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Structural Investigations on the Non-starchy Polysaccharides of Apples

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SUMMARY

Stepwise extraction of cell wall materials from apples with water, aqueous ammonium oxalate and dilute alkali yields polysaccharide preparations from which three components have been isolated on further fractionation in sufficiently homogeneous form for more detailed investigation. Structural studies have furnished evidence for (a) a highly branched α-L-arabinofuranan; (b) pectin fractions of the same general type in which linear 4-linked α-D-galacturonan chains carry α-L-arabinofuranan sub-units; and (c) a fucogalactoxyloglucan.

INTRODUCTION

The term dietary fibre refers to a chemically heterogeneous group of substances consisting largely of non-starchy polysaccharides, which are only slowly digested in the upper gastro-intestinal tract and exert various beneficial physiological effects (Burkitt & Trowell, 1975; Kay, 1982). These effects differ from one material to another amongst cereal, fruit and vegetable sources, and are probably associated with different polysaccharide components, either individually or in combination. 'Fibre' from various sources may include not only fibrous cellulose and associated polysaccharides, but also gel-forming matrix polysaccharides, and even actual water-soluble polysaccharides. In this and the following paper (Aspinall & Carpenter, 1984) we describe experiments to charac-

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terise the major polysaccharide components of two food materials in sufficient detail to establish their chemical identities.

The non-starchy polysaccharides from plants fall into a limited number of structural families (Aspinall, 1980) which include cellulose, other β-D-glucans of the 3-linked type (callose) and of the mixed 3- and 4-linked type from cereals, xyloglucans, the various xylans, the pectic substances, i.e. galacturonans and rhamnogalacturonans, and associated arabinans, galactans and arabinogalactans of type I (4-linked), arabinogalactans of type II (3,6-linked), galactomannans and glucomannans. However, determinations of individual sugar constituents are rarely sufficient to establish the nature of the parent polysaccharides since the same sugars occur in various combinations and linkage types in different polysaccharides. Wherever possible it is desirable to isolate individual polysaccharides for detailed structural analysis. The fractionation of all polysaccharides from a given source is not always readily achieved, but it is often possible to identify polysaccharides in incompletely separated mixtures, and even in cell wall preparations, through compositional analysis supported by analysis of linkage types by the methylation procedure.

The first major task is to obtain a plant preparation, such as a cell wall material (CWM) (Selvendran, 1975; O'Neill & Selvendran, 1980), from which non-polysaccharide cytoplasmic substances have been removed with minimum loss of the more soluble polysaccharide. A second task, which may sometimes be performed at the same time, is that of starch removal which may be achieved by preferential solubilisation or selective enzymic depolymerisation. Some of the problems encountered here are discussed in the following paper (Aspinall & Carpenter, 1984). Once a suitable CWM or similar material has been prepared, graded extractions may be performed with water alone or with added chelating reagents to obtain pectic substances, and then with dilute alkali to obtain the so-called hemicelluloses. There is no simple or even consistent relationship between solubility or ease of extraction and detailed chemical structure. However, these 'broad spectrum' classes of polysaccharides are operationally convenient and more chemically homogeneous preparations may often be obtained on further fractionation. We have previously reported (Aspinall *et al.*, 1983) such an approach to the non-starchy polysaccharides of carrots together with some compositional data of CWM from apples. We describe here a more detailed examination of the apple polysaccharides.

RESULTS AND DISCUSSION

Apple pectin is well known as a commercial material and numerous chemical studies of sugar composition of different pectin preparations have been reported, especially in relation to fruit ripening and food processing (Knee, 1970; Knee *et al.*, 1975; Rombouts & Pilnik, 1978; de Vries *et al.*, 1981), and to biosynthesis (Barrett & Northcote, 1965). There is, however, little information reported on the structural chemistry of apple pectin and even less on the nature of other polysaccharides in apples. We describe here an examination of three polysaccharide components of apples which gives information on polysaccharides based on galacturonic acid, arabinose and xylose, which, together with glucose (mainly but not exclusively from cellulose), are the main sugar constituents of carbohydrate polymers of apples.

We have reported previously (Aspinall *et al.*, 1983) the preparation from apples of cell wall material (CWM) and of an alcohol insoluble

TABLE 1

Sugar Composition (%) of Apple CWM and AIR, and Selected Polysaccharide Fractions Extracted with (A) Cold Water, (B) Hot Water, (C) Ammonium Oxalate and (E) Sodium Hydroxide

Source (extract):	*CWM*	*(B)*	*(C)*	*(E)*	*AIR*	*(A)*	*(B)*	*(C)*
Yield from source (%):	*100*	*17*	*10*	*14*	*100*	*6*	*14*	*12*
Sugar								
Rhamnose	2	3	3	2	2	3	2	3
Fucose	2	–	<1	7	2	2	<1	2
Arabinose	12	23	22	10	12	9	23	21
Xylose	6	2	4	29	8	2	1	3
Mannose	1	–	1	4	1	1	<1	–
Galactose	6	5	14	11	6	6	5	8
Glucose[a]	2	1	3	31	2	3	3	1
	(23)				(18)			
Uronic acid[b]	15	55	37	6	17	54	58	64

[a] Figures in parentheses indicate values obtained after digestion with 72% sulphuric acid, followed by dilution and hydrolysis.

