Extraction of pectic polysaccharides from sugar-beet cell walls

Mazz Marry,[1†] Maureen C McCann,[1*] Frank Kolpak,[2] Alan R White,[3] Nicola J Stacey[1] and Keith Roberts[1]

[1]Department of Cell Biology, John Innes Centre, Colney, Norwich NR4 7UH, UK
[2]Hercules Incorporated Research Centre, 500 Hercules Road, Wilmington, DE 19808-1599, USA
[3]Department of Botany, North Dakota State University, Fargo, ND 58105-5517, USA

Abstract: Previous methods of extracting pectin from sugar-beet have used pulp as the starting material. As the temperature and pressure of the pulping process may modify the architecture of the cell wall, we have adapted a relatively non-disruptive method to characterise cell wall material (CWM) isolated directly from the sugar-beet. Cell walls from mature sugar-beets (*Beta vulgaris* L Aztec) were sequentially extracted four times with imidazole and twice with sodium carbonate to produce six heterogeneous pectic polysaccharide extracts, and with KOH to produce a hemicellulosic extract which was predominantly xylans. Heterogeneity of the extracted pectins was indicated by differences in FTIR spectra, uronic acid content, % methyl esterification, % feruloylation, % acetylation, molecular weight distribution and neutral sugar composition. The highest proportion of feruloyl esters was found in polysaccharides solubilised by the second sodium carbonate extraction. Anion exchange chromatography of these polysaccharides gave three fractions, one of which contained most of the feruloyl ester. These results indicate that feruloyl esters are not randomly distributed among the different pectic polysaccharides in the sugar-beet cell wall, and that esterification is likely to be dependent on the local sugar sequence or conformation.

Keywords: pectin; sugar-beet; cell walls; methyl esters; Fourier transform infrared (FTIR) microspectroscopy; GC–MS; sugar linkage analysis; ferulic acid; feruloyl esters; acetyl esters

INTRODUCTION

Sugar-beet is a biennial plant of the genus *Beta* and a member of the family *Chenopodiaceae*. Its relatively low cost and availability have long made sugar-beet pulp a potential source of pectin.[1] However, because of the acetylation and feruloylation of the polymers, the applications for sugar-beet pectin in the food industry have been limited. Removal of acetyl groups promotes the gelling of sugar-beet pectin.[1–4]

The pectin network in plant cell walls[5,6] is constructed primarily from two galacturonic acid-rich pectic polysaccharides, rhamnogalacturonan (RG)-I and homogalacturonan (HGA).[7–9] HGA is a helical polymer consisting of (1–4)-α-linked D-galacturonan (GalA). The basic unit structure of RG-I is composed of a backbone of alternating (1–4)-α-linked D-galactopyranosyluranyl and (1–2)-α-linked L-rhamnopyranosyl residues which forms a contorted rod that is considerably more flexible than the helical HGA.[6,7] The side-chains of neutral arabinan, galactan and arabinogalactan are linked to the C-4 and/or C-3 of the rhamnose. Arabinans are mostly 5-linked units of L-arabinofuranosyl residues forming short helical chains, whilst galactans generally have a backbone of (1–4)-β-linked residues with (1–6)-β-linked substitutions.[6] There are two types of arabinogalactans (AGs) associated with RG-I. Type I AG has a (1–4)-β-linked linear chain of D-galactose units onto which short chains of (1–5)-α-linked L-arabinose residues are connected, generally to C-3. Type II AGs are highly branched chains with backbones of (1–3)- and (1–6)-linked β-D-galactose units. The chains are mostly terminated by (1–6)-α-linked L-arabinose residues.[6,7,11,12]

The structures of HGA, RG-I and its side-chains are highly conserved, even in the most evolutionarily diverse plants.[1,6,10–13] HGA and RG-I are spatially in their distribution, both within a single wall and between different cell types, and the relative abundance of each varies widely between dicotyledons, monocotyledons and gymnosperms.[14,15] Two additional substituted HGAs have been characterised,

* Correspondence to: Maureen C McCann, Department of Cell Biology, John Innes Centre, Colney, Norwich NR4 7UH, UK
†Current address: Agricultural, Food and Environmental Chemistry, Glasgow University, Glasgow G12 8QQ, UK
Contract/grant sponsor: Hercules Incorporated, USA
Contract/grant sponsor: Royal Society
Contract/grant sponsor: BBSRC
(*Received 7 May 1999; revised version received 14 July 1999; accepted 29 July 1999*)

xylogalacturonan[6] and rhamnogalacturonan (RG)-II.[8–10,16] RG-II is a minor but extremely highly conserved molecule, and recent evidence suggests that the formation of borate–diester cross-links between RG-II polymers may play a role in the structure of cell walls.[16–20]

The chemical structure of sugar-beet pectin has been characterised by means of acid hydrolysis[2,4,21–25] and enzymatic degradation,[26–32] and chemically characterised following a sequential extraction of wet pulp.[33] Oosterveld *et al*[33] reported that the sugar moieties of pectic polysaccharides (arabinose, rhamnose, galactose and galacturonic acid) account for more than 63% (w/w) of the cell wall material present in pulp, and that approximately 70% of the pectin in sugar-beet pulp consists of branched RG-I, assuming a rhamnose/galacturonic acid ratio of 1:1. Unlike most dicots, the *Chenopodiaceae* have significant levels of ferulic acid in their primary walls.[33–35] The ferulic acid moieties are ester-linked either to arabinose or galactose residues.[21,35,36] These feruloyl groups appear to be mostly located at the non-reducing arabinosyl termini as described for spinach pectin.[4,34]

Previous extraction methods[3,4,22] have used wet or dried pulp that has already been subjected to 80 °C and high pressure during sugar extraction. As such severe conditions may modify pectin structure or change its extractability, we have adapted a method[37] to sequentially extract pectins in as close to their native state as possible from fresh cell walls of mature sugar-beet. We have assayed total and methyl-esterified pectin content, % feruloylation and % acetylation. FTIR microspectroscopy, GC–MS linkage analysis, size exclusion chromatography and immunolabelling indicate that each extract has differences that may play diverse roles in wall architecture as well as different commercial functionalities. In particular, the second sodium carbonate extract contains a highly feruloylated pectin which can be further resolved by anion exchange chromatography.

MATERIALS AND METHODS

Cell wall material

Mature sugar-beets (*Beta vulgaris* L Aztec) were washed, cut into pieces about 1 cm^3 in size, frozen in liquid nitrogen and stored at −20 °C. The tissues were coarsely chopped in a food blender and then ground to a fine powder in liquid nitrogen in a mortar pre-cooled to −80 °C. This powder was washed with distilled water over four layers of Miracloth (Calbiochem, USA) to obtain cell wall material (CWM). For some experiments, CWM was boiled in 80% methanol (BDH, UK) at 80 °C for 30 min prior to sequential extraction (adapted from Ref 38).

Sequential extraction of sugar-beet pectin

CWM was extracted with 2 M imidazole pH 7.0 (Sigma) at 20 °C for 6 h followed by an overnight incubation in fresh 2 M imidazole.[39] Both these steps were repeated prior to an overnight incubation at 4 °C in 0.05 M sodium carbonate and 20 mM $NaBH_4$ followed by a 3 h incubation in fresh solution at 20 °C. The cell wall residue was further incubated in 1 M KOH and 10 mM $NaBH_4$ at 20 °C for 2 h (adapted from Ref 37). Supernatants containing extracted wall polymers from each extraction step were neutralised with HCl, dialysed (12 000 MW cut-off) against deionised water and concentrated using an Amicon filter (10 000 MW cut-off) apparatus to a final volume of 15–20 ml. Each extract was then frozen in liquid nitrogen, freeze-dried and resuspended in sterile distilled water (dH_2O) at a concentration of 1 mg ml^{-1}.

FTIR spectroscopy

Spectra were obtained on a Bio-Rad FTS40 FTIR spectrometer equipped with a Spectra-Tech IR-Plan microscope accessory. All spectra were obtained at a resolution of 8 cm^{-1}, with 256 interferograms co-added for a high signal-to-noise ratio. Microscope aperture dimensions of $100 \times 100\,\mu m^2$ were used. Aliquots (40 µl) of each soluble pectin extract were dried in a layer on the barium fluoride window (13 mm diameter × 2 mm thickness) at 37 °C for 2 h. FTIR spectra were collected in transmission mode as previously described.[40] The wavenumbers corresponding to chemical species of interest in a plant cell wall have been previously characterised,[40,41] including the ester region (1740–1720 cm^{-1}), carboxylate ion stretches (1600 and 1414 cm^{-1}) and amide peaks (1650 and 1550 cm^{-1}).

