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Compositional Analysis of Chinese Water Chestnut (*Eleocharis dulcis*) Cell-Wall Material from Parenchyma, Epidermis, and Subepidermal Tissues

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Supporting Information

ABSTRACT: Chinese water chestnut (*Eleocharis dulcis* (Burman f.) Trin ex Henschel) is a corm consumed globally in Oriental-style cuisine. The corm consists of three main tissues, the epidermis, subepidermis, and parenchyma; the cell walls of which were analyzed for sugar, phenolic, and lignin content. Sugar content, measured by gas chromatography, was higher in the parenchyma cell walls (931 μg/mg) than in the subepidermis (775 μg/mg) or epidermis (685 μg/mg). The alkali-extractable phenolic content, measured by high-performance liquid chromatography, was greater in the epidermal (32.4 μg/mg) and subepidermal cell walls (21.7 μg/mg) than in the cell walls of the parenchyma (12.3 μg/mg). The proportion of diferulic acids was higher in the parenchyma. The Klason lignin content of epidermal and subepidermal cell walls was ∼15%. Methylation analysis of Chinese water chestnut cell-wall polysaccharides identified xyloglucan as the predominant hemicellulose in the parenchyma for the first time, and also a significant pectin component, similar to other nongraminaceous monocots.

KEYWORDS: *cell wall, diferulic acid, Eleocharis dulcis, epidermis, ferulic acid, parenchyma, xyloglucan*

INTRODUCTION

Chinese water chestnuts (*Eleocharis dulcis* (Burman f.) Trin ex Henschel) of the family Cyperaceae are edible corms that grow both naturally and by cultivation in many parts of Asia (Figure 1). The Chinese water chestnut (CWC) is widely known to retain a crisp texture when thermally treated, and this has been related to the presence of ferulic acid dimers.[1−6]

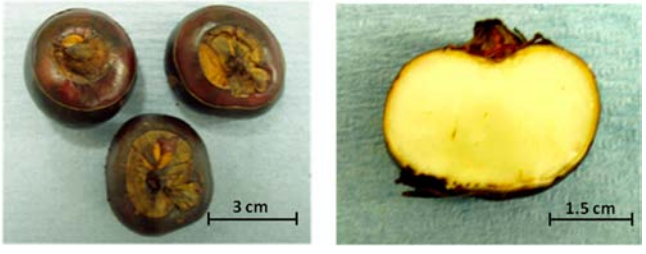

Figure 1. Whole (left) and vertically cut (right) CWC.

CWC plants grow in paddylike ponds and are therefore beset by a wide range of pathogenic organisms, protection from which is provided by a thick (0.75 mm) epidermal layer. Apart from damage due to animal feeding, actively growing CWC appears to be susceptible to only a few pathogens such as rust (*Uromyces* sp.), stem blight (*Cylindrosporium eleocharidis*), and water chestnut wilt caused by a specific *Fusarium oxysporum*. However, they are readily damaged during harvest allowing saprophytic fungi and bacteria access to the inner tissue.[7]

Phenolic acids, such as ferulic acid ((*E*)-3-(4-hydroxy-3-methoxy-phenyl)prop-2-enoic acid) and *p*-coumaric acid ((*E*)-3-(4-hydroxyphenyl)-2-propenoic acid), are found esterified to a range of cell-wall polysaccharides particularly arabinoxylans in many monocotyledonous plants and pectic polysaccharides in a few dicotyledonous species. For example, destarched maize bran and sugar beet pulp can contain as much as 5.5% and 1.0% (w/w) of phenolic acids, respectively.[8] Ferulic acid molecules may be oxidatively coupled through the formation of a radical in the presence of peroxidase and H_2O_2, to form dimers, trimers, and tetramers of ferulic acid.[9]

The detection of the 5,5′-linked ferulic acid dimer (diferulic acid) by Markwalder and Neukom[10] was followed by Ishii et al.[11] isolating a 5,5′-diferulic acid esterified at both ends to cell-wall hemicellulose. Studies involving a horseradish peroxidase (EC 1.11.1.7, type II), H_2O_2, ferulic acid, and coniferyl alcohol in vitro revealed the possibility of additional diferulic acids (DiFA), in particular, 8,5′-DiFA, 8-*O*-4′-DiFA, and 8,8′-DiFA, which were subsequently identified in plants.[12,13] These diferulic bonds have been shown to reduce cell wall extensibility in maturing cells,[14] inhibit digestibility by ruminants,[15] inhibit growth during exposure to light,[16] maintain tissue texture during cooking,[17] limit cell wall degradability in forage grasses,[18] act as nucleation sites for lignin,[19,20] and promote cell−cell adhesion.[21]

Phenolics have been shown to increase in response to mechanical damage in asparagus[22] and pathogenic attack by *F. oxysporum* in date palm.[23] Pathogen resistance is reduced in plants that are compromised in their phenolic supply via suppression of certain genes in the phenyl propanoid

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pathway.[24] Research on the CWC has previously concentrated on cell-wall material made from the edible parenchyma. However, the CWC is surrounded by protective epidermal tissues. Given the important role they play in protecting CWC corms in the aqueous environment in which they grow, this investigation seeks to evaluate the similarities and differences between the inner parenchyma and the epidermal layers.

In this paper, we report and compare the compositions of the edible parenchyma, the outer dark brown epidermal tissues, and the layer of intermediary tissue, containing pale brown vascular tissue and some parenchyma (subepidermis). The use of methylation analysis on the cell-wall polysaccharides provides a novel insight into the polysaccharide composition of CWC parenchyma, highlighting the presence of xyloglucan and pectic polysaccharides.