[b] Estimated as D-galacturonic acid with the 3-hydroxydiphenyl reagent (Blumenkrantz & Asboe-Hansen, 1973).

residue (AIR). The results of overall sugar analyses of these materials and of polysaccharide fractions isolated from them are summarised in Table 1. The following salient points may be observed: (1) the general similarity in composition of CWM and AIR indicating only small losses of polysaccharides during the preparation of the former material; (2) the general similarity in composition (mainly galacturonic acid and arabinose) of polysaccharide fractions isolated by extraction with water and aqueous ammonium oxalate, pointing to the presence of pectin, possibly associated with neutral polysaccharide, as the major component; and (3) the presence of xylose together with glucose as the main sugar constituents of the alkali-soluble hemicellulose fraction from CWM. These polysaccharide fractions were examined as representative of the major polysaccharide components of apples.

The polysaccharide fraction AIR-B, which was arbitrarily chosen as a typical pectin-rich material, was fractionated by ion-exchange chomatography on DEAE-Sephacel (acetate form) (Table 2). Elution

TABLE 2

Sugar Composition (%) of Polysaccharide Fractions Obtained from Pectin-rich Preparation AIR-B by Ion-exchange Chromatography on DEAE-Sephacel (Acetate Form)

Fraction:	*B1*	*B2*	*B3*	*B4*
Eluent:	*H_2O*	*0·1 M KOAc*	*0·2 M KOAc*	*0·5 M KOAc*
Yield (%):	*18*	*10*	*11*	*28*
Sugar				
Rhamnose	–	1	3	2
Arabinose	85	42	20	8
Xylose	2	3	1	2
Mannose	1	1	–	–
Galactose	4	11	5	2
Glucose	5	2	1	1
Uronic acid[a]	–	30	66	83
Degree of esterification[b]	–	73	42	27

[a] Estimated as D-galacturonic acid with 3-hydroxydiphenyl reagent (Blumenkrantz & Asboe-Hansen, 1973).

[b] Based on determination of methoxyl content.

with water gave a neutral polysaccharide fraction AIR-B1 with arabinose as the major constituent sugar. This fraction afforded an essentially pure arabinan (arabinose, 96%; galactose 3%) on selective precipitation with cetyltrimethylammonium hydroxide (Rees & Richardson, 1966). Further elution of the column with potassium acetate buffers of increasing concentration gave three pectin fractions (AIR-B2, AIR-B3 and AIR-B4) which differed in the proportions of uronic acid residues, in the degree of esterification of those residues, and in the proportions of the same neutral sugar constituents, of which arabinose was the most abundant. Small-scale methylation studies indicated the presence of similarly linked neutral sugar constituents in these fractions. It is noteworthy that when fraction B3 was re-chromatographed on DEAE-Sephacel it was eluted in a single fraction, unchanged in acidic and neutral sugar composition. It may be concluded therefore that the neutral sugars are integral constituents of the acidic polysaccharide and do not arise from associated neutral polysaccharide such as the arabinan despite the demonstration (*vide infra*) of similarly linked arabinofuranose residues in neutral and acid polysaccharides. It is also probable that the pectin fractions are discrete polysaccharides of the same general type but with different proportions of sugar constituents.

The arabinan had a strongly negative optical rotation, $[\alpha]_D = -180°$, characteristic of other α-L-arabinofuranans. The ^{13}C n.m.r. spectrum of the polysaccharide was very similar to that reported by Joseleau *et al.* (1977) for an arabinan from *Rosa glauca* and the following features provided strong evidence for a branched structure containing α-L-arabinofuranose residues: (1) three signals for anomeric carbons at $\delta_C = 109{\cdot}1$, 108·7, and 107·9; (2) eight resonances between δ_C values of 85·6 and 78·5, probably due to C-2, C-3 and C-4 of differently situated residues, but an absence of resonances in the range 78–70 ppm normally present with glycopyranose residues; and (3) a group of incompletely resolved signals centred at 68·2 and a sharp signal at 62·8 ppm in the approximate ratio of 2:1 which probably arose from glycosylically substituted and unsubstituted (at O-5) C-5 carbons. The highly branched nature of the polysaccharide was substantiated by methylation analysis which furnished the sugars listed in Table 3, which were identified by gas-liquid chromatography–mass spectrometry (g.l.c./m.s.) of the derived partially methylated alditol acetates (Lindberg, 1972).

In order to obtain information on the distribution of the most abundant linkages, namely those of the $1 \rightarrow 5$ and $1 \rightarrow 3$ types, in the

TABLE 3

Relative Proportions (% of total) of Methyl Ethers of Arabinose Formed on Hydrolysis of Methylated Arabinan[a] (A) and Mixture of Methylated Arabinosylglycerols (B) Derived from Smith Degradation

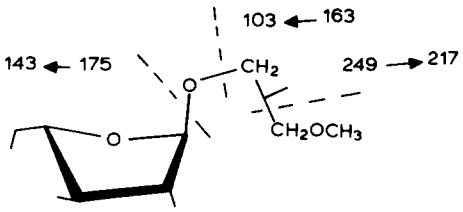
Sugar[b]	*A*	*B*
2,3,5-Me_3 Arabinose	36	59
2,3-Me_2 Arabinose	32	28
2,5-Me_2 Arabinose	4	7
3,5-Me_2 Arabinose	–	3
2-Me Arabinose	20	3
Arabinose	8	–

[a] No methyl ethers of galactose were detected. It is concluded that the small proportion of these sugar residues in the arabinan preparation arose from a separate polysaccharide.

