JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Characterization and Distribution of Phenolics in Carrot Cell Walls

YOON-HAN KANG,† CHARLOTTE C. PARKER, ANDREW C. SMITH, AND KEITH W. WALDRON*

Sustainability of the Food Chain Exploitation Platform, Institute of Food Research, Norwich Research Park, Colney Lane, Norwich NR4 7UA, United Kingdom

Carrot cell walls have been shown to contain significant quantities of esterified *p*-hydroxybenzoic acid, which is presumed to be esterified to cell wall polymers. The purpose of this study was to investigate the distribution of *p*-hydroxybenzoic acid and related phenolics among carrot cell wall polysaccharides. Cell wall material was prepared from fresh carrot root tissues and extracted sequentially with water, imidazole, cyclohexane-*trans*-1,2-diamine-*N,N,N′,N′*-tetraacetate, Na_2CO_3, and KOH (0.5, 1, and 4 M) to leave a cellulose-rich residue. The fractions were analyzed for their carbohydrate and phenolic acid components. Selected soluble fractions were subfractionated further by graded precipitation in ethanol. The majority of the polymer fractions comprised pectic polysaccharides, with varying quantities of neutral sugars (arabinose and galactose). Hemicellulosic polymers were generally found only in the strong alkali extracts (4 M KOH). *p*-OH-benzoic acid was the predominant phenolic ester and was associated with most fractions analyzed; *p*-OH-benzaldehyde was also detected in the fractions at much lower levels. Principal components analysis of the chemical data indicated that the *p*-OH-benzoic acid was associated predominantly with the branched pectic polysaccharides, in contrast to the *p*-OH-benzaldehyde. The possible roles and functional properties of these phenolics are discussed.

KEYWORDS: Carrot; *Daucus carota*; cell walls; sequential extraction; *p*-hydroxybenzoic acid; pectic polysaccharides

INTRODUCTION

The cell walls of edible vegetables are important determinants of food structure and texture and provide important sources of dietary fiber (*1*−*4*). They are principally composed of polysaccharides. In mature carrot, for example, the cell walls are rich in cellulose (25% of the cell wall dry matter) and hemicelluloses (10−15%) such as xyloglucans, xylans, and pectic material (45−50%) with associated galacturonic acid (GalA)-, arabinose-, and galactose-containing polysaccharides (*5, 6*). Many of these wall polymers are probably complexed with proteins and polyphenolic material (*7*). Furthermore, the chemical compositions of the numerous fractions that may be obtained suggest that a range of pectic complexes is present (*5*).

In addition to the main polysaccharide components, the nonlignified cell walls of edible plant tissues often contain small quantities of phenolics, many of which are esterified to the cell wall polysaccharides. These may be quantified by reverse-phase high-performance liquid chromatography (HPLC) after extraction with alkali (*8*). In some cases, these phenolics can confer properties that are seemingly out of proportion to their levels. For example, the relatively small quantities of esterified ferulic acid in the cell walls of Chinese water chestnut and sugar beet can be peroxidatively cross-linked to create diferulic acids, which cross-link cell wall polymers and enhance cell adhesion. This results in a reduction in cell separation during thermal cooking and maintains a crisp texture (*9*). In addition, many of these phenolics play important roles in disease and wound responses (*8*). By extracting cell wall polymers of a number of plant tissues sequentially while minimizing their degradation (*10*), we have previously demonstrated that the ferulic acid moieties, as well as other phenolics, are distributed across a range of cell wall polysaccharide fractions, for example, in tobacco (*11*), brewers' spent grain (*12*), and wheat bran (*12*).

These studies have also highlighted the presence of other nonlignin cell wall phenolics, the functions of which are not clear. A good example of this concerns cell walls of carrot root. Previous studies have revealed substantial quantities of *p*-OH-benzoic acid (*p*-OH-BA) and *p*-OH-benzaldehyde (*p*-OH-BAld) (*13*) in the cell walls. However, there is generally little knowledge concerning the functional properties of these phenolics, nor of the cell wall polymers to which they are attached. The study reported here has used established methods to

* To whom correspondence should be addressed. Tel: +44-1603-255385. Fax: +44-1603-507723. E-mail: keith.waldron@bbsrc.ac.uk.
† Present address: Department of Food Science, Kangnung National University, Gangneung Daehangno 120, Gangneung, Gangwon-do, 210-702, Republic of Korea.

10.1021/jf801540k CCC: $40.75 © 2008 American Chemical Society
Published on Web 08/30/2008

disassemble and fractionate cell wall polysaccharides to investigate the distribution and content of these two phenolics in carrot cell walls.

MATERIALS AND METHODS

Carrots. Carrots (*Daucus carota*) were obtained from a local supplier, washed, and cut into discs (5 mm thick), which were frozen immediately in liquid nitrogen and stored −40 °C prior to chemical analysis.

Preparation of Cell Wall Material (CWM). CWM was prepared using the method of Parker and Waldron (*9*). Carrots (frozen, 100 g) were blended in 150 mL of sodium lauryl sulfate (SDS; 15 g/L) containing 5 mM $Na_2S_2O_5$ with an Ystral homogenizer (Ystral GmbH, Dottingen, Germany) for 5 min. A few drops of octanol were added to reduce foaming. The homogenate was filtered through a 100 μm nylon mesh (John Stannier and Co., Manchester, United Kingdom), and the residue was ball milled (Pascall, 0.5 L pot) at 1−4 °C in 5 g/L SDS containing 3 mM $Na_2S_2O_5$ for 2 h at 60 rpm to release the bulk of the remaining cell contents. After the homogenate had been filtered through 70 μm nylon mesh, the residue was suspended in cold water containing 3 mM $Na_2S_2O_5$, homogenized for 5 min, and refiltered. The procedure was repeated three times until the cell wall residue was free of intracellular contents as assessed by light microscopy after staining in iodine/potassium iodide. The CWM was stored as a frozen suspension at −20 °C.

