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Studies on Apple Protopectin V: Structural Studies on Enzymatically Extracted Pectins

C. M. G. C. Renard,[a,*] A. G. J. Voragen,[a] J.-F. Thibault[b] & W. Pilnik[a]

[a]Agricultural University, Laboratory of Food Chemistry, Bomenweg 2, PO Box 8129, 6700 EV Wageningen, The Netherlands

[b]Institut National de la Recherche Agronomique, Laboratoire de Biochimie et Technologie des Glucides, rue de la Géraudière, BP 527, 44026 Nantes Cedex 03, France

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ABSTRACT

Apple insoluble pectins were extracted by pectolytic enzymes alone and combined with side-chain degrading enzymes or endo-*glucanase. The highest yield of pectic material was obtained with the combination pectin-lyase plus* endo-*glucanase, showing the existence of a connection between rhamnogalacturonan and xyloglucan. The extracts were fractionated on Sephacryl S 500 and DEAE Sepharose CL-6B. Methylation analysis of high-molecular-weight material coming from the 'hairy' regions showed the presence of highly branched arabinans and arabinogalactans of type I and II side-chains, and of terminal xylose residues linked to the rhamnogalacturonan backbone.*

INTRODUCTION

Most of the studies on apple pectins have been concerned with the extractability, composition and physico-chemical properties of soluble pectins (Joslyn & Deuel, 1963; Barrett & Northcote, 1965; Knee, 1973*a*,*b*, 1978; Knee *et al.*, 1975; de Vries *et al.*, 1981; Stevens & Selvendran, 1984). Structural studies have been carried out by Barrett & Northcote (1965), de Vries *et al.* (1982, 1983*a*,*b*) and Aspinall &

*To whom correspondence should be addressed at Institut National de la Recherche Agronomique, Laboratoire de Biochimie et Technologie des Glucides, rue de la Géraudière, BP 527, 44026 Nantes Cedex 03, France.

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Fanous (1984). De Vries *et al.* (1982) established, by degradation experiments using pectolytic enzymes or alkali, that in the soluble pectins the neutral sugars were grouped in 'hairy' regions containing 5% or less of the galacturonic residues, as opposed to 'smooth' homogalacturonic regions containing 95% of the galacturonic residues. Similar results were obtained by Rouau & Thibault (1984) on pectins from apple juice. Such 'hairy' regions have been isolated by degradation of pectins or by extraction of pectins by pectolytic enzymes from cell walls of various origins (Talmadge *et al.*, 1973; Ishii, 1981, 1982; Jarvis *et al.*, 1981; Thibault, 1983; Rombouts & Thibault, 1986; Saulnier & Thibault, 1987; Saulnier *et al.*, 1988; Massiot & Thibault, 1989). Methylation analysis of the 'hairy' regions from apple soluble pectins (de Vries *et al.*, 1983*a*) showed the presence of arabinogalactans of types I and II and suggested the presence of xylogalacturonan. Less attention, however, has been devoted to the 'insoluble' pectins, also called protopectins.

Studies on the protopectin from apple cell walls (Renard *et al.*, 1990, 1991*a*,*b*) showed that degradation of the rhamnogalacturonic backbone was necessary for extraction of significant amounts of galacturonic acid. Pectolytic enzymes, or rather pectolytic systems, active on highly methylated pectins, efficiently extracted pectic material from the cell walls; some synergism was obtained with *endo*-glucanases, suggesting the existence of an association between xyloglucan and pectins (Renard *et al.*, 1991*a*,*b*). However, extraction of apple fucogalactoxyloglucan did not lead to isolation of a fraction corresponding to that association (Renard *et al.*, 1991*c*). Enzymatic extractions were thus carried out on increased amounts of cell walls devoid of soluble pectins in order to identify through structural studies the connection between pectin and xyloglucans.

EXPERIMENTAL

Materials

Cell-wall material devoid of soluble pectins (CDTA Insoluble Residue, CDTA-IR) was prepared from Golden Delicious apples as described previously (Renard *et al.*, 1990*a*). Pectin-lyase, polygalacturonase, pectin-esterase, *endo*-glucanase (*endo*-glucanase IV), arabinofuranosidase, *endo*-arabinanase and *endo*-galactanase were purified as described previously (Renard *et al.*, 1991*a*).

