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Two Pectic Polysaccharides From Kiwifruit Cell Walls

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ABSTRACT

From kiwifruit cell wall material treated with endopolygalacturonase, two polysaccharide fractions were isolated and purified using ion-exchange and gel-filtration chromatography. One of the fractions was shown to be a polysaccharide composed of 32% galacturonic acid, 15% rhamnose, 18% arabinose, and 35% galactose. This polysaccharide is considered to be a rhamnogalacturonan-type polysaccharide, probably rhamnogalacturonan I. The second fraction, of lower molecular weight, was a homogalacturonan composed of 81% galacturonic acid, 6·7% glucose, 2·8% mannose, 1·7% arabinose and 0·5% fucose.

INTRODUCTION

Kiwifruit *Actinidia deliciosa* (A. Chev, C. F. Liang & A. R. Ferguson), formerly *Actinidia chinensis*, originating in China, have been grown in New Zealand for about 80 years (Sale, 1985). Commencing in 1952, the export of kiwifruit has become a major New Zealand industry (Earp, 1988). Kiwifruit quality is primarily determined by maturity of the fruit at harvest, affecting fruit firmness, flavour and texture when eventually ripened (Harman & Hewett, 1981). Ripening in kiwifruit is associated with extensive flesh softening (Arpaia *et al.*, 1987), affecting fruit texture and quality. Pectic substances, integral parts of the cell walls and middle lamella, undergo the greatest change of the cell wall components during fruit ripening (De Vries *et al.*, 1984; Fuke & Matsuoka, 1984; Arpaia *et al.*, 1987) and hence appear to have the greatest influence on the change in texture. Furthermore, pectic substances play important roles in fruit

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and vegetable processing (Van Buren, 1979, 1986) and as components of dietary fibre (Behall & Reiser, 1986).

Pectic substances are a group of closely associated but structurally variable polymers (Aspinall, 1980). The group comprises the acidic polysaccharides, rhamnogalacturonan I (RG-I) and rhamnogalacturonan II (RG-II) and the largely neutral polysaccharides, arabinans, galactans and arabinogalactans (McNeil *et al.*, 1984). The structures of RG-I and RG-II are particularly complex (Spellman *et al.*, 1983; Melton *et al.*, 1986; Lau *et al.*, 1987; Stevenson *et al.*, 1988) which has led to speculation as to their roles in the plant cell wall. Recently, RG-I and RG-II have been shown (Thomas *et al.*, 1989*a*, *b*) to be present in monocot cell walls.

In a concurrent study (Redgwell *et al.*, 1988) the plant cell walls of kiwifruit have been fractionated by using a series of solvent extractions. The monosaccharide composition and the sugar linkages of the polysaccharide fractions were determined. The monosaccharide composition and the linkages suggest that RG-I might be present in some fractions. The unusual sugars — 2-linked glucuronic acid, 3-linked rhamnose, 3-deoxy-D-manno-2-octulonic acid (ketodeoxyoctulonic acid) and apiose — previously found in RG-II (Darvill *et al.*, 1978; York *et al.*, 1985) isolated from cultured sycamore cells by treatment with endopolygalacturonase were identified in certain fractions, indicating that RG-II is probably present in kiwifruit cell walls.

In order to obtain additional evidence for the presence of rhamnogalacturonans in kiwifruit cell walls, the method developed by Albersheim's group (Darvill *et al.*, 1978; McNeil *et al.*, 1980) for separating RG-I and RG-II from culture sycamore cell walls has been adapted for use in this investigation.

EXPERIMENTAL

Kiwifruit were harvested at the DSIR Te Puke Orchard on 20 May 1986 with 8·0% total soluble solids (TSS). The exported graded fruit were packaged and placed in cold storage (0°C) within 24 h of harvest. After 3 weeks' storage the fruit were allowed to warm to 22°C and had the following parameters: 11·6% TSS; weight, 82·5 g; penetrometer firmness, 3·7 kgf (average of 10 fruit).

Preparation of cell wall material

Twenty peeled kiwifruit (1260 g) were homogenised with an equivalent volume of cold distilled water. The resulting slurry was centrifuged at

10 000 g for 10 min at 0°C and the pellet was washed according to the method of Talmadge *et al.* (1973). Cell wall material was dried under vacuum to constant weight (45·1 g) and stored in an air-tight container.