Spectrophotometric assays

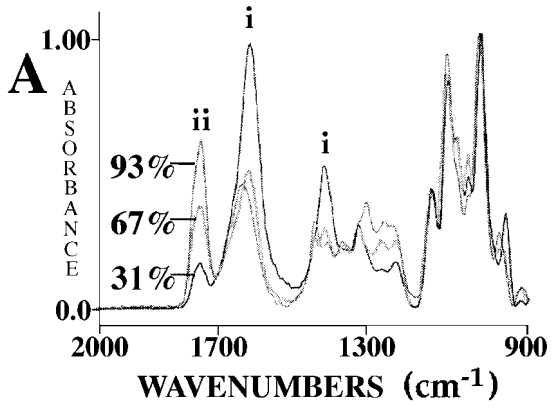
Uronic acid assays for each extract were carried out as described previously.[43,44] Methyl esterification was assayed by measuring release of methanol after saponification,[45] with modifications to increase sensitivity and reproducibility.[46] The feruloyl content of each pectin extract was determined by the method of Guillon and Thibault,[26] except that a standard curve of ferulic acid (Fluka) from 5 to 50 nmol was used to determine feruloyl content. *O*-acetyl content was determined by the Hestrin method.[47]

Ion exchange chromatography

Aliquots (1 ml) of each extract were fractionated by ion exchange chromatography using a Bio-Rad Econo System with a Bio-Rad 1 ml Econo PAC Q Cartridge and twin buffer system of buffer A (25 mM Tris-HCl pH 8.1) and buffer B (25 mM Tris-HCl pH 8.1, 1 M NaCl). The 1 ml fractions collected during the programme (time T (min)/buffer B%: T0/0%, T11/10%, T14/20%, T17/40%, T20/60%, T28/100%; flow rate 1.5 ml min^{-1}) were stored at −20 °C until required.

Immunodot blots

Aliquots (2 µl) of each column fraction were spotted onto a square of Protran BA 85 nitrocellulose membrane with a pore size of 0.45 µm (Schleicher &



pectin standard	Uronic acid (nmol)	% Methyl esterification
31%	1000±14	24±2
67%	921±17	63±4
93%	1036±12	87±4

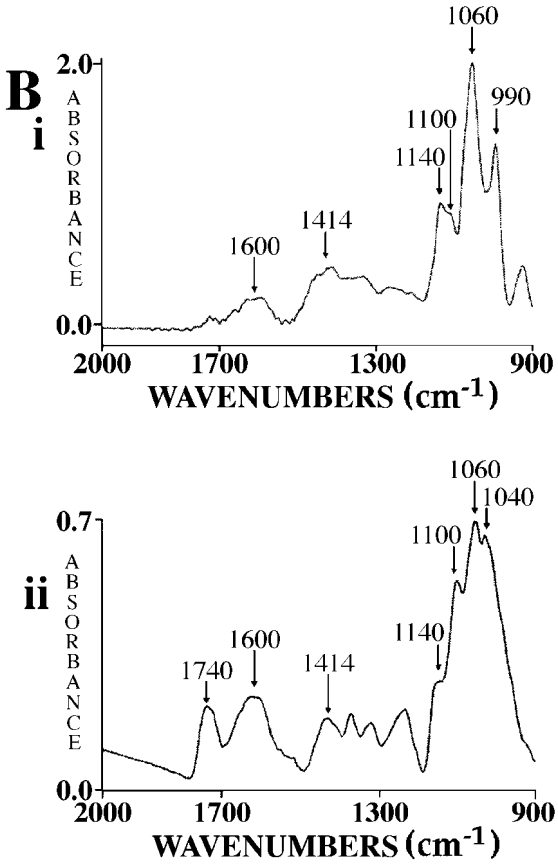


Figure 1. (A) FTIR spectra of citrus peel pectin (Sigma) with methyl esterification values of 31%, 67% and 93%. The spectral features of all three standards were similar except in absorbance intensity of carboxylate ions (1600 and 1414 cm^{-1}) (i) and carboxylic ester (1740–1720 cm^{-1}) (ii). (B) FTIR spectra of sugar-beet pulp (i) and unextracted CWM (ii). The spectrum of the pulp shows peaks at 1600 and 1414 cm^{-1}, indicating the presence of pectic material, together with carbohydrate peaks at 1140, 1100 and 1060 cm^{-1} and a peak at 990 cm^{-1}. The spectrum of the unextracted CWM exhibits more peaks in the 1500–900 cm^{-1} region than that of the pulp, as well as an ester peak at 1740 cm^{-1} which is absent from the spectrum of the pulp.

Schuell) and assayed for the presence of immunoreactive pectic epitopes as described previously.[42] The primary antibodies used, JIM 5 and JIM 7, have also been described previously.[42]

Aqueous size exclusion chromatography

The extracts were chromatographed through a set of TSK-Cel columns (three linear GMPWXL and one G3000PWXL in series) at a flow rate of 1 ml min^{-1}. A sample concentration of 0.2 mg ml^{-1} was used with an injection volume of 200 µl. SEC measurements were performed in a 0.2 M lithium acetate buffer, pH 4, mobile phase with both the columns and the refractive index detector at 30 °C. The molecular weight distribution data are in terms of a polyethylene oxide/polyethylene glycol calibration.

Sugar and linkage analyses

Extracts (2 mg) were derivatised for glycosyl linkage analysis by methylation by *n*-butyllithium.[48] Extracts dried over P_2O_5 were dissolved in 500 µl DMSO, and 200 µl of *n*-butyllithium was added slowly, followed by 500 µl of methyl iodine. Methylated samples were extracted with chloroform ($CHCl_3$) and washed with water, then the $CHCl_3$ phase was evaporated under nitrogen gas to dryness. To 2 mg of each extract, partly methylated as above or unmethylated for sugar composition, was added 1 ml of 2 M trifluoroacetic acid (TFA) (Sigma) containing 0.1 mg ml^{-1} myo-inositol (Sigma) as an internal standard. The extracts were heated at 120 °C for 90 min. Upon cooling, 1 ml of *tert*-butyl alcohol (Sigma) was added and mixed thoroughly. The TFA and *tert*-butyl alcohol were evaporated under nitrogen gas at 45 °C, and the sugars were converted to their corresponding alditol acetates.[48] Other extracts were carbodiimide-activated as described previously.[48] Analysis was carried out on a Hewlett Packard G1800A GCD series gas chromatograph with an electron ionisation detector controlled by HP GCD chemstation G1074A. Alditol acetates were separated in an SP-2380 fused silica capillary column 30 m long × 0.25 mm id × 0.20 µm film thickness (Supelco). Injections were made in the splitless mode with helium as the carrier gas at a flow rate of 1 ml min^{-1}. Column oven temperature was initially 160 °C, programmed to rise at 4 °C min^{-1} to a final holding temperature of 240 °C and maintained at this temperature for 5 min. Detector and injector temperatures were both 250 °C, and derivative structures were deduced as described by Carpita and Shea.[48]

RESULTS

Known pectin standards

In order to determine if the assays used for the estimation of uronic acid content and % methyl esterification were accurate, known standards were investigated. Citrus peel pectin (Sigma) with declared methyl esterification values of 31%, 67% and 93% was assayed for uronic acid and methyl ester content and