Extractions

Enzymatic extractions were performed as described previously (Renard *et al.*, 1991*a*). The suspension of CDTA-IR (1 g in 100 ml of sterilized sodium succinate buffer 0·05 M pH 4·5) was incubated with enzymes for 24 h at 40°C with end-over-end mixing in hermetically sealed Erlen-Meyer flasks. The amounts of enzymes added were calculated to theoretically degrade the corresponding polymer present in 24 h under standard conditions. However, the *endo*-arabinanase, available in minute amounts, was used at half the amount so calculated. The polygalacturonase was used at the same concentration alone and with pectin-esterase, regardless of the degree of methylation of the pectins. After incubation, the suspension was centrifuged for 20 min at 4100 *g*. The enzymes were inactivated by boiling the supernatants for 10 min in a water bath. The pellets were washed three times with distilled water and freeze-dried. Part of the extracts were dialysed. Dialysed and non-dialysed extracts were concentrated on a rotary evaporator and frozen. Aliquots were thawed or freeze-dried as needed. The blank was made under exactly the same conditions.

Chromatography

The concentrated dialysed extracts (1 ml, 5 to 10 mg of carbohydrates) were applied to a 1·3 × 11 cm column of DEAE sepharose CL-6B. The column was washed with 50 ml of succinate buffer (0·005 M pH 4·8) and eluted by a succinate gradient (100 ml, from 0·005 to 0·4 M). Residual material was eluted by 30 ml of 0·4 M buffer followed by 30 ml of 1 M buffer and 50 ml of 0·25 M NaOH. Fractions (2 ml) were collected and assayed for uronic acid and neutral sugars. Appropriate fractions were pooled, dialysed, freeze-dried and analysed for individual neutral sugars.

The concentrated extracts (1 or 5 ml) were also applied to a 1 × 98 or 2·5 × 80 cm column of Sephacryl S 500 eluted by sodium succinate buffer (0·1 M pH 4·5). Fractions (2 or 5 ml) were collected and assayed for galacturonic acid, neutral sugars and absorbance at 280 nm. Appropriate fractions were pooled, dialysed, freeze-dried and analysed for individual neutral sugars.

High-pressure gel filtration chromatography (HPGPC) was performed as described by Schols *et al.* (1990) on Biogel TSK 40XL, 30XL and 20XL columns in series.

Analytical methods

The galacturonic acid (GalA) and total neutral sugars (NS) concentrations were measured by automated *meta*-hydroxydiphenyl (Thibault, 1979) and orcinol assays (Tollier & Robin, 1979), respectively. An arabinose standard was used for the neutral sugars. Corrections were made for the mutual interferences. Individual neutral sugars were analysed as their alditol acetate derivatives by gas chromatography (Englyst & Cummings, 1984) after hydrolysis by 1 M H_2SO_4 at 100°C for 3 h. Proteins were measured in the residues by semi-automatic micro-Kjeldahl (Roozen & Ouwehand, 1978).

Purified polysaccharides were methylated by the Hakamori method (1964) as adapted by Janssen *et al.* (1976) and Harris *et al.* (1984). The sample (5 mg) was dried overnight at 40°C *in vacuo* over P_2O_5. Anhydrous dimethylsulphoxide was added under nitrogen and the mixture was ultrasonicated for 2 h. Potassium methylsulphinylmethanide was then added, and the sample was ultrasonicated for 3 h at 40°C. The flask was cooled in ice and 1 ml methyl iodide was added dropwise. After ultrasonication for 2 h at 20–30°C, the flasks were bubbled with nitrogen to evaporate excess methyl iodide. The mixture was dialysed for 24 h against running tap water and overnight against distilled water. Dialysis retentates were evaporated in a rotary evaporator. Chloroform–methanol (1:1, 1 ml) was added and the sample was concentrated to dryness. The drying was repeated three times. The chloroform–methanol solution was transferred to a Kimax tube and dried under a stream of dry air. After addition of inositol, the methylated polysaccharides were hydrolysed by 2 M trifluoroacetic acid for 1 h at 120°C. The reaction mixture was dried under a stream of dry air. The partially methylated sugars were reduced by 10 mg $NaBH_4$ in 200 μl of 3 M NH_4OH. Acetylation was further performed as described for alditol acetates. Partially methylated alditol acetates were analysed by GLC on a DB 225 wide bore column (15 m × 0·53 mm) (1 min at 160°C, 160 → 230°C at 5°C/min and 3 min at 230°C). Peak areas were corrected by the estimated carbon response coefficients as calculated by Sweet *et al.* (1975).