MATERIALS AND METHODS

Materials. Fresh CWCs were obtained from Golden Gate Grocers (16 Newport Place, London, U.K.); they had been vacuum-packed at source and refrigerated at 4 °C during transport and presale storage. The top and base of each corm were excised, the main body of the corm was peeled with a knife, and then, the parenchyma was chopped into pieces (1 × 1 × 1 cm^3). The peel and parenchyma pieces were frozen separately in liquid N_2 and stored at −20 °C. All reagents were of analytical grade, unless otherwise specified.

Methods have been described in detail by Grassby,[25] so brief descriptions are given below. Samples were analyzed in triplicate, unless otherwise stated.

Microscopy. Transverse sections of the epidermis from fresh CWC were cut by hand and photographed unstained under visible light and under UV light at pH 9.6 (20 mM NH_4OH) using an Olympus BX60 (Olympus, Tokyo, Japan) microscope with Acquis software (Syncroscopy, Cambridge, U.K.). The autofluorescence in unstained sections was recorded using the UV filter cube (U-MWU, exciter filter BP330-385, barrier filter BA420) of the microscope.

Preparation of Parenchyma-Derived Cell-Wall Material. Cell-wall material (CWM) was prepared from frozen CWC parenchyma (∼100 g) as per Parker et al.[26] After ball-milling once for 1 h (180 mL of 0.5% SDS, 60 rpm, 4 °C), the cell-wall suspension was filtered through 100 μm mesh, washing with deionized (DI) water. Trapped starch granules were released by homogenizing for 3 min with an Ystral homogenizer (Ystral GmbH, Ballrechten-Dottingen, Germany). The mixture was filtered and washed with DI water (∼6 L) until no starch was visible under the microscope (staining with I_2/KI solution). The residue was redispersed in ∼70 mL of DI water and frozen (−20 °C).

Preparation of Epidermal CWM. A variation on the above method was also used to produce CWM of the epidermal and subepidermal tissues from the frozen peel (∼100 g) (Figure 2). After ball-milling, the cell-wall suspension was separated by differential rates of sedimentation and decanting into multiple fractions. The densest fraction predominantly consisted of the dark-brown epidermal CWM (ECWM); the second densest fraction contained the lighter-brown subepidermal CWM (SECWM), consisting of vascular and parenchyma cell walls; the remaining fractions containing mostly PCWM and starch were discarded. Starch was removed from the two preparations by homogenizing as above and filtering through a 70 μm nylon mesh, washing with DI water (2−8 L) as required. The preparations were redispersed in DI water and frozen (−20 °C).

Dry CWM material from all tissue types was recovered from the CWM suspensions by filtering (70 μm nylon mesh) and washing 3 times in absolute ethanol (∼300 mL) and finally acetone (150 mL). The CWM was recovered by filtering. Any clumps in the air-dried CWM were removed by milling briefly in a dry mill (Janke & Kunkel, Staufen, Germany).

Neutral Sugar Analysis. Samples of CWM (2−4 mg) were dispersed in 72% (w/w) H_2SO_4 (200 μL) for 3 h at room temperature (Saeman hydrolysis), followed by dilution with DI water (2.2 mL) and hydrolysis at 100 °C for 2.5 h. One hour into the hydrolysis, 0.5 mL was removed for the uronic acid determination (see below). After cooling, 2-deoxyglucose (200 μL, 1 mg/mL) was added as an internal standard. A 1 mL aliquot of each sample was neutralized with $NaBH_4$ and acetylated by the method of Blakeney et al.[27] The samples were then dissolved in acetone (1 mL) and analyzed by gas chromatography (GC) against internal and external standards.[28] To determine the noncellulosic sugars, samples of CWM (∼5 mg) from all tissue types were dispersed in 2.4 mL of 1 M H_2SO_4 and incubated at 100 °C for 2.5 h and then treated as above.

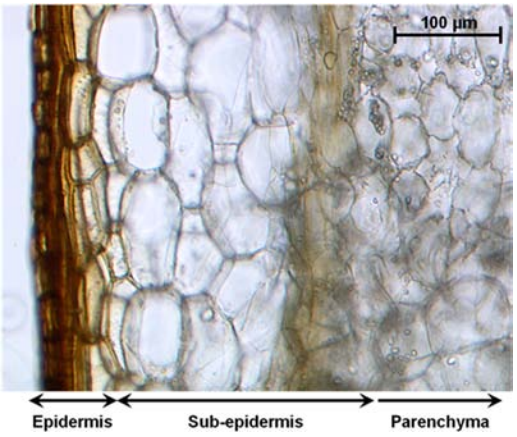

Figure 2. Micrograph of longitudinal transverse section of CWC showing, from left to right, the epidermis, subepidermis, and parenchyma tissues.

Uronic Acid Analysis. Uronic acids were measured colorimetrically, as glucuronic acid equivalents, using an adaptation of the method by Blumenkrantz and Asboe-Hansen.[29] Each hydrolyzate aliquot (see above) was diluted with DI water (2 mL); sulfuric acid reagent (1.2 mL, 25 mM $Na_2B_4O_7{\cdot}10H_2O$ in 96% H_2SO_4) was added to four replicates (0.2 mL) of the diluted samples. The samples were heated for 10 min at 100 °C, before adding 0.15% *m*-phenyl phenol in 0.5% (w/v) NaOH (20 μL) to three replicates; the fourth replicate (background control) had 0.5% (w/v) NaOH (20 μL) added. Absorbance was measured at 490 nm on a microplate reader (Molecular Devices, Sunnyvale, CA).