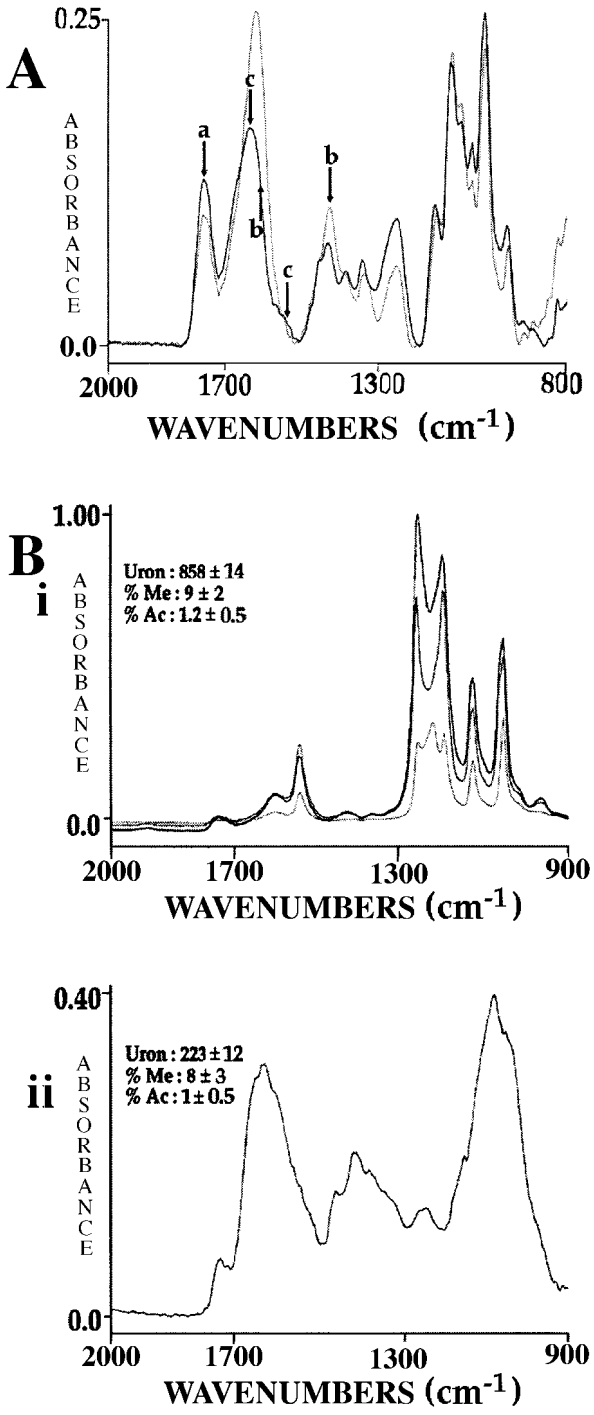


Figure 2. (A) FTIR spectrum of the second sodium carbonate extract from CWM boiled in methanol prior to extraction (black). The spectrum of the same extract from untreated CWM is shown for comparison (grey). Boiling walls in methanol prior to extraction resulted in increased carboxyl ester at 1740 cm^{-1} (a) and decreased carboxylate peaks at 1600 and 1414 cm^{-1} (b) and protein peaks at 1650 and 1550 cm^{-1} (c) relative to the extract from untreated walls. (B) FTIR spectra of pectic polysaccharides extracted from CWM by the methods of Michel *et al*[22] and Faulds and Williamson.[3] Using the Michel *et al* method (three independent experiments) (i), the spectral features of the extract could not be identified. The spectral features of the extract from the method of Faulds and Williamson (ii) contained peaks characteristic of pectic polysaccharides, but also contained protein and other carbohydrates. For chemical analysis the values for % methyl esterification (%Me) and % *O*-acetylation (%Ac) are relative to the nmol uronic acid content (Uron) from 1 mg ml^{-1} starting material.

characterised by FTIR microspectroscopy (Fig 1A). The spectral features of all three pectin standards vary only in amounts of carboxylate ions present and in ester content. The % methyl esterification values were reproducibly determined to be slightly lower than the range stated by Sigma (Fig 1A). This may be due to the compensation for neutral sugar interference used here.

FTIR spectra obtained from sugar-beet pulp and unextracted CWM

In order to determine if the CWM was a better source of pectin material than pulp, both were assayed by FTIR microspectroscopy. The sugar-beet pulp was obtained from Hercules Incorporated, USA. The spectrum of the pulp (Fig 1Bi) shows carboxylate ion peaks at 1600 and 1414 cm^{-1} and carbohydrate peaks at 1140, 1100, 1060 and 990 cm^{-1}. The spectrum of the unextracted CWM (Fig 1Bii) has an ester peak at 1740 cm^{-1}, carboxylate ion peaks at 1600 and 1414 cm^{-1} and carbohydrate peaks at 1140, 1100, 1060 and 1040 cm^{-1}. The increased absorbances at 1740, 1600 and 1414 cm^{-1} in the CWM show that pectin has been lost from the pulp material.

Sequential extraction of sugar-beet pectins

Cell walls prepared from fresh sugar-beet were sequentially extracted with imidazole (four extracts), sodium carbonate (two extracts) and KOH, and FTIR spectra collected for all extracts. The spectral features of the first imidazole extract were very broad, indicating a mixture of components (data not shown), whilst FTIR spectra of successive imidazole and sodium carbonate extracts were progressively similar to an FTIR spectrum of citrus peel pectin. For example, the spectrum of the second sodium carbonate extract (Fig 2A) shows peaks at 1740, 1650, 1550, 1440, 1414, 1380, 1330, 1240, 1190, 1130, 1090, 1044, 992 and 937 cm^{-1} in common with peaks in the spectrum of the citrus peel pectin (Fig 1A).

Table 1. Uronic acid and % methyl ester contents of CWM residues remaining at each step during a sequential extraction. In each case the values for the % methyl esterification are relative to the nmol uronic acid present, adjusted to 1 mg ml^{-1} starting material. All figures shown are the mean of duplicate assays carried out on five separate sequential extractions

Sample	*Uronic acid content (nmol)*	*% Methyl esterification*
Unextracted CWM	2237 ± 21	30 ± 6
CWM after extraction with		
1st imidazole	2005 ± 18	19 ± 3
2nd imidazole	1695 ± 15	8 ± 2
3rd imidazole	1180 ± 12	5 ± 2
4th imidazole	1038 ± 11	4 ± 3
1st Na_2CO_3	900 ± 9	3 ± 2
2nd Na_2CO_3	610 ± 12	0
KOH	426 ± 11	0

Table 2. Uronic acid (UA) and percentage methyl ester (%Me) contents of sequential extracts from untreated CWM (untreated) and extracts from methanol-boiled CWM (methanol). The uronic acid values are given in nmol uronic acid mg^{-1} extract following subtraction of neutral sugar interference.[43] In each case the values for the % methyl esterification are relative to the nmol uronic acid content. All figures shown are the mean of duplicate assays carried out on five separate sequential extractions with standard variance

	Untreated		*Methanol*	
Sample	*UA*	*%Me*	*UA*	*%Me*
1st imidazole	90±11	12±3	292±27	27±5
2nd imidazole	279±31	21±4	227±23	24±4
3rd imidazole	461±29	21±4	270±25	25±5
4th imidazole	86±11	13±3	272±29	22±3
1st Na_2CO_3	89±9	15±3	288±30	25±4
2nd Na_2CO_3	238±16	6±2	272±25	13±4
KOH	30±8	0	88±9	1±1

To ensure that any native esterase activity was eliminated, the CWM was boiled in 80% methanol prior to sequential extraction.[38] By FTIR analysis, polymers from the second sodium carbonate extract of methanol-boiled CWM show significant differences from an untreated extract shown for comparison (Fig 2A). A decrease in carboxylate stretches at 1600 and 1414 cm^{-1} and an accompanying increase in the 1740 cm^{-1} carboxylic ester peak, relative to polymers from untreated walls, were observed. Less protein was extracted.

Comparison of methods for sugar-beet pectin extraction

The sequential extraction used here was compared with two previously published methods for the extraction of pectic polysaccharides from sugar-beet.[3,22] Using the Michel *et al* method,[22] CWM was incubated in dH_2O, adjusted to pH 1.0 with conc HCl for 1 h at 85 °C, cooled, and the pH adjusted to 4.5 with 2 M NaOH. The supernatant was dialysed against dH_2O and freeze-dried. The FTIR spectrum of material extracted by this method had no identifiable characteristics of pectin (Fig 2Bi). Using the Faulds and Williamson method,[3] CWM was incubated in 38 mM HNO_3 pH 1.7 for 1 h at 45 °C, followed by 1 h at 70 °C. The supernatant was neutralised with NaOH, dialysed against dH_2O and freeze-dried, giving an FTIR spectrum with characteristics of a mixture of components (Fig 2Bii). Ester peaks at 1720 and 1740 cm^{-1} were observed. No values of % methyl esterification or % acetylation were published by Faulds and Williamson.[3] However, the values obtained here were similar to those of extracts from untreated CWM by the Michel *et al* method.[22] Only about one-quarter of the uronic acid was extracted with this method relative to the Michel *et al* method.[22]

Pectin remaining in the cell wall residues

A sample of residual CWM from each sequential extraction step was analysed for uronic acid and methyl ester content (Table 1). The uronic acid values for these samples show a consistent reduction in pectic material remaining with successive extraction steps. The value for uronic acid in the KOH-extracted wall residue was 19% of that of the unextracted CWM. The methyl ester content was significantly reduced after the first two imidazole extractions, with the second sodium carbonate and KOH-extracted wall residue samples containing no methyl ester (Table 1).