RESULTS

Yields and compositions of the enzyme extracts

The global yields and the sugar composition of the fractions are given in Table 1. The main sugar in the starting material, CDTA-IR, was glucose

TABLE 1
Sugar Composition of the Polysaccharides Extracted by Various Enzymes

	Yields (% CDTA-IR)	*Sugars (g/100 g extract)*							
		Rha	*Fuc*	*Ara*	*Xyl*	*Man*	*Gal*	*Glc*	*GalA*
Blank	3·2	3·0	0·0	11·5	trace	0·0	5·0	trace	80·5
Pectin-lyase	17·6	3·2	0·0	20·3	2·3	0·0	11·4	1·2	61·6
Polygalacturonase	5·2	3·2	0·0	15·7	trace	0·0	6·9	trace	74·2
Polygalacturonase + pectinesterase	17·4	2·8	0·0	20·1	1·5	0·0	11·4	trace	65·3
endo-Glucanase	11·7	1·8	3·7	7·4	17·3	0·0	10·4	30·0	29·6
Arabinofuranosidase + *endo*-arabinanase + *endo*-galactanase	13·3	0·8	0·0	43·3	0·8	0·0	22·6	1·0	31·5
endo-Glucanase + pectin-lyase	37·5	2·9	1·4	24·1	8·0	0·0	16·1	9·4	38·1
Arabinofuranosidase + *endo*-arabinanase + *endo*-galactanase + pectin-lyase	26·5	2·2	0·0	31·7	2·1	0·0	18·8	1·6	43·6

(33·6%), mostly present as cellulose. It was rich in highly methylated pectins, containing 19·8% galacturonic acid with a degree of methylation of 70. It also contained 11% arabinose, 8·8% galactose, 5·9% xylose, minor amounts of mannose (1·6%), fucose (1·3%) and rhamnose (1%) and some proteins (5·7%). The yields obtained here were slightly higher than in the smaller-scale experiments (Renard *et al.*, 1991*a*), probably because of the change in the incubation conditions. However, the compositions of the extracts were comparable.

The blank contained pectic material probably solubilized by the mechanical effect of grinding. The pectin-lyase had a marked effect, liberating material rich in galacturonic acid, arabinose, galactose and rhamnose. The polygalacturonase, in contrast, freed little more material than the blank. However, addition of the pectin-esterase resulted in yields similar to those of the pectin-lyase. The absence of effect of the polygalacturonase alone was probably due to the high degree of methylation of the pectins of the CyDTA-IR. Yields per sugar were calculated, showing that pectolytic systems active on highly methylated pectins (pectin-lyase, polygalacturonase plus pectin-esterase) liberated more than 50% of the galacturonic acid and rhamnose, accompanied by one-third of the arabinose and a fifth to a quarter of the galactose.

The *endo*-glucanase extract was rich in glucose and xylose, and also contained galactose and fucose. The arabinanases and galactanase extract was rich in arabinose and galactose: this combination freed 53% of the arabinose and 34% of the galactose initially present in the CDTA-IR. The yields of galacturonic acid in these two extracts were 1·5 times that of the blank, showing that, although the extracts contained significant amounts of galacturonic acid, the enzymes were not able to free pectins from the cell wall.

Addition of *endo*-glucanase to the pectin-lyase led to synergistic effects for cell wall degradation. The combination of *endo*-glucanase plus pectin-lyase freed the highest amount of cell-wall material: 84% of the arabinose, 75% of the rhamnose, 72% of the galacturonic acid, 68% of the galactose, 51% of the xylose, 41% of the fucose and 11% of the glucose were liberated in the buffer. The synergistic effects of this combination were more marked for arabinose and galactose than for the sugars most extracted by the two enzymes separately. The addition of the arabinanases and the galactanase to the pectin-lyase gave an extract rich in arabinose, galacturonic acid and galactose; this combination, however, did not lead to synergistic effects, and for all sugars the yields were lower than the sum of the amounts extracted separately by the pectin-lyase and by a mixture of arabinanases plus galactanase.

Gel-filtration on Sephacryl S500

The blank contained high-molecular-weight pectic material poor in neutral sugars (Fig. 1). Two peaks could be seen for the arabinanase plus galactanase extract and the *endo*-glucanase extract; the peak at K_{av} values $\approx 0{\cdot}3$ contained pectic material similar to that of the blank, and the peak at the total volume was composed of neutral oligomers (Table 2). These chromatograms showed the superposition of high-molecular-weight material liberated in the blank and of neutral oligomers. The enzymes freed oligomers, whereas the high-molecular-weight material was already present in the blank. The *endo*-glucanase liberated fucose, xylose, galactose and glucose and the arabinanases and galactanase liberated arabinose and galactose. In the arabinanase plus galactanase extract, the arabinans and arabinogalactans of the high-molecular-weight material were degraded, and this fraction was low in neutral sugars.