[b] Sugars formed were estimated and identified by g.l.c./m.s. of the derived partially methylated alditol acetates.

branched arabinan, the polysaccharide was submitted to a Smith degradation (Rees & Richardson, 1966) involving sequential periodate oxidation, reduction and mild acid hydrolysis, and afforded a syrupy mixture of *O*-glycosylglycerols. Methylation analysis (Table 3) provided information on those sugar units which had resisted periodate oxidation The presence of a much higher proportion of arabinofuranosyl end groups than of non-terminal units and an even smaller proportion of potential branching units implied the presence of an *O*-arabinofuranosylglycerol arising from isolated periodate-resistant residues at branch points. The methylation evidence also indicated that mutually linked arabinofuranose residues were most commonly, but not exclusively, joined by $1 \rightarrow 5$ bonds. The mixture of methylated derivatives was then examined directly by g.l.c./m.s. The three volatile components detected were assigned structures* **1–3** on the basis of the major fragment ions

* In formulae **1**, **2**, **3**, **7**, **8** and **9** for permethylated oligosaccharide alditols substituents are — = OCH_3 and —′ = CH_2OCH_3. *m/e* values are shown for structurally significant fragment ions in the mass spectra.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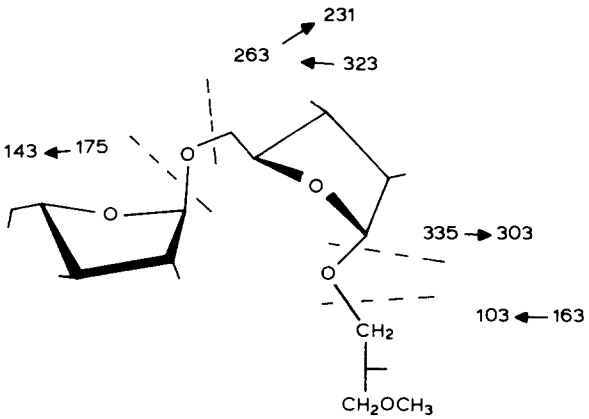
143 ← 175
CH2
249 → 217
O
O
CH2OCH3

1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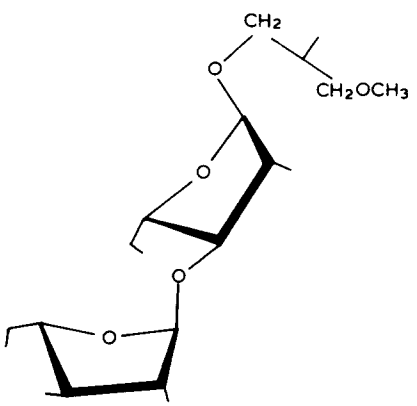
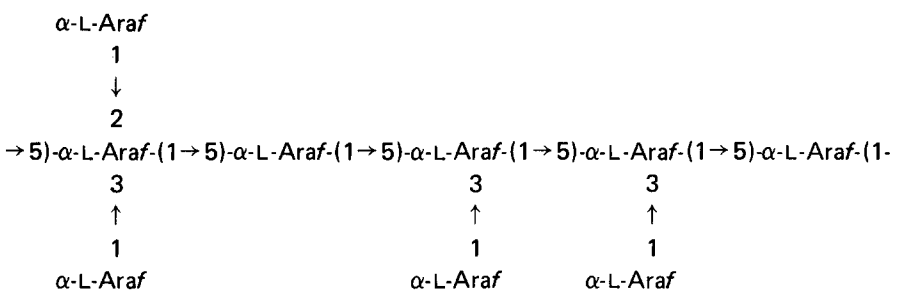
263
← 323
143 ← 175
O
O
O
335 → 303
O
103 ← 163
CH2
CH2OCH3

2


CH2
O
CH2OCH3
O
O
O

3

observed in their mass spectra. Compound **1** was the anticipated arabinofuranosylglycerol. Compounds **2** and **3** were isomeric arabinobiosylglycerols, presumably one with a $1 \rightarrow 5$ and the other with a $1 \rightarrow 3$ intersugar linkage, but the available evidence did not permit individual identification. These two compounds were detected in approximately equimolar proportions, but since the mixture of products formed in the Smith degradation contained a higher proportion of $1 \rightarrow 5$ than of $1 \rightarrow 3$ bonds it may be inferred that higher oligosaccharides containing $1 \rightarrow 5$ bonds only were present. The main conclusion to be drawn from these results is that apple arabinan contains as its major structural feature $(1 \rightarrow 5)$-linked chains of α-L-arabinofuranosyl residues to which, at intervals, single unit side-chains of other α-L-arabinofuranosyl residues are joined by $(1 \rightarrow 3)$-bonds (**4**). In this respect the polysaccharide resembles mustard seed arabinan (Rees & Richardson, 1966) but the



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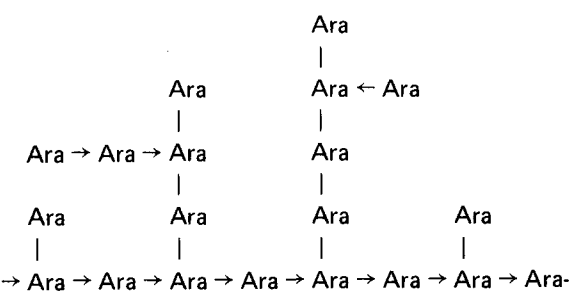


