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Cell Wall Dissolution in Ripening Kiwifruit (*Actinidia deliciosa*)[1]

Solubilization of the Pectic Polymers

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ABSTRACT

Pectic polysaccharides solubilized *in vivo* during ripening, were isolated using phenol, acetic acid, and water (PAW) from the outer pericarp of kiwifruit (*Actinidia deliciosa* [A. Chev.] C.F. Liang and A.R. Ferguson var *deliciosa* 'Hayward') before and after postharvest ethylene treatment. Insoluble polysaccharides of the cell wall materials (CWMs) were solubilized *in vitro* by chemical extraction with 0.05 molar cyclohexane-trans-1,2-diamine tetra-acetate (CDTA), 0.05 molar Na_2CO_3, 6 molar guanidinium thiocyanate, and 4 molar KOH. The Na_2CO_3-soluble fraction decreased by 26%, and the CDTA-soluble fraction increased by 54% 1 day after ethylene treatment. Concomitantly, an increase in the pectic polymer content of the PAW-soluble fraction occurred without loss of galactose from the cell wall. The molecular weight of the PAW-soluble pectic fraction 1 day after ethylene treatment was similar to that of the Na_2CO_3-soluble fraction before ethylene treatment. Four days after ethylene treatment, 60% of cell wall polyuronide was solubilized, and 50% of the galactose was lost from the CWM, but the degree of galactosylation and molecular weight of pectic polymers remaining in the CWMs did not decrease. The exception was the CDTA-soluble fraction which showed an apparent decrease in molecular weight during ripening. Concurrently, the PAW-soluble pectic fraction showed a 20-fold reduction in molecular weight. The results suggest that considerable solubilization of the pectic polymers occurred during ripening without changes to their primary structure or degree of polymerization. Following solubilization, the polymers then became susceptible to depolymerization and degalactosidation. Pectolytic enzymes such as endopolygalacturonase and β-galactosidase were therefore implicated in the degradation of solubilized cell wall pectic polymers but not the initial solubilization of the bulk of the pectic polymers *in vivo*.

Studies detailing the involvement of pectolytic enzymes such as PG[2] in cell wall degradation have been prompted by the observation that the cell wall pectin content of many fruit decreases or changes its form during ripening. Attempts have been made to relate an increase in the activity of a particular cell wall-degrading enzyme to the onset and development of fruit softening. This approach has provided apparent correlations, but most are inconclusive, and one, polygalacturonase involvement in tomato softening, has largely been discredited by results of genetic experiments. PG activity in tomato fruit increases dramatically during ripening because of *de novo* synthesis of the enzyme (3, 5). However, when the PG gene was introduced into a nonripening mutant tomato (9), the rate of softening, ethylene production, and color development did not increase markedly. The authors concluded that PG, although necessary for polyuronide degradation, was not the primary determinant of softening in the transgenic fruit. The findings were confirmed by Smith *et al.* (20) who investigated the role of PG using mutant tomato lines in which the expression of the PG gene was largely suppressed by antisense RNA. Their results also suggested that, although PG may be necessary for *in vivo* depolymerization of the pectic polymers, it was not necessarily the cause of pectin solubilization. More recently, however, DellaPenna *et al.* (6) obtained results that contradict these findings and led them to believe that polyuronide solubilization and depolymerization were separate manifestations of the same process, catalyzed by the PG isozyme, PG1.

Another approach for deducing the nature of the physicochemical and enzymological processes involved in pectin solubilization is to characterize the *in vivo* chemical changes in polyuronide structure that accompany ripening. Changes to individual polymer fractions isolated by chemical methods can be related to the *in vivo* situation, providing precautions are taken to minimize inadvertent degradation of the cell wall during its preparation and fractionation. It is important that changes to a specific fraction are not assessed in isolation but in relation to the changes in all fractions. Otherwise, it is possible to attribute a physiological basis to a change that is in part an artifact of the methodology. To study the transition in the fruit from insoluble cell wall to partially solubilized cell wall, it is necessary that methods make a distinction between the insoluble cell wall and the polymers solubilized *in vivo* during ripening. Cell walls are often prepared as acetone-insoluble powders in which all solubilized polysaccharides

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[2] Abbreviations: PG, endopolygalacturonase; CWM, cell wall material; CDTA, cyclohexane-*trans*-1,2-diamine tetra-acetate; GTC, guanidinium thiocyanate; PAW, phenol:acetic acid:water (2:1:1, w/v/v); SSC, soluble solids concentration; CTAB, hexadecyltrimethylammonium bromide; d.e., degree of esterification.

and cytoplasmic proteins are coprecipitated and recovered as part of the insoluble CWM (19). It is unlikely that the polysaccharides subsequently solubilized by a water or EDTA extraction from the dry preparations are representative of the polymers solubilized *in vivo*. We avoided this problem by separating the soluble from the insoluble polymers during homogenization of the kiwifruit tissue in PAW, a solvent used for CWM preparation because of its effectiveness in inactivating endogenous wall enzymes. PAW has the necessary property of being a poor solvent for insoluble cell wall polymers (8) but a ready solvent for polymers already solubilized during ripening.

It has been demonstrated that the solubilization of pectic polymers during kiwifruit ripening is even more extensive than in tomato (13). However, levels of PG activity reported in ripe kiwifruit were 300-fold less than in tomato (21). The objective of the present study was to characterize the changes to cell wall pectic polymers of ripening kiwifruit that occurred during and after solubilization of the pectic polymers in an effort to clarify mechanisms involved in the solubilization process.

MATERIALS AND METHODS

Plant Material

Fruit were harvested from Te Puke during May 1989. A single tray of 33 kiwifruit (*Actinidia deliciosa* [A. Chev.] C.F. Liang and A.R. Ferguson var *deliciosa* 'Hayward') was picked from three 12-year-old vines on May 4 for immediate analysis, with repeated pickings on May 11, 18, and 23. Henceforth, the term "harvest" fruit will refer specifically to the fruit picked on the May 23 before ethylene treatment. Six additional trays harvested on the May 23 were exposed to ethylene (1000 ppm, 12 h) as previously described (15) and stored at 21°C in the absence of ethylene. A single tray of fruit was taken for analysis 1, 2, 4, 6, 8, and 10 d after ethylene treatment. Each tray was separated into three samples of 11 fruit, and each fruit was measured for SSC and firmness (10) and peeled, and the outer pericarp tissue was cut into 2-cm^2 pieces and frozen in liquid nitrogen.

Isolation and Fractionation of CWMs and PAW-Soluble Polymers

CWMs

Each 11-fruit sample was cryomilled in liquid nitrogen, and 200 g was homogenized in 400 mL of PAW until the tissue had thawed and become a fine slurry. The homogenate was centrifuged at 4000*g* for 10 min and the supernatant filtered through Miracloth. The residue was resuspended in H_2O and the supernatant recovered as before. This step was repeated. The residue was extracted overnight in 90% aqueous DMSO to solubilize starch, and the DMSO-soluble fraction and the CWM were recovered following dialysis and freeze drying.