Polysaccharide Linkage Analysis. Polysaccharide linkage analysis was carried out in duplicate and consisted of the following: methylation using lithium dimsyl reagent as the catalyst;[30,31] carboxyl reduction of one replicate using lithium triethylborodeuteride; hydrolysis of the glycosidic bonds by 2 M trifluoroacetic acid; and acetylation with 1-methylimidazole and acetic anhydride. Before methylation, the samples were cryomilled and dispersed in dry DMSO (1 mL), under argon, by heating at 90 °C for 1 h and then sonicating at 20 °C for 2 h. An acetan polysaccharide from *Acetobacter xylinum*, with a defined structure, was used as an external standard.[32] It went through the same procedure as the samples. Partially methylated alditol acetates were prepared from methyl glycosides to act as additional external standards. Analysis was by GC and gas chromatography−mass spectrometry (GC-MS).[32] The partially methylated alditol acetates (PMAAs) were identified by their mass spectra and their calculated RRTs (to *myo*-inositol or 1,4-Glc) and were quantified using the peak areas obtained on the GC and the response factors of Sweet et al.[33] As the peaks sometimes coeluted (1, 4-Xyl, 1,2-Xyl, and terminal Gal (t-Gal) coeluted, and 1,3,4-Xyl and 1,4-Gal coeluted), the proportion of each linkage was estimated from the relative intensities of appropriate mass spectra. To make comparisons with the sugar compositions derived from 72% (w/w) H_2SO_4 hydrolysis (Table 1), which were quantitatively more accurate than the methylation analysis results, the mole percent recorded for each linkage in the methylation analysis (M) was corrected to reflect the mole percent for that sugar in the total 72% H_2SO_4 results (T) using the formula below. The noncarboxyl-reduced values were used

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Table 1. Monosaccharide Results for CWM from All Three Tissues Compared with Those of Parr et al.[4a]

	PCWM[b]		SECWM[c]		ECWM[d]		PCWM (Parr et al.[4])
sugar	μg/mg	mol %	μg/mg	mol %	μg/mg	mol %	mol %
total sugars[e]							
rhamnose[f]	5.2 (2.2)	0.59	3.3 (0.7)	0.44	5.6 (2.2)	0.83	0.30
fucose[f]	3.4 (0.4)	0.39	2.5 (0.1)	0.34	1.6 (0.5)	0.24	0.53
arabinose[f]	94.2 (2.8)	11.78	96.0 (1.3)	14.18	76.6 (4.0)	12.57	11.79
xylose[f]	157.9 (7.6)	19.74	172.0 (3.4)	25.41	220.4 (4.8)	36.17	27.51
mannose[f]	4.7 (1.4)	0.58	3.9 (0.2)	0.58	3.1 (0.0)	0.51	0.51
galactose[f]	41.4 (2.8)	4.22	33.3 (0.3)	4.00	14.1 (0.4)	1.89	4.81
glucose[f]	501.1 (7.5)	51.07	376.3 (19.1)	45.27	285.4 (1.7)	38.16	48.25
uronic acids[g]	123.8 (2.2)	11.62	88.4 (5.5)	9.79	78.2 (1.3)	9.63	6.31
noncellulosic sugars[h]							
rhamnose[f]	3.1 (0.2)	0.92	3.1 (0.9)	0.83	2.2 (0.2)	0.63	NR
fucose[f]	4.0 (0.3)	1.20	3.7 (0.9)	1.00	1.9 (0.2)	0.55	NR
arabinose[f]	93.6 (1.3)	30.83	104.6 (20.3)	31.77	77.4 (2.7)	24.12	NR
xylose[f]	147.5 (3.7)	48.59	167.0 (37.7)	50.51	214.1 (2.6)	66.72	NR
mannose[f]	1.8 (0.1)	0.60	2.0 (0.4)	0.62	1.6 (0.1)	0.50	NR
galactose[f]	38.2 (0.2)	10.27	36.4 (9.1)	8.96	13.4 (0.3)	3.39	NR
glucose[f]	28.3 (1.4)	7.59	25.7 (8.1)	6.30	16.1 (1.8)	4.09	NR
uronic acids[g]	ND	ND	ND	ND	ND	ND	NR

[a]Standard deviations (in μg/mg) are given in parentheses. ND indicates values were not determined. NR indicates values were not recorded. [b]Parenchyma CWM. [c]Subepidermis CWM. [d]Epidermis CWM. [e]Total sugars extracted in 72% H_2SO_4 initially, values include uronic acids in mol % calculations. [f]Neutral sugars. [g]Acidic sugars. [h]Noncellulosic sugars extracted in 1 M H_2SO_4 initially, values do not include uronic acids in mol % calculations.

for the neutral sugars, and the carboxyl-reduced values were used for the GalA linkages (Table 3, corrected values).

$$\text{corrected linkage mol \%} = \frac{M \times T}{\text{total sugar linkages mol \%}}$$

Extraction of Total Phenolics in Alkali. Phenolics were extracted from CWM (~5 mg) with NaOH (1 mL, 4 M, deoxygenated, N_2-flushed), mixing continuously in darkness for 24 h. The samples were centrifuged (130*g*, 15 min), and the supernatant (0.6 mL) and internal standard (*trans*-cinnamic acid, 50 μL, 0.2 mg/mL 50:50 v/v MeOH/water) were mixed, before acidifying with conc. HCl and extracting with ethyl acetate (3 × 3 mL). The ethyl acetate extracts were dried under N_2 at 40 °C, dissolved in MeOH/water (50:50 v/v; 1 mL), and analyzed by HPLC-DAD using a solvent gradient adapted from Waldron et al.[34]

Sequential Alkali Extraction of Phenolics. Triplicate samples of CWM (~10 mg) were dispersed in a series of NaOH solutions (4 mL, deoxygenated, N_2-flushed) as follows: (i) 0.1 M, 1 h; (ii) 0.1 M, 24 h; (iii) 1 M, 24 h; (iv) 2 M, 24 h; and (v) 4 M, 24 h. They were centrifuged (130*g*, 15 min) to recover the solids, removing the supernatants from each stage using a fine-tipped Gilson pipet. The phenolic content of the supernatants was measured as above.

