Ester-Linked Phenolic Components of Carrot Cell Walls

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The cold alkali-labile cell wall phenolics of carrot storage roots have been examined by diode-array HPLC following sequential treatment of purified wall material with aqueous sodium hydroxide of increasing strength. In addition to 4-hydroxybenzoic acid and ferulic acid, which have previously been reported from this source, a range of other typical wall-bound phenolic aldehydes and cinnamic acid derivatives were also identified. An 8-*O*-4′-linked dehydrodiferulic acid, which has recently also been identified as a major ferulic acid dimer in several monocots, was found to be present in readily measurable quantities, and other putative dimers were also noted. At least *ca.* 30% of the total ferulic acid existed in dimer form. Cell wall phenolics might have a role to play in influencing the physical properties of carrot tissue.

Keywords: *Carrot; cell wall; Daucus carota; dehydrodiferulic acid; phenolics*

INTRODUCTION

There is extensive literature on the phenolics associated with the cell walls of various monocots, particularly grasses (e.g., Hartley and Keene, 1984; Hartley and Morrison, 1991; Ishii, 1991; Grabber *et al.*, 1995; Jacquet *et al.*, 1995). These phenolics may have a general role in limiting cell wall digestibility, and also in various aspects of disease resistance (Eraso and Hartley, 1990; Besle *et al.*, 1994). The phenolic dimers which link together two carbohydrate chains, or a carbohydrate and lignin, are thought to also have a more specific role in influencing the physical and chemical properties of cell walls (e.g., Biggs and Fry, 1987; Jacquet *et al.*, 1995; also references cited therein). One example of this is their role in the thermal stability of texture in monocotyledonous vegetables such as Chinese Water Chestnut (Parker and Waldron, 1995; Parr *et al.*, 1996). A study by Hartley and Harris (1981) revealed that with the exception of the Caryophyllales, dicots typically had a much lower level and complexity of simple phenolics associated with their cell walls, in the absence of pathogen attack, than did monocots. This lower level of phenolics has apparently (though perhaps not necessarily correctly) often been interpreted as indicating that, in general, they have relatively little role to play in unchallenged dicot cell walls, and thus, except in the plant pathogen area, there have been rather few detailed investigations of the simple wall phenolics of dicots outside the order Caryophyllales. Apart from a few forage legumes (e.g., Eraso and Hartley, 1990), perhaps one of the most studied species has been the carrot, *Daucus carota* L., where the studies of Hartley and Harris (1981) indicated the presence of 4-hydroxybenzoic acid (4HBA) in significant amounts, and Massiot *et al.* (1988) reported the occurrence of ferulic acid. Even here, most studies have concentrated on disease-resistance aspects. Thus, the levels of 4HBA have been found to rise following challenge of carrot cells by pathogen-related elicitors (Schnitzler *et al.*, 1992), so possibly implicating this phenolic in defense responses.

Given the range of functions ascribed to cell wall phenolics, we have recently been reinvestigating the wall phenolics of several dicots in order to reassess possible contributions toward the textural properties of the species as foodstuffs, and also to see whether they may contribute specific health benefits to dietary fiber, via an antioxidant function or by virtue of other more specific biological activities. As a significant item in the British diet, and one where some background information already exists, the carrot was chosen for detailed investigation.

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MATERIALS AND METHODS

Plant Material. Carrots, var. Amstrong, were grown at the Institute of Food Research, Norwich, Norfolk, G. B., during the 1995 season. They were cultivated on a sandy soil, and were grown for 6 months to maturity. No pesticides were used.

Preparation of Alcohol-Insoluble Residue (AIR). Carrot samples were extracted for AIR as described by Martin-Cabrejas et al. (1994).

