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Studies on apple protopectin VI: extraction of pectins from apple cell walls with rhamnogalacturonase

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Apple cell walls were extracted by treatment with rhamnogalacturonase, an enzyme that cleaves the rhamnogalacturonic backbone in the rhamnose-rich regions. It solubilised 13·6% of the cell walls after 24 h. The extracted material was composed of galacturonic acid (42%) and neutral sugars (46%), especially arabinose, and had a high rhamnose : galacturonic acid ratio; it was also rich in methyl and acetyl groups (58 and 24 mg/g, respectively). The material was separated into two fractions by anion-exchange chromatography on DEAE Sepharose CL-6B. The neutral, non-retained fraction was composed mostly of arabinose and had a low hydrodynamic volume as shown by its elution pattern on Sephacryl S500. The retained fraction was enriched in galacturonic acid and had a higher hydrodynamic volume. Methylation analysis of the side-chains showed mostly arabinans and type I arabinogalactans. Some terminal rhamnose could be detected, and was relatively more abundant in the non-retained fraction. Admixture with pectinlyase or polygalacturonase plus pectinmethylesterase during treatment of the apple cell walls resulted in degradation of the high-molecular-weight fractions, and with arabinanases and galactanase in degradation of the low-molecular-weight fraction of the rhamnogalacturonase extract.

INTRODUCTION

Much insight into the structure of cell wall polymers, notably pectins, and of cell walls themselves, has been gained by the use of enzymic degradations (Keegstra *et al.*, 1973; Knee *et al.*, 1975; de Vries *et al.*, 1982; Rombouts & Thibault, 1986; Saulnier & Thibault, 1987; Massiot & Thibault, 1989). All pectolytic enzymes hitherto purified degraded pectins in the 'smooth', homogalacturonic regions. Recently, Schols *et al.* (1990) have isolated from a technical preparation of *Aspergillus aculeatus* a pectolytic enzyme able to cleave pectins in the 'hairy regions', which they named rhamnogalacturonase. The rhamnogalacturonase splits the rhamnogalacturonic backbone in rhamnose-rich regions, typically in regions which present a regular alternating sequence of rhamnose and galacturonic acid residues. Its end products have a tetrasaccharide backbone composed of alternating rhamnose and galacturonic acid residues (Colquhoun *et al.*, 1990), with rhamnose (eventually substituted by a galactose residue) at the non-reducing end.

In previous articles, the authors have reported results obtained from apple cell walls using pectolytic and non pectolytic enzymes (Renard *et al.*, 1991*a,b,c*). It appeared that efficient extraction of pectins could only be obtained with enzymes or enzyme mixtures that degraded the methylated pectin backbone (pectinylase, polygalacturonase plus pectin methylesterase). The amount of pectins extracted by, for example, pectinlyase could be increased by admixture with endo-glucanase, which was shown to degrade the (fucogalacto)xyloglucan of apple cell walls (Renard *et al.*, 1992), but not by arabinanases plus galactanase.

Rhamnogalacturonase has been used to degrade 'modified hairy regions' isolated from enzymatically

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liquefied fruits and vegetables in xylogalacturonan, rhamnogalacturonan and arabinan-carrying subunits (Voragen *et al.*, 1992). The present authors report here on the treatment of apple cell walls by rhamnogalacturonase and characterisation of the extracted material.

EXPERIMENTAL

Cell wall material

Cell wall devoid of soluble pectins (cyclohexane diamino tetracetic acid insoluble residue, CDTA-IR) was prepared from Golden Delicious apples by extensive treatment of apple alcohol insoluble solids with 20 mM CDTA (pH 4·8–5) at room temperature, as described by Renard *et al.* (1990).

Enzymes

Rhamnogalacturonase was isolated from Ultra Sp (Novo Ferment AG, Basel, Switzerland), a commercial preparation from *Aspergillus aculeatus*, as described by Schols *et al.* (1990). Endo-pectinlyase (EC 4.2.2.10, PL), endo-polygalacturonase (EC 3.2.1.15, PG), pectinmethylesterase (EC 3.1.1.11, PE), endo-glucanase (EC 3.1.2.4, Endo Glu IV), endo-arabinanase (EC 3.2.1.99, Endo Ara), arabinofuranosidase (EC 3.2.1.55, Arafase) and endo-β-(1 $\rightarrow$ 4)-galactanase (EC 3.2.1.89, Endo Gal) were obtained as described previously (Renard *et al.*, 1991*a*).