KlasonLignin Analysis of CWM. Duplicate samples of CWM (~50 mg) were analyzed for lignin content using the method of Merali et al.[24]

RESULTS AND DISCUSSION

CWC Morphology and Autofluorescence. CWC parenchyma had been shown previously to autofluoresce under alkali conditions, but these investigations did not include the epidermal tissues.[3] Figure 3a shows the morphology and natural pigmentation of the CWC epidermal layers. The cells show a gradual transition from parenchyma (circular cells, ⌀ 80−100 μm, thin colorless cell walls) to epidermis (angular cells, width 5 × 20 μm^2, thick brown cell walls). Figure 3b shows the autofluorescence of the epidermis at pH 9.6; the outer cortex cell walls are turquoise indicating the presence of ferulic acid,[35] and the vascular bundles that serve the buds and scales on the surface in the epidermis are blue indicating the presence of lignin. The autofluorescence appears to taper out toward the surface; this is not due to a lack of phenolics, as the total and sequential extractions of phenolics showed the epidermis contained more than the parenchyma (see below). The lack of fluorescence could be due to a high concentration of pigmented compounds, and perhaps suberin, preventing the incident light from reaching the phenolics, or by blocking/quenching the emission of the fluorescent signal.

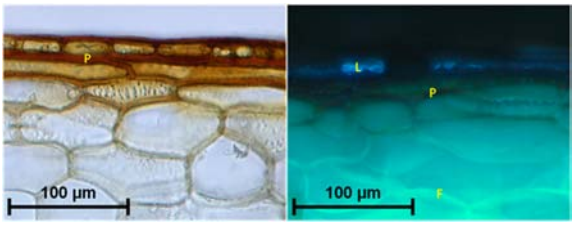

Figure 3. Micrographs of longitudinal transverse sections of CWC illuminated by (a) visible light and (b) UV light at pH 9.6. Thickening and increased pigmentation of the cell walls is observed as the cells transition from parenchyma to epidermal tissues. The bright blue autofluorescence present under UV illumination at neutral (not shown) and alkali pH is suggestive of lignification of the cell walls (L). The green autofluorescence intensified on addition of alkali, indicating the presence of ferulic acid derivatives (F). Lack of green autofluorescence in epidermal cell walls is probably due to interference from the wall pigmentation (P).

Yields of CWM. The yield of PCWM was 5.9 g/kg frozen tissue. The yields of SECWM and ECWM were 17.4 and 8.3 g/kg of frozen peel, respectively. Higher yields of SECWM and ECWM than PCWM would be expected due to the thicker cell walls and smaller cell lumen observed by microscopy. The subepidermis tissues consist primarily of vascular bundles that serve the buds and scales on the surface. The thicker walls are necessary as the vascular bundles are under significantly higher osmotic pressure than the parenchyma cells.

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Table 2. Comparison of the Phenolic and Diferulic Acid Compositions in CWC Tissues[a]

	PCWM[b]		SECWM[c]		ECWM[d]	
	μg/mg	wt %	μg/mg	wt %	μg/mg	wt %
ferulic acids[e]	4.87	39.63	9.79	45.05	10.96	33.85
diferulic acids	6.84	55.68	8.06	37.11	5.44	16.81
p-coumaric acids	0.33	2.67	3.38	15.54	12.85	39.71
other phenolics[f]	0.25	2.02	0.50	2.31	3.12	9.63
total known phenolics	12.29		21.73		32.37	
total unknown phenolics[g]	0.80	6.50	1.56	7.16	3.57	11.04
8,8′-DiFA(AT)	1.03 (0.11)	15.01	0.98 (0.05)	12.21	1.24 (0.24)	22.77
8,8′-DiFA	0.00 (0.00)	0.00	0.05 (0.04)	0.62	0.00 (0.00)	0.00
8,5′-DiFA	0.97 (0.10)	14.22	1.23 (0.12)	15.25	1.31 (0.86)	24.08
5,5′-DiFA	0.84 (0.12)	12.31	1.15 (0.19)	14.22	0.28 (0.13)	5.13
8-*O*-4′-DiFA	2.92 (0.07)	42.72	3.64 (0.30)	45.20	2.25 (0.04)	41.37
8,5′-DiFA(BF)	1.08 (0.08)	15.73	1.01 (0.09)	12.50	0.36 (0.01)	6.65

[a]Standard deviations (in μg/mg) are given in parentheses. [b]Parenchyma CWM. [c]Subepidermis CWM. [d]Epidermis CWM. [e]Ferulic acids includes *t*-ferulic acid and *c*-ferulic acid only. [f]Other phenolics includes *p*-hydroxy benzoic acid, vanillic acid, *p*-hydroxy benzaldehyde, and vanillin. [g]Total wt % of unknown phenolics calculated relative to the total known phenolics; assumes a response factor of 0.2, and a molecular weight of 150 or 386.