Endopolygalacturonase

Endopolygalacturonase was purified from frozen ripe tomato pericarp using a modification of the procedures of Tucker *et al.* (1980) and Ali and Brady (1982). Tomato pericarp (30 g) was homogenised in a Sorvall Omnimixer with an equivalent volume of cold distilled water. The homogenate was centrifuged to 2400 g for 10 min at 0°C. The pellet was resuspended in 1 M NaCl with the pH adjusted to 6·0 with 1 M NaOH (30 ml). The resulting suspension was stirred for 3 h at 0°C and then centrifuged at 2400 g for 10 min. The supernatant was filtered using Whatman No. 541 filter paper. The filtrate was subjected to ammonium sulphate fractionation in the 40–80% saturation range. After centrifugation at 10 000 g for 20 min the precipitate was dissolved in 0·125 M sodium acetate, pH 6·0 (10 ml). The solution was applied to a Sephadex G-100 column (3 × 60 cm) which was eluted with 1 M NaCl adjusted to pH 6·0. As a final purification step the enzyme was eluted from a Concanavalin A–Sepharose 4B column with alpha-methyl-D-mannoside as described by Ali and Brady (1982). Ultrafiltration (with 10 000 Da mol. wt cut-off membrane) was used to separate off the eluent and concentrate the enzyme solution.

Preparation of pectic polysaccharides

The procedure of Darvill *et al.* (1978) was used with some modifications, which are given below. The cell wall material (3·6 g) was suspended in 10 mM sodium acetate buffer pH 5·2 (200 ml) with endopolygalacturonase, incubated at 30°C for 3 h and then centrifuged (10 000 g for 10 min). This procedure was repeated twice. The solubilised material from the three extractions was filtered through GF/C glass filter paper prior to dialysis against distilled water. The dialysed material was lyophilised.

Purification of the pectic polysaccharides was carried out using a DEAE-Trisacryl M column (3 × 35 cm), eluted with 10 mM potassium phosphate buffer, pH 6·5, followed by a linear NaCl gradient (0–0·5 M) in the phosphate buffer and 4 ml fractions collected. This was followed by gel filtration chromatography on a Sephacryl S-300 column (3 × 50 cm) and then on a BioGel P-10 column (2 × 23 cm). Both columns were eluted with 50 mM sodium acetate buffer, pH 5·2. Fractions collected were 2·5 and 1·5 ml, respectively.

Pectic polysaccharide assay

Pectic polysaccharides were quantified as anhydrogalacturonic acid using the *m*-hydroxydiphenyl method at 520 nm (Blumenkrantz & Asboe-Hansen, 1973). Reagents were made up as described by Kintner and Van Buren (1982). A new *m*-hydroxydiphenyl solution was prepared every 28 days.

Neutral sugars were quantified as galactose by the anthrone method at 620 nm (Dische, 1947).

Preparation of derivatives for gas chromatography

Methyl glycosides and methyl ester glycosides of standards and sample material were prepared using the method of Chambers and Clamp (1971). Samples (1 mg) were silylated using 0·5 ml pyridine, 0·1 ml hexamethyldisilazane and 0·05 ml trimethylchlorosilane. To the silylated glycosides an equivalent volume of dichloromethane was added prior to injection into the capillary column. Gas chromatography (GC) was carried out on a capillary column fused silica BP-10 25 m × 0·22 mm (i.d.). The temperature programme used was: 140–160° at 10°/min and 160–275° at 5°/min for 10 min. Apiose was isolated from partial hydrolysates by paper chromatography and quantified by gas chromatography of its alditol acetate (Redgwell *et l.*, 1988).

RESULTS AND DISCUSSION

Cell wall material from kiwifruit was treated with endo-alpha-1,4-polygalacturonase isolated from tomatoes. The endopolygalacturonase-solubilised cell wall material was applied to a DEAE-Trisacryl M ion-exchange column. The column was eluted using a linear sodium chloride gradient (Fig. 1). Three carbohydrate-containing peaks (A, B and C) were eluted from the column within the salt gradient and one further peak (D) was observed when the salt gradient was maintained at 0·5 M sodium chloride until 400 ml eluent had been collected. Fractions corresponding to the peaks were pooled, dialysed against distilled water and freeze dried. The peaks A, B and C corresponded to those obtained with those from endopolygalacturonase-solubilised material from cultured sycamore cells applied to a DEAE-Sephadex column (Darvill *et al.*, 1978; McNeil *et al.*, 1980; Melton *et al.*, 1986). In the work on sycamore cells, RG-I and RG-II were found in the third peak and because of overlapping in the second peak. On this basis the rhamno-