5

α-L-arabinofuranose residues are joined through $(1 \rightarrow 5)$-linkages (→) and $(1 \rightarrow 3)$-linkages (—) [or occasionally $(1 \rightarrow 2)$-linkages]

complete structure cannot be accommodated in a comb-like model. Regions of the structure must include more ramified arrangements of units with some degree of multiple branching as in the partial structure **5**. In connection with the distribution of different linkage types in the branched arabinan the recent isolation and characterisation of an essentially linear 5-linked α-L-arabinofuranan from a commercial sample of apple juice (Churms *et al.*, 1983) is of interest. Such an attenuated arabinan could arise either from incomplete biosynthesis or from selective enzymic breakdown of the complete macromolecule.

In an attempt to define the structural role of the neutral sugar residues in the constitutionally related pectin fractions a pectin preparation B5, which probably contained the above-mentioned pectin fractions AIR-B2, AIR-B3 and AIR-B4, was separated from neutral arabinan by preparative-scale ion-exchange chromatography. Linkage analysis by methylation as applied to pectins is complicated by the twin problems of achieving complete methylation without substantial base-catalysed degradation and of obtaining complete depolymerisation without extensive decomposition during hydrolysis (or other acid-catalysed depolymerisation). Alternatively, preparation of carboxyl-reduced pectic acid, even with complete reduction achieved and unaccompanied by degradation, fails to maintain a distinction between galactose residues originally present and those formed on reduction of galacturonic acid residues, but in other respects provides the basis for an overall linkage analysis profile for the polysaccharide. Both approaches were adopted for pectin fraction B5.

Permethylated apple pectin was prepared by the Hakomori procedure (cf. Lindberg, 1972). Depolymerisation by methanolysis followed by acetylation gave methyl glycoside methyl esters of galacturonic acid derivatives whose qualitative examination by g.l.c. suggested some degree of incomplete methylation in the interior chains. Nevertheless direct hydrolysis to give neutral methylated sugars which were characterised by g.l.c./m.s. of the derived partially methylated alditol acetates (Table 4) appeared to give a reasonably representative assessment of neutral sugars as judged by the relative proportions of methyl ethers of arabinose (the most abundant neutral sugar) present as end groups and branch points. A separate sample of pectin was converted into the carboxyl-reduced polysaccharide by treatment with sodium borohydride and water-soluble carbodiimide (Taylor & Conrad, 1972). Although the reduction was incomplete the relative proportions of

TABLE 4

Relative Proportions of Methylated Sugars Formed on Hydrolysis of Methylated Pectin Fraction AIR-B5 and its Methylated Carboxyl-reduced Derivative AIR-B5-R

Sugar[a,b]	*Methylated polysaccharide*		*Structural unit*
	AIR-B5	*AIR-B5-R*	
2,3,5-Me_3 Ara	9	8	Ara*f* 1-
2,3-Me_2 Ara	7	7	-5 Ara*f* 1-
2-Me Ara	5	5	-3,5 Ara*f* 1-
Ara	4	4	-2,3,5 Ara*f* 1-
3,4-Me_2 Rha	3	2	-2 Rha*p* 1-
3-Me Rha	1	1	-2,4 Rha*p* 1-
2,3,4,6-Me_4 Gal	3	3	Gal*p* 1-
2,3,6-Me_3 Gal	2	2	-4 Gal*p* 1-
		38	-4 Gal*p*A 1-
2,6-Me_2 Gal		3	-3,4 Gal*p*A 1-

[a] Sugars formed were estimated and identified by g.l.c./m.s. of the derived partially methylated alditol acetates.

[b] Relative proportions are normalised according to the total proportions of each neutral sugar constituent in the parent polysaccharide sample (see Table 5).

neutral sugars other than galactose were unchanged indicating that in this respect the modified polysaccharide was representative of the parent pectin (Table 5). The results of methylation analysis of the carboxyl-reduced polysaccharide are shown in Table 4 where the relative proportions of methylated sugars are normalised with reference to those formed from the methylated pectin and to the neutral sugar content of the original pectin. The most striking feature of apple pectin is that residues of arabinose, an obligatory and the main neutral sugar constituent, are present in branched chains. Because of the relative proportions of terminal furanose residues and of those involved in branching, most of the arabinose residues must be mutually linked. The type of structure of this segment of the pectin is generally similar to that of the neutral arabinan although there is at present no evidence

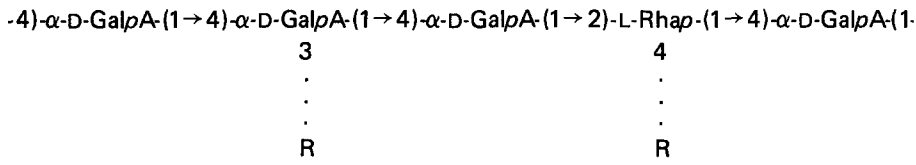
TABLE 5
Sugar Composition (%) of Pectin Fraction AIR-B5 and its Carboxyl-reduced Derivative AIR-B5-R

Sugar	*AIR-B5*	*AIR-B5-R*
Rhamnose	3	3
Arabinose	22	21
Xylose	1	2
Galactose	5	41
Glucose	2	2
Uronic acid[a]	57	6
Degree of esterification[b]	60	–