Neutral Sugars Are Not Generally Associated with the Cellulose Microfibrils. The PCWM, SECWM, and ECWM, respectively, contained 807, 687, and 607 μg/mg CWM of neutral sugars and 124, 88, and 78 μg/mg CWM of uronic acids. These values are in keeping with previous results for PCWM composition,[4] which are detailed in Table 1. Minor differences between the two analyses probably reflect differences in variety, origin, and harvest year. There is a higher yield of uronic acids in the PCWM, and the overall proportion of pectic polysaccharides in the parenchyma cell walls is slightly higher than in those of the epidermal tissues. The yield of neutral sugars, measured by 1 M H_2SO_4 hydrolysis, from PCWM (317 μg/mg), SECWM (343 μg/mg), and ECWM (327 μg/mg) gave an indication of their noncellulosic polysaccharide content. In all CWMs, nearly 95% of the glucose remained unhydrolyzed after 1 M H_2SO_4 treatment. This suggests the majority of the glucose in all three tissues was cellulosic in origin or closely associated with the cellulose microfibrils. In the PCWM, this may be xyloglucan, as some of the xylose was also not extracted by the 1 M H_2SO_4 treatment. The other sugars were extracted in similar amounts as in the concentrated H_2SO_4, indicating they did not originate in crystalline structures but rather in amorphous hemicelluloses and pectins. The cell walls of chufa (*Cyperus esculentus*) have a broadly similar composition to those of CWC, but the chufa parenchyma cell wall has higher amounts of arabinose and lower amounts of glucose than the CWC PCWM.[26]

Ferulic Acid Species and Other Phenolics Extracted from CWM by 4 M NaOH. Diferulic acids were identified by retention time and absorbance spectrum and quantified using the response factors (R_f) published by Waldron et al.[34] The other phenolics were quantified against authentic standards. There was a marked difference between the amounts and compositions of phenolics extracted from parenchyma and epidermal cell wall preparations (Table 2). The ECWM contained the highest levels of phenolic acids (32.4 μg/mg CWM as compared with 21.7 μg/mg in SECWM and 12.3 μg/mg in PCWM). In addition, the phenolics were predominantly monomeric ferulic acid and *p*-coumaric acid. Indeed, the ECWM had a much greater proportion of *p*-coumaric acid than the other cell-wall preparations (Table 2) that probably arises from esterification to the lignin component (see below). Nevertheless, a significant quantity of diferulic acids could be detected. In contrast, phenolics from the PCWM were mostly ferulate-based, with ferulate dimers predominating, reflecting a relatively high degree of cross-linking of the cell-wall polysaccharides. The results for PCWM concur with those published previously for CWC.[4] In general, the composition of SECWM was intermediate between the other two tissues, both in yield of total phenolics and the proportions of diferulic acids and *p*-coumaric acids, although it did have the highest relative amount of ferulic acids. The 8,8′-DiFA (AT), the DiFA thought to be responsible for cell wall adhesion in CWC,[6] was identified in the PCWM and SECWM chromatograms by the distinctive shape of its absorbance spectrum, but the peak at the equivalent relative retention time (RRT) in the ECWM chromatogram had a slightly different spectrum and therefore was not included in the results as 8,8′-DiFA (AT). In addition, a number of compounds not described by Waldron et al.[34] were found in the extracts. At least some of these are expected to be higher oligomers of ferulic acid, as their UV spectra are similar to those of the diferulic acids and triferulic acids described by Dobberstein et al.[36] For comparative evaluation, the R_f of all these unknowns was chosen to be 0.2, the average R_f for the ferulic and diferulic acids. Although this is a rough approximation, these additional compounds may make up almost 10% of the phenolic content of the CWM; these warrant further investigation, as even small amounts may have significant physiological effects if they are concentrated at cell junctions.[3]

Klason Lignin Content Is Highest in ECWM. The yield of Klason lignin was 2.4%, 13.4%, and 16.7% for the PCWM, SECWM, and ECWM, respectively. The predominance of parenchyma cells and the small amount of vascular tissue would account for the low yield in the PCWM, and the greater amount of vascular tissue in the SECWM and secondary cell walls in the ECWM would account for their higher values. These results are quite similar to those of chufa, another member of the Cyperaceae, where the Klason lignin for PCWM and ECWM was less than 2% and greater than 20%, respectively.[26] If ferulic acid is esterified/etherified to lignin, the presence of lignin in the epidermal and subepidermal tissues may have reduced the ability for alkali extraction to remove all the phenolics from the CWM.[37,38]

Overall Composition of CWMs. The compositions of the tissues of CWC corms are summarized in Table 3 and the

Table 3. Overall Composition of CWMs in Weight Percent

	PCWM[a] wt %	SECWM[b] wt %	ECWM[c] wt %
cellulose[d]	47.3	35.1	26.9
neutral sugars[e]	31.6	34.3	32.7
uronic acids[f]	12.4	8.8	7.8
phenolics	1.2	2.2	3.2
Klason lignin	2.4	13.4	16.7
unidentified components[g]	5.1	6.2	12.7

[a]Parenchyma CWM. [b]Subepidermal CWM. [c]Epidermal CWM. [d]Cellulose defined as glucose not hydrolyzed by 1 M H_2SO_4. [e]Neutral sugars detected after hydrolysis by 1 M H_2SO_4. [f]Uronic acids detected after hydrolysis by 72% H_2SO_4. [g]Unidentified components are likely to be protein and ash that have not been quantified in this study.

