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Structural Features of the Neutral Sugar Side Chains of Apple Pectic Substances

J. A. de Vries, C. H. den Uijl, A. G. J. Voragen, F. M. Rombouts & W. Pilnik

Agricultural University, Department of Food Science, De Dreijen 12, 6703 BC Wageningen, The Netherlands

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SUMMARY

The 'hairy regions' of apple pectic substances (fragments of the rhamnogalacturonan chains carrying the neutral sugar side chains) have been subjected to enzymic, acidic and alkaline degradation. The results show that the hairy regions consist of rhamnogalacturonan fragments carrying arabinogalactans and galacturonan fragments carrying single unit xylose side chains. A separate population of molecules is present consisting of galacturonan main chains and side chains of 1,3/1,6-linked galactans. The structural relations of pectic substances from different plant species are discussed.

INTRODUCTION

In two preceding papers (de Vries *et al.*, 1981, 1982), the extraction and purification of pectic substances from apple AIS (Alcohol Insoluble Solids) have been described. The neutral sugar composition of these fractions and fractions obtained by enzymic degradation was determined. From these results a model of apple pectin molecules was proposed describing the intra- and inter-molecular distribution of the neutral sugar side chains. The molecules were thought to consist of 'smooth regions' (blocks of homogalacturonan) and 'hairy regions' (blocks of rhamnogalacturonan carrying side chains composed of arabinose, galactose, xylose and glucose).

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In this paper the structure of the hairy regions is discussed. The results of specific, selective as well as non-selective methods of degradation are reported. β-(1,4)-galactanase (Labavitch *et al.*, 1976) has been employed as a specific degradative agent. Mild acid hydrolysis was used as a selective method. Degradation of the fully esterified hairy regions by chemical β-elimination can be characterised as non-selective. The fragments obtained were fractionated by gel-permeation chromatography and the sugar composition of the fractions was determined. In addition, methylation analysis of some pectin fractions was performed.

MATERIALS AND METHODS

The preparation of purified pectin fractions and purified hairy regions
This was performed as described in the preceding papers (de Vries *et al.*, 1981, 1982).

Gel-permeation chromatography
A sample of (degraded) pectin 10–50 mg in 2 ml of buffer or water) was applied to a Sephacryl S-300 (Pharmacia) column (80 × 2·5 cm) or a Biogel P-2 (Biorad) column (70 × 1·5 cm) and eluted with water or 0·01 M sodium phosphate buffer of pH 5·5. The flow rate (0·3 ml min^{-1}) was controlled by an LKB peristaltic pump and the whole procedure was conducted at room temperature (S-300) or at 50°C (P-2).

Analytical methods
The anhydrouronic acid (AUA, M.W. 176) content of the pectin fractions was determined by an automated carbazole-sulphuric acid assay (van Deventer-Schriemer & Pilnik, 1976). The neutral sugars were analysed gas-chromatographically as their alditol-acetates (Albersheim *et al.*, 1967; Darvill *et al.*, 1975). Neutral sugars in column eluates were monitored by the anthrone assay.

Enzymic degradation
β-(1,4)-galactanase from *Bacillus subtilis* was purified on a DEAE-cellulose column (Labavitch *et al.*, 1976) and on an AH Sepharose-galactan column (Centen, 1979). No glycosidase activity or activity on arabans, xylans, cellulose, carboxy-methylcellulose or pectic acid could be detected. Reaction conditions: 150 mg of substrate and 0·02 units

of enzyme in 50 mM sodium acetate buffer of pH 6·0 (50 ml), 24 h reaction at 30°C.

Partial acid hydrolysis
This was performed with 0·01, 0·05 and 0·09 N TFA (trifluoroacetic acid) solutions for 1 h at 100°C in sealed test tubes containing about 200 mg of substrate in 5 ml of TFA.

β-Elimination
Pectin samples were heated in a 0·05 M sodium phosphate buffer, pH 6·0, for 2 h at 100°C.

Methylation analysis
The method of Hakomori (1964) as described by Talmadge *et al.* (1973) was used.

Carboxyl reduction
This was performed according to Taylor & Conrad (1972) using a Metrohm pH-stat. or by refluxing with $LiAlH_4$ (after methylation) in tetrahydrofuran for 8 h (Lindberg, 1972).