[a] Estimated as D-galacturonic acid with the 3-hydroxydiphenyl reagent (Blumenkrantz & Asboe-Hansen, 1973).
[b] Based on determination of methoxyl content.

concerning the distribution of $1 \rightarrow 5$ and $1 \rightarrow 3$ linkages. Galactose residues in the carboxyl-reduced polysaccharide clearly arise from galacturonic acid residues joined by $1 \rightarrow 4$ bonds in linear chains with branch points at rather infrequent intervals. The small proportion of galactose residues originally present in the pectin contains a high relative proportion of end groups and can only be accommodated as constituents of a heteropolysaccharide, most likely as short side-chains to the galacturonan chain. In common with many pectins the apple polysaccharide contains 2-*O*-substituted rhamnopyranose residues, some of which are probably branch points. No direct evidence is available to confirm whether these residues interrupt galacturonan segments in the main chain, but the general structure of apple pectin may be summarised in formula **6** with some uncertainty as to the points of connection between different regions in the structure.

Polysaccharide fraction CWM-E contained the highest proportion of xylose residues and was selected in order to determine whether this sugar originated from a polysaccharide of the xyloglucan (amyloid) group and/or one of the xylan family. Methylated derivatives of these two types of polysaccharide on hydrolysis afford *inter alia* 3,4- and 2,3-di-*O*-methyl-D-xylose. Characterisation of these sugars by conversion into partially methylated alditol acetates results in the formation of



6

α-D-galacturonan chains with occasional attachment of side-chains, where R = $[\alpha\text{-L-Araf}]_n$ with similar linkage types to those in arabinan **5** or D-Gal*p* in single unit or disaccharide chains

enantiomers whose relative proportions may be determined only from fragment ions in the mass spectra of 1-*d*-labelled alditols formed on treatment with sodium borodeuteride. Since the polysaccharide preparation still contained a small proportion of uronic acid residues which could have arisen from incomplete removal of pectin during earlier extractions, the material was fractionated by ion-exchange chromatography (Table 6). The first two fractions CWM-E1 and E2, eluted with

TABLE 6

Sugar Composition (%) of Polysaccharide Fractions Obtained from Preparation CWM-E by Ion-exchange Chromatography on DEAE-Sephadex (Acetate Form)

Fraction:	*E1*	*E2*	*E3*	*E4*	*E5*
Eluent:	*H_2O*	*0·1 M KOAc*	*0·2 M KOAc*	*0·3 M KOAc*	*0·6 M KOAc*
Yield (%):	*32*	*26*	*5*	*10*	*11*
Sugar					
Rhamnose	–	–	–	4	4
Fucose	6	7	6	3	1
Arabinose	2	2	2	25	18
Xylose	22	26	27	17	16
Mannose	1	7	5	2	1
Galactose	12	13	12	11	8
Glucose	28	39	36	11	7
Uronic acid[a]	–	–	–	22	28

[a] Estimated as D-galacturonic acid with the 3-hydroxydiphenyl reagent (Blumenkrantz & Asboe-Hansen, 1973).

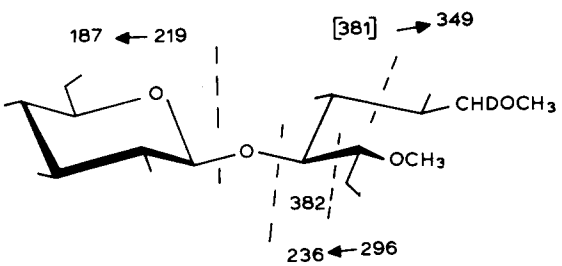
TABLE 7
Relative Proportions of Methylated Sugars Formed on Hydrolysis of Methylated Polysaccharide Fractions E1 and E2

Sugar[a]	*Fraction E1*	*Fraction E2*
2,3,4-Me_3 Fuc[b]	–	–
2,3,4-Me_3 Xyl[b]	23	15
2,3-Me_2 Xyl[c]	–	9
3,4-Me_2 Xyl[c]	12	17
2,3,4,6-Me_4 Gal	6	9
3,4,6-Me_3 Gal	5	4
2,3,6-Me_3 Glc	17	15
2,3-Me_2 Glc	35	20
2,3,6-Me_3 Man	1	6

[a] Sugars formed were estimated and identified by g.l.c./m.s. of the derived partially methylated alditol acetates using columns A and B. A number of minor components whose identities were not confirmed are omitted.
[b] Derivatives of these sugars were not separated but the presence of both derivatives was established from the mass spectra.
[c] The relative proportions of these enantiomeric sugars were determined from the ratios of fragment ions 117/118 and 189/190 in the mass spectra of the 1-*d*-labelled alditols formed on treatment with sodium borodeuteride.

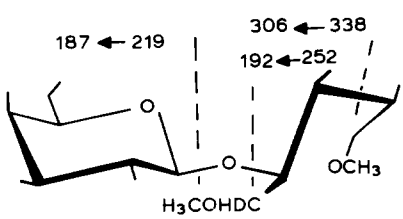
water and with 0·1 M potassium acetate had compositions suggesting the probable presence of a xyloglucan and these fractions were examined by methylation (Table 7). The results confirmed this possibility and showed that the non-terminal xylose residues in fraction E1 were exclusively 2-linked whereas some of those in fraction E2 were 4-linked, suggesting that a 4-linked xylan may be present as a minor polysaccharide component. This latter fraction also contained some 4-linked mannose residues which presumably originated from a galactomannan or glucomannan as a minor component of the polysaccharide mixture.