CWM (1 g) from each of the triplicate samples at each of the 10 sample times was extracted sequentially with H_2O, 0.05 M CDTA, 0.05 M Na_2CO_3, 6 M GTC, and 4 M KOH as described previously (14). All 30 fractionations were done consecutively during 4 weeks under identical conditions. At a later stage, the CWM residue remaining after the 4 M KOH was extracted further in an attempt to solubilize matrix polysaccharides. The triplicate samples for each ripening stage were combined to enable 1 g of CWM residue to be extracted at each of four ripening stages, at harvest and 1, 4 and 10 d after ethylene treatment. The CWM was suspended in 150 mL of 8 M KOH containing 0.1 M $NaBH_4$ (22) and stirred for 4 h at 21°C under argon. The supernatants were recovered by centrifugation, cooled in an ice bath, neutralized with glacial acetic acid, and recovered by dialysis and freeze drying.

PAW-Soluble Fractions

The PAW and H_2O wash supernatants were combined and dialyzed (mol wt cut-off 8000) overnight against running tap H_2O and then for 2 d against distilled H_2O at 4°C. The dialyzed solution was concentrated on a rotary evaporator to approximately 300 mL, dialyzed for an additional 2 d, concentrated to approximately 20 mL, and centrifuged at 23,000*g* for 20 min to sediment out the predominantly proteinaceous precipitate (data not given) that formed during dialysis. The supernatant, henceforth referred to as the PAW-soluble fraction, was recovered and freeze dried.

PAW-soluble fractions at harvest and 1, 4, and 10 d after ethylene treatment (110, 115, 120, and 120 mg, respectively) were deesterified in 25 mL 0.1 N NaOH at 1°C for 2 h to maximize the free carboxylic acid groups available for binding to the DEAE matrix. The solutions were adjusted to pH 5.0 with acetic acid and dialyzed for 2 d against 0.05 M, pH 6.5, phosphate buffer. Each solution was eluted onto a column of DEAE-Sepharose Fast Flow (2.0 × 15 cm), and fractions were recovered with 200 mL phosphate buffer and buffer plus 0.125, 0.25, and 0.5 M NaCl.

General Methods

Monosaccharide composition of polymers was determined by capillary GLC of their alditol acetates following acid hydrolysis in 2 M TFA (14). The column, SP-2330-fused silica (30 m × 0.32 mm), was maintained at 120°C for 2 min, and then the temperature was increased to 220°C at 25°C/min. Uronic acid was measured by the *m*-hydroxydiphenyl assay (2), and column fractions were monitored spectrophotometrically by the phenol-sulfuric acid (7) and *m*-hydroxydiphenyl assays at 490 and 524 nm, respectively. Degree of esterification was determined as previously described (14). Precipitation of the pectic polymers with CTAB was done by the procedure of Scott (18). Protein was determined by the Kjeldahl method ($N \times 6.4$). Viscosity measurements of CWM suspensions were done with a Brookfield Synchro-electric Viscometer as described previously (16).

Gel Filtration

The following columns were used: Sepharose CL-2B (2.5 × 70 cm) for the pectic polymers solubilized from the CWMs and Sepharose CL-6B (2.0 × 90 cm) for the PAW-soluble polymers. Gel media were equilibrated in 0.05 M acetate buffer (pH 6.0) containing 125 mM NaCl. Each polymer fraction (5 mg) was dissolved in 1.0 mL buffer, eluted from the column at 10 mL/h, and 2 mL fractions were collected.

^{13}C-NMR Analysis

Spectra were recorded under conditions of broad-band proton decoupling on a Bruker AM 400 spectrometer at an operating frequency of 100.62 MHz. Native pectic polymers were examined as solutions in D_2O (50 mg in 3 mL solvent) in 10-mm spinning tubes at 55°. Spectra were obtained using 90° pulses with a pulse repetition time of 1.3 s; 64,000 data points and 50,000 transients were acquired. Spectra were processed with exponential multiplication, typically 5 Hz. Spectra were referenced to external 4,4-dimethyl-4-silapentane sodium sulfonate.

RESULTS

Changes to CWM and PAW-Soluble Polymer Fractions

Between the May 4 and and 23 (before ethylene treatment), the fruit showed a decrease in firmness, an increase in SSC, and a loss of starch (DMSO-soluble fraction) from the outer pericarp of kiwifruit (Table I). This was not accompanied by changes in the relative amounts of the CWM and PAW-soluble fractions. Ethylene was used to trigger an accelerated rate of softening and because softening was more uniform among fruit treated with ethylene (10). Following ethylene treatment, there was a more marked decrease in fruit firmness and starch content. An increase in the PAW-soluble fraction was accompanied by a decrease in the amount of CWM. This plateaued 4 d after ethylene treatment when the PAW-soluble fraction represented 49.1% of the CWM, compared with 6.0% at harvest.

The primary constituents of the pectic polymers of kiwifruit are galacturonic acid, galactose, arabinose, and rhamnose. Relative changes for each of these monosaccharides in the CWMs and PAW-soluble fractions derived from 200 g of tissue are shown in Figure 1. Between harvest and 4 d after ethylene treatment, a decrease in CWM uronic acid occurred, which was largely accounted for in the PAW-soluble polymers. A net loss (15%) of total polyuronide took place during this period. The net loss for rhamnose and arabinose was even less marked. Both sugars were lost from the CWM but were recovered in the PAW-soluble fraction. In contrast, there was

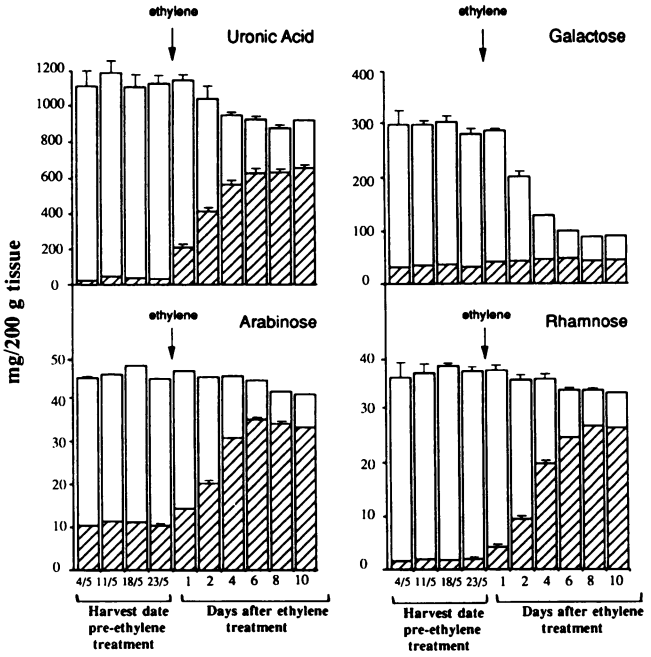

Figure 1. Distribution of uronic acid, galactose, arabinose, and rhamnose among the CWMs and PAW-soluble fractions during ripening. Columns, mean values (error bars, ± SE) for three samples. No SE indicates value is less than width of data column. Shaded, PAW-soluble fraction; unshaded, CWM fraction.

a net loss of nearly 70% of the galactose from the CWM between harvest and 8 d after ethylene treatment, only 17% of which was accounted for in the PAW-soluble fraction. The biggest loss in galactose (approximately 54%) occurred between 1 and 4 d after ethylene treatment.