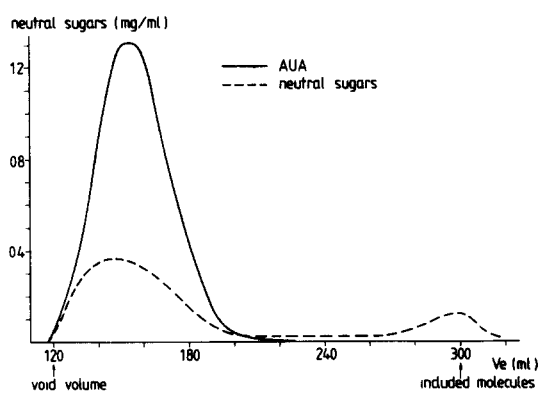

Fig. 1. Gel filtration (Sephacryl S-300) of a β-1,4-galactanase-degraded pectin fraction. AUA, anhydrouronic acid content. Ve, elution volume. The substrate was a DEAE-cellulose-purified pectin from the oxalate extract of AIS from ripe apples. Degradation with β-1,4-galactanase as described in the text. The eluent was water.

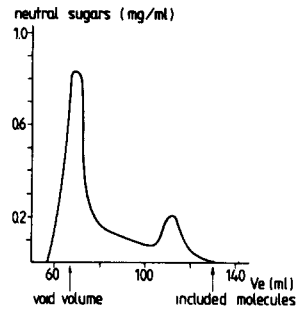

Fig. 2. Gel filtration (Biogel P-2) of the low molecular weight glycan fragments released by β-1,4-galactanase from apple pectin. Ve, elution volume. Substrate, the 'included molecules' shown in Fig. 1. The eluent was water. The second peak elutes from the column in the same elution volume as stachyose (tetramer).

RESULTS AND DISCUSSION

β(1,4)-galactanase action

In Figs 1 and 2 and in Tables 1 and 2, the results of β(1,4)-galactanase action on a pectin preparation are presented. The enzyme removes arabinose and galactose residues from the molecules. No xylose, glucose or rhamnose residues are released, which shows that blocks of arabinogalactans are present in the side chains. The arabinogalactan fragments released are mainly composed of arabinose (Table 1). From their elution volume it can be estimated that the araban side chains of the arabinogalactans have a degree of polymerisation of about 25. Preliminary experiments with a partially purified 1,5-arabanase indicated the presence of 1,5-linked arabans; this was confirmed by methylation analysis.

Methylation analysis and carboxyl reduction

Table 3 shows that about 1/3 of the arabinose residues have branch points. A large part of the arabinose residues are terminal; therefore, the arabans must be highly branched. Methylation analysis of carboxyl-reduced pectin preparations showed that some of the galacturonate

TABLE 1
Sugar Composition of the β-1,4-Galactanase-Degraded Pectin Fractions Shown in Fig. 1 (mg)

Sugar	*Elution volume (ml)*			*% Released**	*%Recovery*
	115–180	*180–280*	*280–320*		
Galacturonic acid	130	trace	trace		84
Rhamnose	1·9	trace	trace	2	71
Arabinose	11·2	7·3	1·4	44	93
Xylose	1·8	trace	trace	3	87
Galactose	2·7	2·4	0·9	55	88

* Present in elution volume 180–320 ml.

TABLE 2
Sugar Composition of the Low Molecular Weight Fraction Released from Apple Pectin by β-1,4-Galactanase and Fractionated as Shown in Fig. 2 (μg)

Sugar	*Elution volume (ml)*	
	60–100	*100–150*
Arabinose	1 220	160
Galactose	225	710

residues carry side chains. Table 3 shows that after reduction by the method of Taylor & Conrad (1972) small amounts of 2,6-dimethylgalactitol and 3,6-dimethylgalactitol appear in the chromatograms, indicating that some galacturonate residues are branched through their C-2 and C-3 atoms. Small quantities of partially methylated alditol acetates, however, can easily result from undermethylation. A complete reduction of the uronic acid residues, however, could not be achieved. The method of Taylor & Conrad (1972) resulted after repeated reaction in about 40% reduction and a separation of products and reagents was

TABLE 3

Methylation Analysis of Apple Pectins. The Substrates were 'Hairy Regions' Obtained by Gel-permeation Chromatography of Pectate Lyase Degraded Pectin Fractions (de Vries *et al.*, 1982)

Sugar residue	*Partially methylated alditol acetate*	*Mole %*		
		*a**	*b**	*c**
Arabinose	2,3,5-tri-O-Me	34·1	26·2	23·8
	2,5-di-O-Me	2·7	4·6	4·1
	2,3-di-O-Me	22·9	8·9	18·3
	2-mono-O-Me	14·0	6·4	11·8
	3-mono-O-Me	1·6	0·5	2·7
	0-O-Me	9·8	7·6	8·3
Galactose	2,3,4,6-tetra-O-Me	2·0	4·3	4·8
	2,3,6-tri-O-Me	7·8	4·1	10·7
	2,4,6-tri-O-Me	–	3·0	–
	2,3,4-tri-O-Me	–	0·7	0·6
	2,4-di-O-Me	–	14·4	3·8
	2,6-di-O-Me	–	–	0·6‡
	3,6-di-O-Me	–	–	0·7‡
	2-mono-O-Me	–	0·9†	–
	2,3-di-O-Me	–	3·1†	
Glucose	2,3,6-tri-O-Me	–	3·9	0·8
Xylose	2,3,4-tri-O-Me	1·7	4·0	2·1
	2,3-di-O-Me	2·3	4·9	–
Deoxyhexose	3,4-di-O-Me	0·3	1·3	3·1
	2,4-di-O-Me	–	1·3	2·7
	3-mono-O-Me	0·7	1·6	2·3