Extractions

Enzymatic extractions of the cell wall material were carried out for 24 h at 40°C in 0·05 M sodium succinate buffer pH 4·5 with solid : liquid ratios of 1 g : 100 ml, as described previously (Renard *et al.*, 1991*a,c*). Small scale extractions were carried out with 50 mg CDTA-IR and 5 ml buffer, and a larger scale extraction with 1 g of starting material. The amount of rhamnogalacturonase used in the small scale assay was 100 μg of enzyme protein per gram of cell walls; it was doubled in the large scale assay so that the reaction was complete in 24 h. The enzymes were inactivated by boiling for 10 min and the suspensions were centrifuged for 20 min at 4100*g*. The extract from the large scale extraction (1 g of CDTA-IR) was dialysed against distilled water, concentrated on a rotary evaporator and frozen. The theoretical cutoff of the dialysis tubing was 6000–8000 Da (peptides); the actual cutoff is much lower for oligosaccharides, notably oligogalacturonates (Mort *et al.*, 1991). Aliquots were thawed or freeze-dried as needed. The extracts from small scale assays, including admixtures with other enzymes, were analysed without dialysis and concentration. The amounts of pectinlyase, polygalacturonase, pectinmetylesterase, endo-glucanase, endo-arabinanase, arabinofuranosidase and endo-β-(1 $\rightarrow$ 4)-galactanase were the same as used previously (Renard *et al.*, 1991*a,b,c*). They were calculated to theoretically totally degrade the amounts of their respective substrates present in the CDTA-IR in 24 h under standard conditions. A cell wall blank was made in exactly the same conditions (24 h incubation with succinate buffer, then 10 min boiling).

Chromatography

High performance gel permeation chromatography (HPGPC) was performed on Biogel TSK columns 20XL, 30XL and 40XL (Bio-Rad Labs, Richmond, CA, USA) in series in combination with a TSK XL guard column, eluted at 30°C by 0·4 M acetate buffer pH 3 at 0·8 ml/min with RI detector as described by Schols *et al.* (1990).

An aliquot of the concentrated extract (5 ml, approximately 20 mg of carbohydrates) was applied to a 1·6 cm $\times$ 18 cm column of DEAE sepharose CL-6B. The column was washed by 50 ml of succinate buffer 0·005 M pH 4·8 and eluted by a succinate gradient (80 ml, from 0·005 to 1 M). Residual material was eluted by 50 ml of 1 M buffer. Fractions (5 ml) were collected and assayed for uronic acid and neutral sugars. Appropriate fractions were pooled and dialysed for further analysis.

The concentrated extract (0·5 ml) and fractions from anion-exchange chromatography were also applied to a 1·6 cm $\times$ 40 cm column of Sephacryl S500 eluted ascendingly by sodium succinate buffer 0·1 M pH 4·5. Fractions (0·5 ml) were collected and assayed for uronic acid and neutral sugars. The void (V_0) and total (V_t) volumes of the column (15 and 31 ml, respectively) were determined with amylopectin ($Mw > 10 \times 10^6$ Da) and glucose, respectively.

Analytical methods

The galacturonic acid (GalA) and total neutral sugars (NS) concentrations were measured by automated *meta*-hydroxy-diphenyl (Thibault, 1979) and orcinol assays (Tollier & Robin, 1979), respectively. Corrections were made for interference of uronic acids in the neutral sugars assay. Individual neutral sugars were analysed as their alditol acetate derivatives by gas chromatography (Englyst & Cummings, 1984) after 3 h hydrolysis by 1 M H_2SO_4 at 100°C. Methyl and acetyl esters were determined according to the method of Voragen *et al.* (1986). The degrees of methylation (DM) and acetylation (DA) were calculated as molar ratios from the contents of methanol and acetic acid, respectively, and galacturonic acid.

Methylation analysis was carried out according to Hakomori (1964), using lithium methylsulphinyl anion with a contact time of 1 h. Acidic fractions were converted into their H^+ form by percolating the solutions through an Amberlite IR-120 resin to ensure dissolution

in dimethyl-sulphoxide. Methylated polysaccharides were extracted with $CHCl_3$-CH_3OH (2:1, v/v), washed three times with distilled water, air-dried at 40°C and then hydrolysed with 2 M trifluoroacetic acid (90 min, 120°C), converted into their alditol acetates using perchloric acid as a catalyst (Harris *et al.*, 1984) and analysed by GLC on:

(a) A fused-silica capillary column (30 m × 0·32 mm) bonded with OV-225; 175°C for 15 min, then 5°C/min to 220°C; injector temperature 210°C; detector temperature 240°C; split 60–80 ml/min; hydrogen as carrier gas at 0.7 bars.
(b) A fused-silica capillary column (30 m × 0·32 mm) bonded with OV-1; 150°C for 10 min, then 2°C/min to 190°C; injector temperature 210°C; detector temperature 240°C; split 60–80 ml/min; hydrogen as carrier gas at 0·7 bars.

Peak identification was based on retention times using inositol as internal standard and on e.i. fragmentation patterns using the OV-225 column coupled to a Delsi-Nermag R10-10C mass spectrometer with a source temperature of 250°C. They were quantified using the effective carbon responses determined by Sweet *et al.* (1975).