Uronic acid content of the PAW-soluble fraction at harvest was 14.3%, indicating that the solvent had solubilized only a small amount of pectic polymer (Table II). One day after ethylene treatment, the polyuronide levels in the PAW-soluble fraction increased to 55.8%. Concurrently, in the CWM there was a decrease in the polyuronide content and its d.e. (Table

Table I. *Changes to Fruit Firmness, SCC, and Yield of Fractions Isolated from 200 g of Kiwifruit at Different Stages of Ripeness*

Values are means ± SE of three fruit samples.

	Fruit Firmness	SSC	CWM	PAW Soluble	DMSO Soluble
	kgf	%		*g*	
Harvest date before ethylene treatment					
5/4	9.80 ± 0.10	6.2 ± 0.10	2.5 ± 0.12	0.18 ± 0.006	12.2 ± 0.15
5/11	8.42 ± 0.16	6.8 ± 0.09	2.43 ± 0.04	0.18 ± 0.004	11.2 ± 0.06
5/18	7.81 ± 0.12	7.9 ± 0.02	2.49 ± 0.04	0.16 ± 0.005	10.6 ± 0.06
5/23	7.73 ± 0.14	8.7 ± 0.10	2.49 ± 0.08	0.15 ± 0.014	9.4 ± 0.32
d after ethylene treatment					
1	5.06 ± 0.04	11.0 ± 0.03	2.44 ± 0.02	0.31 ± 0.007	6.7 ± 0.10
2	2.5 ± 0.16	12.6 ± 0.38	2.06 ± 0.04	0.65 ± 0.036	3.5 ± 0.40
4	1.3 ± 0.03	15.3 ± 0.19	1.79 ± 0.04	0.88 ± 0.029	0.41 ± 0.01
6	0.68 ± 0.01	15.7 ± 0.14	1.80 ± 0.01	0.92 ± 0.003	0.36 ± 0.01
8	0.38 ± 0.01	14.9 ± 0.04	1.62 ± 0.05	0.88 ± 0.016	0.34 ± 0.01
10	0.33 ± 0.01	15.5 ± 0.02	1.76 ± 0.02	0.96 ± 0.010	0.33 ± 0.01

Table II. *Rhamnose, Arabinose, Galactose, Uronic Acid, and Protein Content of CWMs and PAW-Soluble Fractions Isolated from Kiwifruit at Different Stages of Ripeness*

Values are means ± SE of three fruit samples

Fraction	Monosaccharide Composition				Protein	D.e.
	Rha	Ara	Gal	Uronic acid		
	mol %				%	%
CWM						
Harvest date before ethylene treatment						
5/11	1.7 ± 0.08	1.9 ± 0.06	11.5 ± 0.25	38.6 ± 0.67	2.1 ± 0.02	59.8 ± 1.3
5/23	1.7 ± 0.01	1.9 ± 0.01	10.1 ± 0.03	39.4 ± 0.85	2.2 ± 0.13	60.6 ± 1.3
d after ethylene treatment						
1	1.7 ± 0.09	1.8 ± 0.06	10.2 ± 0.27	35.4 ± 0.73	2.1 ± 0.20	53.5 ± 3.8
2	1.6 ± 0.04	1.7 ± 0.02	8.2 ± 0.26	28.1 ± 1.6	2.6 ± 0.16	55.7 ± 2.8
4	1.0 ± 0.06	1.0 ± 0.04	4.5 ± 0.20	20.1 ± 0.90	7.4 ± 0.23	46.6 ± 2.9
8	0.5 ± 0.01	0.6 ± 0.01	2.7 ± 0.07	14.8 ± 0.59	14.5 ± 0.81	33.7 ± 1.8
PAW-soluble						
Harvest date before ethylene treatment						
5/11	1.3 ± 0.09	11.5 ± 0.26	20.2 ± 0.43	13.9 ± 0.61	30.8 ± 2.5	
5/23	1.7 ± 0.11	13.2 ± 0.32	22.4 ± 0.46	14.3 ± 0.35	23.2 ± 1.4	
d after ethylene treatment						
1	1.1 ± 0.03	6.5 ± 0.62	10.8 ± 1.2	55.8 ± 4.2	23.6 ± 1.3	
2	1.5 ± 0.07	4.0 ± 0.12	5.5 ± 0.23	72.8 ± 0.61	20.4 ± 0.77	
4	2.5 ± 0.01	4.7 ± 0.12	4.8 ± 0.20	74.9 ± 1.50	14.8 ± 1.2	
8	3.8 ± 0.06	5.6 ± 0.15	5.1 ± 0.21	76.4 ± 0.27	9.5 ± 0.81	

II) but no change in the levels of galactose or other neutral sugars. Gel permeation profiles on Sepharose-CL-6B of the PAW-soluble fractions at harvest and in each subsequent ripening stage are given in Figure 2. Most of the pectic polymers of the PAW-soluble fraction 1 d after ethylene treatment eluted at the void volume of Sepharose CL-6B, showing that the mol wt was greater than 1,000,000. The elution time on Sepharose CL-2B of the Na_2CO_3-soluble fraction at harvest (the CWM fraction that decreased most during ripening) suggested a similar mol wt. Subsequently, further solubilization and depolymerization of polyuronide occurred up to 8 d after ethylene treatment, at which stage 72% of the polyuronide present in the CWM at harvest was solubilized and the mol wt of the solubilized polyuronides was reduced from approximately 1000,000 to approximately 50,000. During the later stages of ripening (between 4 and 10 d after ethylene treatment), the d.e. of the CWM polyuronide decreased more markedly. Between 1 and 4 d after ethylene treatment, the total carbohydrate profile showed a sharp increase in the proportion of low mol wt polymers. Allied with the fact that the neutral component of the PAW-soluble fraction at harvest contained considerable xylose and glucose (35.5 and 26.6 mol %, respectively), this indicated that partial degradation of a hemicellulose-type polymer may have occurred in the PAW-soluble fraction.