Preparation of Alcohol-Insoluble Residue (AIR). This was prepared prior to analyzing cell wall phenolics to remove any noncovalently bound moieties. Hydrated CWM (20−40 mg) was dispersed in ethanol (up to 10 mL) to a final concentration of 70% (v/v aq) and heated in a closed sovirel tube at 100 °C for 10 min. The alcohol-rich supernatant was removed by decanting, and the residue was extracted under similar conditions but in 100% ethanol. After it was washed once in 100% alcohol, the residue was washed in acetone and air-dried at 40 °C.

Sugars Analysis. Cell wall neutral sugars were analyzed as described previously. CWM was dispersed in 720 g/kg H_2SO_4 for 3 h followed by dilution to 1 M and hydrolyzing for 2.5 h at 100 °C (*14*). All samples were analyzed in duplicate. Neutral sugars were reduced with $NaBH_4$ and acetylated according to the method of Blakeney et al. (*15*) using 2-deoxyglucose (Sigma) as an internal standard. Alditol acetates were quantified by gas chromatography as described in Parr et al. (*13*). UAs were assayed colorimetrically in aliquots of H_2SO_4-hydrolyzed samples according to Blumenkrantz and Asboe-Hansen (*16*).

Phenolic Acid Analysis. Esterified phenolics were extracted and quantified as described by Waldron et al. (*17*) from samples that had been previously extracted in hot alcohol. Each sample was extracted with 4 M NaOH (12 h) under N_2 (O_2-free) at room temperature. The supernatant was filtered (GF-A, Whatman, United Kingdom) and acidified to pH < 2 with HCl. *trans*-Cinnamic acid (Sigma; 100 μL, 1 mg mL^{-1} 500 mL/L methanol) was added to the filtrate as an internal standard, extracted three times with ethyl acetate (3 volumes), and dried under a stream of N_2. The sample was dissolved in 500 mL/L methanol (200 μL; HPLC grade; BDH) and analyzed by HPLC as described in Waldron et al. (*17*).

Wall-bound phenolics were also released by sequential alkaline hydrolysis of wall material under progressively more vigorous conditions. CWM (20 mg) was extracted with 0.1 M NaOH for 1 h at 25 °C under N_2. The suspension was filtered through glass fiber paper, and the residue was retained for further extraction with, sequentially, 0.1 M NaOH for 24 h, 1 M NaOH (24 h), and 2 M NaOH (24 h).

Sequential Extraction of CWM. The methodology for sequentially extracting the CWM was based on that of Redgwell and Selvendran (*10*). CWM (1 g) was sequentially extracted with (a) water (100 mL/g CWM) at 20 °C for 30 min; (b) 2 M imidazole (100 mL/g CWM), pH 7.0 at 20 °C for 16 h; (c) 50 mM cyclohexane-*trans*-1,2-diamine-*N*,*N*,*N*′,*N*′-tetra acetate (CDTA, 100 mL), pH 6.5, at 20 °C for 5 h (CDTA-1); (d) 50 mM CDTA (100 mL) at 20 °C for 2 h (CDTA-2); (e) 50 mM Na_2CO_3 + 20 mM $NaBH_4$ (100 mL) at 0 °C for 20 h (Na_2CO_3-1); (f) 50 mM Na_2CO_3 + 20 mM $NaBH_4$ (100 mL) at 20 °C for 2 h (Na_2CO_3-2); (g) 0.5 M KOH + 20 mM $NaBH_4$ (100 mL) at 20 °C for 2 h; (h) 1 M KOH + 20 mM $NaBH_4$ (100 mL) at 20 °C for 2 h; and (i) 4 M KOH + 20 mM $NaBH_4$ (100 mL) at 20 °C for 2 h. The alkali extractions were carried out with O_2-free solutions under argon. After each extraction, solubilized polymers were separated from the insoluble residue by centrifugation. The alkali extracts were filtered through GFC and then acidified to pH 5 with HOAc. The cellulose-rich residue remaining after the final alkali extraction was also acidified to pH 5, and after recentrifugation, a polysaccharide-containing neutral supernatant was recovered. The filtered extracts and the insoluble cellulose-rich residue were dialyzed exhaustively against water, concentrated by rotary evaporation, and freeze-dried (*18*).

Graded Ethanol Precipitation. Selected sequential extracts were suspended in water and dissolved by stirring at 1 °C. The polymers were then sequentially precipitated by addition of ethanol, the concentration of which was increased in steps from 40 to 80%. Each precipitation step was carried out at 1 °C for 16 h, and the precipitate was collected by centrifugation. To remove the ethanol completely, each precipitate was dissolved in water and rotary-evaporated. The fractions were stored as frozen suspensions at −20 °C. An aliquot of each fraction was freeze-dried for quantification and sugar and phenolic analysis.

Ethanol Extraction of Solubilized Components. Freeze-dried cell wall fractions and ethanol precipitates were extracted in 100% ethanol at 100 °C for 10 min. The alcohol-rich supernatant was removed by decanting, and the residue was extracted under similar conditions but in 100% ethanol. After it was washed once in 100% alcohol, the residue was washed in acetone and air-dried overnight.