Intrinsic viscosity ([η]), ml/g) was calculated by measuring the flow times of solutions of pectins in 0·155 M NaCl at 25·0 ± 0·1°C in an automatic Ubbelohde viscosimeter (Amtec, Nice, France) and by using the double extrapolation to zero concentration based on Huggins and Kramer equations (Axelos & Thibault, 1991).

RESULTS

Characterisation of the rhamnogalacturonase extracts

Rhamnogalacturonase was able to liberate sugars from the CDTA-IR in amounts highly superior to those observed in the blank (Table 1): it liberated 7·2% of the cell wall material after 24 h incubation during the small scale trials, and up to 13·6% in the large scale extraction, for which the proportion of rhamnogalacturonase was doubled. The amounts liberated in the corresponding blanks were of 2·1 and 3·2%, respectively. This increased yield was obtained without noticeable modification of the composition of the extract. The rhamnogalacturonase extracted fragments had a high proportion of galacturonic acid and neutral sugars. The highest extraction yield was obtained for the rhamnose: in the larger scale experiment, 48% of the rhamnose was extracted, together with 35% of the galacturonic acid and 39% of the arabinose. The extract contained a high proportion of arabinose (36·4 mol%) and rhamnose, leading to a high (1:12) rhamnose to galacturonic acid ratio (the corresponding values for pectinlyase and polygalacturonase plus pectinmethylesterase were, respectively, of 1:16 and 1:19) (Renard *et al.*, 1991*c*). The proportions of galactose and also of xylose, relative to the total neutral sugars, were lower than for either pectinlyase or polygalacturonase plus pectinmethylesterase (Renard *et al.*, 1991*c*); only traces of fucose and mannose could be detected. The pectins extracted by rhamnogalacturonase were highly methylated (DM: 76) and had an unusually high degree of acetylation (DA: 17) for apple pectins, assuming that all acetyls were carried by galacturonic acid residues.

The extract from the rhamnogalacturonase shows an elution profile on HPGPC (Fig. 1) differing from those obtained for the other pectolytic enzymes (Renard *et al.*, 1991*b*). It contained a whole range of polysaccharides with elution times ranging from 20 to 27·5 min. There were only two minor peaks in the elution range of pectic oligomers (elution times 32–35 min). The Sephacryl S500 profile (Fig. 2) showed a good correspondence with the HPGPC data. It underlined the heterogeneity of the material extracted by the rhamnogalacturonase, with different patterns for the uronic acid and the

Table 1. Characterisation of the rhamnogalacturonase extract

	Yield[a]	Composition (mg/g)									
		GalA	MeOH (DM)	AcOH (DAc)	Neutral sugars						
					Rha	Fuc	Ara	Xyl	Man	Gal	Glc
CDTA-IR[b]		198	25 (70)	24 (37)	10	13	110	59	16	88	336
Small scale trial:											
Blank	2·1[c]	718	nd	nd	21	0	169	tr	0	71	21
Extract	7·2[c]	516	nd	nd	35	0	313	9	0	117	10
Large scale trial:											
Blank	3·2[d]	805	nd	nd	30	0	115	tr	0	50	tr
Extract	13·6[d]	417	58 (76)	24 (17)	30	0	312	11	tr	97	11

[a]Yield in weight % from the CDTA-IR.
[b]Values from Renard *et al.* (1990).
[c]The yield was calculated from the concentration of the sugars.
[d]The yield was calculated from the weight of the freeze-dried extract.
nd, Not done; tr, traces.

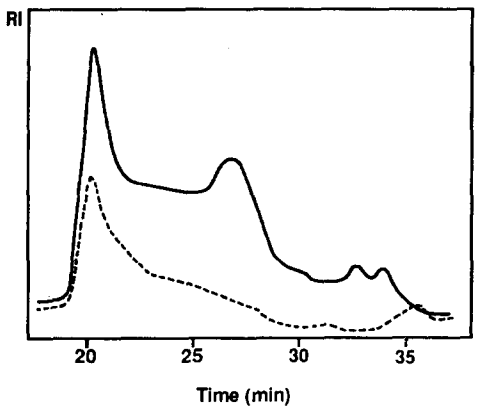

Fig. 1. HPGPC pattern of the rhamnogalacturonase extract on Biogel TSK columns 20XL, 30XL and 40XL: (—) rhamnogalacturonase extract; (---) blank.