Isolation of Cell Wall Material (CWM). Cell wall material was isolated from the main storage root essentially as outlined by Parker and Waldron (1995). Frozen tissue (100 g) was blended in aqueous 1.5% sodium dodecyl sulfate (SDS; Sigma, Poole, U.K.) containing 5 mM $Na_2S_2O_5$ (Sigma) in a Ystral homogenizer (Scientific Instruments Ltd., Eastleigh, U.K.) for 5 min. A few drops of octanol (Sigma) were added to prevent foaming. The homogenate was filtered through 100 μm nylon mesh (John Stanniar and Co., Manchester, U.K.); then the residue was ball-milled (500 mL pot; The Pascall Engineering Co. Ltd., Crawley, U.K.) in cold 0.5% SDS (400 mL) containing 3 mM $Na_2S_2O_5$ for 2 h at 60 rpm at 1 °C to remove the bulk of the intracellular compounds. The homogenate was filtered through 75 mm nylon mesh, and the residue was resuspended 3 times in 1 L of cold distilled water containing 3 mM $Na_2S_2O_5$, and homogenized with a Ystral homogenizer for 5 min. This procedure was repeated until the residue was free of starch as checked by KI/I_2 (Sigma). The cell wall material was further extracted with hot ethanol to remove any alcohol-soluble phenolics, washed 3 times with acetone (Sigma), and then air-dried.

Analysis. Cell wall sugars and uronic acids were determined as described previously (Coimbra *et al.*, 1995). Sugars were released from CWM by dispersing in 72% H_2SO_4 followed by dilution to 1 M and hydrolyzing for 2.5 h at 100 °C. All samples were analyzed in duplicate. Neutral sugars were reduced with $NaBH_4$ and acetylated by the method of Blakeney *et al.* (1983) using 2-deoxyglucose as an internal standard. Alditol acetates were quantified by gas chromatography on a Carlo Erba Vega gas chromatograph after automatic injection by a Carlo Erba A 200 autoinjector. Alditol acetates were

separated with base line resolution on a Restek RT_x 225 WCOT column (15 m × 0.32 mm i.d.; 0.25 μm film) using an oven temperature program of 90 °C for 1 min, 45 °C per minute to 150 °C, 150 °C for 1 min, 2 °C per minute to 210 °C, and 210 °C for 1.5 min. The carrier gas was helium at a column head pressure of 60 kPa. Detection was by flame ionization. Data were collected and integrated on a Spectra Physics SP4400 integrator; reintegration was handled by a Spectraphysics Winner data handling station. Total uronic acid content was determined colorimetrically by the method of Blumenkrantz and Asboe-Hansen (1973) after dispersal in 72% H_2SO_4, dilution to 1 M, and hydrolysis for 1 h at 100 °C.

Wall-bound phenolics were released by sequential alkaline hydrolysis of wall material under progressively more vigorous conditions as described by Hartley and Morrison (1991). Cell wall material (200 mg) was extracted with 0.1 M NaOH (Sigma) 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). *trans*-Cinnamic acid was added to all filtrates as an internal standard, and the solutions were then acidified with concentrated HCl and extracted 3 times with ethyl acetate (3 volumes). The phenolic extracts were evaporated to dryness under a stream of N_2, and the samples were redissolved in 200 μL of 50% (v/v) methanol (Sigma) prior to analysis by HPLC using the methods of Parr *et al.* (1996) and Waldron *et al.* (1996). A Thermo Separation Products (Fremont, CA) UV3000 scanning UV detector was used to give spectral information on the phenolics as they eluted from the column.

Phenolics. Monomeric phenolic standards were obtained from Aldrich Chemical Co., Gillingham, U.K.; the various dehydrodiferulic acid isomers either were synthesized chemically as described by Ralph *et al.* (1994) and Parr *et al.* (1996) or else were kind gifts from Dr. J. Ralph. Vanillin glucoside was isolated from uncured (green) *Vanilla* pods by semipreparative HPLC using methods based on those of Kometani *et al.* (1993).