Extracted pectins

Table 2 shows the total uronic acid content and percentage methyl ester for polymers extracted from untreated and methanol-boiled CWM. The % methyl ester values were highest in the imidazole extracts and decreased through the sodium carbonate and KOH extracts. The % methyl ester and uronic acid values of dialysed extracts were reduced relative to undialysed extracts (data not shown), suggesting that hydrolysis of pectin may occur during dialysis. The extracts from methanol-boiled CWM showed no significant differences between dialysed and undialysed extracts (data not shown) and had the highest % methyl ester content (Table 2). This suggests that boiling CWM in methanol prior to sequential extraction reduces native esterase activity in the extracts. Extracts from methanol-boiled CWM also had the highest % feruloylation and % acetylation (Table 3). The second sodium carbonate extract contained higher amounts of fer-

Table 3. Percentage feruloylation (%Fe) and acetylation (%Ac) of sequential extracts from untreated CWM (untreated) and extracts from methanol-boiled CWM (methanol). In each case the values for the % feruloylation are given in nmol ferulic acid relative to the nmol of arabinose and galactose in each extract, obtained from their mole percentages given in Tables 4A and 5A. The values for the % acetylation are given in nmol acetic acid relative to the nmol uronic acid content given in Table 2. All figures shown are the mean of duplicate assays carried out on three separate sequential extractions

	Untreated		*Methanol*	
Sample	*%Fe*	*%Ac*	*%Fe*	*%Ac*
1st imidazole	0.135	1±1	0.319	1±1
2nd imidazole	0.154	1±1	0.481	2±1
3rd imidazole	0.188	1±1	0.599	2±1
4th imidazole	0.164	0	0.377	2±1
1st Na_2CO_3	0.109	0	0.581	2±1
2nd Na_2CO_3	0.675	2±1	1.703	4±1
KOH	0.271	0	0.319	0

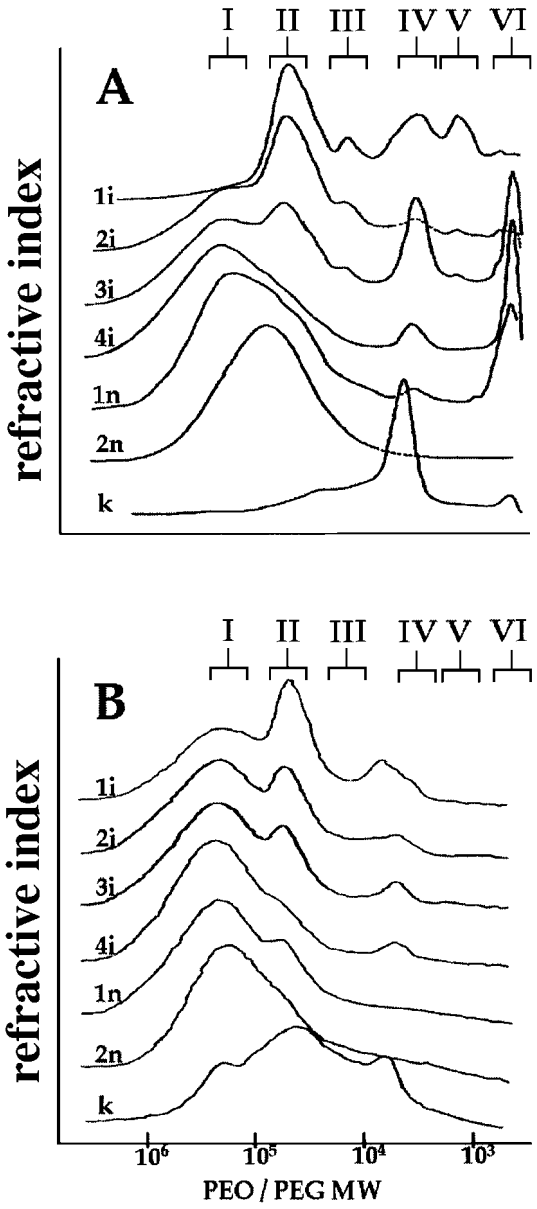


Figure 3. Size exclusion chromatograms, calibrated with PEO/PEG, of the polymers sequentially extracted from untreated CWM (A) and from CWM boiled in methanol prior to extraction (B); 1i–4i = imidazole extracts, 1n–2n = sodium carbonate extracts and k = KOH extract. Peaks of molecular weights (MW) 200 000–250 000 (I), 100 000–50 000 (II), 15 000 (III), 3000–5000 (IV), 1500 (V) and 400 (VI) are indicated.

uloyl and *O*-acetyl groups than all other extraction steps. Autoclaving of polymer extracts, as an alternative to methanol boiling of the CWM, was found to reduce substantially the yield of pectins (data not shown).

Aqueous size exclusion chromatography

Chromatograms (Fig 3A) of the polymers sequentially extracted from untreated CWM show that, while the molecular weight (MW) distributions of these polymers are different in each extract, they appear to be composed of varying amounts of six identifiable macromolecular populations. For example, the first imidazole extract mainly consisted of polymers with an MW of 50 000–100 000 (II), but also contained polymers with MW populations of 15 000 (III), 3000–5000 (IV) and 1500 (V). Successive imidazole extracts were enriched in polymers of increasing MW, primarily in the 200 000–250 000 population (I). The third and fourth imidazole extracts were also enriched in the 400 MW population (VI). Chromatograms of the polymers from CWM boiled in methanol prior to sequential extraction (Fig 3B) differ from their untreated counterparts, with all the extracts becoming enriched in polymers of higher molecular weight, primarily in the 200 000–250 000 population. Thus the lower-MW components observed in extracts from untreated CWM are probably products of enzymatic cleavage of larger molecules during dialysis.

Sugar and linkage analyses

In all experiments the extracted polymers and CWM were first partially reduced by 1-cyclohexyl-3-(2-morpholinyl-4-ethyl) carbodiimide (CMC) to determine the proportion of uronic acid to its corresponding neutral sugar. Table 4A shows the sugar composition of each extract from untreated cell wall material expressed as relative mole percentage (mol%). The amounts of rhamnose and galacturonic acid in each extract increased steadily to a maximum value in the second sodium carbonate extract and were much reduced in the KOH extract. The levels of arabinose in the first two imidazole extracts were significantly higher than in all other extracts. Levels of fucose, mannose, galactose and glucose varied only slightly between extracts. Low levels of xylose were observed in the four imidazole and two sodium carbonate extracts, whereas over half of the total sugar of the KOH fraction consisted of xylose (Table 4A).

The presence of different polysaccharides in each extract can be deduced from the detected linkages (Table 4B). The first and second imidazole extracts consist largely of an unbranched homogalacturonan (4-GalA) and 5-linked arabinan with small amounts of type II arabinogalactan (t-, 6- and 3,6-Gal), a glucomannan (4-Glu, 4-Man), a 4-linked xylan and xyloglucan (t-Xyl, 2-Xyl, t-Fuc, 4,6-Glu). The third and fourth imidazole extracts also contained a branched rhamnogalacturonan (4-GalA, 2-Rha, 2,4-Rha). The two sodium carbonate extracts were similar in composition to the third/fourth imidazole extracts but did not contain glucomannan. These two extracts also contained branched 2,5-arabinans. The KOH extract contained a large proportion of 4-linked xylan (4-Xyl, 2,4-Xyl) and a small proportion of a branched rhamnogalacturonan and xyloglucan. The 2,4- and 4,6-GalA linkages observed in this study are characteristic of under-methylation and are probably artefactual.