The pectin-lyase extract was composed of two fractions of different molecular weight (Fig. 2) and composition (Table 2). The high-molecular-weight fraction was rich in neutral sugars, notably arabinose (49 mol%) and galactose, and had a high rhamnose:galacturonic acid ratio (1:3·5). The included material contained almost exclusively

galacturonic acid. The chromatogram of the combination polygalacturonase plus pectin-esterase (not shown) was identical to that of the pectin-lyase. The extracts of the combinations of pectin-lyase and non-pectolytic enzymes contained the same two fractions. The *endo*-glucanase plus pectin-lyase combination freed high-molecular-weight material rich in arabinose, galactose and with a high rhamnose:galacturonic ratio, like the pectin-lyase alone. However, the proportion of galacturonic acid in the high-molecular-weight fraction was slightly higher: in the *endo*-glucanase plus pectin-lyase extract 20% of the galacturonic acid eluted at $K_{av} < 0{\cdot}6$, versus 7·5% in the pectin-lyase extract. The included material showed a superposition of the low-molecular-weight products of the two enzymes. The combination of arabinanases plus galactanase plus pectin-lyase gave a high-molecular-

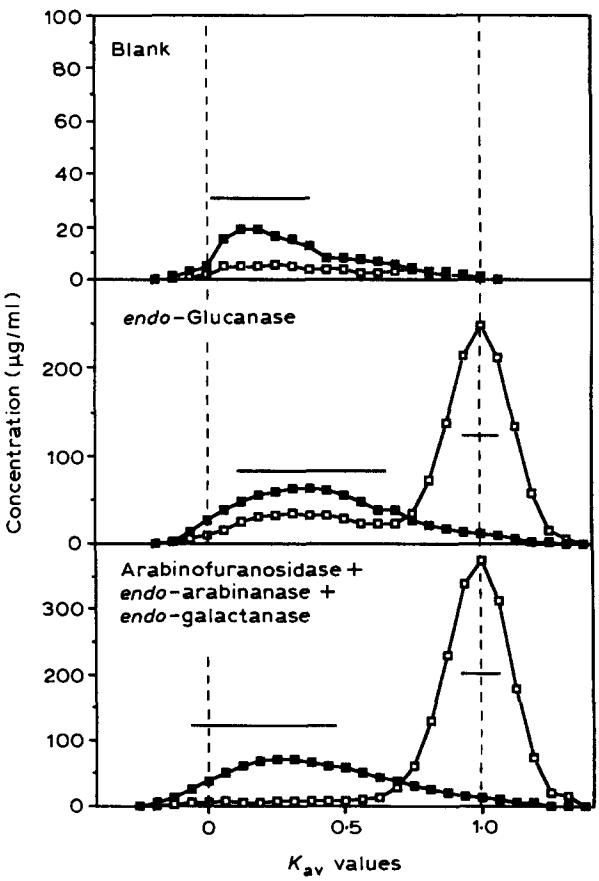

Fig. 1. Gel-filtration on Sephacryl S500 eluted with succinate buffer (0·1 M, pH 4·5) of extracts without pectolytic enzymes. ■, Galacturonic acid; □, neutral sugars.

TABLE 2
Composition (mol%) of Gel-filtration Fractions of the Extracts

Blank		endo-*Glucanase*		*Arabinofuranosidase* + endo-*arabinanase* + endo-*galactanase*		*Pectin-lyase*		endo-*Glucanase* + *pectin-lyase*		*Arabinofuranosidase* + endo-*arabinanase* + endo-*galactanase* + *pectin-lyase*	
($K_{av} \approx 0.3$)		*$K_{av} \approx 0.3$*	*$K_{av} \approx 1$*	*$K_{av} \approx 0.3$*	*$K_{av} \approx 1$*	*$K_{av} \approx 0.4$*	*$K_{av} \approx 1$*	*$K_{av} \approx 0.4$*	*$K_{av} \approx 1$*	*$K_{av} \approx 0.4$*	*$K_{av} \approx 1$*
Rha	1·9	2·4	0·0	2·2	1·1	4·8	1·3	4·3	0·5	11·6	1·1
Fuc	0·0	0·0	7·0	0·0	0·0	0·0	0·0	0·0	3·8	0·0	0·0
Ara	9·8	18·8	1·3	2·0	68·8	49·3	2·3	44·4	2·3	8·5	35·1
Xyl	3·9	3·6	29·0	2·2	0·7	5·1	1·9	7·2	15·2	13·0	1·5
Man	0·0	0·8	0·0	1·1	0·0	0·0	0·3	0·6	0·0	1·1	0·3
Gal	3·4	7·5	11·5	1·9	25·5	22·5	1·1	21·1	6·0	13·8	16·3
Glc	3·6	1·8	47·1	2·5	1·9	1·5	3·9	1·9	20·8	5·9	2·4
GalA	77·4	65·1	4·1	88·1	2·0	16·8	89·2	20·5	51·4	46·1	43·3