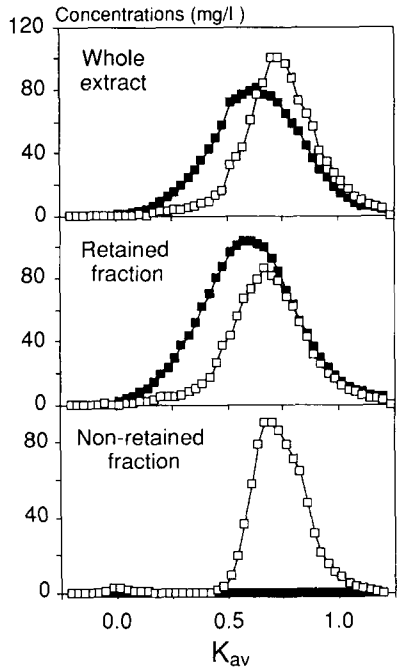

Fig. 2. Gel-filtration chromatography of the rhamnogalacturonase extract and its fractions from anion-exchange chromatography on Sephacryl S500 eluted with 0·1 M sodium succinate buffer pH 4·5: (■) uronic acids; (□) neutral sugars.

neutral sugars. The uronic acid eluted as a broad peak with a maximum at $K_{av} \sim 0{\cdot}6$, and the neutral sugars around K_{av} 0·8.

The elution patterns obtained here are also in agreement with the low value (107 ml/g) obtained for the intrinsic viscosity of the rhamnogalacturonase extract. Values around 600 ml/g are commonly found for pectins from apple juice (Knee, 1978; Rouau & Thibault, 1984) or 'mild' extractions (Renard & Thibault, 1993). Although this intrinsic viscosity does point to some degradation of the pectins, it is very close to values found by Thibault *et al.* (1993) for 'smooth regions' isolated after mild acid hydrolysis.

The rhamnogalacturonase thus differed from the other pectolytic enzymes in respect to the relative size of the molecules in which the neutral sugars and the galacturonic acid are extracted. Both pectinlyase or polygalacturonase plus pectinmethylesterase liberated oligogalacturonates and high-molecular-weight material rich in neutral sugars (Renard *et al.*, 1991*c*; see also Fig. 4). In contrast, with the rhamnogalacturonase the galacturonic-acid-rich moieties are in molecules with higher hydrodynamic volumes than the neutral-sugars-rich fractions.

Structure of the pectins extracted by rhamnogalacturonase from apple CDTA-IR

The material extracted by the rhamnogalacturonase was split into two fractions on DEAE Sepharose CL-6B (Fig. 3). The non-retained fraction (A) contained about 30% of the neutral sugars, and was composed almost exclusively of neutral sugars (93 mol%), mainly arabinose. It was also rich in xylose and glucose, relative to the initial extract, and contained traces of fucose and some mannose; concentration of these two sugars in this fraction probably explains why they could be quantified here. The fact that this fraction was not retained in spite of still containing about 7% uronic acids might be due to complete methylation of carboxyl groups (i.e. DM ~100), as found by de Vries *et al.* (1982) in 'hairy regions'. The retained fraction (B) contained virtually all the galacturonic acid (98%), and eluted at an ionic strength in good agreement with its degree of methylation. Although arabinose was still the main neutral sugar, there were more rhamnose and galactose than in the non-retained fraction, relative to the arabinose. This fraction was, however, not homogeneous, as shown by the slightly different elution patterns obtained for the neutral sugars and uronic acids.

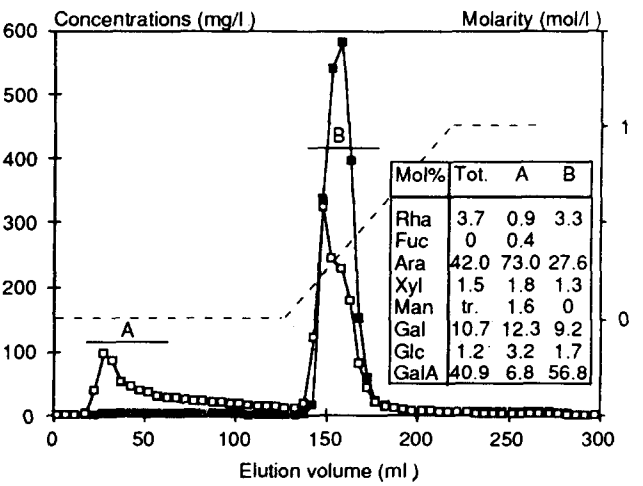

Fig. 3. Fractionation of the rhamnogalacturonase extract on DEAE Sepharose CL-6B eluted with sodium succinate buffer pH 4·8 (Tot., composition of the injected material): (■) uronic acids; (□) neutral sugars; (---) buffer molarity.

Methylation analysis of the whole extract (Table 2) showed the presence of terminal rhamnose (13% of the rhamnose residues), resulting from the action of the rhamnogalacturonase, as it was never found in the previous extracts (Renard *et al.*, 1991*c*). The rhamnose residues were otherwise 2-linked (50%) or 2,4-linked (36%), as seen earlier (Renard *et al.*, 1991*c*). The main component of the neutral sugar fraction was (1 → 5)-linked arabinan; approximately 1/4 of the residues in the main chain carried ramifications on O-3, and a fraction was doubly substituted on O-3 and O-2. The side-chains mostly constituted single residues, with some (short) (1 → 3)-linked chains. As was seen earlier for apple pectins (Renard *et al.*, 1991*c*), there were traces of arabinopyranose. The galactose was present mostly as type I, i.e. (1 → 4)-linked galactan, with a minor fraction of type II galactan.