Release of *p*-Hydroxybenzoic Acid from the Cellulose-Rich Fraction. *Acid Hydrolysis.* The cellulose-rich fraction was hydrolyzed in trifluoroacetic acid (TFA) under three sets of conditions: mild [(a) 0.1 M TFA at 100 °C], strong [(b) 1 M TFA at 100 °C], and (c) 1 M TFA at 120 °C each for 2 h. In each case, 5 mg of the cellulose fraction was suspended in 2 M TFA (2 mL) in a soviril tube with vortexing and heated in a hot block at 100 or 120 °C, after which is was cooled on ice and then centrifuged to separate the insoluble residue from the TFA hydrolysate. The latter was recovered, measured for volume, and then divided into two equal portions. One was acidified and partitioned into ethyl acetate to recover free phenolics. The other was subjected to saponification overnight (as described for phenolic acid analysis above), and phenolics were analyzed as described above. The residue was also analyzed for phenolics as described above.

Enzymatic Digestion with Driselase. Driselase was obtained from Sigma. Driselase was purified as described in the method of Fry et al. (*19*). An aliquot was desalted in a diafiltration cell (Amicon PM10 membrane) and lyophilized.

The cellulose-rich residue (20 mg, dry) was treated with Driselase for 18 h at 37 °C in 25 mM sodium acetate buffer, pH 5.0 (final enzyme concentration, 0.6%), with constant shaking. Following incubation, the hydrolyzed material was centrifuged (13000 rpm, 10 min), and the supernatant was filtered through a microfilter (0.45 μm). The hydrolysate was assessed for phenolics as described above after saponification in 4 M NaOH.

Principal Components Analysis. Chemical compositional data [neutral sugars, uronic acids (UAs), and phenolics] of sequentially extracted and alcohol-precipitated fractions were subjected to principal components analysis using Multi Variate Statistical Package software V3.1 (Kovak Computing Services, Anglesey, Wales).

RESULTS AND DISCUSSION

Preparation and Characterization of CWM. The CWM was prepared as described by the method of Parker and Waldron (*9*). The use of SDS in conjunction with cellular disruption by ball-milling was considered to be sufficient to solubilize and remove intracellular proteins and low molecular weight components. The yield of CWM was 26.8 g/kg of fresh carrot, which is similar to that obtained previously (*13*), and is higher than that from Chinese water chestnut (*9*). The carbohydrate compositions of the CWM are shown in **Table 1** and are similar to results obtained previously (*6, 13*). The high level of GalA reflects the substantial quantity of

Table 1. Carbohydrate and Phenolic Compositions of Carrot CWM and Sequential Extracts[a,b]

		cell wall sugars (μg/mg)										phenolics[c]	
fraction	yield (% CWM)	Rha	Fuc	Ara	Xyl	Man	Gal	Glc	UA	total sugars	ratio UA:LSC	*p*-OH-BA (μg/mg)	*p*-OH-BAld (μg/g)
CWM	100	12.0	2.1	47.5	10.3	12.7	41.5	215.	410	751	5	**Table2**	**Table2**
water	0.3	3.8	3.2	41.6	7.5	11.1	49.0	20.7	283	420	3	0.17	82.3
imidazole	1.8	8.0	6.3	147	5.8	17.8	29.3	19.0	463	695	3	0.18	13.9
CDTA-1	11.1	15.3	5.8	28.1	3.6	1.3	21.0	18.4	906	999	20	0.03	10.9
CDTA-2	3.1	4.2	3.5	23.8	3.3	2.1	22.7	9.9	369	439	8	0.02	6.3
Na_2CO_3-1	11.1	6.6	5.0	58.9	3.8	2.1	50.0	12.0	339	477	3	0.17	9.0
Na_2CO_3-2	3.1	22.6	4.8	111	3.5	0.8	88.3	12.4	508	752	3	1.36	9.9
0.5 M KOH	2.8	25.4	5.1	128	6.3	5.1	102	16.9	487	776	2	2.10	12.7
1 M KOH	1.9	23.6	5.5	123	22.8	20.7	92.2	28.0	488	804	2	1.14	9.0
4 M KOH	2.3	11.9	8.4	83.5	125.1	49.4	63.4	101	270	712	2	0.46	8.1
neutral supt.	1.5	22.9	3.3	155	9.8	1.5	122	9.1	513	836	2	0.32	8.8
final residue	14.3	1.6	0.5	25.0	4.8	14.4	20.8	573	62.9	703	1	0.72	9.0

[a] Values are expressed as μg of anhydrosugar/mg dry polymers. [b] LSC, likely side chain sugar (arabinose + galactose). [c] CWM and fractions extracted as AIR prior to phenolic analysis.

Table 2. Wall-Bound Phenolics Released from Carrot CWM and AIR (in Parentheses) by Sequential Alkaline Treatments at Room Temperature

	μg/g				
phenolics	0.1 M NaOH (1 h)	0.1 M NaOH (24 h)	1.0 M NaOH (24 h)	2.0 M NaOH (24 h)	Total
p-OH benzoic acid	747 (391)	391 (437)	462 (891)	434 (259)	2034 (1978)
p-OH benzaldehyde	6.2 (3.2)	1.5 (1.6)	2.2 (4.0)	2.2 (1.9)	12.1 (10.7)
vanillin	14.5 (5.7)	3.2 (6.9)	16.5 (19.3)	23.7 (24)	57.9 (55.9)
ferulic acid	3.0 (1.6)	2.4 (2.3)	3.2 (6.8)	7.1 (2.9)	15.7 (13.6)