Table of Contents graphic. The parenchyma cell wall is almost completely accounted for (within experimental error). The protein and ash contents were not quantified, and this may account for the higher proportion of unidentified components in the epidermal and subepidermal CWM, although it may also be due to these materials being less finely divided than the PCWM, reducing their accessibility for hydrolysis. The differences in cell-wall compositions of the three tissues of the CWC corms reflect their physiological roles. As CWC epidermis has such high levels of phenolics, *p*-coumaric acid in particular, there may be an important physiological role for them in this tissue. Lattanzio et al. have shown that the presence of high amounts of ferulic acid (and other phenolics) may inhibit the growth of the fungus *F. oxysporum*, one of the known pathogens of CWC.[39] Mandal and Mitra[40] have shown that tomato (*Solanum lycopersicum*) roots produce phenolic acids, particularly ferulic acid, in response to *F. oxysporum* in the environment, and this could potentially be the cause of the high levels detected in CWC epidermis. The increased levels of *p*-coumaric acid in the epidermis tissues in tandem with the higher lignin content agrees with the findings of Ralph et al. that *p*-coumaric acid is often associated with lignin (via ester bonds) in maize internodes.[41] Ferulic acid, in monomeric and dimeric form, is associated with the poorly lignified parenchyma tissue.

Stability of Phenolic–Polysaccharide Ester Linkages in Alkali. Figure 4 shows a subset of the results from the sequential phenolic extractions for the PCWM, ECWM, and SECWM; a complete set of results can be found in the Supporting Information, Table S1. The total phenolics extracted by the sequential phenolic extraction were similar to those for total phenolic extraction for the SECWM (22.3 μg/mg) and ECWM (30.2 μg/mg) (see above), but the value for PCWM (14.5 μg/mg) was a little higher. As found with the total phenolic extraction, the ECWM chromatograms had a peak with an RRT corresponding to 8,8′-DiFA (AT) but with a slightly different spectrum shape, so it has not been presented as 8,8′-DiFA (AT). The overall trends highlighted for total phenolics were reflected in the sequentially extracted phenolics. However, the extraction profiles differed between the different tissues. In the PCWM, the bulk of the phenolics were extracted by 0.1 M NaOH for 1 h and then 24 h. In contrast, in the ECWM and SECWM, harsher saponification conditions were required to release the phenolics, with significant quantities of ferulic acid and *p*-coumaric acid being released by 1 M NaOH, perhaps due to a close association with lignin, as seen with chufa (*C. esculentus*).[26] Parker et al showed that CWC tissue strength (cell-wall adhesion) reduces at the same point in a

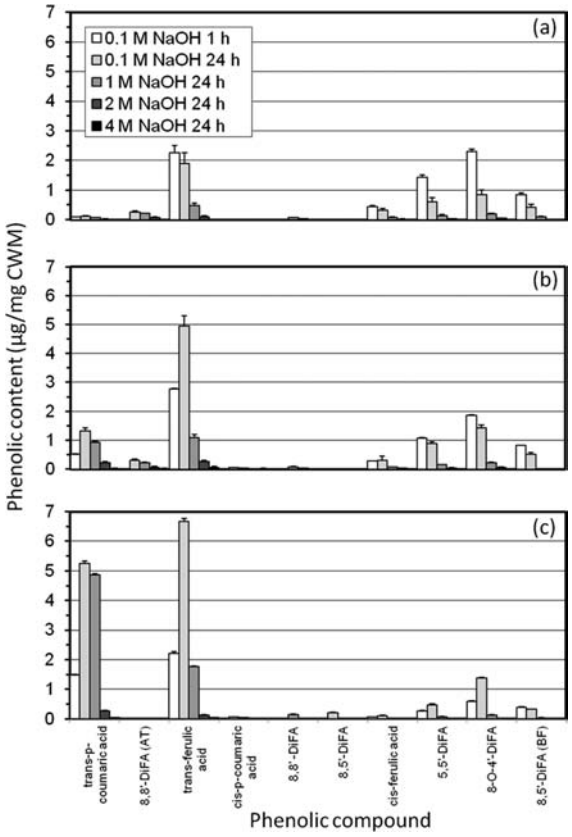

Figure 4. Results of sequential extraction of phenolics in alkali. (a) PCWM, (b) SECWM, and (c) ECWM; the five columns show from left to right the amounts extracted at the following stages (i) 0.1 M NaOH, 1 h; (ii) 0.1 M NaOH, 24 h, (iii) 1 M NaOH, 24 h, (iv) 2 M NaOH, 24 h; and (v) 4 M NaOH, 24 h in the phenolic sequential extraction. Errors are standard deviations from three determinations.

sequential alkali extraction procedure as the 8,8′-DiFA (AT) is extracted from the wall.[6] Recordable amounts of 8,8′-DiFA (AT) were measured in the 0.1 M NaOH 24 h (0.26 μg/mg), 1 M NaOH 24 h (0.21 μg/mg), and 2 M NaOH 24 h (0.07 μg/mg) fractions in the PCWM. In the SECWM, the equivalent values were 0.31, 0.23, and 0.06 μg/mg, indicating that, in accordance with the previous study, much of the structural effect of 8,8′-DiFA (AT) is lost after extraction in 1 M NaOH. A diarabinosyl-8,8′-DiFA (AT) has been isolated from maize bran, indicating that the 8,8′-DiFA (AT) can form links between polysaccharide chains.[42] The small amounts of 8,8′-DiFA were not released until the 1 and 2 M NaOH extractions in all CWMs, and this may indicate a minor role for 8,8′-DiFA in cell adhesion. For the other diferulic acids, at least 90% of the total was removed by the 0.1 M NaOH extractions, suggesting they are not important in cell adhesion. Other monomeric phenolics were also released, particularly from ECWM (see the Supporting Information). As some were released by the higher concentrations of alkali, they are presumably esterified into the cell wall, via their alcohol groups, as suggested by Weber et al.[43]