Table 2. Linkage analysis of the whole extract and of the anion-exchange chromatography fractions

Methyl ethers	Extract	Non-retained fraction	Retained fraction
2,3,5-Arabinose[a]	15·9[b]	11·9	16·7
2,3,4-Arabinose	0·8	0·3	1·1
2,3-Arabinose	29·1	43·7	28·1
2,5-Arabinose	4·4	4·1	3·9
3,5-Arabinose	1·5	0·5	0·9
2-Arabinose	10·5	14·8	10·9
Arabinose	6·6	3·0	4·9
Total	68·8 (71·2)[c]	78·3 (78·4)	66·3 (64·1)
2,3,4,6-Galactose	1·6	tr.	2·5
2,3,6-Galactose	10·2	11·2	11·8
2,4,6-Galactose	5·3	4·4	4·9
2,3,4-Galactose	0·4	0	0·9
2,3-Galactose	1·6	0·7	0·9
2-Galactose	0·7	0	1·4
Total	19·7 (18·1)	16·3 (13·2)	22·4 (21·4)
2,3,4-Rhamnose	0·7	0·3	0·4
3,4-Rhamnose	2·8	0·5	5·0
3-Rhamnose	2·0	0·3	4·0
Total	5·5 (6·2)	1·1 (1·0)	9·4 (7·6)
2,3,4-Xylose	1·8	0·5	0·9
2,3- and 3,4-Xylose	1·0	0·7	0
Total	2·8 (2·5)	1·2 (2·0)	0·9 (3·0)
2,3,6-Glucose	1·0	1·3	0
2,3-Glucose	2·3	1·5	1·1
Total	3·2 (2·0)	2·8 (3·5)	1·1 (4·0)
2,3,4-Fucose	tr.	0·3	0
Total	tr.	0·3 (0·4)	0

[a]2,3,5-Arabinose denotes 1,4-di-*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).
[c]Values in parenthesis: mol% of the parent sugar as determined by alditol acetates.

The two fractions from anion-exchange chromatography were further analysed by gel filtration chromatography (Fig. 2) and methylation analysis (Table 2). The profiles on Sephacryl S500 showed that the non-retained fraction was composed almost exclusively of low-molecular-weight material. The profiles also confirmed that the retained fraction was not homogeneous, as it showed different elution patterns for uronic acid and neutral sugars.

The non-retained fraction contained relatively more terminal rhamnose (~1/4) than the whole extract, and almost no terminal galactose. Its glycosyl linkage composition was otherwise very similar to that of the whole extract. The derivatives obtained for fucose, xylose and glucose indicted presence of traces of fucogalactoxyloglucan. The absence of a noticeable peak corresponding to a 'substituted' rhamnose freed from the main chain, which would give a 1,4,5-tri-*O*-acetyl-2,3-di-*O*-methyl-rhamnitol, indicates that the rhamnogalacturonase was probably only able to split the rhamnogalacturonan backbone at a site free of side-chains. Identification by Colqhoun *et al.* (1990) of the oligomers of the type Gal-(1 → 4)-Rha-(1 → 4)-GalA-(1 → 2)-Rha-(1 → 4)-GalA in a rhamnogalacturonase digest was probably due to the fact that they started from acid-treated material, where the side-chains had been reduced to their monogalactosyl root. In the retained fraction there was in contrast less terminal (4%) and more disubstituted (42%) rhamnose. There was also more terminal galactose, and the xylose was only present as terminal residues. The authors have already shown (Renard *et al.*, 1991*c*) that terminal xylose in apple pectins was not carried by the arabinan or arabinogalactan side-chains, and probably substituted galacturonic acid residues, and Voragen *et al.* (1992) have isolated xylogalacturonan from rhamnogalacturonase-treated 'modified hairy regions'.