* a, Substrate from the acid extract of apple AIS; b, substrate from the cold buffer extract of apple AIS (de Vries *et al.*, 1981); c, substrate from the hot buffer extract of apple AIS.

† Deuteride label at C-6 atom (present only after reduction with LiAlD).

‡ Present only after carboxyl reduction (by the method of Taylor & Conrad, 1972).

not readily achieved. Reduction of the methylated pectin preparations with lithium aluminium hydride in tetrahydrofuran (Lindberg, 1972) also did not result in complete reduction. For this reason, reduction with lithium aluminium deuteride was applied. This showed that some galacturonate residues are branched through their C-3 atoms (Table 3). Table 3 also shows that galactans are present in two types: 1,4-linked (as confirmed by galactanase attack) and 1,3/1,6-linked galactans. Both types have frequently been found in plants (Clarke *et al.*, 1979). Zosterine (see below), panaxan (Solov'eva *et al.*, 1969) and jeol gum and cholla gum (Aspinall, 1969) contain 1,3/1,6-linked galactan. Talmadge *et al.* (1973) showed the presence of both types of galactans in cell wall preparations. In a previous paper (de Vries *et al.*, 1981) it was shown that the relative amounts of neutral sugars in pectin fractions from different extracts were constant with the exception of galactose. Methylation analysis revealed that in pectin fractions containing relatively high amounts of galactose, the galactans were predominantly 1,3/1,6-linked. These pectin fractions occur predominantly in the cold buffer extracts of the apple AIS (de Vries *et al.*, 1981). It can be concluded from these facts that a pectin fraction exists which consists of galacturonan residues which carry only 1,3/1,6-linked galactan side chains and which have a very high degree of esterification and, which are water-extractable. These pectin fractions are hardly degraded by the β(1,4)-galactanase. About 5% of the pectic substances present in the extracts of apple AIS belong to this fraction. A similar pectin fraction may be present in potato, onion and radish (Ishii, 1976, 1978). It is possible that this pectin fraction is part of the middle lamella pectin.

Xylose residues are mainly present as terminal residues, but also 1,4-linked xylose residues have been observed in methylation analysis. More information about the position of the xylose residues have been obtained by β-elimination experiments.

β-elimination

Figure 3 and Table 4 give the results of a typical experiment; β-elimination performed in an aqueous solution is accompanied by saponification and is very incomplete (Albersheim *et al.*, 1960; Fielding, 1975). In this case, the incompleteness of the reaction is an advantage, because only partial degradation can provide information about the distribution of

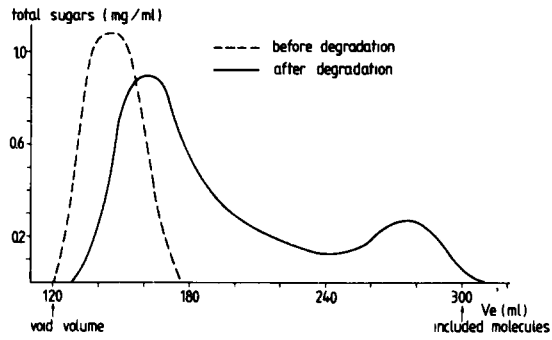

Fig. 3. Gel filtration (Sephacryl S-300) of apple pectin 'hairy regions' after β-elimination. Ve, elution volume. Substrate, hairy regions were prepared by pectate lyase degradation of pectins from the oxalate extract of AIS from ripe apples (de Vries *et al.*, 1982). Total sugars, addition of the values from the anthrone and the carbazole assays. The eluent was water.

the side chains in the hairy regions. The hairy regions cannot be degraded by *Aspergillus* pectin lyase or (after saponification) by *Pseudomonas* pectate lyase.