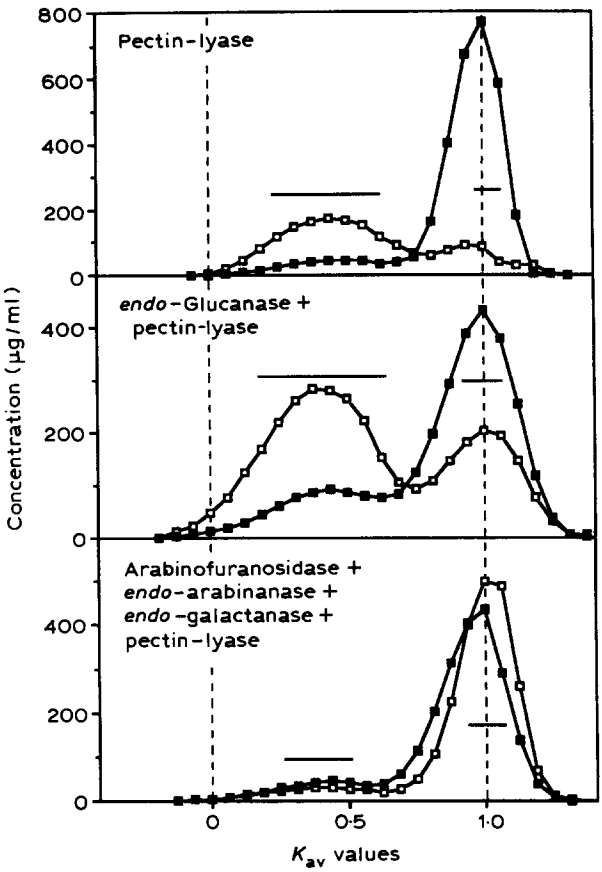

Fig. 2. Gel-filtration on Sephacryl S500 eluted with succinate buffer (0·1 M, pH 4·5) of extracts with pectolytic enzymes. ■, Galacturonic acid; □, neutral sugars.

weight material with low proportions of arabinose and galactose. The amounts of the other sugars were increased in proportion, but their ratios were similar to those of the high-molecular-weight material from the pectin-lyase: the xylose:galacturonic acid ratio were 1:3·5 and 1:3·3, and the rhamnose:galacturonic acid 1:3·9 and 1:3·5, respectively. The galactans were slightly less degraded than the arabinans, as the arabinose:galacturonic acid ratio decreased from 2·9:1 to 0·2:1 and the galactose:galacturonic acid ratio only from 1·3:1 to 0·3:1. The included material peak again showed a superposition of the low-molecular-weight products of the enzymes.

Ion-exchange chromatography

Ion-exchange chromatography was performed on dialysed extracts, so that the oligomers present in the raw extract did not appear. The pectin-lyase extract (Fig. 3) showed two main fractions on DEAE Sepharose CL-6B. The non-retained fraction (A) was very small and composed mostly of glucose, xylose and arabinose (Table 3). The main peak (B) was composed of acidic material poor in neutral sugars and had a low molecular weight (1000–2000 Da as measured by HPGPC). The second retained peak (C) contained neutral sugars-rich material with high proportions of arabinose and galactose and a high rhamnose:galacturonic acid ratio. The tail of that peak (D) was enriched in xylose and