Effect of admixtures

In order to investigate possible synergisms, apple cell walls were treated with admixtures of rhamnogalacturonase and other enzymes. The yields of these extractions are given in Table 3. There was no synergism for liberation of cell wall polysaccharides, i.e. the yields with the admixtures were not superior to the sum of the yields of the mixtures without RGase plus the yield obtained with RGase alone, except maybe for neutral sugars in the combination with pectinlyase. Synergism would have been expected with polygalacturonase plus pectinemethylesterase or arabinanases plus galactanase, as those enzymes either (pectinmethylesterase) decreased the substitution of the galacturonic acids or (arabinanases plus galactanases) eliminated the side-chains in the 'hairy regions', thus making the rhamnogalacturonic backbone more accessible to the rhamnogalacturonase (Schols *et al.*, 1990). The higher activity

Table 3. Yields (% of the CDTA-IR) of neutral sugars and galacturonic acid in the supernatant from incubation of admixtures with rhamnogalacturonase

	RG	RG + PL	RG + PG + PE	RG + Endo Glu	RG + Arafase + Endo Ara + Endo Gal
Neutral sugars	3·9 (0·7)	10·7 (5·2)	6·2 (4·2)	9·8 (5·3)	9·6 (7·8)
Galacturonic acid	3·4 (1·4)	12·4 (9·2)	9·9 (9·6)	4·0 (2·1)	4·5 (2·1)

Values in parenthesis are those obtained without the rhamnogalacturonase.
PL, Pectinlyase; PG, polygalacturonase; PE, pectinmethylesterase, Endo Glu, endo-glucanase; Arafase, arabinofuranosidase; Endo Ara, endo-arabinanase; Endo Gal, endo-β-(1 → 4)-galactanase.

observed by Schols *et al.* (1990) on chemically deesterified modified hairy regions might, however, be due to elimination of the acetyl groups rather than of the methyl esters. A rhamnogalacturonan acetyl esterase has been isolated from the same source as the rhamnogalacturonase, and the two enzymes are thought to act synergistically (Searle-van Leeuwen *et al.*, 1992).

The extracts from the admixtures were analysed by HPGPC (Fig. 4). When conventional pectolytic enzymes, such as pectinlyase or the combination polygalacturonase plus pectinmethylesterase, were added to the rhamnogalacturonase, the elution profile on HPGPC showed a diminution of the excluded peaks compared to the elution patterns obtained with the rhamnogalacturonase on the one hand (Fig. 1) and with the pectinlyase or the combination polygalacturonase plus pectinmethylesterase on the other (Fig. 4, upper and lower right). This was specially marked in the admixture with polygalacturonase plus pectinmethylesterase. For both combinations there was an increase in the amounts of polymers eluting from 22 to 30 min, and in the amounts of oligomers (elution times 30–35 min). It appears that in this case there was a mutual degradation of the high hydrodynamic volume fractions, the pectinlyase or polygalacturonase plus pectinemethylesterase degrading the high-molecular-weight acidic fraction extracted by the rhamnogalacturonase (Fig. 1), and the rhamnogalacturonase the neutral-sugars-rich fraction liberated from apple cell walls by pectinlyase and polygalacturonase plus pectinmethylesterase (Renard *et al.*, 1991*b*). More pronounced effects in the presence of pectinmethylesterase could still be due to deesterification of the pectins. However, most of the degradation led to reaction products of intermediary size rather than oligomers. Adding side-chain degrading enzymes (arabinofuranosidase plus endo-arabinanase plus endo-β-(1 → 4)-galactanase) to the rhamnogalacturonase led to the disappearance of the lower-molecular-weight fraction from the rhamnogalacturonase, which was converted to oligomers, as could be expected from the results of structural characterisation (Fig. 4, upper left). The extract obtained in combination with an endo-glucanase showed only superposition of the profiles of the extracts of the rhamnogalacturonase and of the endo-glucanase (Figs 1 and 4, lower left); there was no interaction between the two enzymes or between one enzyme and the products liberated from the cell walls by the other enzyme.

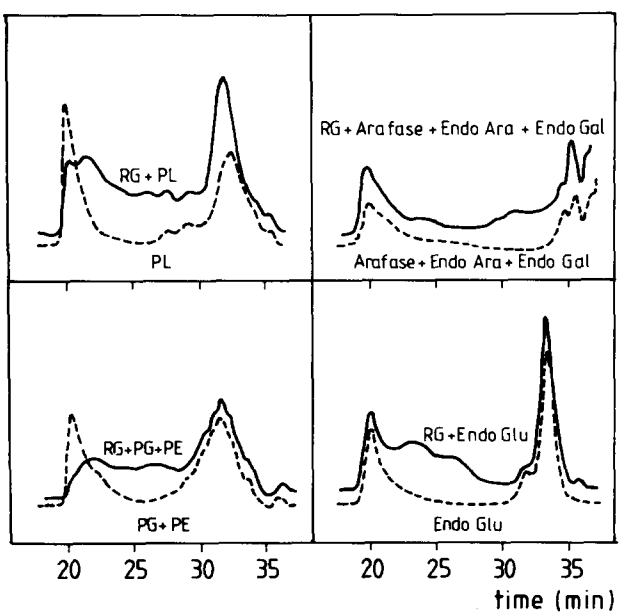

Fig. 4. HPGPC pattern of extracts with admixtures on Biogel TSK columns 20XL, 30XL and 40XL: (—) with rhamnogalacturonase; (---) without rhamnogalacturonase. PL, pectinlyase; PG, polygalacturonase; PE, ppectinmethylesterase; Endo Glu, endo-glucanase; Arafase, arabinofuranosidase; Endo Ara, endo-arabinanase; Endo Gal, endo-β-(1 → 4)-galactanase.

