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Thermal Stability of Texture in Chinese Water Chestnut May Be Dependent on 8,8′-Diferulic Acid (Aryltetralyn Form)

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Ferulic acid (FA) cross-links have been implicated in the thermal stability of texture in Chinese water chestnut (CWC) tissues. The aim of the current study has been to investigate this concept further. CWC tissue strips were measured for their mechanical properties before and after extraction in increasing strengths of alkali. The mechanical properties were related to the associated mode of fracture (cell separation or breakage) at the fracture surfaces and the phenolic composition of the cell walls. CWC tissue softened after prolonged extraction in cold alkali due to an increase in the ease of cell separation. Analysis of wall-bound phenolics demonstrated that most FA moieties, including five of the six dehydrodimers, were released before tissue strength was reduced. Loss of strength was, however, coincident with the loss of 8,8′-diferulic acid, aryltetralin (AT) form. It has been suggested that this dehydrodimer may be particularly concentrated at the edge of the cell faces. These results provide further evidence for the involvement of this dehydrodimer in conferring thermal stability of cell−cell adhesion and hence texture in CWC. However, they do not exclude the other diferulates from involvement in cell adhesion.

KEYWORDS: Cell walls; Chinese water chestnut; cell adhesion; dehydrodiferulic acid; phenolics; texture

INTRODUCTION

Chinese water chestnut (CWC) is prized for its crispness. The vegetable fails to soften during canning or cooking (*1*) due to thermal stability of cell adhesion (*2*). Parker and Waldron (*3*) provided evidence that phenolic acid cross-links could be responsible for this property. Sequential extraction methods, which had been originally developed to solubilize cell-wall polymers, were used to cleave selectively cell-wall chemical bonds in CWC tissue. Vortex-induced cell separation (VICS) could be brought about after extraction in alkali. Analysis of extracted components indicated that ferulic acid (FA)-containing moieties were contributing to cell−cell adhesion and therefore texture in CWC tissue. In addition, FA exhibited a pH-dependent autofluorescence [PDA (*4*)], which was lost during alkali-induced VICS. The association of cell separation with the loss of ferulic acid from the cell wall implied that FA cross-links could contribute to the thermal stability of texture in CWC tissue. The possible role of FA cross-links was strengthened by the knowledge that FA can be peroxidatively cross-linked (*5*) and that such cross-linking of cell-wall polysaccharides is likely to modify the mechanical properties of the cell wall (*6*, *7*). Furthermore, a number of coupled dehydroferulates have been discovered in grass stems (*8*). Parr et al. (*9*) analyzed the cell walls of CWC for their phenolic composition and reported that ∼1% (w/w) of the CWC cell wall consists of FA moieties and that ∼40% of the FA is present as dehydrodimers, including 5,5′-, 8-*O*-4′-, and 8,5′-diferulic acid (DiFA), 8,8′-DiFA [both open and aryltetralin (AT) forms], and 5,8′-DiFA (benzofuran form).

Parker and Waldron (*3*) demonstrated that cell separation could be induced in CWC after treatment with mild, hot alkali (50 mM KOH or Na_2CO_3), without the total loss of PDA. This treatment left a pattern of PDA in the walls, highlighting the junctions with surrounding cells and the position of the intercellular spaces. The phenolic components remaining in these samples were released by saponification methods and analyzed by high-performance liquid chromatography (HPLC) (*10*). The remaining phenolics were found to be predominantly monomeric in nature, with only 10% of the phenolics detected as dehydrodimers. However, the remaining dehydrodimers were rich in 8,8′-DiFA (AT form) (*11*). These results demonstrated significant heterogeneity in the alkali lability of the FA moieties, which may reflect differences in the chemistry of the linkage into the cell walls. In addition, the results suggested that distinct FA moieties such as 8,8′-DiFA (AT form) are distributed at specific locations of the cell wall such as the edges of the cell faces. Because turgor drives isodiametric cells to become spherical, cell separation will be initiated at the edge of each face—exactly where there is a buildup of FA moieties (*12*). The inferred concentration of the 8,8′-DiFA (AT form) at the perimeter of the cell faces and the fact that it has a lower degree of alkali lability raise the possibility that this dehydrodimer may play a key role in conferring thermal stability of cell−cell adhesion and hence texture in the CWC (*11*).