pectic polysaccharides. Arabinose (Ara) and galactose (Gal) were the main noncellulosic sugars and will have originated from branched rhamnogalacturonan pectic moieties (*5*). Alkali-soluble phenolic components were quantified both for CWM and for AIR (prepared from the CWM). AIR was prepared to ensure that no noncovalently bound phenolics remained in the CWM (*13*). The results are shown in **Table 2**. As expected (*13*), *p*-OH-BA was the most abundant phenolic acid at levels of nearly 2034 μg/g CWM. In addition, several other phenolics were also detected including vanillin, ferulic acid, and *p*-OH-BAld. Interestingly, preparation of AIR made no difference to the overall recovery of the detected phenolic components, confirming that the phenolics present in the wall preparations were not loosely associated. However, the AIR extraction process made a considerable difference to their ease of saponification in increasing concentrations of alkali. This may reflect the changes in interpolymeric associations in the cell wall as a result of the AIR dehydration process, resulting in a reduction in the extent of saponofication in 0.1 M NaOH, and a concomitant increase in phenolics released at the higher alkali concentrations (**Table 2**).

Sequential Extraction of Cell Wall Polymers. So as to explore the distribution of wall-bound phenolics among the cell wall polymers, CWM was sequentially extracted using an approach that minimized polymer degradation derived from that of Regwell and Selvendran (*10*). In contrast to the approach used by Stevens and Selvendran (*5*), this approach avoids thermal treatments, which will cause β-eliminative degradation of pectic moieties and associated depolymerisation (*1*). The yield and carbohydrate compositions are presented in **Table 1**. Imidazole is thought to remove very weakly bound pectic polysaccharides held in the wall by Ca^{2+} only. CDTA is a chelating agent and solubilizes any remaining pectic polysaccharides held in the wall by Ca^{2+} alone. Na_2CO_3 is considered to solubilize pectic polysaccharides by hydrolysis of weak ester cross-links (*10*), while KOH solubilizes hemicelluloses by disrupting hydrogen bonding with cellulose. Hemicelluloses with different branching characteristics are extracted by different concentrations of alkali (*1*).

Recoveries of extracts were not quantitative (50%), and it is likely that some material was lost during the dialysis process and possibly during the filtration of hemicellulosic extracts. However, it should be noted that recovery using this type of approach is usually less than 75−80%. The weight and carbohydrate recoveries are shown in **Figure 1**.

The imidazole, CDTA, Na_2CO_3, 0.5 and 1.0 M KOH extractions released predominantly pectic polysaccharides. The bulk of pectic polysaccharides held in the wall by Ca^{2+} only was extracted in CDTA and was composed mainly of GalA. A small proportion of Ca^{2+}-bound pectic polymers were solubilized by imidazole. It is probable that the bulk of the Ca^{2+} cross-linked polysaccharides are of middle lamella origin (*5*). Extracting with increasing strengths of alkali resulted in the release of more highly branched pectic polymers as indicated by the increase in the relative proportions of likely side chain (LSC) sugars (Ara + Gal), GalA, and an increase in the ratio of Rha: GalA (**Figure 2**). The 4 M KOH-extracted components contained significant levels of Xyl and Glc in addition to branched pectic polymers. This might normally be considered to be indicative of xyloglucan hemicelluloses, which are regularly found in cell walls of dicotyledonous fruits and vegetables (*1*). However, previous work (*5*) has demonstrated that the bulk of carrot cell wall Xyl is (1−4)-linked. The observation that pectic polymers are present to significant levels in the 4 M KOH extract is consistent with their attachment to xylan hemicelluloses, which would require such conditions for solubilization. The remaining insoluble cellulosic residue was neutralized. This resulted in the release of further, small quantities of highly branched pectic polymers (**Figure 1**).

Phenolic Acid Analysis. The sequentially extracted cell wall polysaccharides were also analyzed for alkali-labile phenolics. The freeze-dried samples were extracted in hot alcohol to ensure removal of any noncovalently bound phenolics and then saponified as described. The most prominent and quantifiable phenolics were *p*-OH-BA and lower levels of *p*-OH-BAld (**Table 1**). The water- and CDTA-extracted polymers contained relatively low levels of *p*-OH-BA, which was mainly associated with the alkaline-extracted polymers, particularly those in the 0.5 M KOH fraction. *p*-OH-BA was also found in considerable quantities in the cellulose-rich residue. *p*-OH-BAld was found

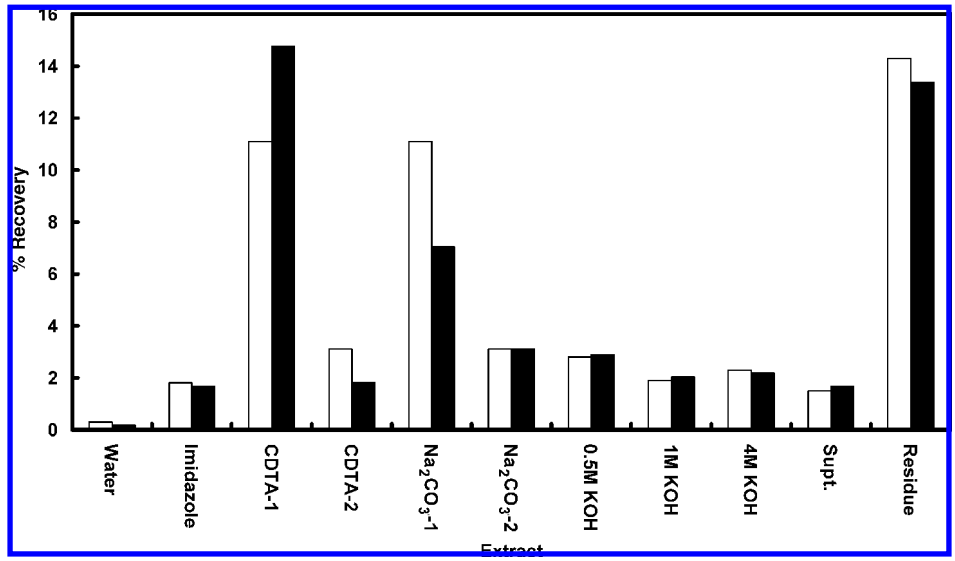

Figure 1. Recoveries of extracted cell wall components. White bars, % weight recovery as a function of cell wall weight; black bars, % recovery of carbohydrate as a function of cell wall carbohydrate.