Selected PAW-soluble fractions were fractionated further by ion-exchange chromatography on Fast Flow DEAE-Sepharose to remove the hemicellulosic polysaccharides. Fractions were eluted from the column with increased molarities of NaCl. The small amounts of pectic polymer solubilized by PAW at harvest were characteristically different from those recovered in PAW after ethylene treatment (Table III). The former contained much greater amounts of arabinose and galactose and lesser amounts of rhamnose and uronic acid. One day after ethylene, two fractions containing >91% uronic acid were recovered in 0.25 and 0.5 M NaCl. Four and 10 d after ethylene treatment, the major PAW-soluble pectic fractions contained 93.7 and 95.4% uronic acid, respectively.

CWMs contained protein that increased in amount as the fruit ripened (Table II). There was a concomitant decrease in the protein content of the PAW-soluble fractions (Table II). An increase in the protein content of the CWMs paralleled a decrease in the d.e. of the CWM polyuronide (Table II). As more free carboxylic acid groups are generated, the capacity of the cell wall pectic substances to bind basic proteins would increase. CWMs and the PAW-soluble fractions at several stages of ripening were hydrolyzed in 6 N HCl, and the amino acid composition was examined by thin-layer electrophoresis/chromatography (1). The amino acid compositions were identical (data not given).

Changes in Yield and Composition of Solubilized CWM Fractions

CWMs were fractionated by sequential extraction with H_2O, CDTA, 0.05 M Na_2CO_3, 6 M GTC, and 4 M KOH. There was some variation in the amounts of each fraction among the pre-ethylene samples (Table IV) but no trend that would indicate changes to the chemical state of the cell wall. The percentage of each fraction in the CWM derived from 200 g tissue at harvest was 0.98, 6.9, 23.6, 8.4, 16.9,and 43.2% for the H_2O-, CDTA-, Na_2CO_3-, GTC-, and KOH-soluble and CWM residue fractions, respectively. One day after ethylene treatment, there was a 25% reduction in the amount of

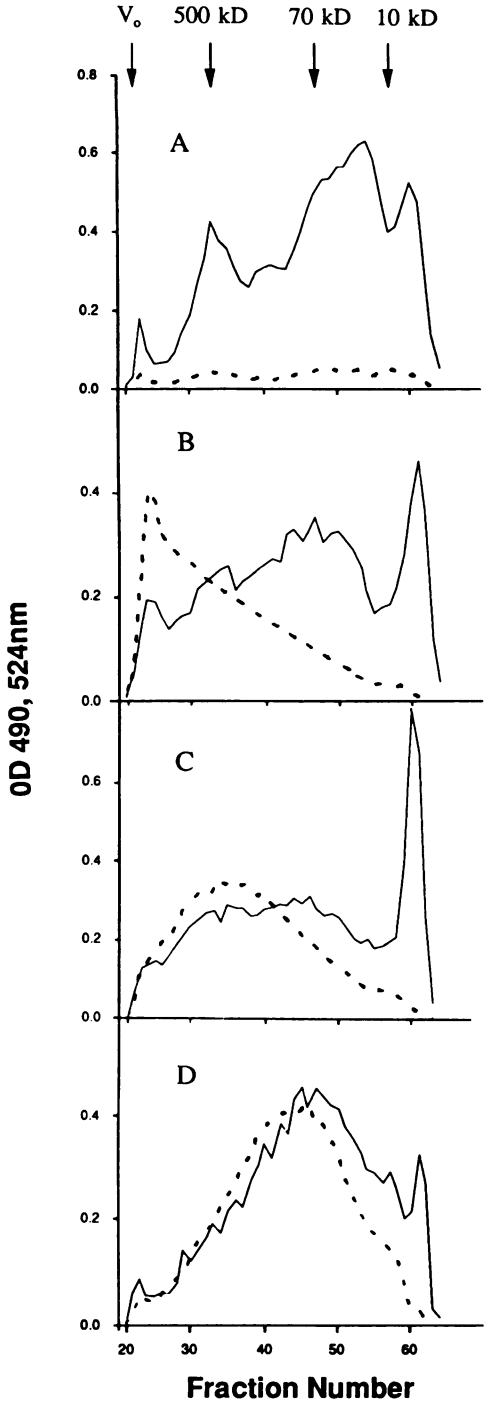

Figure 2. Gel permeation profiles of PAW-soluble fractions at different stages of ripening on Sepharose CL-6B. Column fractions were assayed for total carbohydrate using the phenol-sulfuric acid method (7) (A_{490}, solid line) and for polyuronide using the *m*-hydroxybiphenyl method (2) (A_{524}, dotted line). A, Harvest; B, 1 d after ethylene treatment; C, 4 d after ethylene treatment; D, 8 d after ethylene treatment.

Na_2CO_3-soluble fraction and a 54% increase in the amount of CDTA-soluble fraction relative to the harvest sample (Table IV). Amounts of the other fractions did not change during the same period. As fruit ripened, the Na_2CO_3-soluble fraction diminished further, until 76.5% of the fraction had been lost 8 d after ethylene treatment. After the same time, there were also reductions in the GTC- and KOH-soluble fractions (45.9 and 45.3%, respectively). The amount of the CDTA-soluble fraction was reduced by 25.8% after 8 d when compared with the 1-d sample but was still 14.5% higher than the harvest value.

Compositions of all fractions were determined by GLC of their alditol acetates following hydrolysis in 2 M TFA. Changes to the galactose, rhamnose, arabinose, and uronic acid contents of each fraction at all stages of ripening are shown in Figure 3. Uronic acid was lost from all fractions except the CDTA-soluble fraction 1 d after ethylene treatment. The Na_2CO_3-soluble fraction which accounted for approximately 60% of the cell wall polyuronide lost the most (35%). By the final stages of ripening, the Na_2CO_3-, GTC-, and KOH-soluble and CWM residue fractions contained 4.2, 11.7, 18.9, and 20.6% of their harvest levels of polyuronide, respectively. Between 1 and 4 d after ethylene treatment, 66% of the galactose was lost from the wall. Of this, 82% was lost from the KOH-soluble and CWM residue pectic fractions, which contained the more highly branched pectic polymers (14, 16). More was lost from the CWM residue fraction than the KOH-

Table III. *Rhamnose, Arabinose, Galactose, and Uronic Acid Content of PAW-Soluble Polymers Fractionated by Ion-Exchange Chromatography*

Fraction	Fraction Amount	Composition			
		Rha	Ara	Gal	Uronic acid
	mg[a]	mol %[b]			
Harvest					
Neutral	41.1	1.4	9.1	15.2	7.8
0.125 M	17.8	2.8	33.0	47.0	11.5
0.25 M	7.4	1.5	25.3	40.4	25.6
0.5 M	1.9	1.2	9.1	14.1	66.1
d after ethylene treatment					
1					
Neutral	26.2	1.5	8.1	4.6	8.7
0.125 M	11.7	3.2	30.9	46.3	13.8
0.25 M	6.3	0.9	1.8	3.0	91.6
0.5 M	5.3	1.3	1.6	1.7	91.3
4					
Neutral	39.1	4.3	11.1	11.0	37.1
0.125 M	12.2	12.8	35.7	27.0	20.9
0.25 M	35.8	1.2	1.4	2.1	93.7
0.5 M	5.2	1.6	1.5	2.9	89.2
10					
Neutral	28.8	7.2	15.7	14.0	30.7
0.125 M	13.2	14.4	28.2	22.2	33.2
0.25 M	62.3	1.4	1.3	1.2	95.4
0.5 M	3.7	1.8	1.8	0.7	92.7

[a] Polymers recovered in buffer (neutral) and buffer plus 0.125, 0.25, and 0.5 M NaCl. [b] Values are averages of two determinations.