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In this study, the potential role of FA moieties in determining the mechanical and hence textural properties of CWC has been investigated further. CWC parenchyma tissue has been altered in chemical composition by sequential extraction techniques to assess how the removal of phenolic components relates to the tensile strength and strain of the tissue. The mechanical properties have then been compared with the mode of fracture (cell separation or breakage) at the fracture surface of samples at each stage of extraction, together with phenolic composition. The results provide further evidence for a precise role of 8,8′-DiFA(AT) in cell adhesion in CWC.

MATERIALS AND METHODS

Materials. Fresh and canned (Sharwoods) CWC were obtained from a local supplier. Slices of 3 mm thickness were cut transversely from CWC with a diameter >30 mm. Each slice was then cut into rectangular strips using a scalpel in conjunction with a strip cutter designed in our laboratory. The dimensions of the strips were typically 30 mm long, 3 mm wide, and 1 mm thick.

Sequential Extraction of Cell-Wall Components. *Extraction Containers.* Purpose-made extraction containers were designed and built in our laboratory. These consist of a 1-L Perspex pot with inner and outer chambers. The inner chamber contains a magnetic propeller that, when driven by an external magnetic stirrer, results in a flow of liquid in a circular motion. Perforations in the wall dividing the two chambers transfer the flow of liquid to the outer chamber. The strips were placed in the outer chamber, ensuring a constant flow of extracting solution over each surface without damage from the propeller.

Sequential Extraction. Strips were treated sequentially in the extracting containers with 2 M imidazole for 24 h at 20 °C, with 50 mM cyclohexane-*trans*-1,2-diamine-*N*,*N*,*N*′,*N*′-tetraacetate (CDTA), pH 6.5, for 8 h at 20 °C, and with 50 mM Na_2CO_3 containing 20 mM $NaBH_4$ at 1 °C for 36 h and then at 20 °C for 2 h. This was followed by one of two extraction treatments in alkali. Sequential extraction series A involved the following: 2-h extraction treatments with 0.05, 0.1, 0.25, 0.5, 1.0, and 4.0 M KOH, each containing 20 mM $NaBH_4$ at 20 °C. Sequential extraction series B consisted of the following: 0.25 M KOH for 2 and 4 h, 0.5 M KOH for 1, 2, and 3 h, and 1 M KOH for 1 h. Treatments were terminated by neutralization. All control samples were stirred for the equivalent length of time and temperature in water containing 3 mM $Na_2S_2O_5$ and 0.02% (w/v) sodium azide. All solutions contained 0.02% (w/v) sodium azide to prevent microbial growth.

Mechanical Properties—Tensile Test. The mechanical properties of control and sequentially-extracted CWC strips were measured using a texture analyzer (TA) (Stable Microsystems, Godalming, U.K.). The TA, equipped with a 5 kg load cell, was used to record force data as a function of distance. Rectangular strips were single-edge notched across their width at the midpoint of their length using a razor blade. The precise thickness of each strip was measured accurately using a micrometer screwgauge (Mitutoyo) to calculate the cross-sectional area (thickness × un-notched width). Strip ends were glued to stainless steel plates using cyanolit adhesive (cyanoacrylate super rapide 223F, Eurobond Adhesives Ltd., Sittingbourne, U.K.). The plates were clamped at a set distance apart to ensure minimum additional forces to each sample during loading in the tensile jaws of the TA. The dimensions of CWC tissue used in tensile tests were typically 17 mm long, a 1 mm notch in the 3 mm width, and 1 mm thick. Upward movement of the crosshead at a speed of 0.5 mm/s caused the tissue to fail. A minimum of 10 replicates were analyzed from each extraction time.

Notch Sensitivity. To determine the notch sensitivity of CWC tissue, strips of canned samples were tested with notch sizes of 0.5, 1, 1.5, and 2 mm.

Mechanical Properties. The strength and failure strain, which characterize each specimen, were calculated from the force deflection curve

$$\text{strength} = F_{max}/A\ (\text{N}\cdot\text{m}^{-2})$$

where F_{max} is the force at failure (maximum force) and A is the un-notched cross-sectional area.

$$A = tw$$

where t is the strip thickness and w is the un-notched strip width.

$$\text{failure strain (deformation)} = (L_{max} - L)/L$$

where L_{max} is the length at which failure occurred and L is the initial length.