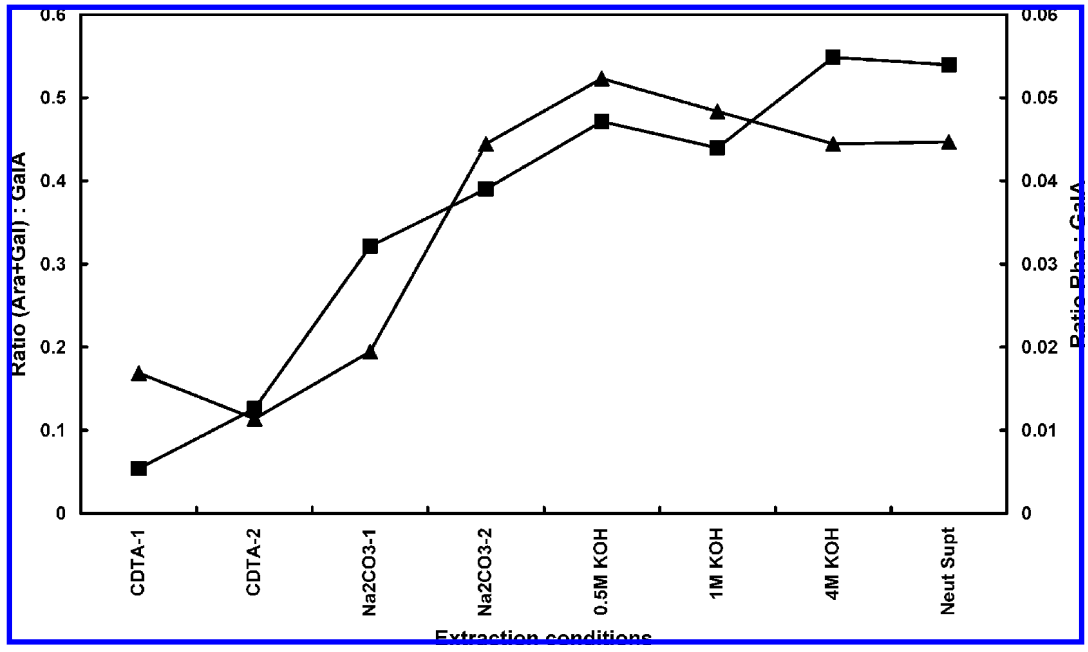

Figure 2. Ratios of UA to specific and groups of LSC sugars in carrot cell wall sequential extracts. Symbols: ratio of LSC neutral sugar (Ara + Gal) to GalA (■); ratio of Rha to GalA (▲).

at much lower levels (presented as μg/g in **Table 1**). Other phenolics, including ferulic acid and vanillin, were detected at very low levels but not quantified.

Graded Alcohol Precipitation. The higher-yielding, water-soluble polymer fractions (CDTA-1, Na_2CO_3-1 and 2, and 0.5 M KOH) were subfractionated further by subjecting them to graded ethanol precipitation, after which the recovered freeze-dried fractions were characterized for their carbohydrates and phenolics. The results are shown in **Table 3**. CDTA-1- and Na_2CO_3-derived polymers each gave rise to several fractions with sugar compositions broadly similar to the parent mixtures except for the nonprecipitating supernatants that were low in sugars. *p*-OH-BA acid was not uniformly distributed across the precipitated subfractions and was found mainly in the 40% (v/v) ethanol-soluble Na_2CO_3 and 0.5 M KOH subfractions and in the 80% (v/v)-ethanol-soluble Na_2CO_3-2 and 0.5 M KOH subfractions. It is not clear why so much of the extracted, highly branched polymers remain soluble in high concentrations of alcohol and may indicate that the polymers are of relatively low molecular weight or complexed with other, unidentified hydrophobic moieties.

Release of *p*-OH-BA from the Cellulose-Rich Residue and AIR Fraction. Significant amounts of *p*-OH-BA were retained in the cellulose-rich residue. In an attempt to release oligosaccharide fragments of cell wall polymers to which *p*-OH-BA might be attached and to obtain further information on the lability of this moiety, the cellulose-rich residue was subjected to further chemical and biochemical extraction procedures.