The relative mole percentages of sugars from each extract from CWM boiled in methanol prior to extraction are given in Table 5A. The amount of rhamnose in each extract again increased steadily to a maximum value in the second sodium carbonate

Table 4A. Sugar analysis of sequential extracts from untreated sugar-beet CWM. The values are given as relative mole percentages of sugars and are the mean of duplicate assays carried out on three separate sequential extractions

	Relative mole percentage of sugar							
Sugar	*CWM*	*1st Im*	*2nd Im*	*3rd Im*	*4th Im*	*1st Na*	*2nd Na*	*KOH*
Rha	4	3	4	6	8	7	9	3
Fuc	2	1	1	1	1	1	1	0
Ara	33	47	42	27	25	26	22	18
Xyl	4	4	6	8	6	4	2	58
Man	4	5	3	6	4	3	1	4
Gal	18	10	8	6	14	10	7	5
Glu	11	8	5	12	8	8	7	4
GalA	24	21	32	35	35	43	51	8

Table 4B. Linkage analysis, given as relative mole percentage, of sequential extracts from untreated sugar-beet CWM (nd = not detected)

Sugar	*Linkage*	*1st Im*	*2nd Im*	*3rd Im*	*4th Im*	*1st Na*	*2nd Na*	*KOH*
Rha	t-	3	4	4	3.2	3.8	4.1	0.6
	2-	nd	nd	1	3.6	1.8	3.9	0.8
	2,4-	nd	nd	1	1.2	1.4	1	1.6
Fuc	t-	1	1	1	1	1	1	0
Ara	t-	8	10.5	8.7	7	7	5.5	2
	2-	1.7	3.5	2.1	1	2	1.5	0.3
	3-	3.4	1.8	2	1	1.1	1	0.2
	5-	24	18.6	13.7	10	12	10	10
	2,5-	nd	nd	nd	nd	1.8	2	nd
	3,5-	5.9	7.5	0.5	6	1.9	2	5.5
Xyl	t-	0.8	1	1.45	1.5	1.7	0.8	1
	2-	1	2.2	2.9	2.5	0.4	0.3	5.1
	4-	2.2	2.8	3.6	2	1.9	0.9	50.7
	2,4-	nd	nd	nd	nd	nd	nd	1.2
Man	t-	3	1.2	4.5	2.9	3	1	4
	4-	0.8	1	1.5	1.1	nd	nd	nd
	2,4-	1.2	0.8	nd	nd	nd	nd	nd
Gal	t-	1.2	0.8	1.8	7.9	2.8	2.2	0.2
	6-	1.7	1.4	2	3.6	4.8	3.5	2.8
	3,6-	4.6	3.7	1	2.5	2.4	1.3	2
	3,4,6-	2.5	2.1	1.2	nd	nd	nd	nd
Glu	t-	0.8	1	0.8	1.2	0.8	1.2	0.6
	4-	6.2	2.8	9.2	5.4	5.2	4.6	2.4
	4,6-	1	1.2	1.2	1.4	2	2	1
GalA	4-	21	32	27.3	28.1	35.8	42.4	8
	2,4-	nd	nd	3.5	2.3	2.2	3.8	nd
	4,6-	nd	nd	4.2	4.6	5	4.8	nd

extract and was much reduced in the KOH extract. GalA levels increased to a maximum in the third imidazole extract and were reduced by the second sodium carbonate extract, whilst the KOH extract did not contain GalA. Low but constant levels of mannose and glucose were observed throughout the extracts, and fucose was present only in the second sodium carbonate extract. Almost constant levels of xylose were present in the four imidazole and two sodium carbonate extracts, whereas this sugar constituted 94% of the KOH extract. The level of arabinose in the third imidazole extract was lower than the constant levels found in the other extracts, except for the KOH extract which contained a significantly reduced amount. Galactose decreased significantly from the first imidazole extract.

The linkage analyses of these methanol-boiled extracts (Table 5B) show that the first two imidazole extracts consisted mainly of an unbranched homogalacturonan, 5-linked arabinan and type II arabinogalactan, with increased amounts, relative to extracts from untreated CWM, of a 2-linked arabinan, a 4-linked xylan, xyloglucan and a glucomannan. The third and fourth imidazole extracts and the two sodium carbonate extracts were similar in composition to extracts from untreated CWM. The KOH extract contained only a large proportion of 4-linked xylan and a small proportion of xyloglucan.

Table 5A. Sugar analysis of sequential extracts from methanol-boiled CWM. The values are given as relative mole percentages of sugars and are the mean of duplicate assays carried out on three separate sequential extractions

	Relative mole percentage of sugar							
Sugar	*CWM*	*1st Im*	*2nd Im*	*3rd Im*	*4th Im*	*1st Na*	*2nd Na*	*KOH*
Rha	5	2	2	2	2	5	7	1
Fuc	2	0	0	0	0	0	2	0
Ara	32	32	34	23	34	31	31	2
Xyl	4	10	10	10	7	10	9	94
Man	4	1	1	1	1	1	1	1
Gal	15	18	6	7	7	11	12	1
Glu	9	4	2	2	2	3	2	1
GalA	20	33	48	54	47	39	36	0

Table 5B. Linkage analysis, given as relative mole percentage, of sequential extracts from methanol-boiled CWM (nd = not detected)

Sugar	*Linkage*	*1st Im*	*2nd Im*	*3rd Im*	*4th Im*	*1st Na*	*2nd Na*	*KOH*
Rha	t-	2	2	0.5	0.6	1.8	1.6	1
	2-	nd	nd	1.5	1.2	2	4.2	nd
	2,4-	nd	nd	nd	nd	1.2	1.2	nd
Fuc	t-	nd	nd	nd	nd	nd	2	nd
Ara	t-	8	8	5	6.3	9.6	6	1
	2-	9	10	5.2	6.8	nd	nd	nd
	3-	1	nd	nd	1.7	1.2	0.8	nd
	5-	11	10.3	8.8	12.6	15.5	18.4	1
	2,5-	nd	nd	nd	nd	1.3	1.6	nd
	3,5-	4	5.7	4	6.6	3.4	4.2	nd
Xyl	t-	1.6	2	3.8	2	1.8	2.2	3.4
	2-	1	1	1.2	2.3	3.4	2.7	7.3
	4-	7.4	7	5	1.5	2.4	3	79.2
	2,4-	nd	nd	nd	1.2	2.4	1.1	4.1
Man	t-	nd	nd	nd	nd	1	1	1
	4-	0.4	0.5	0.5	0.4	nd	nd	nd
	2,4-	0.6	0.5	0.5	0.6	nd	nd	nd
Gal	t-	2	1	1	1	1.4	1.3	0.4
	6-	4	3	1.8	2.4	6.2	7.5	0.3
	3,6-	5.5	2	2.2	1.8	3.4	3.2	0.3
	3,4,6-	6.5	nd	2	1.8	nd	nd	nd
Glu	t-	0.5	nd	nd	0.4	0.8	0.5	nd
	4-	2.5	1.2	1.3	0.6	1.4	1.1	0.3
	4,6-	1	0.8	0.7	1	0.8	0.4	0.7
GalA	4-	33	48	54	47	39	36	0

Anion exchange chromatography

Ion exchange chromatography was used to further fractionate the polymers solubilised at each extraction step. By immunodot blot analysis, each extract contained more than one species of molecule. An example is shown for the fractions of the second sodium carbonate extract for untreated CWM and CWM boiled in methanol prior to extraction (Fig 4), cross-reacting with the antibodies JIM 5 (unesterified pectin) and JIM 7 (methyl-esterified pectin). The fourth column fraction, when rerun down the column, eluted at its original position. The data suggest that boiling the CWM in methanol prior to extraction preserves epitopes within individual fractions, shown by the JIM 5 staining of fractions 4, 7–21 and 26–29 relative to their untreated counterparts. There was also an increase of JIM 7 staining of fractions 4–12, 19, 20 and 25–29 (Fig 4).

All fractions from extracts from untreated (Fig 5A) and methanol-boiled CWM (Fig 5B) were assayed for % feruloylation and % acetylation. Feruloyl groups were present in untreated extract fractions 30–32 only, with the highest percentage observed in fraction 30. Fraction 30 did not contain any *O*-acetyl groups, but these were observed in fractions 31 and 32. Small but consistent levels of *O*-acetyl groups were observed in fractions 6–8 and 17–19.