Scanning Electron Microscopy (SEM) of the Fracture Surface of Sequentially-Extracted CWC Tissue. Strips (control and extracted) were fixed in 30 g L^{-1} glutaraldehyde in 0.05 M cacodylate buffer (pH 7.2) overnight, dehydrated in an ethanol series, transferred to acetone, and critical point dried using liquid carbon dioxide. They were then mounted, fractured surface uppermost, onto aluminum pin stubs using silver conducting paint and coated with a layer of gold, ∼25 nm thick.

Samples were examined and photographed in a Leica Cambridge Stereoscan 360 SEM using the secondary electron detector.

Preparation of Hot Alcohol-Insoluble Residues (AIR) from CWC Strips. To solubilize low molecular weight components including intracellular and non-covalently bound phenolics from the tissue strips, a hot alcohol extraction was used (*9*). AIR was prepared by grinding CWC strips in ethanol (final concentration = 85%, v/v, aq) with a pestle and mortar. The suspension was placed in a Sovril tube and heated at 85 °C for 2 min and then centrifuged at 1500*g* for 2 min. This procedure was repeated twice, resuspending the residue in fresh 85% (v/v) aqueous ethanol (8 mL) each time. The residue was then resuspended and centrifuged in boiling absolute alcohol (twice) and acetone (twice) and then air-dried, after which it was stored in a sealed container at room temperature.

Analysis of Alkali-Labile Phenolics. Wall-bound phenolics were released from AIR by sequential alkaline hydrolysis under progressively more vigorous conditions, based on the approach described by Hartley and Morrison (*13*) and adapted by Waldron et al. (*10*).

Cell-wall esterified phenolic acids were identified and quantified by reverse phase HPLC with diode array detection (HPLC-DAD) as described by Waldron et al. (*10*). Phenolics were quantified by HPLC using an Inertpak ODS2 reverse phase column (25 cm × 4.6 mm i.d., 5 μm; Capitol HPLC Ltd., Broxburn, West Lothian, U.K.). Elution was performed using a gradient system, which progressively increased methanol/acetonitrile levels in 1 mM trifluoroacetic acid (TFA). The gradient profile used for separation of wall-bound phenolics was formed using solvent A [10% (v/v) aqueous acetonitrile plus TFA to 1 mM] and solvent B [40% (v/v) aqueous methanol, 40% aqueous acetonitrile, and 20% water plus TFA to 1 mM] in the following program: initially, A 90%, B 10% linear gradient over 25 min to A 25%, B 75%; exponential gradient over 5 min to A 0%, B 100%; exponential gradient over 10 min to A 90%, B 10%; held isocratically at A 90%, B 10% for a further 2 min. The flow rate was maintained at 1 mL min^{-1}. The solvents were sparged with helium prior to use. Phenolics were detected using a Perkin-Elmer 235C DAD. Quantitation was by integration of peak areas at 280 nm, with reference to calibrations made using known amounts of pure compounds. All chemicals used were of far-UV quality HPLC grade purity when available.

RESULTS

Notch Sensitivity. To control the location in which failure takes place and thus obtain a breaking load on a cross-sectional area, test strips were notched (at their midpoint) to define the failure zone (*14*). The notch directs the fracture and, depending on the material, it may also cause a stress concentration (*14*). By varying the notch length, notch sensitivity can be studied. The material within a notch-insensitive specimen is not weakened by a notch and maintains a constant strength when expressed relative to the un-notched cross-sectional area (*15*). **Figure 1** shows the plot of strength as a function of relative notch length for the canned CWC samples. The best-fit straight

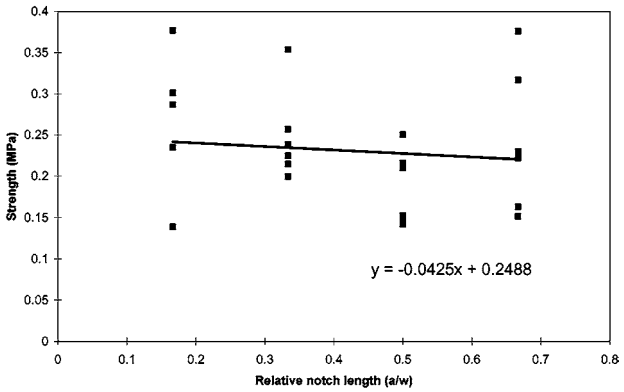

Figure 1. Notch sensitivity of CWC tissue (solid line represents best line of fit).