Table IV. *Yields of Fractions Solubilized from CWMs Isolated from 200 g of Kiwifruit at Different Stages of Ripeness*
Values are means ± SE of three samples CWM.

	Fraction					
	H_2O	CDTA	Na_2CO_3	GTC	KOH	Residue
	mg					
Harvest date before ethylene treatment						
5/4	30.0 ± 1.8	174.0 ± 18.0	600.0 ± 58.2	197.7 ± 15.0	409.4 ± 7.7	1007.3 ± 28.9
5/11	22.5 ± 1.1	158.8 ± 5.3	520.2 ± 14.5	145.7 ± 2.3	375.2 ± 4.6	890.4 ± 21.8
5/18	17.7 ± 3.9	146.6 ± 4.6	543.4 ± 8.6	182.9 ± 3.7	366.6 ± 18.9	937.8 ± 6.7
5/23	22.7 ± 4.1	160.1 ± 5.1	547.8 ± 5.5	193.8 ± 8.1	390.2 ± 5.8	1001.4 ± 41.3
d after ethylene treatment						
1	22.2 ± 1.5	247.0 ± 4.9	407.8 ± 4.4	180.9 ± 2.5	381.7 ± 3.8	998.5 ± 10.1
2	25.1 ± 3.1	233.5 ± 10.2	263.8 ± 4.8	140.7 ± 5.7	285.2 ± 13.4	988.2 ± 12.8
4	38.5 ± 7.7	232.7 ± 15.7	151.4 ± 3.2	125.0 ± 1.7	233.1 ± 8.4	1009.3 ± 28.0
6	24.0 ± 1.7	213.9 ± 11.7	168.5 ± 4.8	128.8 ± 1.7	243.0 ± 1.2	956.9 ± 13.5
8	18.4 ± 1.8	183.4 ± 12.5	128.9 ± 4.3	105.0 ± 5.7	213.6 ± 7.0	849.7 ± 46.0
10	18.4 ± 0.40	196.3 ± 2.9	148.5 ± 5.0	121.2 ± 2.7	215.3 ± 1.2	967.3 ± 71.4

soluble fraction. Rhamnose and arabinose loss paralleled that of the galactose.

Between harvest and 1 d after ethylene treatment, every CWM fraction except the CWM residue showed an increase in the galactose level (Fig. 3). The CWM residue, however, lost galactose during the same period. The amount lost from the CWM residue was accounted for by the galactose gained in other fractions, particularly the Na_2CO_3-soluble fraction (13).

Changes to Mol Wt and Degree of Galactosidation of Pectic Polymer Fractions

The CDTA-soluble fraction was the only CWM pectic fraction to show a marked reduction in mol wt during ripening (Fig. 4). From 1 d after ethylene treatment, the mol wt of the fraction diminished and continued to do so until 8 d after ethylene treatment (Fig. 4).

Previously, we reported that an increase in the mol wt and the galactose, rhamnose, and arabinose content of the Na_2CO_3-soluble pectic polymers occurred at an advanced stage of ripening, despite a net loss of the Na_2CO_3-soluble fraction from the cell wall (16). Gel permeation profiles on Sepharose CL-2B of the Na_2CO_3-soluble fractions at selected ripening stages (Fig. 5) show the intermediate stages. Two days after ethylene treatment, there was a broadening of the peak on the high mol wt side which developed two distinct peaks after 4 d. The fraction 6 d after ethylene treatment (Fig. 5D) contained mostly protein, hence its weak carbohydrate reaction with the phenol-sulfuric acid reagent.

Confirmation that the increased galactose of the Na_2CO_3-soluble fractions was accommodated in β-1→4-galactan side chains was provided by the ^{13}C-NMR spectra of the Na_2CO_3-soluble fractions at harvest and 2 d after ethylene treatment. Signal assignments were based on published spectra of several plant pectins (4, 12, 17). Chemical shifts at 106.2, 73.8, 75.2, 79.5, 76.3, and 62.7 ppm corresponded to C1, C2, C3, C4, C5, and C6 of β-1→4-D-galactopyranosyl residues, respectively. There was approximately a sixfold increase in the intensity of each of these signals in the 2-d Na_2CO_3-soluble pectic fraction compared with the signals in the harvest fraction. Integration of the anomeric carbon resonances at 101.0 (galacturonic acid) and 106.2 ppm (galactose) gave a galacturonic acid:galactose ratio of 25:1 in the harvest sample and 5:1 in the 2-d sample, which is in approximate agreement with the compositions given in Table V.

Pectic polymers in the GTC- and KOH-soluble fractions at selected ripening stages were separated from the hemicelluloses by CTAB precipitation. Additional pectic polymers were solubilized from the CWM residue in 8 M KOH and after dialysis were subjected to CTAB precipitation. There was an increase in the degree of branching of the pectic polymers as they became more difficult to solubilize from the CWM. Thus, the ratio of galactose:uronic acid at harvest was 1:74, 1:20, 1:2, 0.9:1, and 0.8:1 for the CDTA-, Na_2CO_3-, GTC-, and KOH-soluble and CWM residue pectic polymers (Table V). Compared with the harvest polymers, a doubling of the galactose, arabinose, and rhamnose contents of the Na_2CO_3-soluble polymers occurred 1 d after ethylene treatment, and a tripling occurred 4 d after ethylene treatment. The CDTA-soluble fraction followed a similar trend. During ripening, the ratio of galactose:uronic acid in the pectic polymers of the CDTA-, Na_2CO_3-, and GTC-soluble and CWM residue fractions either did not decrease markedly or increased when compared with the harvest level. The exception was the KOH-soluble fraction, which appeared to show a reduction in the ratio of galactose to uronic acid. However, KOH-soluble fractions contained very high levels of xylose, which probably originated from the 4-*O*-methylglucuronoxylan (Table V) (16). The acidic hemicellulosic polymer would also have been precipitated by CTAB. Its proportion of the KOH-soluble fraction increased markedly as the pectic polymers were lost from the fraction, making it difficult to assess the neutral sugar to uronic acid ratio of the pectic polymer content.