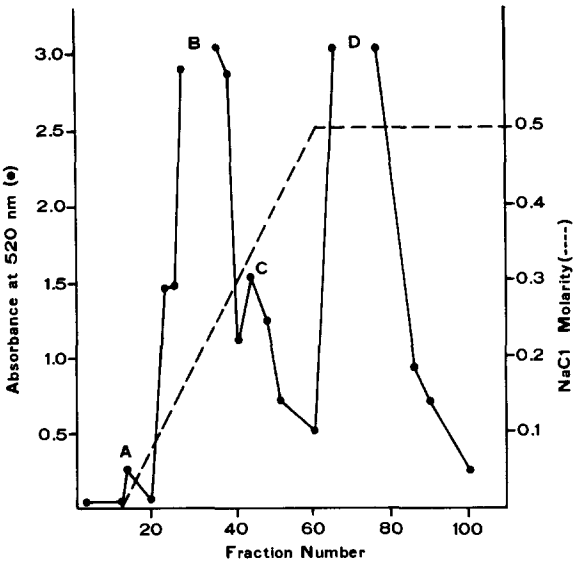

Fig. 1. Chromatography of endopolygalacturonase solubilised cell wall material on a DEAE-Trisacryl M column. Column fractions were assayed for uronosyl residues by the *m*-hydroxydiphenyl method.

galacturonans were assumed to be in peaks B and C and no further work was done on peaks A and D. The freeze-dried material in peaks B and C (101 mg an 30 mg, respectively) were separately dissolved in acetate buffer and subjected to gel filtration on Sephacryl S-300 columns. Each column produced two carbohydrate-containing peaks (Figs 2 and 3). The appropriate fractions were pooled, dialysed against distilled water and freeze dried. The higher-molecular-weight peaks from fraction B (7·9 mg) and fraction C (0·5 mg) were characterised by a high neutral sugar content relative to the uronic acid content (Figs 2 and 3). The higher-molecular-weight peak obtained by McNeil *et al.* (1980) on an agarose A-5m column was composed of RG-I and contained a relatively high amount (63%) of neutral sugar for a pectic polysaccharide. The lower-molecular-weight peaks from fraction B (0·5 mg) and fraction C (7·5 mg) were characterised by low neutral sugar contents relative to uronic acid content. In contrast, the lower-molecular-weight peak, comprising RG-II, obtained by Darvill *et al.* (1978) on an agarose A-5m column contained 66% neutral sugar content. The higher molecular weight fractions from Sephacryl S-300 gel filtration of fractions B and C were combined, dissolved in 50 mM sodium acetate buffer (5 ml, pH 5·2) and applied to a BioGel P-10 which produced a single peak (Fig. 4a). After pooling the peak fractions, dialysis and freeze drying, the yield was

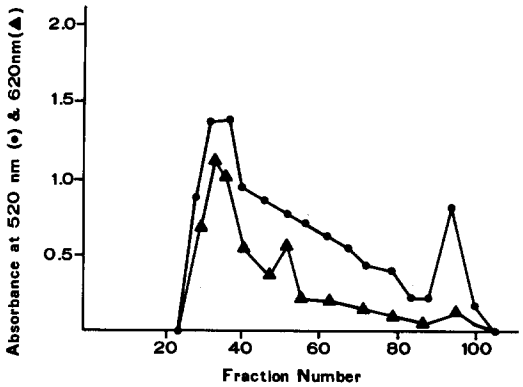

Fig. 2. Chromatography, on Sephacryl S-300 column, of material in fraction B from DEAE-Trisacryl M column. Column fractions were assayed for neutral glycosyl residues by the anthrone method (at 620 nm) and for uronosyl residues by the *m*-hydroxydiphenyl method (at 520 nm).