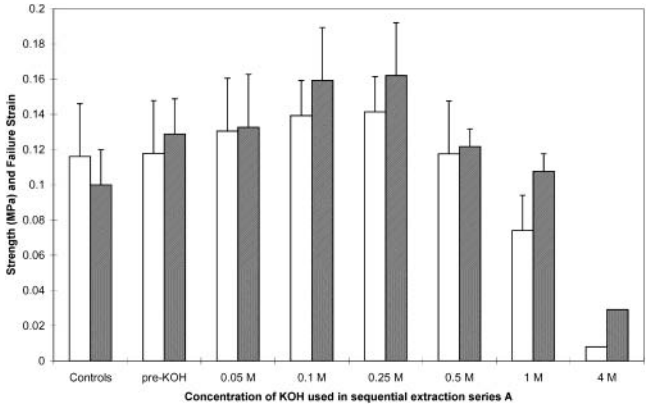

Figure 2. Strength (□) and failure strain (■) of fresh CWC tissue during sequential extraction series A, which involves extraction of CWC tissues in increasing concentrations of alkali. Vertical bars mark the standard deviation of each parameter.

line was produced and the gradient calculated. The line is almost horizontal so CWC parenchyma can be considered to be a notch-insensitive material as strength was almost constant as the width decreases with notching. This indicates that any local minor imperfections created during strip preparation would not have influenced its ability to fracture. Data for any samples that did not fail at the notch were discarded. All strengths presented have been calculated from data with a fixed notch length and so are suitable measurements of the fracture properties of CWC.

Mechanical Properties of CWC Tissue after Sequential Extraction of Cell-Wall Components. The force and displacement were recorded from each tensile result and used to calculate the mean tensile strength and strain, respectively.

Fresh CWC. Changes in the mechanical properties of fresh CWC strips during sequential extraction series A are shown in **Figure 2**. Preliminary experiments showed that extractions with CDTA or Na_2CO_3 did not affect the tensile properties of the CWC tissue (results not shown). As a result, mechanical tests were performed only on samples at the KOH stage of the full sequential extraction. Samples labeled "pre-KOH" refer to strips extracted up to and including 50 mM Na_2CO_3 treatment at 20 °C. The vertical bars mark the standard deviation of each parameter.

The strength (white bars) of fresh CWC tissue did not change notably during subsequent extractions in KOH up to 0.5 M (**Figure 2**). Extractions above this concentration resulted in a decrease in tissue strength, which became appreciably weaker at concentrations of 1 M KOH. There was a rise in failure strain (black bars) up to 0.25 M treatment, after which it decreased

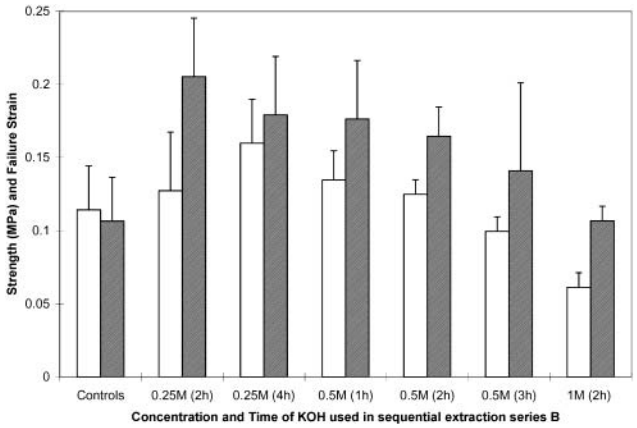

Figure 3. Strength (□) and failure strain (■) of fresh CWC tissue during sequential extraction series B, which involves extraction of CWC tissues in increasing concentrations of alkali. Vertical bars mark the standard deviation of each parameter.