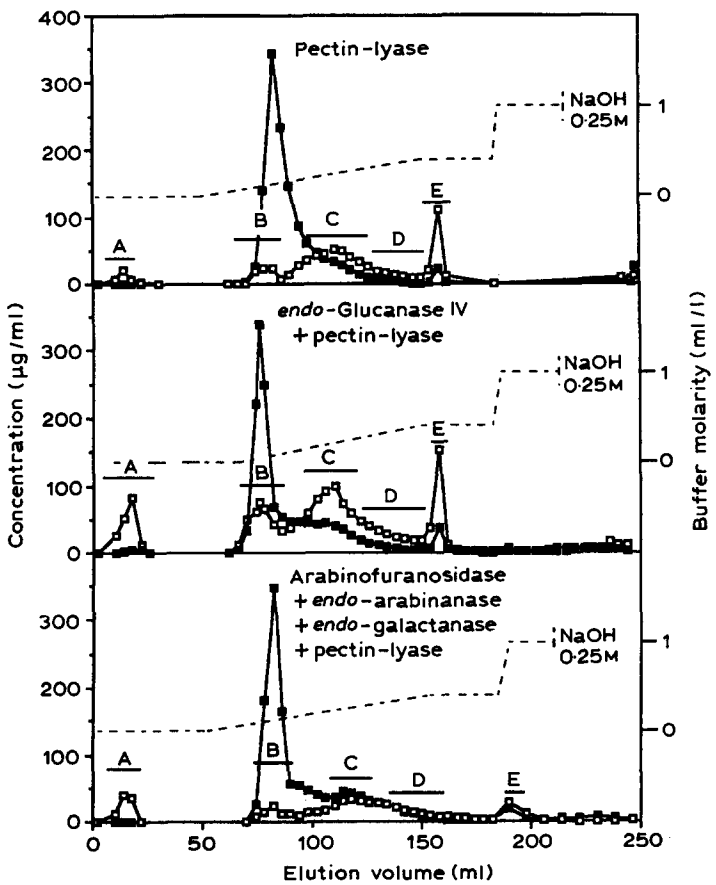

Fig. 3. Ion-exchange chromatography on DEAE Sepharose CL-6B eluted by sodium succinate buffer (pH 4·8) of enzyme extracts. ■, Galacturonic acid; □, neutral sugars; – – –, buffer molarity.

TABLE 3
Composition (mol%) of Ion-exchange Fractions

	Pectin-lyase					endo-*Glucanase* + *pectin-lyase*					*Arabinofuranosidase* + endo-*arabinanase* + endo-*galactanase* + *pectin-lyase*				
	A	*B*	*C*	*D*	*E*	*A*	*B*	*C*	*D*	*E*	*A*	*B*	*C*	*D*	*E*
Rha	8·4	1·0	6·2	10·8	6·2	trace	2·7	4·8	2·2	2·2	8·0	1·5	5·9	16·6	9·5
Fuc	0·0	0·0	0·0	0·0	0·0	6·7	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0
Ara	18·6	7·2	35·0	40·8	48·9	16·8	21·4	38·3	38·8	47·0	31·6	10·2	5·1	9·5	7·0
Xyl	21·9	3·2	5·9	10·5	6·9	22·2	3·7	6·4	8·7	7·3	15·6	3·0	8·0	15·6	10·5
Man	2·1	0·0	0·0	0·0	0·0	0·0	0·4	0·7	2·8	1·2	6·8	0·5	1·5	6·1	4·7
Gal	13·7	4·9	10·9	11·3	19·1	19·3	10·1	13·6	14·8	20·4	14·4	3·0	8·3	13·6	7·9
Glc	35·3	0·2	0·8	2·9	1·7	26·8	1·4	1·0	3·2	1·6	22·2	6·1	12·3	15·5	38·8
GalA	0·0	83·5	41·2	23·7	17·2	8·6	60·3	35·2	29·5	20·3	1·4	74·7	58·7	23·1	21·6

glucose. The extract of the combination of arabinanases plus galactanase plus pectin-lyase showed an elution pattern similar to that obtained with the extract of the pectin-lyase, but the proportions of arabinose and galactose in the bound material were lower. On the other hand, for the combination *endo*-glucanase plus pectin-lyase the height of the neutral sugars-rich retained peak (C) and the proportion of neutral sugars in this material were higher than for the pectin-lyase alone. Here too, the tail of the retained peak (D) contained a material enriched in xylose and glucose. In both these combinations, the neutral fractions were low, due

TABLE 4
Partially Methylated Alditol Acetates from 'Hairy' Regions

Derivatives	*Pectin-lyase*	*Pectin-lyase + pectin-esterase*	endo-*Glucanase IV + pectin-lyase*	*OHSP*
2,3,5-Me_3-Ara[a]	20·7[b]	23·1	17·1	18·2
2,3,4-Me_3-Ara	1·5	0·2	0·6	0·2
2,3-Me_2-Ara	26·2	32·4	26·2	31·4
2,5-Me_2-Ara	3·3	3·7	5·2	3·6
3,5-Me_2-Ara	0·3	0·3	0·5	0·2
2-Me-Ara	14·0	15·0	15·7	17·4
5-Me-Ara			0·2	
Ara	4·1	6·8	7·9	8·4
2,3,4,6-Me_4-Gal	3·2	2·1	3·3	3·9
2,3,4-Me_3-Gal	0·3	0·0	0·4	0·0
2,3,6-Me_3-Gal	6·9	6·0	6·8	5·6
2,4,6-Me_3-Gal	2·1	1·9	3·5	2·9
2,3-Me_2-Gal	2·0	0·4	1·1	
2,4-Me_2-Gal	0·2	0·1	0·2	0·3
2,6-Me_2-Gal	1·3	0·8	1·4	0·9
3,6-Me_2-Gal	1·9	1·0	2·5	0·8
2-Me-Gal	1·0	0·3	0·6	0·3
6-Me-Gal	0·7	0·9	1·1	1·1
Gal	0·5	0·9	1·3	0·8
2,3,4-Me_3-Xyl	2·2	0·6	0·6	0·9
2,3-Me_2-Xyl	trace	0·3	0·8	0·2
Xyl		0·1		0·6
3,4-Me_2-Rha	1·0	0·7	trace	0·4
3-Me-Rha	1·5	1·1	1·4	0·9
2,3,6-Me_3-Glc	0·6	0·5	0·2	
2,3,6-Me_3-Man	trace	trace	trace	