Fraction E1 was therefore taken as the most highly purified xyloglucan and a sample was depolymerised by partial acetolysis, as described for other xyloglucans (Aspinall *et al.*, 1977). The mixture of sugar acetates thus generated was de-*O*-acetylated, reduced with sodium borodeuteride and methylated. Examination of the products by g.l.c./m.s. showed the presence of three oligosaccharide derivatives whose structures (**7**, **8** and **9**) may be inferred from the major fragment ions shown and from the known linkage types of sugar residues in the polysaccharides whose structures are shown in parenthesis in shorthand form. Compound **7** was indistinguishable from synthetic permethylated cellobiitol-1-*d*. Compounds **8** and **9** arise from side-chain units attached to the 4-linked β-D-glucan core. Although no oligosaccharides containing xylose and glucose residues were detected as products of partial depolymerisation, linkage analysis data indicated attachment of side-chains through O-6 of glucose residues as the sole branch points in the polysaccharide. Structure **10** may therefore be advanced for the fucogalactoxyloglucan



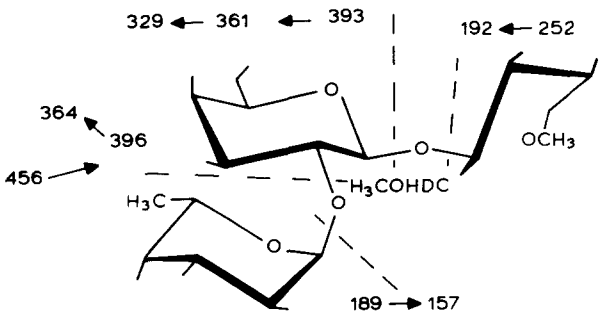
7

[β-D-Glc*p*-(1 → 4)-D-Glc*p*-(1-]



8

[β-D-Gal*p*-(1 → 2)-D-Xyl-(1-]



9

[α-L-Fuc*p*-(1 → 2)-β-D-Gal*p*-(1 → 2)-D-Xyl-(1-]

→4)-β-D-Glc*p*-(1→4)-β-D-Glc*p*-(1→4)-β-D-Glc*p*-(1→4)-β-D-Glc*p*-(1→4)-β-D-Glc*p*-(1-

6 6 6
↑ ↑ ↑
1 1 1
α-D-Xyl*p* α-D-Xyl*p* α-D-Xyl*p*
2 2
↑ ↑
1 1
β-D-Gal*p* β-D-Gal*p*
2
↑
1
α-L-Fuc*p*

10

Representative structure for fucogalactoxyloglucan in which anomeric configurations have been assigned by analogy with other polysaccharides of this type, and in which spacing of side-chains is arbitrary

as the main xylose-containing polysaccharide in apples. Similar xyloglucans have been isolated from several sources including rapeseed hulls (Aspinall *et al.*, 1977), mung beans (Kato & Matsuda, 1980*a*,*b*) and runner beans (O'Neill & Selvendran, 1983).

The results of this study indicate that, in addition to cellulose whose presence is inferred rather than directly proven, three polysaccharides,

an arabinan, a pectin (largely an arabinogalacturonan) and a fucogalactoxyloglucan, account for the main carbohydrate polymers in apples and are the materials of which account must be taken in an assessment of the dietary fibre content in apples. It may be pointed out that these conclusions could not have been reached on the basis of sugar compositional data alone without extensive polysaccharide fractionation supported by reasonably detailed structural examination. Parallel studies in this laboratory on carrots (Aspinall *et al.*, 1983) showed some general similarities in overall sugar composition. Both materials contain pectin as the main non-cellulosic polysaccharide and some associated neutral arabinan. Whereas apple pectin is largely an arabinogalacturonan, carrot pectin, which is under further examination, contains a much lower proportion of neutral sugar residues with galactose as the main component. The xylose-containing polysaccharides are quite different with an acidic xylan accounting for most of the sugar in carrots, whereas this sugar in apples arises largely from the fucogalactoxyloglucan.

After the preparation of this manuscript, de Vries *et al.* (1983) reported the results of a study of the neutral sugar sidechains of apple pectic substances. These authors reached similar but more extensive conclusions to those described here concerning these aspects of the pectin structure.

EXPERIMENTAL

General methods

Gas-liquid chromatography was performed with a Perkin-Elmer Sigma 3B gas chromatograph fitted with a flame ionisation detector using (A) a packed column of 3% of silicone gum Silar 10 CP on Chromosorb W-HP (100/200 mesh); (B) a S.C.O.T. column coated with silicone gum OV-225; or (C) a packed column of 3% silicone-polyester copolymer ECNSS-M on Gas Chrom Q. A Perkin-Elmer Data System Sigma 10B was used for peak integration. For g.l.c./m.s. columns (B), (C) or (D), a glass capillary column of silicone DB5-15N (permanently bonded OV-54) were used in a Pye-Unicam series 204 gas chromatograph connected by a jet separator to a VG Micromass 16F mass spectrometer and VG data system 2000, operated with an inlet temperature of

~ 250°C, an ionisation potential of 70 eV, and an ion-source temperature of ~ 250°C. ^{13}C n.m.r. spectra were measured with a Bruker WH-400 spectrophotometer operating at 100·6 MHz. Optical rotations were measured with a Perkin-Elmer model 141 polarimeter at 20 ± 2°. Evaporations were carried out under diminished pressure at temperatures of 40°C or less.