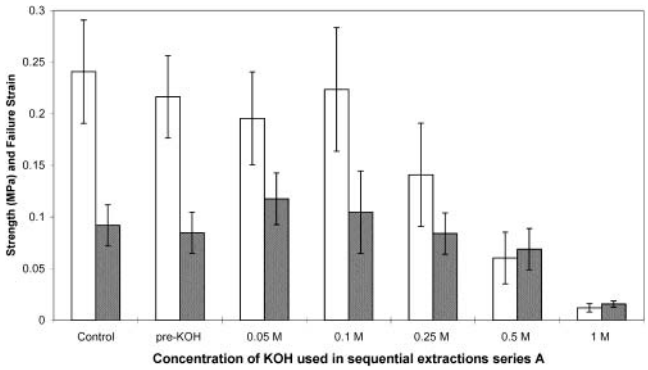

Figure 4. Strength (□) and failure strain (■) of canned CWC tissue during sequential extraction series A, which involves extraction of CWC tissues in increasing concentrations of alkali. Vertical bars mark the standard deviation of each parameter.

rapidly. Only one 4 M KOH treated strip was tested because the rest disintegrated during handling.

This approach was repeated using extended extraction times with 0.25 and 0.5 M KOH (described as sequential extraction series B) to expand the gradient of tissue weakening. The mechanical properties of the fresh CWC strips at different stages of this sequential extraction are shown in **Figure 3**. The strength (white bars) of CWC tissue decreased with increasing 0.5 M KOH extraction times. After an initial increase from control samples, the failure strain (black bars) decreased.

The small but repeatable increase in strength and strain between control and alkali concentrations up to 0.25 M KOH in extraction series A and B may relate to changes in component alignment in the wall as a result of the alkali treatment. Subsequent alkali extractions result in tissue softening and cell separation.

Samples (20 strips) from each step of sequential extraction series B were retained for phenolic analysis.

Canned CWC. Mechanical properties of extracted strips of canned CWC tissue are shown in **Figure 4**. Strength decreased notably during 0.25, 0.5, and 1 M KOH treatments. The failure strains of all samples were similar with the exception of samples treated with 1 M KOH. Canned CWC samples were also collected at each stage of the sequential extraction to identify which phenolic acids remained in the tissue. The gradation of tissue softening was considered to be broad enough for comparison with phenolic analysis.

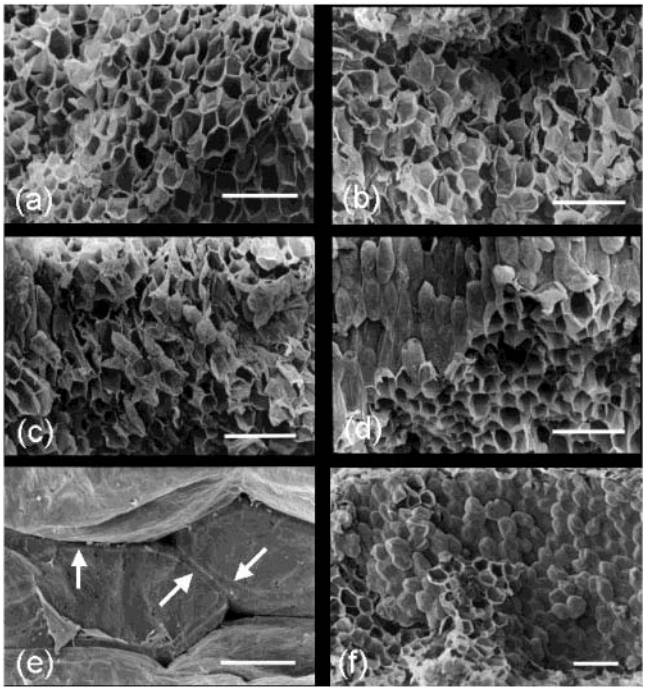

Figure 5. Fracture surfaces of fresh CWC strips at different stages of sequential extraction series B: (a) control (untreated); (b) 0.25 M KOH treatment for 2 h; (c) 0.5 M KOH treatment for 2 h; (d, e) 0.5 M KOH treatment for 4 h [(e) is a magnified region of (d) with arrows showing the imprint of neighboring cells on the surface of separated cells]; (f) 1 M KOH treatment for 2 h. Bars = (a–d) 200 μm, (e) 20 μm, and (f) 500 μm.