CONCLUSION

Rhamnogalacturonase was able to liberate approximately 14% of the apple cell walls, i.e. it was able to act on native 'hairy regions' in whole (isolated) cell walls, although it has been shown to be noticeably more active on saponified and/or acid-treated than on native pectins (Schols *et al.*, 1990). This indicates that the rhamnogalacturonic backbone stays accessible to enzymes in the cell wall. The rhamnogalacturonase liberated material rich in neutral sugars and of low molecular weight, and material rich in uronic acids and of larger size, in contrast to that obtained with other pectolytic enzymes (Renard *et al.*, 1991*c*). A noticeable proportion of the pectins are retained in the apple cell wall only because they form part of a larger molecule, pointing out the

rarity of the cross-links. In particular, whole 'smooth regions' were freed simply by cutting them off from the 'hairy regions' of larger pectic molecules. It would be interesting to see if such a phenomenon would also occur in cell walls not treated with a chelating agent, i.e. where calcium cross-link might exist between pectin molecules, providing a check on the extent of this particular type of cross-link in the cell wall. Liberation of homogalacturonic sequences by rupture of the rhamnogalacturonic backbone also implies that those two entities (homogalacturonan and 'rhamnogalacturonan I') were indeed part of the same molecule in the cell wall; the homogalacturonan forms the 'smooth regions', and the rhamnogalacturonan the 'hairy regions' of the complex pectin molecule.

A fraction enriched in arabinose could be isolated from the rhamnogalacturonase extract by ion-exchange chromatography, but it was not devoid of galacturonic acid and rhamnose, showing that its origin was the 'hairy regions'. The results from ion-exchange chromatography support the hypothesis that 'hairy regions' are not homogeneous in structure, both for the relative proportion of rhamnose and galacturonic acid and for the nature (and possibly size) of the attached side-chains (Saulnier & Thibault, 1987; Voragen *et al.*, 1992). The structure of the polysaccharides extracted by rhamnogalacturonase was characterised by the presence of non-reducing terminal rhamnose residues, caused by the action of the enzyme. The xylose and glucose (and fucose) that were present in the rhamnogalacturonase extract appeared to originate partly from some fucogalactoxyloglucan (in the non-retained fraction). In the retained fraction the xylose was only found as terminal residues, i.e. not offering a possibility of further glycosidic linkage to other cell wall polysaccharides. No synergism was seen in admixtures with other enzymes for liberation of polysaccharides from apple cell walls. There were, however, qualitative changes in the extracts (except with endo-glucanase), notably degradation of the highest size fractions with other pectolytic enzymes, and of the low-molecular-weight fraction with arabinanases and galactanase.

REFERENCES

Axelos, M.A.V. & Thibault, J.-F. (1991). Influence of the substituents of the carboxyl groups and of the rhamnose content on the solution properties and flexibility of pectins. *Int. J. Biol. Macromol.*, **13**, 77–82.

Colquhoun, I.J., de Ruiter, G.A., Schols, H.A. & Voragen, A.G.J. (1990). Identification by n.m.r. spectroscopy of oligosaccharides obtained by treatment of the hairy regions of apple pectins with rhamnogalacturonase. *Carbohydr. Res.*, **206**, 131–44.

Englyst, H.N. & Cummings, J.H. (1984). Simplified method for the measurement of total non-starch polysaccharides by gas–liquid chromatography of constituent sugars as alditol acetates. *Analyst*, **109**, 937–42.

Hakomori, S.I. (1964). A rapid methylation of glycolipids and polysaccharide catalyzed by methylsulfinyl carbanion in dimethylsulfoxide. *J. Biochem.*, **55**, 205–8.

Harris, P.J., Henry, R.J., Blakeney, A.B. & Stone, B.A. (1984). An improved procedure for the methylation analysis of oligosaccharides and polysaccharides. *Carbohydr. Res.*, **127**, 59–73.

Keegstra, K., Talmadge, K.W., Bauer, W.D. & Albersheim, P. (1973). The structure of plant cell walls III: A model of the walls of suspension-cultured sycamore cells based on the interconnections of the macromolecular components. *Plant Physiol.*, **51**, 188–96.

Knee, M. (1978). Properties of polygalacturonate and cell cohesion in apple fruit cortical tissue. *Phytochemistry*, **17**, 1257–60.

Knee, M., Fielding, A.H., Archer, S.H. & Laborda, F. (1975). Enzymic analysis of cell wall structure in apple fruit cortical tissues. *Phytochemistry*, **14**, 2212–22.