RESULTS AND DISCUSSION

Purity of Cell Wall Material. Initial experiments investigated alcohol-insoluble residues (AIR's) of carrot. AIR's are often used in the analysis of the carbohydrate composition of plant cell walls since they are relatively quick to prepare. They have been used for analyzing the phenolic components in other plant cell walls such as grapes (Weber et al., 1995). However, our preliminary experiments showed that carrot AIR's contained many impurities possibly arising from precipitated intracellular compounds. Repeatable and meaningful results were only obtained if cell wall material (CWM) was prepared by homogenizing and ball-milling in SDS followed by exhaustive washing. This effectively removed intracellular components, leaving a highly pure water-insoluble CWM. The differences in carbohydrate composition of a typical carrot AIR and CWM are shown in Figure 1. On a Fwt basis, the yield of AIR was approximately 4.5% (this varied), and comprised 79% carbohydrate. In contrast, the yield of CWM was approximately 3%, and comprised 91% carbohydrate. The lower uronic acid level in the CWM reflects the solubilization of poorly-branched water-soluble pectic polysaccharides during the aqueous extraction as discussed in detail elsewhere (Ng and Waldron, 1997). In both AIR and CWM, the low level of Glc released by hydrolysis in 1 M H_2SO_4 is consistent with the lack of starch in the preparations.

Phenolic Composition. Phenolics were released by saponification, acidified and partitioned into organic solvent, and then analyzed by diode-array HPLC (see Materials and Methods). The data shown in Table 1 consist of the means of duplicate analyses; the standard deviations of these were less than 2%, and are not shown. Although it is possible that some cell wall phenolics may have been attached to the water-soluble pectic polysaccharides lost during preparation of the CWM, measurable quantities of phenolics could readily be identified in the CWM, unlike in the AIR where the chromatogram was very confused. As expected, 4-hydroxybenzoic acid was identified as the major wall phenolic, at levels of nearly 500 μg/g of cell wall carbohydrate. The report by Massiot *et al.* (1988) of the occurrence of ferulic acid could also be confirmed, with a significant quantity apparently existing in the *cis* form. More importantly, a large number of additional phenolics were also noted (Figure 2). Among those, it was possible to identify, on the basis of their chromatographic and spectral (UV, MS) properties: vanillic acid, 4-hydroxybenzaldehyde, vanillin, and *p*-coumaric acid (Table 1). The occurrence of 4-hydroxybenzaldehyde and vanillin is interesting in that these compounds were detected in significant amounts even after alkaline hydrolysis with only 0.1 M NaOH for 1 h, a procedure unlikely to cleave the phenolic glycoside linkage which is one way in which these compounds could be linked into the wall. Treatment with even 1 M NaOH for 1 h was found to hydrolyze only approximately 5% of free vanillin glucoside (data not shown). Possibly some of the aldehydes in the cell wall are ester-linked through their phenolic hydroxyl group to the carboxyl groups on

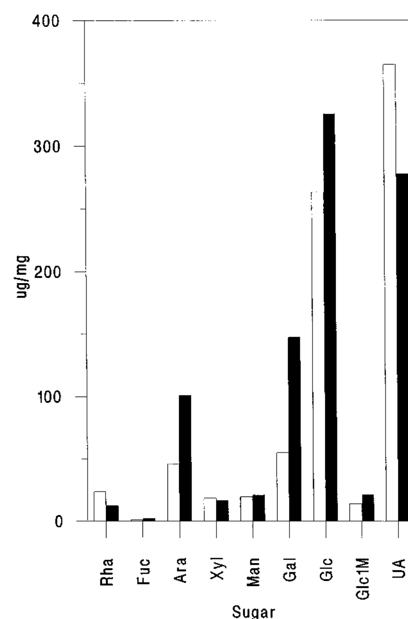


Figure 1. Carbohydrate composition of alcohol-insoluble residue (AIR, white bars) and cell wall material (CWM, black bars) of carrots.

Table 1. Levels of Phenolics Released from Purified Carrot Cell Walls by Sequential Treatments at Room Temperature with Alkali of Increasing Strength

	phenolic released (μg/g of wall carbohydrate)				
	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
4-hydroxybenzoic acid	74	80	238	92	484
vanillic acid	3.5	6.7	5.5	1.2	16.9
4-hydroxybenzaldehyde	0.8	0.4	1.4	0.2	2.8
vanillin	4.1	4.2	7.3	3.4	19
p-coumaric acid	0.1	0.1	0	0	0.2
trans-ferulic acid	1.5	2.1	9.1	6.1	18.8
cis-ferulic acid	1.0	0.8	5.2	3.3	10.3
8-*O*-4′-dehydrodiferulic acid	1.0	1.0	2.8	6.4	11.2
unknown ("peak 11")	0.6[a]	0.5[a]	1.6[a]	2.1[a]	4.8[a]

[a] Assuming response factor equal to that of ferulic acid.

