylene glycol and potassium iodide.

Methylation of xylogalacturonan. The polysaccharide (fraction 2:6; 800 mg as potassium salt) in water (20 ml) was methylated under nitrogen at room temperature for 6 h with methyl sulphate (10 ml) and 30 % potassium hydroxide (40 ml) added in portions. The solution was acidified, dialysed against running tap water and treated with Dowex 50W (H^+) ion exchange resin. After concentration and precipitation with acetone, there was obtained 685 mg of a white powder. This was methylated with iodomethane and silver oxide in dimethyl formamide to give an oily product which was refluxed with excess lithium hydridoaluminate in tetrahydrofuran. After adding water and aqueous phosphoric acid to the reaction mixture, the product was extracted with benzene. It was further methylated twice with iodomethane and silver oxide in dimethyl formamide to give a product, that on extraction with light petroleum, left a glass (314 mg) with $[\alpha] + 92°$ (*c*, 0.5 in chloroform) and OCH_3 45.6 %. On taking the extract to dryness, it yielded a sticky material (192 mg) that gave the same pattern of methylated sugars on hydrolysis as the glass but evidently also contained degradation products. The two fractions were combined and hydrolysed using 90 % formic acid at 100° for 30 min, followed by 0.25 M sulphuric acid for 14 h at 100°. After removal of insoluble material, the hydrolysate was concentrated to give a mixture of methylated sugars as a clear syrup (336 mg). The mixture was resolved on a carbon-Celite column (3 × 23 cm) by eluting with a gradient of 8 → 35 % aqueous ethanol, to give the following fractions:

Fract. 1 (19 mg) contained mainly monomethyl ethers, some giving the pentose colour with anisidine. Approximately half of the fraction was 6-*O*-methyl-D-galactose by visual examination of the chromatogram.

Fract. 2 (82 mg) was chromatographically and ionophoretically pure and indistinguishable from 2,6-di-*O*-methyl-D-galactose. Twice crystallised from ethyl acetate, it had m.p. and mixed m.p. 120 – 122° and $[\alpha] + 90°$ (*c*, 0.6).

Fract. 3 (12 mg). 2,6-Di-*O*-methyl-D-galactose constituted approximately one third of the fraction by visual examination of chromatograms. Other constituents were not identified.

Fract. 4 (61 mg) was by chromatography pure 2,3,6-tri-*O*-methyl-D-galactose with $[\alpha] +93°$ (*c*, 0.5). The product from bromine oxidation gave, on distillation at 0.1 mm Hg, 2,3,6-tri-*O*-methyl-D-galactonic lactone with m.p. and mixed m.p. 99 – 100.5°. 2,4,6-Tri-*O*-methyl-D-galactose was not present on examination of the fraction by paper chromatography using butanone saturated with water as solvent. This ether gives a well defined spot in that solvent while 2,3,6-tri-*O*-methyl-D-galactose gives a streak.

Fract. 5 (39 mg) had $[\alpha]_D +77°$ and was shown to contain by chromatography 2,3,4,6-tetra-*O*-methyl-D-galactose and 2,3,4-tri-*O*-methyl-D-xylose. From reported rotations of these ethers,[11] $+118°$ and $+18°$, respectively, the fraction contained 60 % of tetramethylgalactose and 40 % of trimethylxylose. Treatment of the fraction with aniline gave 2,3,4,6-tetra-*O*-methyl-D-galactosyl-N-phenylamine m.p. and mixed m.p. 195 – 197°.

Fract. 6 (96 mg) was chromatographically pure 2,3,4-tri-*O*-methyl-D-xylose with $[\alpha] +18°$ (*c*, 1.0). Twice crystallised from ether-light petroleum it had m.p. 91 – 92°, in agreement with reported values.[10]