Sugar and other analyses

Hydrolyses of polysaccharide fractions and cell wall preparations were performed (a) directly with trifluoroacetic acid (Albersheim *et al.*, 1967); and (b) after digestion with 72% sulphuric acid and dilution, with M sulphuric acid (Adams, 1965). D-Allose was added as an internal standard and the sugar mixtures were converted into alditol acetates (Sloneker, 1972) for analysis by g.l.c. on column (A). Uronic acid determinations were carried out spectrophotometrically with the 3-hydroxydiphenyl reagent (Blumenkrantz & Asboe-Hansen, 1973). In the case of insoluble materials determinations were performed after digestion with 72% sulphuric acid and appropriate dilution. Estimation of degree of esterification of uronic acid residues was based on methoxyl determination by the micro-Zeisel method.

Methylation analyses

Polysaccharide samples were methylated by the Hakomori procedure as described by Lindberg (1972). In the case of acid polysaccharides, especially for pectins, de-esterification was first carried out at 4°C with sodium hydroxide at pH 12 and, in order to minimise base-catalysed degradations, methylations were performed with restricted proportions of reagents (Conrad, 1972). Completeness of methylation was checked by the absence of hydroxyl bands in the infrared spectra. Neutral methylated sugars formed on hydrolysis were converted into the derived partially methylated alditol acetates for analysis by g.l.c. alone using columns (B) or (C) or with confirmations of identities by g.l.c./m.s. on columns (B) or (C). Methylated acid polysaccharides were methanolysed with methanolic 3% hydrogen chloride for 16 h at 100°C in sealed tubes, and the resulting methyl glycosides were acetylated for g.l.c. examination on column (B).

Isolation of polysaccharide fractions

The preparation of apple CWM and AIR, and the extraction of pectin-rich fractions CWM-B and CWM-C, and AIR-A, AIR-B and AIR-C have been described previously (Aspinall *et al.*, 1983). The CWM residue C which remained after extraction of pectin fraction C was suspended in water (1·8 litres) and delignified by treatment with sodium chlorite (5·4 g) and glacial acetic acid (22 ml) at 70° in an inert atmosphere. The suspension was stirred for 15 min and the residue was separated by centrifugation. The procedure was repeated twice and the residue D was washed three times with water. The combined extracts and washings were dialysed against distilled water and freeze-dried to give polysaccharide fraction D (2·0 g). This fraction, which was not examined further, had the following sugar analysis (percentages): rhamnose, 2; arabinose, 31; xylose, 6; mannose, 1; galactose, 11; glucose, 4; uronic acid, 21. Residue D was treated with aqueous sodium hydroxide at pH 12 at 4°C in order to ensure pectin de-esterification with minimum base-catalysed degradation. The residue was then extracted by stirring in M sodium hydroxide (1·5 litres) for 2 h at room temperature. The residue was separated by centrifugation, extracted again in like manner and separated. The combined extracts were neutralised with acetic acid, dialysed against distilled water, and freeze-dried to give polysaccharide fraction E (4·6 g) (see Table 1).

Fractionation of pectin-rich preparation AIR-B

Polysaccharide fraction AIR-B (2·0 g) was chromatographed on a column (3·6 × 60 cm) of DEAE-Sephacel (acetate form) which was eluted successively with water, and with 0·2 M, 0·3 M and 0·5 M potassium acetate buffered at pH 5·5 to give, respectively, fractions B1 (350 mg), B2 (200 mg), B3 (220 mg) and B4 (550 mg). In each case the eluates were dialysed as required and the fractions were isolated by freeze-drying. The results of analyses of neutral sugars, uronic acid and methoxyl content (and hence degree of esterification) are given in Table 2. A portion of fraction B3 was re-chromatographed with the same sequence of eluents. The polysaccharide was recovered in 95% yield and unchanged in composition on elution with 0·3 M potassium acetate. Samples of fractions B2, B3 and B4 were methylated and

examination of the neutral methylated sugars formed on hydrolysis after conversion into partially methylated alditol acetates for g.l.c. analysis showed the same sugar constituents in each fraction (as for B5 in Table 4). A further sample of a representative pectin B5 (460 mg) (see Table 5) was obtained by elution with 0·5 M potassium acetate buffered at pH 5·5 after elution of fraction B1 (220 mg) with water.

Examination of fraction B1 and characterisation of arabinan

Cetyltrimethylammonium hydroxide solution (10% w/v, 3 ml) was added to fraction B1 (350 mg) in water (40 ml). On addition of M sodium hydroxide (3 ml) a flocculent precipitate formed and was removed by centrifugation. The precipitate was dissolved in water (40 ml) containing sufficient acetic acid for neutralisation and the polysaccharide was reprecipitated on addition of M sodium hydroxide. The precipitate was again dispersed in dilute acetic acid and the solution was deionised by successive treatments with Dowex 1X4 (HCO_3^-) and 50X8 (H^+) resins, and freeze-fried to give arabinan (150 mg) – found: arabinose 96%, galactose, 3%; $[\alpha]_D = -180°$ (*c.* 0·43 in water); n.m.r. data (D_2O): δ_C = 109·1, 108·7, 107·9 (C-1), 85·6, 85·5, 83·9, 83·8, 83·0, 82·9, 82·5, 78·5 (various C-2, C-3 and C-4), 68·5, 68·2 (C-5 substituted), 62·8 (C-5 unsubstituted). A sample of arabinan was methylated and the results of g.l.c./m.s. analysis of the derived 1-*d*-labelled partially methylated alditol acetates are shown in Table 3.