Figure 3 and Table 4 provide evidence in favour of the presence of xylogalacturonan regions, in which the xylose residues are directly linked to the galacturonate residues: a high proportion of xylose residues is present in the lower molecular weight fractions. In the xylogalacturonan regions, xylose is present as single unit side chains, but may also be present (1,4-linked) in very short side chains. The pseudo-

TABLE 4

Sugar Composition (Percentage of Total Amount of the Sugar Residue Present) of Alkaline-Degraded Pectin Fractions (Fractionated as Shown in Fig. 3)

Sugar	*Elution volume (ml)*		
	125-180	*180-250*	*250-300*
Galacturonic acid	64	16	20
Rhamnose	82	11	7
Arabinose	72	17	11
Xylose	42	26	32
Galactose	85	8	7

aldobiuronic acid xylosylgalacturonic acid has been detected several times in pectic substances (Bouveng, 1965; Aspinall *et al.*, 1967*a*,*b*; Stoddart *et al.*, 1967; Aspinall *et al.*, 1968; Foglietti & Percheron, 1968). It may also be present in apple pectic substances (Barret & Northcote, 1965; Pfister, 1977). In soya sauce polysaccharides, single unit side chains of xylose linked to galacturonan have been observed by Kikuchi & Sugimoto (1976). A pure xylogalacturonan was isolated from Mountain pine pollen by Bouveng (1965). Tragacanthic acid is a xylogalacturonan with some fucose and galactose residues linked to the single unit xylose side chains (Aspinall, 1969).

Structural features of galacturonic acid-containing polysaccharides

Ovodov and coworkers have been investigating the pectic substances of marine plants; for the pectic substance of plants of the genus *Zostera*, zosterine, a model was constructed (Ovodov, 1975). In this model, zosterine consists of several homogalacturonan- and apiogalacturonan-like regions. Aspinall (1969) divided the galacturonic acid-containing polysaccharides into three groups, one of which has blocks of homogalacturonan and blocks of rhamnogalacturonan. Although the methods applied in our studies do not give the exact sequence of the sugar residues, it can be concluded from our results that apple pectic substances can be thought to consist of homogalacturonan, xylogalacturonan and rhamnogalacturonan regions. All these results suggest that all galacturonic acid-containing polysaccharides are constructed from a limited number of building stones. In apple pectic substances, homogalacturonan ('smooth region') is the most important building stone, but in tragacanthic acid xylogalacturonan is the basic building stone.

In this scheme, zosterine represents a polysaccharide with several building stones. It should be realised, however, that each polysaccharide may also contain some very specific fragments of a complex structure. Such fragments may play a role in the host–parasite relation of plants and fungi or insects as 'elicitor' (Albersheim *et al.*, 1981).

Partial acid hydrolysis

The results as shown in Figs 1, 2 and 3 and in the corresponding Tables have been confirmed by partial acid hydrolysis experiments. Figure 4

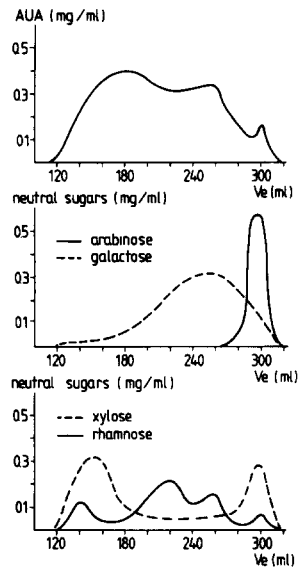

Fig. 4. Gel filtration (Sephacryl S-300) of partially hydrolysed pectin fractions. Ve, elution volume. Substrate, see Fig. 1. AUA, anhydrouronic acid content. Neutral sugars determined by GLC as described in the text. Degradation with 0·05 N TFA for 1 h at 100°C. The eluent was water.

represents an example of such experiments. The pattern of xylose is typical of short side chains, while the pattern of galactose indicates the presence of rather long galactan chains. On hydrolysis in 0·01 N TFA, almost all of the arabinose residues occur as dimers or trimers, while all the other residues still present are high-molecular (results not shown); it can, therefore, be concluded that arabans represent the outer branch of the arabinogalactan side chains. As can be seen in Fig. 4, galacturonic acid and rhamnose show similar patterns. Comparison of the patterns of the different sugar residues, provides evidence that the xylose residues are not bound to the arabinogalactan side chains. Pure arabinogalactans are present as side chains. The role of glucose residues could not be elucidated in these experiments; one of the reasons is that the columns of DEAE-cellulose and Sephacryl S-300 were 'glucose-bleeding'.

The data published in this paper about methylation analysis are in agreement with literature data (Rees & Wight, 1969; Talmadge *et al.*, 1973; Kikuchi & Sugimoto, 1976; Siddiqui & Wood, 1976; Yakovlev

& Gorin, 1977; Simson & Timell, 1978). In the cell wall model of Keegstra *et al.* (1973), xyloglucans are covalently linked to arabinogalactans which, in turn, are covalently linked to rhamnogalacturonans. In our experiments, xyloglucan fragments could not be detected.

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