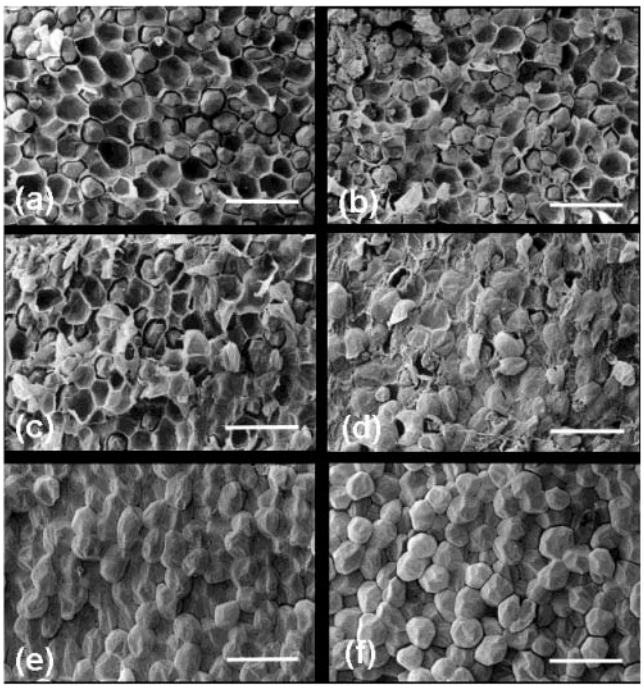

Figure 6. Fracture surfaces of canned CWC strips at different stages of sequential extraction series A: (a) control (untreated); (b) pre-KOH treatment; (c) 0.1 M KOH treatment; (d) 0.25 M KOH treatment; (e) 0.5 M KOH treatment; (f) 1 M KOH treatment. Bars = 200 μm.

SEM of the Fracture Surfaces. SEM images of fracture surfaces of both fresh and canned CWC are shown in **Figures 5** and **6**, respectively. Cell breakage was the only mode of tissue fracture in all fresh CWC samples and after extractions up to 0.25 M KOH (**Figure 5a**,**b**). However, cell separation was increasingly evident in all samples treated with 0.5 M KOH for 2 h (**Figure 5c**) or more (**Figure 5d**,**e**) and was the main mode of fracture after 1 M KOH for 2 h (**Figure 5f**). This indicates that decreases in tensile strength were associated with, and probably due to, a decrease in cell adhesion. Interestingly, the edge-of-face structures associated with cell adhesion (*16*) can be seen on the surfaces of cells that separate after 0.5 M KOH (**Figure 5e**). In canned CWC, all samples treated up to the 0.25 M KOH stage of sequential extraction A failed due to cell breakage (**Figure 6a**−**d**). Samples treated with either 0.5 M KOH (**Figure 6e**) or 1 M KOH (**Figure 6f**) failed due to cell separation alone. Therefore, as for fresh CWC, decreases in the tensile strength occurred in association with cell separation.

Analysis of Wall-Bound Ester-Linked Phenolics in Sequentially Extracted Fresh and Canned CWC Tissue. Esterified phenolics were released from the AIR of sequentially extracted CWC tissues by saponification (*10*). They were identified and quantified by HPLC in conjunction with analysis of their absorbance spectra obtained with a diode array scanning detector (*10*). The recovery of esterified phenolic acids extracted from the AIR of both fresh and canned CWC samples was less than half that reported by Parr et al. (*9*) on a weight basis. This is due to a diluting effect of starch in the AIRs. This was confirmed by sugar analysis of the AIR of canned CWC, which showed large amounts of glucose residues (data not shown). However, the ratios of the different phenolic components present in fresh and canned CWC (results not shown) were broadly similar to that reported by Parr et al. (*9*). FA was the most abundant phenolic component, comprising ~60% of the total phenolic component in both fresh and canned CWC. Approximately 35% of this was the *cis*(= *Z*)-isomer. This may have resulted from light-induced isomerization (*17*, *18*), although care had been taken to avoid exposure to light. Waldron et al. (*19*) reported that direct exposure of alkaline extracts to intense light increases the proportion of *cis*-FA but has no discernible effect on the other components in sugar beet. Parr et al. (*9*) reported that saponification of FA standard had little effect on the *cis*/*trans* ratio, indicating that the levels of *cis*-FA may not be an artifact. The bulk of the remaining phenolic components consisted of FA dehydrodimers. The most prominent of these were 8-*O*-4′-DiFA, 5,5′-DiFA, and 8,5′-DiFA (benzofuran form). Small amounts of a number of other phenolics were also detected including *p*-coumaric acid, vanillin, *p*-hydroxybenzoic acid, vanillic acid, and *p*-hydroxybenzaldehyde.