Arabinan (135 mg) was oxidised in 0·04 M sodium metaperiodate (50 ml) in the dark at room temperature, and the consumption of oxidant was monitored spectrophotometrically by withdrawal of aliquot portions and dilution (× 250) (Aspinall & Ferrier, 1957). Consumption of reagent reached a constant value corresponding to 0·67 mol per mol arabinose residue after 48 h, excess of oxidant was destroyed by the addition of a calculated quantity of ethanediol, sodium borohydride (5 mmol) was added and the solution was kept for 48 h. The solution was deionised successively with Amberlite resins IR-120 (H^+) and IR-45 (OH^-), and concentrated to dryness by co-distillation with toluene and then methanol. The residue was kept in 0·5 M sulphuric acid (4 ml) at room temperature for 24 h, and the solution was neutralised with barium carbonate, filtered and concentrated. The dry residue was methylated to furnish a mixture of permethylated *O*-glycosyl-

glycerols. The methylated derivatives were examined directly by g.l.c./m.s. on column B at 210°C, and three components were detected with retention times relative to that of the first component: $T = 1.00$ with fragment ions at $m/e = 249, 217, 205, 175, 163, 143$ and 103; $T = 3.2$ with fragment ions at $m/e = 335, 323, 303, 263, 231, 175, 143$ and 103; and $T = 3.9$ with fragment ions at $m/e = 335, 323, 263, 231, 175, 163, 143$ and 103. A portion of the mixture of methylated glycosylglycerols was hydrolysed and the products were converted into partially methylated alditol acetates for analysis by g.l.c./m.s. (see Table 3).

Examination of pectin fraction AIR-B5

Pectin fraction B5 was methylated and a portion of the methylated pectin was methanolysed, and the products were acetylated and examined by g.l.c. on column B to show the presence *inter alia* of methyl ester methyl glycoside acetates of 2,3-di-*O*-methylgalacturonic acid. A further sample of methylated pectin was hydrolysed and the resulting neutral methylated sugars were analysed as the derived partially methylated alditol acetates by g.l.c./m.s. on column B (Table 4).

A sample of pectin fraction B5 was treated three times with water-soluble carbodiimide and sodium borohydride (Taylor & Conrad, 1972) to give carboxyl reduced pectin AIR-B5-R whose sugar analysis is compared with that of the parent pectin in Table 5. The results of methylation analysis of the carboxyl-reduced polysaccharide are given in Table 4.

Examination of polysaccharide fraction CWM-E and characterisation of fucogalactoxyloglucan

Polysaccharide fraction CWM-E (350 mg) was chromatographed on a column (2·2 × 30 cm) of DEAE-Sephacel (acetate form) which was eluted successively with water, and with 0·1 M, 0·2 M, 0·3 M and 0·6 M potassium acetate to give respectively fractions E1 (110 mg), E2 (91 mg), E3 (18 mg), E4 (36 mg) and E5 (40 mg) whose sugar analyses are reported in Table 6. The results of methylation analyses of fractions E1 and E2 are given in Table 7. Fraction E1 (fucogalactoxyloglucan) was chosen for further examination and had $[\alpha]_D + 43°$ (*c.* 0·14 in water).

Fucogalactoxyloglucan (100 mg) was converted into the polysaccharide acetate (110 mg) (Carson & Maclay, 1948). The acetylated poly-

saccharide was added with stirring to a mixture of acetic anhydride (3 ml), acetic acid (3 ml) and concentrated sulphuric acid (0·3 ml) at 0°C and the mixture was stirred at room temperature. After 24, 48, 100 and 200 h, samples were withdrawn, poured into ice-water and the mixtures were extracted with chloroform, the chloroform extracts were washed with aqueous sodium hydrogen carbonate, dried and concentrated. Methanolic 0·5 M sodium methoxide was added dropwise to the syrupy acetates in methanol (2 ml) to maintain permanent alkalinity. The mixtures were kept for 1 h, treated with Amberlite IR-120 (H^+) resin to remove sodium ions, the filtered solutions were concentrated, the residues were reduced with sodium borodeuteride and the products were methylated. The resulting mixtures of permethylated alditol derivatives were examined by g.l.c./m.s. on column D at 210°C. In each case three oligosaccharide alditol derivatives were detected. Compound **7** was assigned the proposed structure on the basis of fragment ions at m/e = 382, 349, 297, 236, 219 and 187 and was shown to be indistinguishable from permethylated cellobiitol-1-*d*. Compound **8** was assigned the proposed structure on the basis of fragment ions at m/e = 382, 338, 306, 252, 219, 192, 187 and 160. Compound **9** was assigned the proposed structure on the basis of fragment ions at m/e = 456, 396, 393, 364, 361, 329, 252, 192, 189 and 157.

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