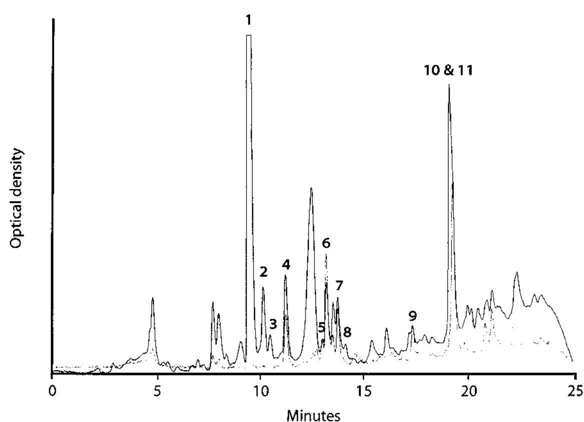
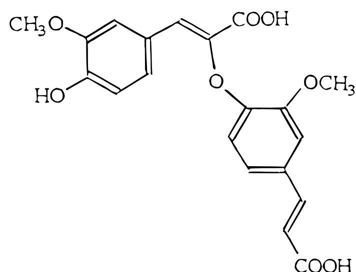


Figure 2. HPLC trace (monitored at 280 nm, boldface line; and at 325 nm, dashed line) of carrot cell wall phenolics released by 0.1 M NaOH at room temperature for 1 h. Peak identification: 1 = 4-hydroxybenzoic acid; 2 = vanillic acid; 3 = 4-hydroxybenzaldehyde; 4 = vanillin; 5 = *trans-p*-coumaric acid; 6 = *trans*-ferulic acid; 7 = *cis*-ferulic acid; 9 = 8-*O*-4′-diferulic acid; 10 = internal standard (*trans*-cinnamic acid).

the uronic acids of pectin (cf. Das *et al.*, 1984). The possibility that some might also arise from alkali-induced degradation of labile phenolic moieties in the wall also cannot be excluded, though the ease of release makes it unlikely that *p*-coumaric and ferulic acids are the phenolics involved. The absence of caffeic acid indicates that contamination of the wall preparations by free cytoplasmic phenolics is minimal, various caffeoyl quinate esters being the major intracellular phenolics of carrots (Babic *et al.*, 1993).

Several peaks with *trans*-ferulic acid-like spectra, but generally later-running than ferulic acid itself, were consistently noted. These did not correspond to isoferulic (3-methoxy-4-hydroxycinnamic acid) or 3,4-dimethoxycinnamic acid (data not shown). A very similar situation is observed in monocots (Parr *et al.*, 1996; Waldron *et al.*, 1996), where it has recently been realized that in addition to the well-known 5,5′-linked diferulic acid dimer (4,4′-dihydroxy-5,5′-dimethoxy-3,3′-bicinnamic acid), a number of other oxidatively dimerized ferulic acids are also present (Ralph *et al.*, 1994; Parr *et al.*, 1996). This suggested that the ferulic acid-like peaks in carrot hydrolysates might represent various diferulic acid isomers or derivatives. Comparison of UV spectra and HPLC retention times with standards of various dehydrodiferulic acid dimers indicated that one of the more important peaks is the oxygen-linked 8-*O*-4′-dehydrodiferulic acid (β-[4-(2-carboxyvinyl)-2-methoxyphenoxy]-4-hydroxy-3-methoxycinnamic acid,



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I). This is the first time that 8-*O*-4′-linked dehydrodiferulic acid has been identified from dicots outside

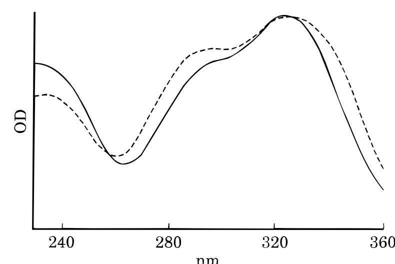

Figure 3. UV spectrum of major unknown phenolic from carrot cell walls (peak 11, dashed line) in relation to that of ferulic acid (solid line).