Minor components for which identities were not confirmed are omitted.
[a]2,3,5-Me_3-Ara denotes 1-*O*-acetyl-2,3,5-tri-*O*-methyl-arabinitol, etc.
[b]Percentage of the total area of the surfaces of identified peaks corrected by ECR (Sweet *et al.*, 1975).

to the loss during dialysis of the oligomers produced by the *endo*-glucanase, the arabinanases or the galactanase. However, some of the oligomers persisted in both extracts, as can be seen from the composition of the non-retained fractions compared to the non-retained fraction of the pectin-lyase extract.

Methylation analysis

High-molecular-weight fractions of the pectin-lyase extract, of the polygalacturonase plus pectin-esterase extract and of the *endo*-glucanase plus pectin-lyase extract were isolated and analysed by methylation analysis (Table 4). The high-molecular-weight fraction of OHSP, a pectin fraction extracted by 0·05 M sodium hydroxide from CDTA-IR (Renard *et al.*, 1990), was also analysed in order to compare the effects of chemical and enzymic extractions. The methylation was not complete for the *endo*-glucanase plus pectin-lyase extract or for OHSP; however, the main structural features of these fractions could be distinguished. The high-molecular-weight fractions were very similar in the different extracts. The main sugar in the side chains was arabinose. It was mostly present as highly ramified (1→5)-linked arabinans; about a third of the arabinose residues in the main chains carried ramifications on O-3. Traces of arabinopyranose could be detected in all the extracts. Galactose was present in both types of galactans. Galactans of type I ((1→4)-linked) were predominant, but (1→3)-linked galactose was also present in significant proportions. About 60% of the rhamnose residues, inserted in the rhamnogalacturonan backbone by a (1→2)-linkage, carried ramifications on the O-4. The xylose was mostly present as terminal residues.

DISCUSSION

Structure of the cell-wall polysaccharides

The included peaks of the *endo*-glucanase and the arabinanases plus galactanase extracts contained neutral oligomers (Renard *et al.*, 1991*b*) corresponding to their substrates in the cell wall. The arabinanases and the galactanase freed arabinose and galactose oligomers. The *endo*-glucanase liberated fucose, xylose, galactose and glucose in molar ratios similar to those obtained for apple fucogalactoxyloglucan (Renard *et al.*, 1991*c*). Although *endo*-glucanases are most active on cellulose in model

systems, Hayashi *et al.* (1984) have shown that in the cell walls they degrade xyloglucans first. Here the oligomers freed by the *endo*-glucanase come from the fucogalactoxyloglucan which is the main hemicellulose from apples (Stevens & Selvendran, 1984; Aspinall & Fanous, 1984; Voragen *et al.*, 1986; Renard *et al.*, 1991*c*). The non-pectolytic enzymes, notably the arabinanases and the galactanase, did not free significant amounts of galacturonic acid or rhamnose in the buffer. This is not in agreement with the idea that pectins are bound to the rest of the cell wall through arabinogalactan side chains.

The pectolytic enzymes extracted high-molecular-weight material carrying numerous side-chains, coming from the 'hairy' regions, and oligogalacturonides, coming from the 'smooth' regions, as reported by Massiot & Thibault (1989) and Saulnier & Thibault (1987). Here, as for carrots (Massiot & Thibault, 1989), higher yields were obtained with the pectolytic enzymes than with chemical extractions (hot acid, cold alkali) (Renard *et al.*, 1990*a*), in contrast to what was reported by Saulnier & Thibault (1987) for grape berries. This may be related to the fact that the highest yields for the chemical extractions were obtained with cold alkali for carrots (Massiot *et al.*, 1988) and apple (Renard *et al.*, 1990*a*) cell walls, and with hot acid for grape berries (Saulnier & Thibault, 1987). The composition and structure of the arabinogalactan side chains of pectins extracted by pectolytic enzymes and by dilute sodium hydroxide were identical, whereas Saulnier *et al.* (1988) found different proportions of arabinans and arabinogalactans in the pectin-lyase extract and in the chemical extracts.