The mol wt of the GTC- and KOH-soluble and CWM residue pectic fractions at harvest and 4 d after ethylene treatment were compared by gel permeation chromatography on Sepharose CL-2B (Fig. 6). There was little apparent reduc-

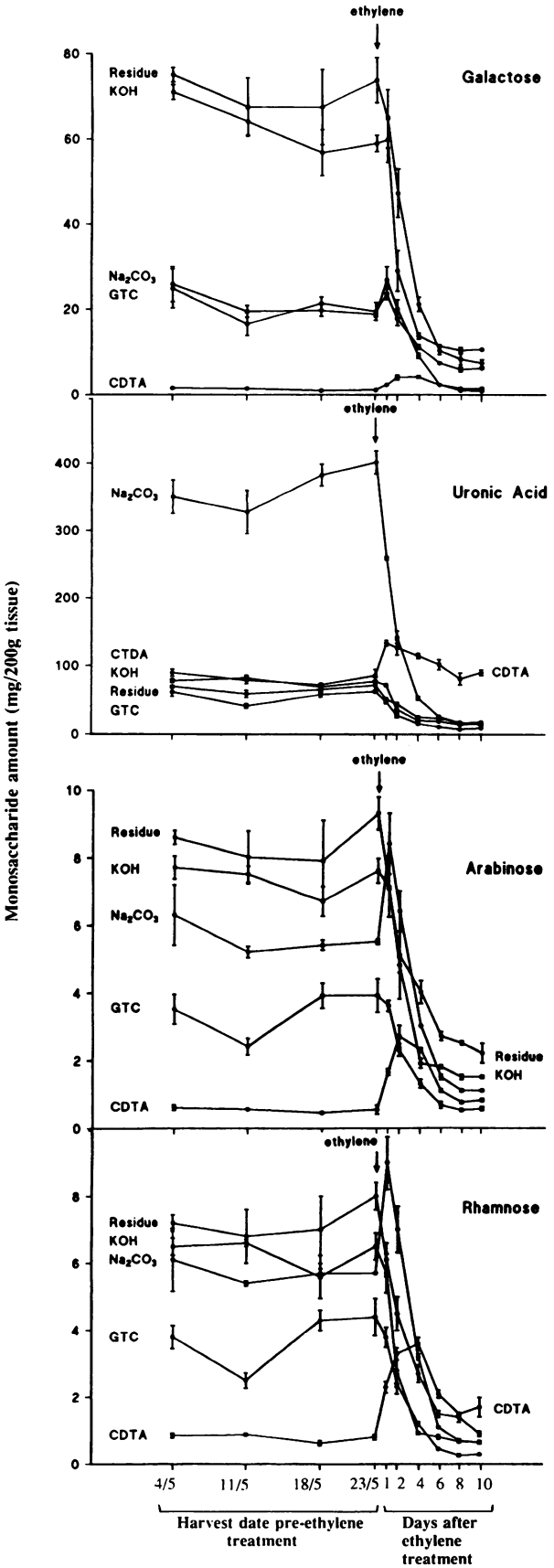

Figure 3. Changes to the amount of uronic acid, galactose, arabinose, and rhamnose in the CDTA-, Na_2CO_3-, GTC-, and KOH-soluble and CWM residue fractions during ripening. Points, mean values (error bars, ± SE) for three samples.

tion in mol wt of the GTC- and KOH-soluble fractions, but there was a reduction in the proportion of the high mol wt molecules in the CWM residue fraction.

Swelling of the CWMs

During preparation of the CWMs, it was noticeable that aqueous suspensions of CWM became thicker as the fruit ripened. A 1.25% suspension of the CWMs on May 5 and 23 (pre-ethylene samples) had viscosities of 6.0 centipoise. The viscosities 1, 2, 4, 6, 8, and 10 d after ethylene treatment were 12.5, 375, 1180, 520, 680, and 340 centipoise, respectively. Samples, therefore, reached maximum viscosity 4 d after ethylene treatment, when the CWM suspension was a viscous gel. However, 1 d after ethylene treatment, there was a measurable increase in the viscosity of the CWM and its ability to swell in water.

DISCUSSION

A recent series of papers has detailed our investigations of changes to the cell wall pectic and hemicellulosic polysaccharides at three stages of kiwifruit ripening (15, 16). In the present work, more ripening stages were taken to determine the sequence of cell wall changes leading to pectin solubilization. The present study focused on two aspects of pectin degradation: the solubilization of the pectic polymers and the loss of galactose residues from the pectic molecule. We considered changes taking place simultaneously in the insoluble cell wall, represented by the CWMs, and in the solubilized pectic polymers, contained in the PAW-soluble fractions.

Isolation of CWMs

In a previous paper (15), we did not report changes to the mol wt of pectic polymers solubilized *in vivo* because the SDS solution used for CWM preparation solubilized too much of the CWM, making it impossible to distinguish pectic polymer originating from *in vivo* solubilization and that derived from SDS solubilization. In addition, it was believed that the pH of the SDS-extracting solution encouraged interaction between intracellular proteins and the soluble anionic pectic polymers to form insoluble complexes. The suspension of the cryomilled tissue directly in PAW was an attempt to overcome these problems. It is an excellent solvent for proteins but a poor solvent for insoluble pectic polymers. The low pH of the PAW/tissue homogenate meant that the carboxylic acid groups would not be ionized and would, therefore, be less likely to interact ionically with basic cytoplasmic proteins.

Changes to Na_2CO_3- and CDTA-Soluble Pectic Fractions

In a previous paper (16), it was reported that the mol wt and degree of galactosylation of the Na_2CO_3-soluble fraction increased during ripening. We speculated that this phenome-