Acid Hydrolysis. The contents of *p*-hydroxybenzoic acid in carrot cellulose-rich residue were subjected to acid hydrolysis with trifluro acetic acid solutions: 0.1−2 M TFA, 100−120 °C, for 2 h. *p*-OH-BA remaining in the residue was released by saponification, recovered by partitioning the acidified extract into ethyl acetate, and quantified by HPLC. Solubilized material was subjected to direct analysis by HPLC or after saponification (see the Materials and Methods). The results (**Figure 3**) show

Table 3. Carbohydrate and Phenolic Composition of Fractions Obtained by Graded Precipitation of Cell Wall Extracts

		sugar composition										phenolic composition
fraction	yield (% parent fraction)	Rha	Fuc	Ara	Xyl	Man	Gal	Glc	UA	total sugar[a]	ratio[b] UA:LSC	*p*-OH-BA[c]
CDTA-40	22.2	8.7	4.5	17.8	5.5	6.2	32.2	11.2	490	577	10	0.01
CDTA-60	42.9	12.8	6.2	20.8	1	6.1	31.6	9.2	905	993	23	
CDTA-80	14	6.9	8.6	15.9	6.7	6.3	19.2	7.3	524	595	15	
CDTA-Supt	10.1	1.6	3.8	1.8	1.4	1.4	5.4	4	43.1	62.5	6	0.02
Na_2CO_3(1)-40	4.7	17	3.5	64	1.5	1.3	70.6	4.6	552	715	4	0.28
Na_2CO_3(1)-60	57.8	16.3	4	75	1	0.3	75.9	2.1	591	765	4	0.04
Na_2CO_3(1)-80	0.9											
Na_2CO_3(1)-Supt	29.7	0.9	2.5	4.8	0.9	0.3	1.5	1.5	0	12.4		0.30
Na_2CO_3(2)-40	5.5	24.5	5.6	117	1.3	1.3	119	7.6	639	917	3	1.34
Na_2CO_3(2)-60												
Na_2CO_3(2)-80												
Na_2CO_3(2)-Supt	31.8	13.1	4.7	71.5	0.9	1	76.2	3.4	409	580	3	0.91
0.5 M KOH-40	21.1	19.1	3.5	115	1	1.2	112	11.1	484	748	2	2.09
0.5 M KOH-60												
0.5 M KOH-80												
0.5 M KOH-Supt	51.2	23.1	5.1	128	1.2	10.2	128	23.4	571	890	2	2.06

[a] Values are expressed as μg of anhydrosugar/mg dry polymers. [b] LSC, likely side chain sugar (arabinose + galactose). [c] Values are expressed as μg of phenolic acid/mg dry polymers.

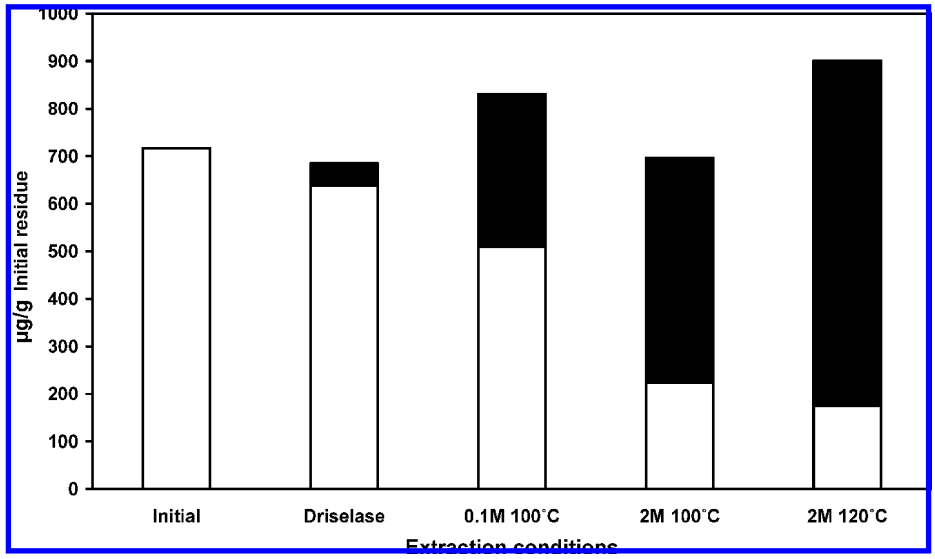

Figure 3. Solubilization of free *p*-OH-BA from cellulose-rich residue by Driselase digestion or hot dilute acid (TFA for 2 h) treatment. White bars, *p*-HO-BA esterified to cellulose-rich residue; black bars, *p*-OH-BA released by the treatment.

the release of monomeric *p*-OH-BA (i.e., measured directly by HPLC). Increasing the concentration of acid and temperature released increasing quantities of *p*-OH-BA, up to approximately 80% after hydrolysis in 2 M TFA at 120 °C. Saponification of the acid-hydrolyzed material did not increase the yields (results not shown). This indicates that even dilute, hot acid hydrolysis is sufficient to hydrolyze the inferred ester linkage.

Enzymatic Hydrolysis. Driselase has been used for the enzymatic hydrolysis of hemicellulosic polysaccharides, particularly to release fragments of cell wall sugars to which are attached phenolic acids. This provides a route for the purification and characterization of substituted sugars. In this study, Driselase was purified and used to degrade the polymers in the cellulose-rich residue. The results (**Figure 3**) show that only about 7% of the *p*-OH-BA could be released, and all of it was in monomeric form (as evaluated by direct HPLC analysis; saponification of the Driselase-solublized did not have any effect on the levels quantified). This indicates that there may be a specific esterase-like activity in Driselase, which will release *p*-OH-BA but which is not active on other phenolics such as ferulic acid.