the order Caryophyllales. Some of the minor peaks also showed similarities in spectrum and retention time to other known dimers, but were present at too low a level to be confident of the identifications. The other major hydroxycinnamic acid-like peak present in chromatograms (peak 11, Figure 2) currently remains unidentified, but on the basis of the spectral similarities (Figure 3), it too may be an oligomeric ferulic acid derivative. Quantitation relative to standards shows that roughly 30% of the ferulic acid present is in the form of the 8-*O*-4′-linked dimer (Table 1). This proportion has been found to rise during maturation of the storage roots in the field, being 20% in immature plants (10-week-old roots) and 35% in fully mature plants (Ng *et al.*, 1998). Given that the true level of ferulic acid in dimer form is likely to be even greater than this, although absolute levels of ferulic acid in the cell wall are relatively low, the cross-linking of carbohydrate chains which results from dimer formation (Van-Huystee and Zheng, 1993; Parker and Waldron, 1995) must have significant structural implications. Interestingly, the 8-*O*-4′-linked dimer has now been shown to be the major dimer in several species (e.g., Ralph *et al.*, 1994; Parr *et al.*, 1996; Waldron *et al.*, 1996), which may indicate that early analyses of both monocots and dicots, which focused solely on the 5,5′-linked dehydrodiferulic acid isomer, are in need of reexamination.

In addition to the ferulic acid-like components already discussed, several other minor unidentified peaks were consistently noted. Given the extensive dimerization of ferulic acid detected, and the relative abundance of C6−C1 phenolics present, it is possible that some of these in the later portion of the chromatogram are benzoic acid-type dimers rather than cinnamic acid-type dimers, and this aspect is currently under investigation. Certainly one of the components (peak 8, Figure 2) has a spectrum closely resembling that of vanillic acid, but showing a bathochromic shift of about 2 nm, suggestive of increased electronic conjugation.

CONCLUSIONS

Numerous cold alkali-releasable phenolic acids and aldehydes have here been detected from carrot cell walls, though in general smaller quantities than found in the cell walls of monocots. The analysis of underivatized phenolics by HPLC, coupled to the spectral information obtained from the use of a scanning or diode array detector, has proved very powerful, and this may explain why a more complete picture has been obtained than by earlier workers. There is also an indication that certain components are quite strongly bound to the cell wall, and are only slowly released by 1 M NaOH, which is the highest concentration of alkali used in several reported studies.

The range of typical wall phenolics present, coupled to the occurrence of a high degree of ferulic acid

dimerization (even if not to the more studied 5,5′-linked isomer), would rather suggest that the rather low attention given to the wall phenolics of carrots may not be totally justified. Preliminary analysis of a number of other dicot leaves and fruit indicates that many other species contain a range of readily measurable phenolic wall constituents, though the balance of components often differs considerably from that of carrots. These results imply that in many cases wall phenolics could well have a significant role to play in determining the mechanical properties of dicot tissues and/or in influencing the biological activity of dicot-derived dietary fiber. The presence of vanillin in carrot cell walls is, for example, of interest in a dietary context since this compound has been shown to stimulate certain DNA repair mechanisms (Sasaki *et al.*, 1990). It should also be noted that certain of the ferulic acid dehydrodimers now being discovered in cell walls (Ralph *et al.*, 1994; Parr *et al.*, 1996) have a lignan-type skeleton. Given the growing understanding of the anticancer and other biological activities of certain lignans (e.g., Middel *et al.*, 1995), this observation may also have some relevance to possible beneficial effects of cell wall-derived phenolics in the diet.

ABBREVIATIONS USED

AIR, alcohol-insoluble residue; CWM, cell wall material; CHO, carbohydrate; Rha, rhamnose; Fuc, fucose; Ara, arabinose; Xyl, xylose; Man, mannose; Gal, galactose; Glc, glucose; Glc1M, glucose released by 1 M acid hydrolysis; UA, uronic acid.

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