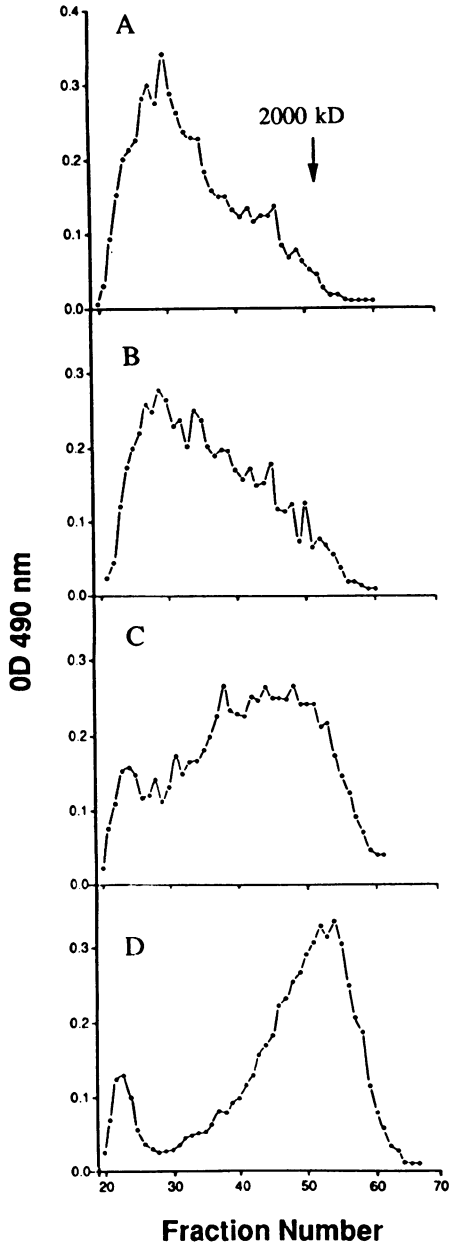

Figure 4. Gel permeation profiles of CDTA-soluble fractions at different stages of ripening on Sepharose CL-2B. Column fractions were assayed for carbohydrate using the phenol-sulfuric acid method (7) (A_{490}). A, Harvest; B, 1 d after ethylene treatment; C, 4 d after ethylene treatment; D, 8 d after ethylene treatment.

non was caused by preferential solubilization of some smaller, less galactosylated polymers and/or reallocation of some larger, more highly branched pectic polymers from later fractions to the Na_2CO_3-soluble fraction. Results of the present study suggest that the phenomenon is a result of both factors. One day after ethylene treatment, polyuronide with a low degree of branching and similar mol wt to that of the Na_2CO_3-soluble fraction at harvest was recovered in the PAW-soluble fraction, implying that depolymerization was not required for initial pectin solubilization. Concurrently, the only CWM

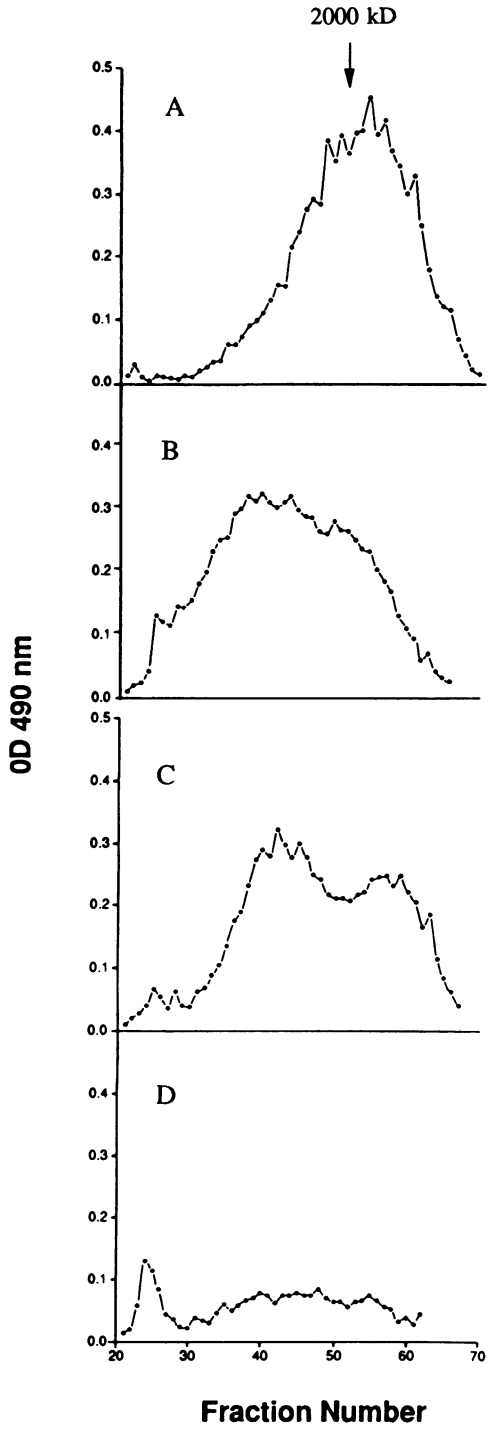

Figure 5. Gel permeation profiles of Na_2CO_3-soluble fractions at different stages of ripening on Sepharose CL-2B. Column fractions were assayed for carbohydrate using the phenol-sulfuric acid method (7) (A_{490}). A, Harvest; B, 2 d after ethylene treatment; C, 4 d after ethylene treatment; D, 6 d after ethylene treatment.

Table V. *Rhamnose, Arabinose, Galactose, Xylose, and Uronic Acid Content of CDTA-, Na_2CO_3-, GTC-, 4 M KOH-, and 8 M KOH-soluble Pectic Fractions of Kiwifruit CWM at Different Stages of Ripeness*

Fraction and Time after Ethylene Treatment	Composition				
	Rha	Ara	Xyl	Gal	Uronic acid
	mol %[a]				
CDTA-soluble					
Harvest	1.0	0.8	0.2	1.3	95.8
1 d	1.9	1.5	0.2	1.8	93.9
2 d	3.0	2.5	0.4	3.1	90.4
4 d	3.3	2.4	0.5	3.5	89.7
Na_2CO_3-soluble					
Harvest	1.5	1.6	0.4	4.6	91.4
1 d	3.4	3.5	0.4	9.3	83.1
2 d	4.5	4.5	0.4	11.9	78.2
4 d	5.3	5.6	1.2	13.8	73.2
GTC-soluble					
Harvest	6.6	6.0	2.1	27.7	55.1
1 d	6.9	5.9	2.0	27.9	54.8
2 d	7.4	6.8	2.7	30.8	49.3
4 d	5.9	5.9	7.0	23.3	51.4
4 M KOH-soluble					
Harvest	4.9	4.9	22.1	33.1	30.6
1 d	4.8	4.2	20.3	35.8	32.4
2 d	2.1	2.0	60.3	11.1	19.7
4 d	1.9	2.0	64.6	5.6	21.7
8 M KOH-soluble					
Harvest	6.0	6.0	3.3	45.2	37.3
1 d	6.1	6.3	5.8	46.6	32.3
2 d	6.8	6.8	8.8	41.4	33.5
4 d	6.5	7.1	27.9	32.2	22.0

[a] Values are averages of two determinations.

fraction to show a marked loss of pectic polymer was the Na_2CO_3-soluble fraction (Table IV). In addition, 1 d after ethylene treatment, there was no net loss of neutral sugars from the CWM but a measurable shift of the neutral sugars normally associated with the pectic polymers from the CWM residue to the CDTA- and Na_2CO_3-soluble fractions.