GENERAL DISCUSSION

This study has shown unequivocally that the most abundant carrot cell wall phenolics, *p*-OH-BA acid and *p*-OH-BAld, are closely associated with a range of cell wall polymers. Preliminary attempts using cell wall-degrading enzymes and hot, dilute acid hydrolysis to release fragments that might enable identification of the presumed sugar(s) to which these phenolics may be attached have not been fruitful. However, the data for extracted components from **Tables 1−3** have been analyzed further using principal components analysis with MVSP software. This approach can provide some information on the likely association between variables within the data tables. The results are presented in **Figure 4**. The black circles represent the "cases" (here, the extraction conditions), and the vectors (arrowed lines) represent the variables (the individual chemical components). The closer that circles or vectors are to each other, the more they are likely to have an association. Hence, the extracts/cases represented by black circles in cluster 1 are more similar to each other than they are to the circles in cluster 2 (and vice versa). Likewise, the chemical moieties (sugars and phenolics)

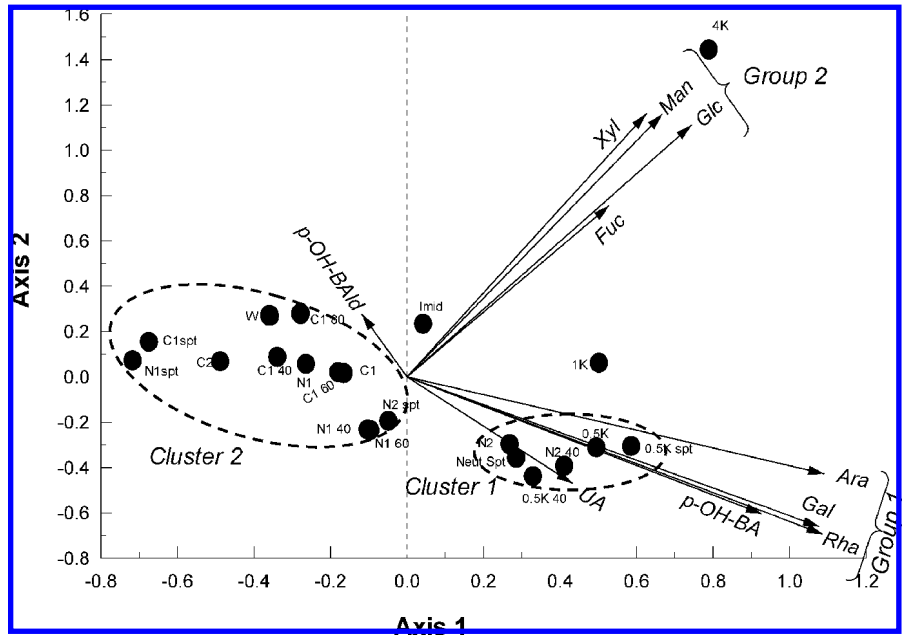

Figure 4. Principal components analysis to show the possible relationship between phenolics and polysaccharides in carrot cell walls residue. Legend for extract labels: W, water; C1, CDTA-1; C1 40, CDTA-1 40% (v/v) ethanol precipitate; C1 60, CDTA-1 60% (v/v) ethanol precipitate; C1 80, CDTA-1 80% (v/v) ethanol precipitate; C1 spt, CDTA-1 80% (v/v) ethanol supernatant; C2, CDTA-2; N1, Na_2CO_3-1; N1 40, Na_2CO_3-1 40% (v/v) ethanol precipitate; N1 60, Na_2CO_3-1 60% (v/v) ethanol precipitate; N1 spt, Na_2CO_3-1 80% (v/v) ethanol supernatant; N2, Na_2CO_3-2; N2 40, Na_2CO_3-2 40% (v/v) ethanol precipitate; N2 spt, Na_2CO_3-2 80% (v/v) ethanol supernatant; 0.5K, 0.5 M KOH; 0.5K 40, 0.5 M KOH 40% (v/v) ethanol precipitate; 0.5K spt, 0.5 M KOH 80% (v/v) ethanol supernatant; 1K, 1 M KOH; 4K, 4 M KOH; and Neut spt, soluble components from neutralized cellulose-rich residue.

represented by vectors in group 1 are more closely associated with each other than with those in group 2. The results thus indicate that the *p*-OH-BA is closely associated with Ara-, Gal-, and Rha-containing (and therefore branched) rhamnogalacturonan pectic polysaccharides (group 1 arrows), and these polysaccharides are predominantly found in the extracts positioned in cluster 1. In contrast, *p*-OH-BAld does not appear to be associated with these polymers or with its sister phenolic *p*-OH-BA. The extracts represented in cluster 2 appear to be much more poorly branched pectic polymers. As expected, the Xyl, Glc, and Fuc components (group 2 vectors) are closely associated with the 4 M KOH extract in which they are mostly found.

Hence, *p*-OH-BA in carrot cell walls appears to be attached to the more highly branched pectic polysaccharides, possibly to either Ara or Gal, while *p*-OH-BAld may not be. The functional properties of polysaccharides substituted with these phenolics are not clear. Intracellular (soluble) *p*-OH-BA is associated with wound responses (*20*). Soluble esters of *p*-OH-BA (parabens) have antimicrobial activity and are widely used as preservatives in food, cosmetics, and pharmaceutical products (*21*). Cvikrova et al. (*22*) have shown that *p*-OH-BA acid increases in the cell walls of Norway spruce during infection. Monomeric phenolic components in cell walls are also considered to play an essential role as precursors of suberin and lignin in the prevention of infection and in healing mechanisms (*23*). The presence of these cell wall phenolics is therefore consistent with the requirements for carrots to lie dormant underground for a substantial period while minimizing their metabolic activity, possibly providing an antimicrobial function.

ABBREVIATIONS USED

CWM, cell wall material; CDTA, cyclohexane-*trans*-1,2-diamine-*N*,*N*,*N*′,*N*′-tetra acetate; *p*-OH-BA, *p*-OH-benzoic acid; p-OH-BAld, p-OH-benzaldehyde; AIR, alcohol-insoluble residue; TFA, trifluoroacetic acid; UA, uronic acid; LSC, likely side chain; GalA, galacturonic acid.