In the second sodium carbonate extract from methanol-boiled CWM a small peak of feruloyl groups was observed in fractions 3–6 but was not present in the untreated fractions. Small but significant amounts of feruloyl groups were also observed in fractions 3–6

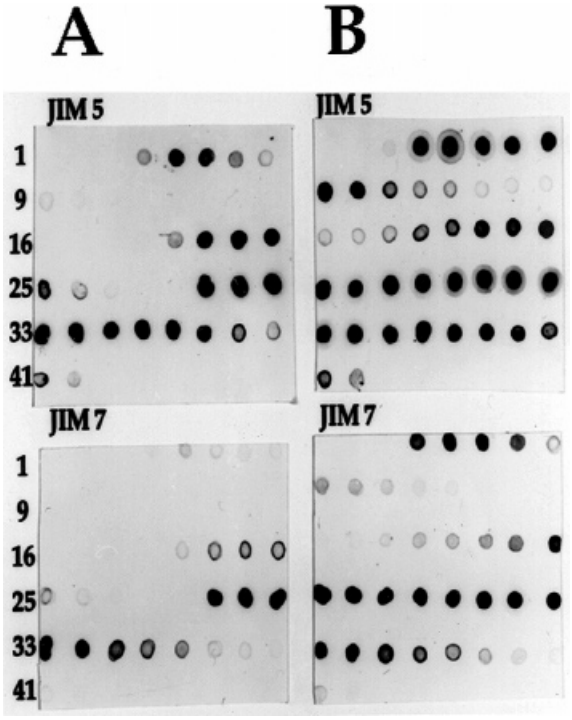


Figure 4. Immunodot blot analysis of anion exchange column fractions of the second sodium carbonate extract from both untreated and methanol-boiled CWM. Aliquots of 2 μl of the fractions collected were used for analysis. The fractions contain both unesterified (JIM 5) and methyl-esterified (JIM 7) pectin epitopes, and the binding pattern suggests that the fractions contain more than one species of molecule. Fractions from the methanol-boiled CWM extract contain two species of pectin, compared with three in the untreated counterpart. The dot intensities suggest that the fractions from CWM boiled in methanol before extraction contain more pectin. Samples loaded left to right and top to bottom as 1–8, 9–16, 17–24, 25–32, 33–40, 41–42.

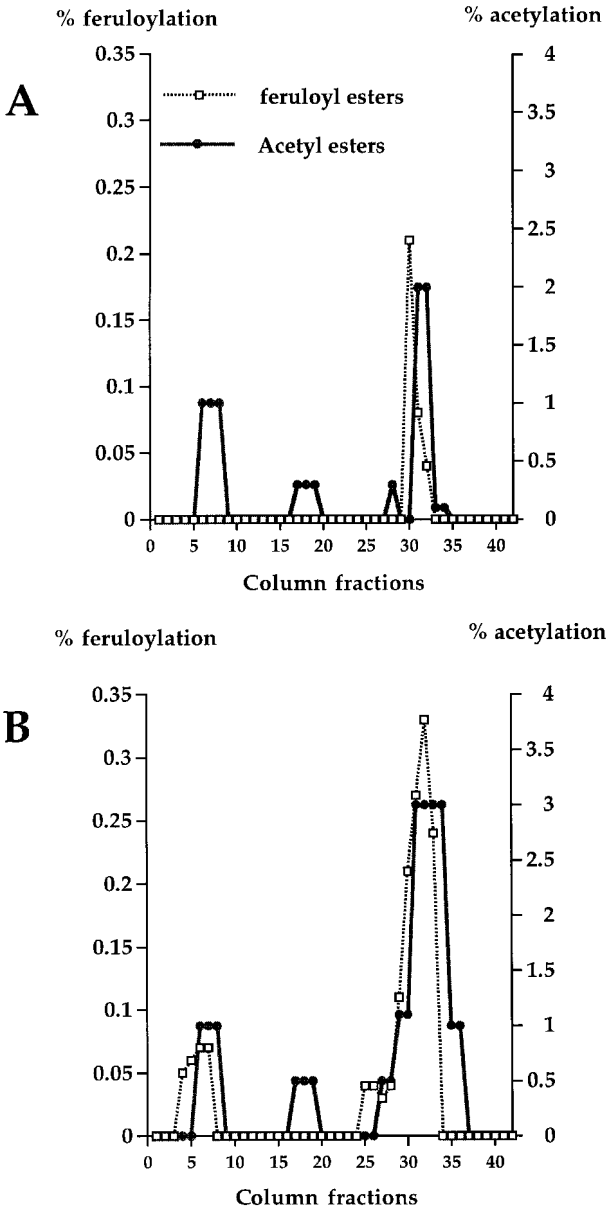


Figure 5. Feruloyl ester and *O*-acetyl ester contents of anion exchange chromatography fractions of the second sodium carbonate extract from both untreated (A) and methanol-boiled (B) CWM. The values of the feruloyl esters for both untreated and methanol-boiled CWM extracts are relative to the galactose and arabinose content of each fraction. The values of the *O*-acetyl esters for both untreated and methanol-boiled CWM extracts are relative to the amount of uronic acid present in each fraction. Methanol boiling prior to extraction appears to preserve esters in many fractions relative to their untreated counterparts.

and 25–28, and substantial amounts in fractions 29 and 33. Fraction 31 contained the highest feruloyl content. A marked increase in the presence of *O*-acetyl groups was seen between fractions 27 and 36, with a maximum in fractions 31–34.

The FTIR spectrum of fraction 30 (Fig 6) shows intense 1600 and 1414 cm^{-1} peaks, characteristic of the carboxylate ion stretches for pectin, and peaks in the 1500–1300 and 1200–900 cm^{-1} regions, similar to a spectrum of citrus peel pectin. The spectrum also showed an ester peak at 1740 cm^{-1}, which is consistent with the JIM 7-reactive epitope binding patterns (Fig 4) of this fraction. A phenolic ester peak at 1720 cm^{-1} was also present, presumably that of a feruloyl ester (Fig 6).

DISCUSSION

Each successive extraction consistently removed a proportion of pectic polysaccharides with different characteristics

We have adapted a relatively mild method to sequentially extract pectic polysaccharides from mature sugar-beet cell walls. Sugar-beet pulp shows regions in its FTIR spectrum characteristic of pectic material. However, the FTIR spectrum of sugar-beet CWM has stronger absorbances of peaks associated with pectic polysaccharides.[40,41] The FTIR spectra, uronic acid and methyl ester assays of material solubilised from each sequential extract of sugar-beet CWM show that each of the successive treatments consistently removes a defined proportion of uronic acid and methyl ester from the cell wall. Each extract contained different proportions of feruloyl and acetyl esters, with the second sodium carbonate extract containing the highest proportion of both. Successive imidazole and sodium carbonate extracts were seen to become progressively similar to an FTIR spectrum of

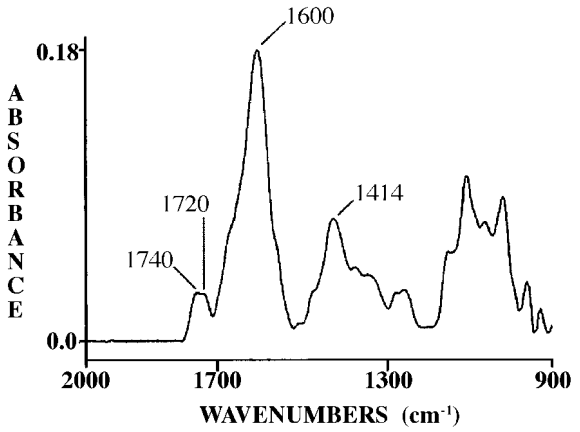


Figure 6. FTIR spectrum of anion exchange chromatography fraction 30 of the second sodium carbonate extract from untreated CWM. The spectral features of this fraction show intense 1600 and 1414 cm^{-1} peaks, characteristic of carboxylate ion stretches for pectin, and peaks in the 1500–1300 and 1200–900 cm^{-1} regions, with characteristics similar to a spectrum of citrus peel pectin. The spectrum also has ester peaks at both 1740 and 1720 cm^{-1}.

citrus peel pectin. The sugar analysis and the colorimetric uronic acid assay both indicate the presence of a small quantity of pectic material in the KOH extract. Analysis of the cell wall residue after the KOH extract shows that about 80% of the uronic acid-containing polymers are removed by this method.