The CDTA-soluble fraction, which is believed to contain the middle lamella pectin, demonstrated both a decrease in mol wt and an increase in total amount during ripening. In previous work (15), the amount of CDTA-soluble fraction in the CWM decreased by 80%, a fact consistent with EM studies (I. C. Hallet, E. A. MacRae, and T. F. Wegrzyn, unpublished data) which showed dissolution of the middle lamella during ripening. The conflicting results are caused by the different solvent properties of PAW and SDS (13), particularly the ability of SDS to dissolve moderate amounts of the Na_2CO_3-soluble fraction from the cell wall. In the present study, 1 d after ethylene treatment, the CDTA-soluble fraction increased by 54%, whereas the only other fraction to change markedly was the Na_2CO_3-soluble fraction which decreased by 25.6%. The Na_2CO_3-soluble fraction was, therefore, the most likely source of the extra polymers recovered in the CDTA-soluble fraction.

Because the CDTA-soluble polymers are among the largest pectic molecules in the wall, any reallocation of the smaller molecules of the Na_2CO_3-soluble fraction would effectively decrease the mol wt of the CDTA-soluble polymers. The assumption that the reduction in mol wt of the CDTA-soluble fraction (Fig. 4) resulted from pectin hydrolysis may, therefore, be misleading. Nevertheless, the homogalacturonan-type structures found in the CDTA-soluble fraction would provide an ideal substrate for PG, and the possibility must be considered that the decrease in mol wt of the CDTA-soluble fraction resulted from the solubilization of middle lamella pectin by PG action.

Degalactosidation of the Pectic Polymers

The first polyuronides were solubilized 1 d after ethylene treatment, but this was not accompanied by a decrease in the

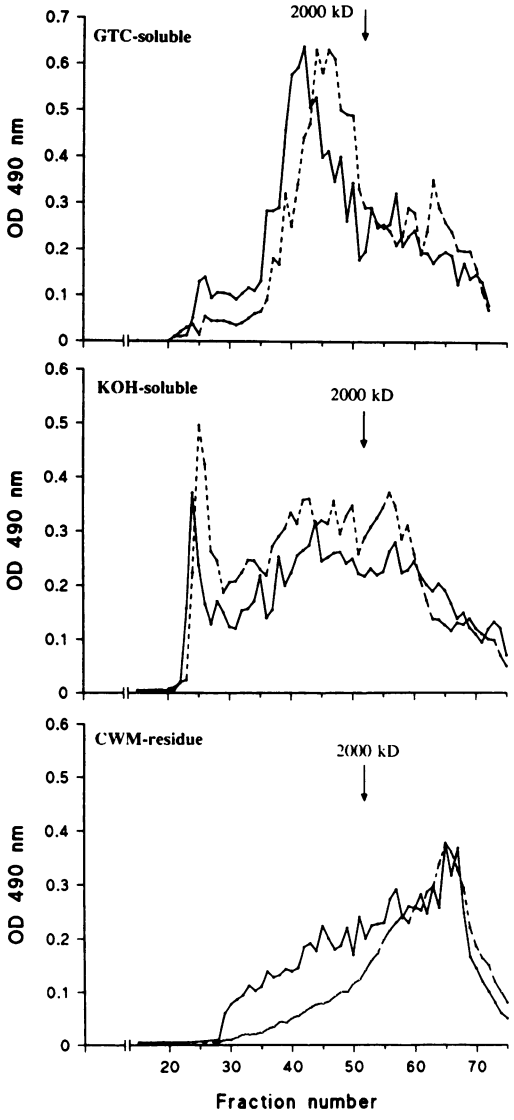

Figure 6. Gel permeation profiles on Sepharose CL-2B of the pectic polymers of the GTC- and KOH-soluble and CWM residue fractions at harvest and 4 d after ethylene treatment. Column fractions were assayed for carbohydrate using the phenol-sulfuric acid method (7) (A_{490}). Solid line, harvest; dotted line, 4 d after ethylene treatment.

galactose content of the CWM. This signified that removal of pectic side chains was not necessary for the initial solubilization of some pectic polymers. There appear to be no other reports that polyuronide solubilization during fruit ripening has occurred independently of galactose (or other neutral sugar) loss. Loss of galactose residues appeared to occur as the pectic polymers were being solubilized or soon after. This was supported by the fact that some highly galactosylated pectic galactans were not solubilized and remained in the CWM at an advanced stage of ripening. Either specific pectic galactans in the cell wall were resistant to attack by a galactosidase or degalactosidation could only occur after solubilization. β-Galactosidase from kiwifruit has recently been characterized (11). It is plausible that in unripe fruit potential substrates for β-galactosidase are present but are inaccessible because of their location or physical state in the wall. Solubilization of the pectic polymers renders them susceptible to enzyme attack. The difficulty with this theory is that in the pre-ethylene-treated PAW-soluble fraction there were small amounts of highly galactosylated pectic polymers. If they represented *in vivo* solubilized polymers, why were they not subject to the same degalactosidation? A real possibility is that, although PAW is not a good solvent for pectic polymers, it is solubilizing some of the pectic polymers of the CWM during homogenization. The highly galactosylated polymers shown in Table III may *in situ*, therefore, still be part of the insoluble cell wall.

The results of this study support some of the conflicting ideas mooted by both Smith *et al.* (20) and DellaPenna *et al.* (6) with regard to the role of PG in pectin solubilization in tomato. In our study of kiwifruit, there was chemical evidence implying that PG and β-galactosidase played a role in degrading the solubilized pectic polymers but were not necessarily involved in the process of solubilizing the bulk of the primary wall polymers. This supported the findings of Smith *et al.* (20). On the other hand, there was tentative evidence to suggest that the CDTA-soluble fraction was depolymerized while part of the insoluble CWM, supporting the claim of DellaPenna *et al.* (6) that PG also mediated solubilization of some pectic polymers. If, despite the chemical evidence, PG and glycosidases were responsible for the solubilization of the bulk of primary cell wall pectic polymers in kiwifruit, then their action must be extremely restricted and selective.

Swelling of the CWM

Swelling was detected 1 d after ethylene treatment and appeared to be in synchrony with both pectin solubilization and a decrease in the mol wt of xyloglucan during ripening (13). After the cell walls have swollen, the aqueous environment of individual polymer chains is modified. Smaller, less branched polymers immobilized within the wall by the compactness of the structure in unripe fruit may be able to move within the looser more hydrophilic structure formed during ripening and, hence, be the first pectic molecules to be solubilized. More branched polymers, which at harvest were only solubilized in 4 M KOH, may become susceptible to solubilization in less severe solvents at later stages of ripening. Thus, the solubilization of the pectic polymers and their reallocation to different chemical fractions during ripening may be different manifestations of the same process. After the wall has swollen, the environment may also be more favorable for polysaccharide-degrading enzymes to act on pectic side chains. We do not know the causes of the loosening and swelling of the cell wall during kiwifruit ripening. If it is a manifestation of changes to the hemicellulose/cellulose polymers, then it invites the speculation that pectin solubilization in ripening fruit may not promote cell wall dissolution but be a consequence of it.

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