LITERATURE CITED

(1) Brett, C. T.; Waldron, K. W. *Physiology and Biochemistry of Plant Cell Walls*; Kluwer Academic: New York, 1996; 272pp.

(2) Ng, A.; Waldron, K. W. Effect of cooking and pre-cooking on cell-wall chemistry in relation to firmness of carrot tissues. *J. Sci. Food Agric.* **1997**, *73*, 503–512.

(3) Greve, L. C.; Shackel, K. A.; Ahmadi, H.; McArdle, R. N.; Gohlke, J. R.; Labavitch, J. M. Impact of heating on carrot firmness; contribution of cellular turgor. *J. Agric. Food Chem.* **1994**, *42*, 2896–2899.

(4) Greve, L. C.; McArdle, R. N.; Gohlke, J. R.; Labavitch, J. M. Impact of heating on carrot firmness; changes in cell wall components. *J. Agric. Food Chem.* **1994**, *42*, 2900–2906.

(5) Stevens, B. J. H.; Selvendran, R. R. Structural features of cell wall polysaccharides of the carrot *Daucus carota*. *Carbohydr. Res.* **1984**, *129*, 197–207.

(6) Massiot, P.; Rouau, X.; Thibault, J. F. Isolation and characterization of the cell-wall fibres of carrot. *Carbohydr. Res.* **1988**, *172*, 217–227.

(7) Massiot, P.; Rouau, X.; Thibault, J. F. Characterization of the extractable pectins and hemicellulose of the cell wall of carrot. *Carbohydr. Res.* **1988**, *172*, 229–242.

(8) Waldron, K. W.; Selvendran, R. R. Ferulic acid dehydrodimers in the cell walls of *Beta vulgaris* and their possible role in texture. *J. Sci. Food Agric.* **1997**, *74*, 221–228.

(9) Parker, M. L.; Waldron, K. W. Texture of Chinese water chestnut: Involvement of cell wall phenolics. *J. Sci. Food Agric.* **1995**, *68*, 337–346.

(10) Redgwell, R.; Selvendran, R. R. Structural features of cell wall polysaccharides of onion. *Carbohydr. Res.* **1986**, *157*, 183–199.

(11) Merali, Z.; Mayer, M. J.; Parker, M. L.; Michael, A. J.; Smith, A. C.; Waldron, K. W. Metabolic diversion of the phenylpropanoid pathway causes cell wall and morphological changes in transgenic tobacco stems. *Planta* **2007**, *225*, 1165–1178.

(12) Mandalari, G.; Faulds, C. B.; Sancho, A. I.; Saija, A.; Bisignano, G.; LoCurto, R.; Waldron, K. W. Fractionation and characterisation of arabinoxylans from brewers' spent grain and wheat bran. *J. Cereal Sci.* **2005**, *42*, 205–210.

(13) Parr, A. J.; Ng, A.; Waldron, K. W. Ester-linked phenolic components of carrot cell walls. *J. Sci. Food Chem.* **1997**, *45*, 2468–2471.

(14) Saeman, J. F.; Moore, W. E.; Mitchell, R. L.; Millett, M. A. Techniques for the determination of pulp constituents by quantitative paper chromatography. *TAPPI,* **1954**, *34*, 336–343.

(15) Blakeney, A. B.; Harris, P. J.; Henry, R. J.; Stone, B. A. A simple and rapid preparation of alditol acetates for monosaccharide analysis. *Carbohydr. Res.* **1983**, *113*, 291–299.

(16) Blumenkrantz, N.; Asboesen-hansen, G. New method for quantitative determination of uronic acids. *Anal. Biochem.* **1973**, *54*, 484–489.

(17) Waldron, K. W.; Parr, A. J.; Ng, A. Cell wall esterified phenolic dimers: identification and quantification by reverse phase high performance liquid chromatography and diode array. *Phytochem. Anal.* **1996**, *7*, 305–312.

(18) Waldron, K. W.; Selvendran, R. R. Cell wall changes in immature *Asparagus* stem tissue after excision. *Phytochemistry* **1992**, *31*, 1931–1940.

(19) Fry, S. C.; Dumville, J. C.; Miller, J. G. Finger printing of polysaccharides attacked by hydroxyl radicals in vitro and in cell walls of ripening pear fruit. *Biochem. J.* **2001**, *357*, 729–737.

(20) Babic, I.; Amiot, M. J.; Nguyen-The, C.; Aubert, S. Changes in phenolic content in fresh ready-to-use shredded carrots during storage. *J. Food Sci.* **1993**, *58*, 351–356.

(21) Soni, M. G.; Carabin, I. G.; Burdock, G. A. Safety assessment of esters of *p*-hydrozybenzoic acid (parabens). *Food Chem. Toxicol.* **2005**, *43*, 985–1015.

(22) Cvikrova, M.; Mala, J.; Hrubcova, M.; Eder, J. Soluble and cell wall-bound phenolics and lignin in *Ascocalyx abietina* infected Norway spruces. *Plant Sci.* **2006**, *170*, 563–570.

(23) Rhodes, M. J. C.; Wooltorton, L. S. C. Changes in activity of hydroxycinnamyl CoA—Quinate hydroxycinnamyl transferase and in levels of chlorogenic acid in potatoes and sweet potatoes stored at various temperatures. *Phytochemistry* **1978**, *17*, 1225–1229.

Received for review May 19, 2008. Revised manuscript received July 12, 2008. Accepted July 15, 2008. This work was supported by a postdoctoral fellowship program from Korea Science & Engineering Foundation (KOSEF) and the UK Biotechnology and Biological Sciences Research Council (BBSRC).

JF801540K