Boiling the CWM in methanol prior to extraction alters the properties of the extracts

Consistently higher degrees of methyl, feruloyl and acetyl esterification were seen in all extracts from methanol-boiled CWM relative to their untreated counterparts. Boiling CWM in methanol preserves esters present on the extracted polysaccharides. Also, the total yield of pectic polysaccharide for methanol-boiled CWM was greater than by other extraction methods.[3,22] Differences in the sugar composition and linkage analysis were evident in each extract, and these were also dependent upon the treatment of CWM prior to extraction. For example, the second sodium carbonate extract from untreated CWM contained the highest proportion of GalA, whilst from methanol-boiled CWM the third imidazole extract contained most of the GalA. The proportions of mannose, glucose and xylose were different in extracts from methanol-boiled CWM relative to untreated CWM. A similar sequential extraction of Oosterveld *et al*[33] using sugar-beet pulp produced extracts containing much higher proportions of rhamnose and arabinose but lower proportions of all other neutral sugars than in the method used here. Higher proportions of GalA were obtained by our method. Other methods where linkage data were obtained from sugar-beet pulp did not detect fucose.[4,21,24–26,28,33]

Unlike previous studies that used autoclaving to extract pectin,[33] our data indicate that this process partially degrades the native pectins. Using CWM rather than pulp as the starting material, stopping hydrolytic activities by boiling the CWM in methanol, and using a relatively mild sequential extraction rather than harsh acidic conditions at high temperature, may all contribute to preserving native polymer structures. Boiling the CWM in methanol altered the relative abundance of linkages in all extracts (Tables 4B and 5B) but not their types, from which we deduce that the polymers present in each extract were essentially the same as from untreated CWM, although relative amounts changed. The four imidazole and two sodium carbonate extracts from untreated and methanol-boiled CWM all contained small amounts of xyloglucan, arabinans and type II arabinogalactans. Only the four imidazole extracts contained glucomannan and only the two sodium carbonate extracts contained branched 2,5-arabinans. The first and second imidazole extracts contained no RG-I, only unbranched homogalacturonan. Although Oosterveld *et al*[33] found RG-I in all fractions, a higher proportion of RG-I was found in the third and fourth imidazole and both sodium carbonate extracts in this study. The sugar linkages of arabinose and galactose are probably present as side-chains of RG-I and are similar to those reported by Oosterveld *et al.*[33] The polymer to which the terminal fucose residues are attached cannot be determined from these data, as such residues could either be (1–2)-α-linked to a xylose residue in xyloglucan or (1–2)-linked as terminal units on RG-I.[49,50] No type I arabinogalactans were detected, consistent with data from sugar-beet pulp that only type II arabinogalactans are present.[50] The presence of xylogalacturonan (an equimolar ratio of t-Xyl Xyl and 3,4-GalA) has been deduced from linkage analysis of pectin from pulp;[33] however, these linkages were not present in any of our extracts.

Size exclusion chromatography showed that populations of molecules of different sizes are present in each extract

The proportion of polymers of high MW (200 000–250 000) extracted from untreated CWM increased with successive imidazole treatments and the first sodium carbonate treatment. The second sodium carbonate extract, the extract with the highest degree of feruloylation, from untreated CWM only contained polymers with MWs of between 50 000 and 100 000 (Fig 2A). Extracted pectins from sugar-beet pulp with MWs of between 50 000 and 100 000 have been previously reported to contain high levels of feruloyl esters.[33] However, the MW distribution of the polymers extracted from CWM boiled in methanol prior to extraction showed a consistent shift to higher MWs, with the second sodium carbonate extract containing polymers predominantly in the 200 000–250 000 molecular weight population. In extracts from untreated CWM, six different size populations are seen, but the smallest populations are not represented in extracts from methanol-boiled CWM, whilst the third smallest is generally reduced. Thus these three discrete size

classes must be products of hydrolytic enzyme activities.

Hydrolytic enzymes were active during dialysis

To thoroughly remove the chemical extractants, each extract was dialysed for long periods of time. During this time, esters were degraded and potential epitopes were lost. When extracted tomato pectic polysaccharides are in solutions of CDTA or sodium carbonate, these polymers are not degraded (McCann, personal communication). Enzyme activities may be inhibited by CDTA or sodium carbonate until these extractants are removed by dialysis.

Colorimetric assays indicated that methyl, feruloyl and acetyl esterase activities are inactivated by methanol boiling of CWM prior to extraction. The KOH extract from the untreated CWM had a relative mole percentage of xylose of 48%, which dramatically increased to 94% in the extract from methanol-boiled walls. Size exclusion chromatography showed that the MW range of this extract increased from a single peak between 3000 and 5000 to a range between 5000 and 250 000 in the KOH extract from methanol-boiled CWM, indicating either that xylanases are inactivated following methanol boiling or that cross-links between smaller xylans are cleaved, or both. The higher MWs of the polymers in the imidazole and sodium carbonate extracts from methanol-boiled CWM indicate that various classes of pectinases are also present in the mature sugar-beet. The increased JIM 5 labelling and total uronic acid content of the anion exchange chromatography fractions of the second Na_2CO_3 extract from methanol-boiled CWM relative to untreated CWM add further evidence for the inactivation of pectinases following methanol boiling.

The fractions with the highest degree of feruloyl esters appear to be acidic pectins

Anion exchange chromatography suggests that feruloyl and acetyl esters are located close to each other, which is consistent with previously published data on pectin extracts from pulp.[33] Previously published data have suggested that feruloyl esters are ester-linked either to arabinose units or to galactose units.[4,25,35,36] Such areas would be highly exposed domains of the pectin molecule and therefore more accessible to wall peroxidases, which could catalyse oxidative cross-linking, via diferulate bridges, which may strengthen the cell wall. However, the data from the anion exchange chromatography indicate that the fractions with the highest degree of feruloyl esters are acidic pectic polysaccharides. It is possible that the majority of the neutral sugar moieties with which the feruloyl esters are associated were still linked to the acidic pectin backbone when it was eluted from the column. Indeed, the FTIR spectrum of fraction 30 from the untreated extract exhibits a phenolic ester peak at $1720\,cm^{-1}$.

The linkage analyses show that the second sodium carbonate extract from both untreated and methanol-boiled CWM contains the highest relative proportion of acidic pectins. This extract also contains the highest levels of feruloyl esters. The increase in feruloyl esters between the second sodium carbonate extract from untreated and methanol-boiled CWM is proportional to the increase in 5-linked arabinose between these two extracts. This is consistent with the NMR data of Colquhoun *et al*[36] and the observations of Guillon and Thibault,[4] which suggest that most of the feruloyl ester is bound to arabinose.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge the assistance of Jan Peart and Anna Cullingford with production of JIM 5 and JIM 7 antibodies; Sue Bunnewell for photographic assistance; Jim Wade, Grant Calder and Jordi Chan for useful discussions; and Nicholas Carpita for assistance with GC–MS and useful discussions. MM is funded by Hercules Incorporated, USA. MCM gratefully acknowledges the support of a Royal Society University Research Fellowship. KR and NJS acknowledge financial support from BBSRC.

REFERENCES

1 Pilnik W, Pectin, a many splendoured thing. In *Gums and Stabilisers for the Food Industry 5*, Ed by Phillips GO, Wedlock DJ and Williams PA, IRL Press at Oxford University Press, Oxford, pp 209–221 (1989).

2 Dea ICM and Madden JK, Acetylated pectic polysaccharides of sugar-beet. *Food Hydrocolloids* **1**:71–88 (1986).

3 Faulds CB and Williamson G, Effects of enzymes derived from orange peel on citrus and sugar beet pectins. In *Gums and Stabilisers for the Food Industry 5*, Ed by Phillips GO, Wedlock DJ and Williams PA, IRL Press at Oxford University Press, Oxford, pp 277–280 (1989).

4 Guillon F and Thibault J-F, Oxidative cross-linking of chemically and enzymically modified sugar-beet pectin. *Carbohydr Polym* **12**:353–374 (1990).

5 McCann MC and Roberts K, Architecture of the primary cell wall. In *The Cytoskeletal Basis of Plant Growth and Form*, Ed by Lloyd CW, Academic Press, London, pp 109–129 (1991).

6 Carpita NC and Gibeaut DM, Structural models of primary cell walls in flowering plants—consistency of molecular structure with the physical properties of the walls during growth. *Plant J* **3**:1–30 (1993).

7 McNeil M, Albersheim P and Darvill AG, The pectic polysaccharides of primary cell walls. In *Methods in Plant Biochemistry*, Vol 2, Ed by Dey PM, Academic Press, London, pp 415–441 (1992).

8 Stevenson TT, Darvill AG and Albersheim P, 3-Deoxy-D-lyxo-2-heptulosaric acid, a component of the plant cell-wall polysaccharide rhamnogalacturonan II. *Carbohydr Res* **179**:269–288 (1988).

9 Stevenson TT, Darvill AG and Albersheim P, Structural features of the plant cell-wall polysaccharide rhamnogalacturonan II. *Carbohydr Res* **182**:207–226 (1988).