Massiot, P. & Thibault, J.-F. (1989). Enzymic analysis of carrot cell wall polysaccharides. *Carbohydr. Res.*, **190**, 121–36.

Mort, A.J., Moeerschbacher, B.M., Pierce, M.L. & Maness, N.O. (1991). Problems encountered during the extraction, purification, and chromatography of pectic fragments, and some solutions to them. *Carbohydr. Res.*, **215**, 219–27.

Renard, C.M.G.C. & Thibault, J.-F. (1993). Structure and properties of apple and sugar-beet pectins extracted by chelating agents. *Carbohydr. Res.*, **244**, 99–114.

Renard, C.M.G.C., Voragen, A.G.J., Thibault, J.-F. & Pilnik, W. (1990). Studies on apple protopectin I: Extraction of insoluble pectin by chemical means. *Carbohydr. Polym.*, **12**, 9–25.

Renard, C.M.G.C., Searle-van Leeuwen, M.J.F., Voragen, A.G.J., Thibault, J.-F. & Pilnik, W. (1991*a*). Studies on apple protopectin II: Apple cell wall degradation by pure polysaccharidases and their combinations. *Carbohydr. Polym.*, **14**, 295–314.

Renard, C.M.G.C., Schols, H.A., Voragen, A.G.J., Thibault, J.-F. & Pilnik, W. (1991*b*). Studies on apple protopectin III: Characterization of the material extracted by pure polysaccharidases from apple cell walls. *Carbohydr. Polym.*, **15**, 13–32.

Renard, C.M.G.C., Voragen, A.G.J., Thibault, J.-F. & Pilnik, W. (1991*c*). Studies on apple protopectin V: Structural studies on enzymatically extracted pectins. *Carbohydr. Polym.*, **16**, 137–54.

Renard, C.M.G.C., Lomax, J.A. & Boon, J.J. (1992). Apple fruit xyloglucan: A comparative study of enzyme digests of whole cell walls and of alkali extracted xyloglucans. *Carbohydr. Res.*, **232**, 303–20.

Rombouts, F.M. and Thibault, J.-F. (1986). Enzymic and chemical degradation and the fine structure of pectins from sugar-beet pulp. *Carbohydr. Res.*, **154**, 189–203.

Rouau, X. & Thibault, J.-F. (1984). Apple juice pectic substances. *Carbohydr. Polym.*, **4**, 111–25.

Saulnier, L. & Thibault, J.-F. (1987). Enzymic degradation of pectic substances from the pulp of grape berries. *Carbohydr. Polym.*, **7**, 329–43.

Schols, H.A., Geraeds, C.C.J.M., Searle-van Leeuwen, M.F., Kormelink, F.J.M. & Voragen, A.G.J. (1990). Rhamnogalacturonase: A novel enzyme that degrades the hairy regions of pectins. *Carbohydr. Res.*, **206**, 105–15.

Searle-van Leeuwen, M.J.F., van den Broek, L.A.M., Schols, H.A., Beldman, G. & Voragen, A.G.J. (1992). Rhamnogalacturonan acetylesterase: A novel enzyme from *Aspergillus aculeatus*, specific for the deacetylation of hairy (ramified) regions of pectins. *Appl. Microbiol. Biotechnol.*, **38**, 347–9.

Sweet, D.P., Shapiro, R.H. & Albersheim, P. (1975). Quantitative analysis by various g.l.c. response factors theories for partially methylated and partially ethylated alditol acetates. *Carbohydr. Res.*, **40,** 217–25.

Thibault, J.-F. (1979). Automatisation du dosage des substances pectiques par la méthode au méta-hydroxydiphenyl. *Lebensm. -Wiss. u. -Technol.*, **12,** 247–51.

Thibault, J.-F., Renard, C.M.G.C., Axelos, M.A.V., Roger, P. & Crépeau, M.-J. (1993). Studies of the length of homogalacturonic regions in pectins by acid hydrolysis. *Carbohydr. Res.*, **238,** 271–86.

Tollier, M.-T. & Robin, J.-P. (1979). Adaptation de la méthode à l'orcinol sulfurique au dosage automatique des oses neutres et totaux. *Ann. Technol. Agric.*, **28,** 1–15.

Voragen, A.G.J., Schols, H.A. & Pilnik, W. (1986). Determination of the degree of methylation and acetylation of pectins by hplc. *Food Hydrocolloids,* **1,** 65–70.

Voragen, A.G.J., Schols, H.A. & Gruppen, H. (1992). Structural studies on plant cell wall polysaccharides using enzymes. In *Proc. Int. Symp. on Plant Polymeric Carbohydrates*, Berlin, 1–3 July 1992.

de Vries, J.A., Rombouts, F.M., Voragen, A.G.J. & Pilnik, W. (1982). Enzymic degradation of apple pectins. *Carbohydr. Polym.*, **2,** 25–33.