Sugar Linkages Indicate Xyloglucan Is a Major Polysaccharide in PCWM and SECWM. The composition of monosaccharide linkages from the polysaccharides of PCWM are given in Table 4. The proportion of each polysaccharide was estimated following a similar procedure to that used by Smith and Harris.[44] These are presented in Table 5. In the case of PCWM, cellulose was the most abundant wall

Table 4. Methylation Analysis Data, both Raw and Corrected Relative to Total Sugars Analysis

		PCWM[a]			SECWM[b]		
sugar	linkage	lithium dimsyl (mol %)	lithium dimsyl with carboxyl reduction (mol %)	corrected value (mol %)[c]	lithium dimsyl (mol %)	lithium dimsyl with carboxyl reduction (mol %)	corrected value (mol %)[c]
Rha	all[d]			0.59			0.44
Fuc	t-	0.41		0.39	0.36		0.34
Ara-f	t-	0.49	2.87	3.52	4.57	5.72	9.63
Ara-f	(1−3)				0.82	8.62	1.73
Ara-f	(1−5)	1.15	2.44	8.26			
Ara-p	t-				0.15		0.32
Ara-p	(1−4)				1.19	4.56	2.51
Xyl	t-	5.96	5.18	8.01	5.80	4.10	4.78
Xyl	(1−4)	3.34	3.22	4.49	18.48	32.94	15.23
Xyl	(1−2)	1.60	1.48	2.15			
Xyl	(1−3,4)	3.78	5.32	5.08	4.99	14.12	4.11
Xyl	(1−2,4)				0.95	2.11	0.78
Xyl	unmeth[e]				0.61	3.57	0.50
Man	(1−4)	1.23	0.66	0.58	1.37		0.58
Gal	t-	1.51	1.90	2.22	0.36	1.63	1.62
Gal	(1−6)				0.22		0.99
Gal	(1−4)	1.01	0.69	1.49			
Gal	(1−2)	0.35		0.51			
Gal	(1−4,6)				0.11		0.49
Gal	(1−3,6)				0.2		0.90
Gal	unmeth[e]						
Gal(A)	(1−4)		5.39	10.2		5.28	9.79
Gal(A)	(1−2,4)		0.75	1.42			
Glc	t-				0.59		0.45
Glc	(1−4)	55.67	52.50	35.91	49.60	14.11	37.54
Glc	(1−4,6)	12.13	12.34	7.82	6.89	3.22	5.21
Glc	(1−2,4)				1.10		0.83
Glc	(1−3,4)	7.96	3.36	5.13			
Glc	(1−3,4,6)	2.08	0.74	1.34	0.80		0.61
Glc	(1−2,4,6)	0.80	0.75	0.52	0.41		0.31
Glc	unmeth[e]	0.53	0.42	0.34	0.43		0.33
		100	100	100	100	100	100

[a]Parenchyma CWM. [b]Subepidermal CWM. [c]Corrected as per equation given in text. [d]Rhamnose was detected in small amounts, see Table 1, but methylated derivatives were not detected. [e]Unmeth stands for unmethylated.

Table 5. Proposed Polysaccharide Composition for PCWM and SECWM

	PCWM[a]		SECWM[b]	
polysaccharide	mol %	degree of branching (%)	mol %	degree of branching (%)
cellulose[c]	35.1	–	34.5	–
xyloglucan[d]	19.0	50	11.2	50
arabinoxylan	11.5	44	25.0	24
RG I or RG I/HG[e]	20.5		10.2	–
AG II	–	–	4.1	34
mannans[c]	0.6	–	0.6	–
total accounted for[f]	86.7		85.6	

[a]Parenchyma CWM. [b]Subepidermal CWM. [c]Assumed to be unbranched. [d]Xyloglucan is calculated on the basis that the substitution pattern is XXFG and/or XXXG.[45,49] [e]Assumed that branches are present, but linkages not identified in linkage analysis (Table 4). [f]Some linkages are not accounted for as they were not assigned to a polysaccharide for the calculations; some linkages did not have their whole mol % used in the calculations.

polymer as inferred from the sugars analysis above and the high proportion of 1,4-Glc. However, there was also a considerable quantity of 1,4,6-Glc (7.8 mol %). This, in conjunction with significant quantities of t-Xyl (7.4 mol %) and the presence of t-Fuc (0.4 mol %), 1,2-Gal (0.4 mol %), and 1,2-Xyl (0.4 mol %) indicates that 19 mol % of the cell-wall polysaccharide is composed of xyloglucan, when applying a similar pattern of xyloglucan substitution as in *Cyperus papyrus*, another Cyperaceae.[49] Nevertheless, the levels of t-Ara (3.5 mol %), 1,4-Xyl (4.5 mol %), and 1,3,4-Xyl (3.5 mol %) imply arabinoxylan (~11.5 mol %) is also present; the high degree of arabinose substitution (44%) is in keeping with the cell-wall ferulic acid complement and the role of arabinoxylan in cell adhesion as determined previously.[3,6,26] However, no glucuronic acid could be detected, and the uronic acid complement was found to be mainly pectic in origin. The proportions of GalA, Rha, Ara, and Gal suggest two possible structural configurations. The first assumes that the polysaccharide is rhamnogalacturonan I (RG I) with significantly more GalA residues than Rha residues in the backbone and long, predominantly Ara and Gal side chains. Alternatively, there may be sections of the polysaccharide that are predominantly homogalacturonan (HG) interspersed with regions of RG I.