10 Albersheim P, An JH, Freshour G, Fuller MS, Guillen R, Ham KS, Hahn MG, Huang J, O'Neill M, Whitcombe A, Williams MV, York WS and Darvill AG, Structure and function studies of plant cell wall polysaccharides. *Biochem Soc Trans* **22**:374–378 (1994).

11 Fry SC, *The Growing Plant Cell Wall: Chemical and Metabolic Analysis*, Longman Scientific and Technical Press, Harlow, Essex (1988).

12 Lau JM, McNeil M, Darvill AG and Albersheim P, Structure of the backbone of rhamnogalacturonan I, a pectic polysaccharide in the primary cell walls of plants. *Carbohydr Res* **137**:111–125 (1985).
13 Schols HA, Structural characterisation of pectic hairy regions isolated from apple cell walls. *PhD Thesis*, Wageningen Agricultural University, pp 1–9 (1995).
14 McCann MC, Wells B and Roberts K, Complexity in the spatial localisation and length distribution of plant cell wall matrix polysaccharides. *J Microsc* **166**:123–136 (1992).
15 Roberts K, Structures at the plant cell surface. *Curr Opin Cell Biol* **2**:920–928 (1990).
16 Ishii T and Matsunaga T, Isolation and characterisation of a boron–rhamnogalacturonan-II complex from cell walls of sugar beet pulp. *Carbohydr Res* **284**:1–9 (1996).
17 Carpita NC, McCann MC and Griffing LR, The plant extracellular matrix: news from the cell's frontier. *Plant Cell* **8**:1451–1463 (1996).
18 Hu HI and Brown PH, Localisation of boron in cell walls of squash and tobacco and its association with pectin—evidence for a structural role of boron in the cell wall. *Plant Physiol* **105**:681–689 (1994).
19 Kobayashi M, Matoh T and Azuma J, Two chains of rhamnogalacturonan-II are cross-linked by borate–diol ester bonds in higher plant cell walls. *Plant Physiol* **110**:1017–1020 (1996).
20 Matoh T, Sayaka K and Kobayashi M, Ubiquity of a boron–rhamnogalacturonan II complex in the cell walls of higher plants. *Plant Cell Physiol* **37**:636–640 (1996).
21 Rombouts FM and Thibault J-F, Feruloylated pectic substances from sugar beet pulp. *Carbohydr Res* **154**:177–187 (1986).
22 Michel F, Thibault J-F, Mercier M, Heitz F and Pouillaude F, Extraction and characterisation of pectins from sugar beet pulp. *Food Sci* **50**:1499–1501 (1985).
23 Renard CMGC, Thibault J-F, Liners F and Cutsem PV, Immunological probing of pectins isolated or *in situ*. *Acta Bot Neerl* **42**:199–204 (1993).
24 Renard CMGC, Crepeau M-J and Thibault J-F, Structure of the repeating units in the rhamnogalacturonic backbone of apple, beet and citrus pectins. *Carbohydr Res* **275**:155–165 (1995).
25 Guillon F and Thibault J-F, Methylation analysis and mild acid hydrolysis of the 'hairy' fragments of sugar beet pectins. *Carbohydr Res* **190**:85–96 (1989).
26 Guillon F and Thibault J-F, Enzymic hydrolysis of the 'hairy' fragments of sugar beet pectins. *Carbohydr Res* **190**:97–108 (1989).
27 Matora AV, Korshunova VE, Shkodina OG, Zhemerichkin DA, Ptitchkina NM and Morris ER, The application of bacterial enzymes for extraction of pectin from pumpkin and sugar beet. *Food Hydrocolloids* **9**:43–46 (1995).
28 Rombouts FM and Thibault J-F, Enzymic and chemical degradation and the fine structure of pectins from sugar-beet pulp. *Carbohydr Res* **154**:189–203 (1986).
29 Sakamoto T, Yoshinaga J, Shogaki T and Sakai S, Studies of protopectinase-C mode of action: analysis of the chemical structure of the specific substrate in sugar beet protopectin and characterisation of the enzyme activity. *Biosci, Biotechnol Biochem* **57**:1832–1837 (1993).
30 Sakamoto T, Hours RA and Shogaki T, Enzymic pectin extraction from protopectins using microbial protopectins. *Process Biochem* **30**:403–409 (1994).
31 Sakamoto T and Sakai S, Analysis of structure of sugar-beet pectin by enzymic methods. *Phytochemistry* **39**:821–823 (1995).
32 Zhemerichkin DA and Ptitchkina NM, The composition and properties of pumpkin and sugar beet pectins. *Food Hydrolloids* **9**:147–149 (1995).
33 Oosterveld A, Beldman G, Schols HA and Voragen AGJ, Arabinose and ferulic acid rich pectic polysaccharides extracted from sugar-beet pulp. *Carbohydr Res* **288**:143–153 (1996).
34 Fry SC, Feruloylated pectin substances from the primary cell wall: their structure and possible functions. *Planta* **157**:111–123 (1983).
35 Micard V, Renard CMG and Thibault J-F, Studies on enzymic release of ferulic acid from sugar-beet pulp. *Lebensm Wiss Technol* **27**:59–66 (1994).
36 Colquhoun IJ, Ralet MC, Thibault J-F, Faulds CB and Williamson G, Structure identification of feruloylated oligosaccharides from sugar-beet pulp by NMR spectroscopy. *Carbohydr Res* **263**:243–256 (1994).
37 Redgwell RJ and Selvendran RR, Structural features of cell wall polysaccharides of onion *Allium cepa*. *Carbohydr Res* **157**:183–199 (1986).
38 Talbot LD and Ray PM, Molecular size and separability features of pea cell wall polysaccharides: implications for models of primary cell walls. *Plant Physiol* **98**:357–368 (1992).
39 Mort AJ, Moerschbacher BM, Pierce ML and Maness NO, Problems encountered during the extraction, purification and chromatography of pectic fragments and some solutions to them. *Carbohydr Res* **215**:219–227 (1991).
40 McCann MC, Hammouri M, Wilson R, Belton P and Roberts K, Fourier-transform infrared microspectroscopy is a new way to look at plant cell walls. *Plant Physiol* **100**:1940–1947 (1992).
41 Séné CFB, McCann MC, Wilson RH and Grinter R, Fourier-transform Raman and Fourier-transform infrared-spectroscopy—an investigation of 5 higher plant cell walls and their components. *Plant Physiol* **106**:1623–1631 (1994).
42 Knox JP, Linstead PJ, King J, Cooper C and Roberts K, Pectin esterification is spatially regulated both within cell walls and between developing tissues of root apices. *Planta* **181**:512–521 (1990).
43 Filisetti-Cozzi TMCC and Carpita NC, Measurement of uronic acids without interference from neutral sugars. *Anal Biochem* **197**:157–162 (1991).
44 McCann MC, Shi J, Roberts K and Carpita NC, Changes in pectin structure and localisation during the growth of unadapted and NaCl-adapted tobacco cells. *Plant J* **5**:773–785 (1994).
45 Wood PJ and Siddiqui IR, Determination of methanol and its application to measurement of pectin ester content and pectin methyl esterase activity. *Anal Biochem* **39**:418–428 (1971).
46 Carpita NC and McCann MC, Some new methods to study plant polyuronic acids and their esters. In *Techniques in Glycobiology*, Ed by Townsend RR and Hotchkiss AT, Marcel Dekker, New York, pp 593–611 (1996).
47 Downs F and Pigmann W, Determination of *O*-acetyl groups by the Hestrin method. *Meth Carbohydr Chem* **7**:241–243 (1976).
48 Carpita NC and Shea EM, Linkage structure of carbohydrates by GC–MS of partially methylated alditol acetates. In *Analysis of Carbohydrates by GLC and M-S*, Ed by Biermann CJ and McGinnis GD, CRC Press, Boca Raton, FL, pp 157–216 (1989).
49 Puhlmann J, Bucheli E, Swain MJ, Dunning N, Albersheim P, Darvill AG and Hahn MG, Generation of monoclonal antibodies against plant cell wall polysaccharides. 1. Characterisation of a monoclonal antibody to a terminal α(1–2)-linked fucosyl-containing epitope. *Plant Physiol* **104**:699–710 (1994).
50 Voragen AGJ, Pilnik W, Thibault J-F, Axelos MAV and Renard CMGC, *Food Polysaccharides and Their Applications*, Ed by Stephen AM, Marcel Dekker, New York, pp 287–339 (1995).