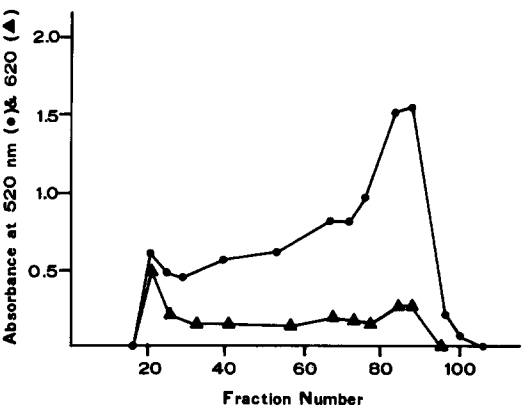

Fig. 3. Chromatography, on Sephacryl S-300 column, of material in fraction C from DEAE-Trisacryl M column. Column fractions were assayed for neutral glycosyl residues by the anthrone method (at 620 nm) and for uronosyl residues by the *m*-hydroxydiphenyl method (at 520 nm).

3·4 mg. Similarly, the combined lower-molecular-weight peaks from fractions B and C eluted from a BioGel P-10 column as a single peak (Fig. 4b) with a yield of 3·6 mg.

Freeze-dried material from the BioGel P-10 columns underwent methanolysis and was derivatised to form the trimethylsily (TMSi) derivatives, allowing GC analysis of the glycuronosyl and glycosyl components of the isolated polysaccharides. The higher-molecular-weight fraction contained rhamnose, arabinose, galactose and galactu-

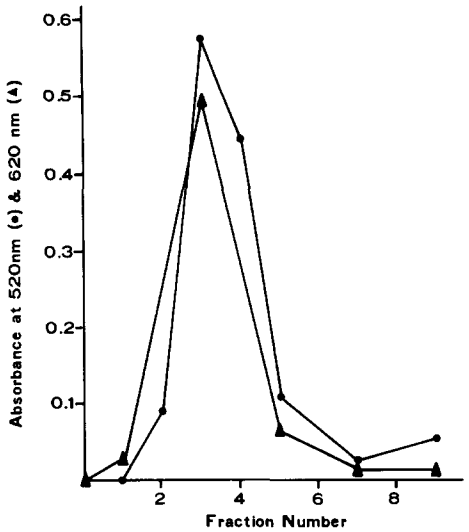

Fig. 4a. Chromatography, on Bio Gel P-10 column, of the combined high-molecular-weight fractions from Sephacryl S-300. Fractions were assayed as in Figs 2 and 3.

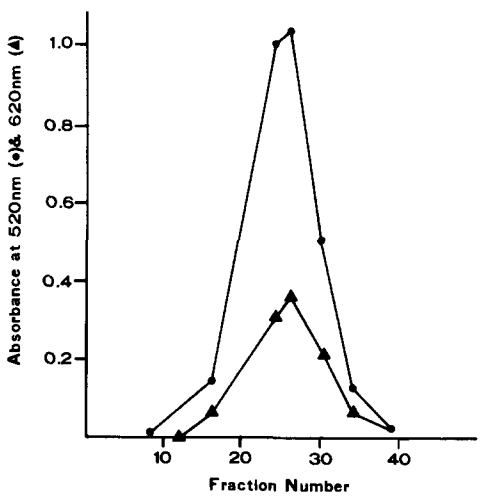

Fig. 4b. Chromatography, on Bio Gel P-10 column (2 × 23 cm), of the combined low-molecular-weight fractions from Sephacryl S-300. Fractions were assayed as in Figs 2 and 3.

ronic acid, comprising in mol%: 15%, 18%, 35% and 32%, respectively (Table 1). The polysaccharide appears to be a rhamnogalacturonan-type polysaccharide. The glycosyl composition of the higher-molecular-weight fraction from this present study compares favourably with the

TABLE 1
Chemical Composition of RG-I, RG-II and the Two Isolated Polysaccharides from Kiwifruit Cell Wall Material (Results are expressed in % mol)

Glycosyl residues	*RG-I*[a]	*RG-II*[b]	*High-molecular-weight peak*	*Low-molecular-weight peak*
Apiose		7	ND[c]	ND
Arabinose	24	14	18	1·7
Fucose	2	4	ND	0·5
Galactose	20	12	35	ND
Galacturonic acid	36	29	32	81
Glucose	1	2	ND	6·7
Glucuronic acid		3	ND	ND
Mannose			ND	2·8
2-*O*-methyl fucose		5	ND	ND
2-*O*-methyl xylose		4	ND	ND
Rhamnose	15	18	15	ND
Xylose	1		ND	ND
Unknown (other)	1	2	ND	7·3