Enzymic degradation of xylogalacturonan. Xylogalacturonan (1.03 g as potassium salt) from fraction 2:5 + 7 was dissolved in 0.1 M sodium acetate buffer of pH 4.2 (200 ml) and 200 mg of pectinase (from mould concentrate; a product of Mann Research Laboratories Inc.) was added. After 40 h at 25° with occasional shaking insoluble material making up the bulk of the enzyme, was removed by filtering. The filtrate was treated with Dowex 50W (H^+), concentrated and poured into ethanol to give a precipitate (250 mg). Non-precipitated material (554 mg) was fractionated on a Sephadex G-25 column. In order to obtain the maximum yield of oligosaccharides, the higher dextrins

from the Sephadex column and the above precipitate were treated again with enzyme and the digest fractionated, this being repeated until not more than 16 mg of higher oligosaccharides remained. After suitable purification, the following were obtained:

Monosaccharide A (115 mg) which, after removal of traces of other monosaccharides by chromatography on thick filter paper, gave a glass (105 mg) with [α] +47° (*c*, 1.5). It was chromatographically identical to D-galacturonic acid and was converted by oxidation with bromine to galactaric acid, m.p. 220–222°.

Disaccharide XA (224 mg) with [α] +36.4° (*c*, 1.1) giving xylose and galacturonic acid on hydrolysis. A sample (70 mg) was methylated with methyl sulphate in aqueous potassium hydroxide and then esterified with diazomethane and reduced with sodium hydridoborate in aqueous ethanol in the presence of Dowex 1 ion exchange resin (acetate form). Esterification and reduction were repeated once, whereupon exhaustive methylation followed using iodomethane/silver oxide in dimethyl formamide. Hydrolysis gave two components which were separated on thick filter papers into:

(a) *2,4,6-Tri-O-methyl-D-galactose* (15 mg) indistinguishable from authentic material on paper chromatography. Although the ether crystallised on seeding with an authentic sample of 2,4,6-tri-*O*-methyl-D-galactose, it was best characterised as the aniline derivative, m.p. 166–167.5°, undepressed on admixture with authentic 2,4,6-tri-*O*-methyl-D-galactosyl-N-phenylamine.

(b) *2,3,4-tri-O-methyl-D-xylose* (16 mg), indistinguishable from authentic material on chromatography and having m.p. 88–91°, undepressed on admixture with the 2,3,4-tri-*O*-methyl-D-xylose isolated from the methylated polysaccharide. In addition 2,4-di-*O*-methyl-D-galactose (3 mg) was obtained and identified by paper chromatography. This ether probably arose from incomplete methylation.

Disaccharide A_2 which travelled with the same speed as XA on a Sephadex G-25 column but could be separated from it on thick filter papers to give a glass (19 mg) with [α] +128° (*c*, 0.8). It was rapidly further hydrolysed into galacturonic acid by the pectinase and was found to be chromatographically identical to the digalacturonic acid isolated from a pectin hydrolysate. From this and from the methylation data on the original polysaccharide, indicating the main part of the galacturonic acid residues to be combined through (1→4)-linkages, its structure was deduced to be 4-*O*-α-D-galactopyranuronosyl-D-galacturonic acid.

Trisaccharide XA_2 which was obtained as a glass (25 mg) with [α] +104° (*c*, 1.0) after separation of small amounts of XA and higher dextrins on thick filter papers. Treatment with 0.25 M sulphuric acid at 100° for 2 h gave, as principal products, xylose and disaccharide A_2. Starting material, XA and A were present in small amounts. Treatment with pectinase for 40 h gave, besides minor amounts of starting material, XA and A. After reduction with sodium hydridoborate it was only attacked very slowly by the enzyme, giving A as the only reducing sugar. A sample (3 mg) was oxidised for 48 h with excess sodium periodate. Ethylene glycol was added and the solution (3 ml) was added to the top of a 2.5 × 29 cm column of Sephadex G-10 (a gift from Dr. Kirsti Granath, AB Pharmacia, Sweden). The oxidised saccharide was located by testing one drop of each fraction with orcinol-sulphuric acid. The separation from iodate ion was checked by adding 1 drop of 20 % potassium iodide and 1 drop of starch solution to 1 drop of each fraction. Fractions containing oxidised oligosaccharide were combined, reduced with sodium hydridoborate and hydrolysed. No galacturonic acid could be detected on a chromatogram, whereas a non-oxidised but otherwise similarly treated sample gave a bright galacturonic acid spot. From this XA_2 appears to have a 4-*O*-α-D-galactopyranuronosyl-(3-*O*-β-D-xylopyranosyl)-D-galacturonic acid structure.