The proportions of 'hairy' regions were much higher in the insoluble pectins than in the soluble pectins studied by de Vries *et al.* (1982), who found 5% or less of the galacturonic acid in the 'hairy' regions with degradation extents of only 4–5%. Here 7·5% of the galacturonic acid was present in the high-molecular-weight fractions of the pectin-lyase extract and 20% in the *endo*-glucanase plus pectin-lyase extract. The overall structural features of the side chains of the insoluble pectins are similar to those of the soluble pectins as reported by de Vries *et al.* (1983*a*). Higher amounts of type II galactans were present and a higher proportion of rhamnose residues carried ramifications. Aspinall & Fanous (1984) did not report the presence of type II galactans in apple pectins. The 'hairy' regions isolated by Schols *et al.* (1990) from apple juice obtained by the liquefaction treatment were also comparable to the 'hairy' regions isolated here. However, methylation analysis of these molecules showed an increase in the proportion of type II galactans and a decrease of the degree of branching of the arabinans. These modifications were probably due to the polysaccharidases, notably galactanases,

present in the enzyme mixture used for liquefaction. Apple 'hairy' regions, whether isolated from soluble pectins (de Vries *et al.*, 1983*a*), from insoluble pectins or from apple juice obtained by liquefaction (Schols *et al.*, 1990), show similar rhamnose:galacturonic ratios (1:3·5, 1:3·5 and 1:3·3, respectively). Oligomers presenting alternating rhamnose and galacturonic acid residues were isolated by Schols *et al.* (1990), but zones richer in galacturonic acid are also present in the 'hairy' regions.

Xylose residues were present as single units or short side chains. The 'hairy' regions isolated after treatment with arabinanases and galactanase had the same xylose:galacturonic acid ratio as those obtained with the pectin-lyase alone, and no xylose was found in association with the arabinose and galactose oligomers. The terminal xylose residues are thus not carried by the arabinogalactan side chains but directly by the rhamnogalacturonic backbone. The 'hairy' regions contained more xylose than rhamnose, so that it seems that the larger part of the xylose was linked directly to the galacturonic residues. The existence of such xylose side chains was already mentioned by Barrett & Northcote (1965), who isolated the pseudoaldobiouronic acid xylose–galacturonic acid from acid hydrolysates of apple pectins. Such a possibility was also suggested by de Vries *et al.* (1983*a*). Results from acidic treatments and methylation analysis of the 'hairy' regions isolated from apple juice (Schols *et al.*, 1990) also give evidence for presence of xylogalacturonan sub-regions.

Interactions between cell-wall polysaccharides

The composition of the oligomers liberated by the *endo*-glucanase confirm the hypothesis (Renard *et al.*, 1991*b*) that the effects of the *endo*-glucanase are due to degradation of the fucogalactoxyloglucan, which is the main hemicellulose from apple cell walls (Renard *et al.*, 1991*c*). The addition of this enzyme to the pectin-lyase led to a synergism in cell-wall degradation. However, this synergism was due mostly to an increase in the extraction of 'hairy' regions: the highest effects on the yields were obtained for arabinose and galactose, and the proportion of high-molecular-weight material markedly increased in the extracts. If the only limit to the proportion of pectin (notably 'hairy' regions) that could diffuse to the buffer had been mechanical enmeshment, the amounts extracted by the combination *endo*-glucanase plus pectin-lyase should be the same as with the pectin-lyase alone, as the *endo*-glucanase can neither affect the size of the 'hairy' region fragments nor the size of the pores of the extensin network (Lamport & Epstein,

1983; Fry, 1986). However, a possibility that should not be ruled out here is the existence of a network formed by xyloglucan molecules interlinking cellulose microfibrils. It thus appears that there is an association between xyloglucans and pectins, and that this association is most likely to be near or in the 'hairy' regions.

Many results point to the fact that the arabinogalactan side chains of the pectins cannot be the site of this connection. In fact, a large part of these side-chains and actually entire 'hairy' regions appear to be 'loose', 'free' in the cell wall, i.e. connected only to the rhamnogalacturonic backbone. Hydrolysis of the side-chains by hot acid (Renard *et al.*, 1990) or by arabinanases and galactanase failed to free significant amounts of pectic material from the cell walls, and the addition of arabinanases and galactanase to pectin-lyase did not enhance the effect of this enzyme on liberation of rhamnose and galacturonic acid. The absence of effect of the side-chain degrading enzymes might, however, be due to the lack of an enzyme able to degrade the type II arabinogalactans of apple cell walls. In any case, glycosidic bonds are impossible between the end-groups of the arabinogalactan side-chains of the pectins and of the side chains of the xyloglucan, both non-reducing. Moreover, the apple cell-wall material contained no phenolic acids (Renard *et al.*, 1990), ruling out the possibility of diphenolic crosslinks, which might have provided reticulation points (Fry, 1986).

CONCLUSION

Results of enzymatic extraction of apple protopectin indicated the existence of a direct connection between the rhamnogalacturonan backbone of the insoluble pectins and xyloglucans. This connection seems to be located in or near the 'hairy' regions, but does not involve the arabinogalactan side chains. Further work is necessary to elucidate the nature of this connection.

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