[a]McNeil *et al.* (1980).
[b]Darvill *et al.* (1978).
[c]ND = not detected.

literature values for RG-I and not for RG-II (Table 1). The hydrolysate was tested for the presence of both 2-*O*-methyl xylose and apiose, as being indicative of RG-II. Neither 2-*O*-methyl xylose nor apiose was detected in the higher-molecular-weight fraction. Therefore the higher-molecular-weight fraction was not an RG-II type polysaccharide. McNeil *et al.* (1980) using a similar endopolygalacturonase extraction from cell-wall material of sycamore cells, isolated a high-molecular-weight polysaccharide (RG-I) and a lower-molecular-weight polysaccharide (RG-II). Having used a similar procedure in this work it might be expected that the isolated higher-molecular-weight fraction is RG-I and the lower-molecular-weight fraction is RG-II. McNeil *et al.* (1980) found with RG-I that fractions eluted early were rich in arabinosyl residues relative to the later fractions that were rich in rhamnosyl and galacturonosyl residues. On chromatography of the higher-molecular-weight fraction on BioGel P-10 (Fig. 4a), it was found that fractions eluted early had a higher proportion of neutral sugars (68%) compared to uronic acid than fractions eluted later. This is consistent with the work of McNeil *et al.* (1980).

Hence, from the evidence we have obtained, it appears that the higher-molecular-weight fraction (peak B) could be an RG-I-type polysaccharide.

The lower-molecular-weight fraction was shown to contain mainly galacturonic acid and smaller amounts of arabinose, mannose, fucose and glucose, comprising in mol%: 81%, 1·7%, 2·8%, 0·5% and 6·7%, respectively (Table 1). This accounted for 92·7 mol% of the glycosyl residues present, with the remaining 7·3% unidentified. While the unidentified GC peak had a relative retention in the region of the methyl glycoside silylated derivatives of uronic acids, it did not correspond to galacturonic or glucuronic acid. Galactose, rhamnose and 2-*O*-methyl xylose were absent in GC analysis of the derivatised hydrolysate. Apiose could not be detected using a separate procedure. The major component of this polysaccharide — galacturonic acid, comprising 81% mol of the hydrolysate residues — is consistent with a pectic polysaccharide. The absence of rhamnose and galactose is evidence that the polysaccharide is not a rhamnogalacturonan, as rhamnose and galactose comprise 35% and 30% of the glycosyl residues of RG-I (McNeil *et al.*, 1980) and RG-II (Darvill *et al.*, 1978), respectively.

The symmetry of the elution profile from chromatography of the lower-molecular-weight polysaccharide on BioGel P-10 (Fig. 4b) is suggestive of homogeneity. The high galacturonic acid content (81%) is consistent with that found for homogalacturonans (McNeil *et al.*, 1984). Pure homogalacturonans have only been isolated from the primary cell wall after treatment that was likely to cleave covalent bonds (McNeil *et al.*, 1984). This is consistent with the current work where the enzyme endopolygalacturonase was applied to cell-wall material. Nothnagel *et al.* (1982) reported a homogalacturonan consisting of 84% galacturonic acid and lesser amounts of rhamnose, xylose, mannose and glucose. More recently, Thomas *et al.* (1989*a*) obtained a galacturonan during the BioGel P-10 separation of RG-I and RG-II from endopolygalacturonase treatment of rice cell walls.

In spite of the lower-molecular-weight materials' behaviour on the ion-exchange and gel-permeation columns, it is clearly not RG-II. That RG-II was not obtained by the established method is surprising, considering that when using a different separation procedure we were able to show that RG-II is probably present in the cell walls of kiwifruit (Redgwell *et al.*, 1988). Thomas *et al.* (1989*a*) have stressed that de-esterification as well as endopolygalacturonase treatment are required in order to obtain RG-I and RG-II. Since we did not incorporate a specific de-esterification step in our procedure, this may explain the apparent absence of RG-II. A ubiquitous mucilage is found in the kiwifruit plant, but its composition of D-glucuronic acid, D-mannose, L-fucose, L-arabinose and D-galactose in the molar ratios of 1·0:1·0:1·5:2·0:4·0 (Redgwell *et al.*, 1986) means that it cannot be the lower-molecular-weight material.

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