Tetrasaccharide XA_3 which was obtained pure as a glass (33 mg) with [α] +133° (*c*, 1.2) on refractionating the proper fractions on a Sephadex G-25 column. Acid hydrolysis as above gave xylose and a trigalacturonic acid, indistinguishable from the trigalacturonic acid in a pectic hydrolysate. Minor constituents of the hydrolysate were starting material, XA_2, XA together with A_2 and A. Enzymolysis gave XA_2, XA, and A. Enzymolysis of a reduced sample gave only XA and A. XA_3 thereby possesses a *O*-α-D-galactopyranuronosyl-(1→4)-*O*-(3-*O*-β-D-xylopyranosyl)-α-D-galacturonosyl-(1→4)-D-galacturonic acid structure. Some properties of these oligosaccharides are summarised in Table 5.

Dextrins (16 mg). A neutralised sample was treated with 0.03 M sodium hydroxide at room temperature for 48 h. Xylose and galacturonic acid were present in the reaction

product as shown by chromatography, the latter in relatively larger amounts. Partial hydrolysis gave xylose, galacturonic acid and A_2.

Partial hydrolysis of xylogalacturonan. Fractions with varying amounts (60—80 %) of xylogalacturonan (5.34 g) were treated first with 0.125 M sulphuric acid at 100° for 28 h. Of the product, the material (920 mg) precipitable with ethanol gave almost exclusively galacturonic acid on total hydrolysis. This material was further hydrolysed with 0.125 M sulphuric acid at 100° in six steps of 6 h each with the intermediate removal of di- and tri-saccharides by means of a Sephadex G-25 column. Four different di- and trisaccharides, designated A_2, A_2', A_3, and A_3' were obtained in low yield (the degradation of galacturonic acid residues lay within the range 60—80 %). The disaccharides (90 mg) were partly resolved on Sephadex G-25 and further purified on thick filter papers. The trisaccharides (35 mg) were fractionated on thick filter papers to give pure A_3 and A_3'. Some properties of the oligosaccharides are summarised in Table 5. As indicated by the low optical rotations of the disaccharides (values in the range 140—150° were expected) some impurities still remained after the purification process. The disaccharides were characterised as follows.

A_2 (40 mg, [α] +119°) was chromatographically identical to 4-*O*-α-D-galactopyranuronosyl-D-galacturonic acid. It gave only galacturonic acid on hydrolysis. A sample was dissolved in 0.04 M sodium hydroxide (*c*, 0.47) and the change in optical rotation was followed polarimetrically. After 140 h the ratio starting material to galacturonic acid was examined qualitatively by paper chromatography. The disaccharide was degraded with a similar rate to authentic 4-*O*-α-D-galactopyranuronosyl-D-galacturonic acid.

A_2' (19 mg) had [α] +101° (*c*, 0.6) and gave galacturonic acid only on hydrolysis. It was only very slowly hydrolysed by pectinase. It was degraded 3—4 times faster by 0.04 M sodium hydroxide than A_2, indicating that it possessed a 3-*O*-α-D-galactopyranuronosyl-D-galacturonic acid structure.

A_3 (19 mg) was chromatographically indistinguishable from the trigalacturonic acid formed on partial hydrolysis of citrus pectin. A partial hydrolysate (0.25 M sulphuric acid, 3 h, 100°) contained, besides starting material, only A_2 and galacturonic acid. Its structure is therefore *O*-α-D-galactopyranuronosyl-(1→4)-*O*-α-D-galacturonosyl-(1→4)-D-galacturonic acid.

A_3' (9 mg) gave on partial hydrolysis, besides galacturonic acid, A_2' and A_2. A sample reduced with sodium hydridoborate gave on partial hydrolysis only A_2'. A sample (3 mg) was oxidised with periodate and further processed as described for XA_2 above. Galacturonic acid was present in the final hydrolysate. It was hydrolysed by pectinase slower than XA_2 and XA_3. The products were comparable amounts of galacturonic acid and A_2'. These findings suggest the following as possible structure: *O*-α-D-galactopyranuronosyl-(1→3)-*O*-α-D-galactopyranuronosyl-(1→4)-D-galacturonic acid.

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