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The sum of Rha (0.6 mol %), 1,4-GalA (10.2 mol %), 1,5-Ara (8.3 mol %), and 1,4-Gal (1.5 mol %) indicates ~20.5 mol % of RG I/HG.

In addition, there are small quantities of mannans (~0.6 mol %) that may be inferred from the 1,4-Man residues detected, although it is difficult to determine whether they are glucomannans, galactomannans, or galactoglucomannans from the information available. The small quantities of 1,3,4-Glc, 1,3,4,6-Glc, 1,2,4,6-Glc, and unmethylated Glc residues were probably generated by undermethylation, perhaps due to steric crowding within the cellulose microfibrils.

Interestingly, the abundance of hemicellulosic xyloglucans and GalA-rich pectic polysaccharides in the PCWM, as determined by the methylation analysis, suggests that the cell walls of CWC have a similar composition to the cell walls of the fleshy stems of immature, edible asparagus, which is a noncommelinid monocot.[46,47]

The polysaccharide composition for the SECWM was similarly inferred from the linkage analysis data (Table 3). SECWM has twice the amount of arabinoxylan, with some 1,2,4-Xyl, and much less xyloglucan than the PCWM. The uronic acid linkage detected was 1,4-GalA (9.8 mol %); the absence of 1,5-Ara and 1,4-Gal implies HG is the principal pectin present. The 1,6-Gal, 1,4,6-Gal, and 1,3,6-Gal and 1,3-Ara could form ~4.1 mol % of arabinogalactan II (AG II), which may indicate the presence of arabinogalactan proteins. Solubility in DMSO was an issue with the ECWM samples (possibly due to the lignin content), even though they had been cryo-milled to a fine powder, this resulted in severe undermethylation of the glucose residues, the results of which are not shown.

This investigation has shown that xyloglucan is the predominant hemicellulose in CWC parenchyma, with significant levels of arabinoxylan and a small amount of mannan. The significant pectin component is rhamnogalacturonan I, probably with some homogalacturonan domains distributed throughout the chain. Rhamnogalacturonan II is found in most cell walls, making it likely that it is present in small amounts in CWC cell wall, although it was not detected in PCWM. The hemicelluloses in SECWM are generally similar to those of the PCWM, although arabinoxylan is the predominant hemicellulose in SECWM. A similar investigation of cell-wall polysaccharides was carried out on pineapple (*Ananas comosus*) by Smith and Harris; their results for pineapple flesh were similar to CWC SECWM, although they endeavored to circumvent the vascular bundles.[44] The sugar compositions of the PCWM and ECWM are similar to those found in the same tissues of another Cyperaceae, chufa (*C. esculentus*).[26] The amount of xyloglucan in CWC PCWM is particularly high for a monocot and would be considered quite high for a dicot.[48] By using the system of Fry et al,[49] the repeating units of CWC xyloglucan (as calculated) would be XXXG with some XXFG, which is a pattern found in other members of the Cyperaceae, such as papyrus (*C. papyrus*).[45] Perhaps the high level of xyloglucan in the cell wall indicates that it is being used as storage carbohydrate, as it is in nasturtium seeds and tamarind.[50] The presence of only 1,3,4-Xyl branching points in the PCWM arabinoxylan implies it is also like those of the Poaceae (i.e., Oat spelt). The SECWM arabinoxylan had both 1,2,4-Xyl and 1,3,4-Xyl. McNeil et al used in vitro binding experiments to show that the ratio of arabinose to xylose in barley arabinoxylans that bind to cellulose is 0.44 (22% branching), in those that do not bind to cellulose the ratio is 1.38 (65% branching, half of which are doubly branched).[51] In arabinoxylan from CWC parenchyma, the ratio of arabinose to xylose is 0.44 (44% branching), making it ideal for binding to cellulose. Highly substituted xylans are more likely to be sufficiently flexible to support intramolecular formation of ferulic dimers.[52] If the degree of substitution of PCWM arabinoxylan was assumed to be uniform, it would seem to have an intermediate degree of substitution that may hinder the formation of dimers within the cell wall. However, as ferulic acid has been shown to be localized at the edges of cell wall faces,[3] there may also be some localization in the substitution patterns of the arabinoxylan, making the formation of ferulic acid dimers more likely. The same study suggested that the ferulic and diferulic acids are linked to the arabinoxylans;[3] however, there may be a proportion linked to the xyloglucans.

The dimers are probably involved in interpolymeric cross-linking of arabinoxylan, pectin, or xyloglucan. The highest amount of dimers was found in the SECWM, as this is vascular tissue; perhaps more dimers are necessary to maintain cell adhesion in order to counteract the additional forces produced by osmotic pressure. The higher levels of ferulic acid in SECWM and ECWM coincide with their higher arabinoxylan contents, strengthening the supposition that ferulic acids in CWC are esterified to arabinose residues in arabinoxylans. Formation of the 8,8′-DiFA (AT) dimer was tissue specific and may therefore be controlled indirectly by the sugar residues it is linked to and the flexibility of the associated polysaccharide chains.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Table of complete sequential phenolic extraction data from PCWM, SECWM, and ECWM. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS USED

CWM, cell-wall material; PCWM, parenchyma-derived cell-wall material; ECWM, epidermal cell-wall material; SECWM, subepidermal cell-wall material; CWC, Chinese water chestnut;

DI, deionized; DiFA, diferulic acid; AT, aryltetralin form; BF, benzofuran form; R_f, response factor; HPLC-DAD, high-performance liquid chromatography coupled to a diode array detector; GC-MS, gas chromatography coupled to a mass spectrometer; DMSO, dimethyl sulfoxide; RRT, relative retention time

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