Fresh CWC. **Figure 7** shows the wall-bound ester-linked phenolics remaining in the cell walls after each stage of sequential extraction series B. The levels of esterified phenolic acids in the AIR of CWC strips after the 0.25 M KOH treatment for 2 h had decreased by 95% compared to the pre-KOH-treated samples. Only 4.6% of the original FA monomeric components and 5.7% of the total diferulic acid moieties remained. The only dehydrodimer identified in the CWC samples at this stage was the 8,8′-DiFA (AT form) (**Figure 8**). The level of this dehydrodimer began to decrease after 3 h of extraction with 0.5 M KOH.

There was little change in the mechanical strength of the fresh CWC tissue during the loss of the majority of the phenolic components after 0.25 M KOH treatment for 2 h (**Figure 8**). However, subsequent general decreases in the mechanical strength of the fresh CWC tissue during later stages of the 0.5 M KOH extractions coincided with a decrease in the level of 8,8′-DiFA (AT form).

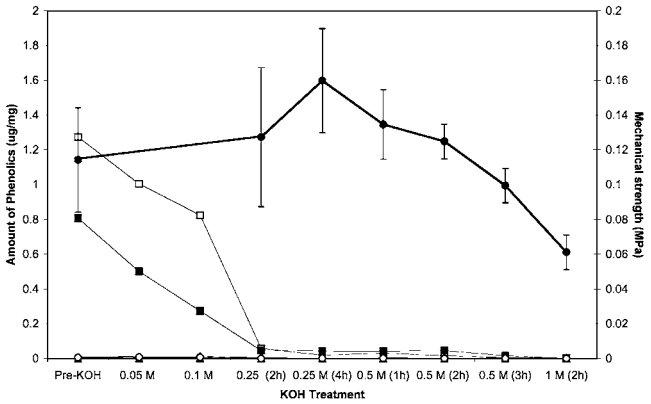

Figure 7. Wall-bound ester-linked phenolics remaining after each stage of the sequential extraction series B of fresh CWC strips: (□) total ferulic acid; (■) total diferulates; (◊) *p*-coumaric acid; (♦) *p*-hydroxybenzoic acid; (△) vanillic acid; (▲) *p*-hydroxybenzaldehyde; (○) vanillin; (●) mechanical strength.

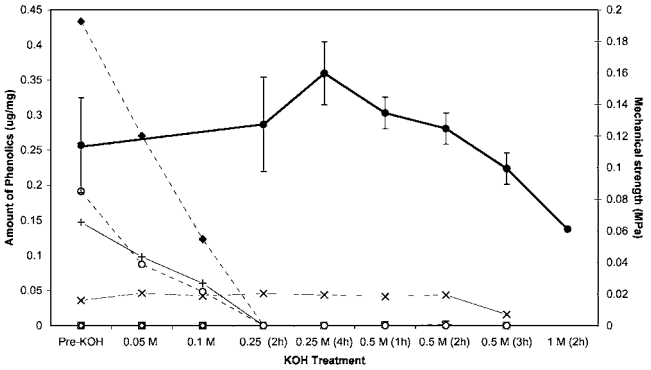

Figure 8. FA dehydrodimers remaining after each stage of sequential extraction series B of fresh CWC tissue: (×) 8,8′-DiFA (aryltetralin form); (□) 8,8′-DiFA; (◊) 8,5′-DiFA; (+) 5,5′-DiFA; (♦) 8-*O*-4′-DiFA; (○) 8,5′-DiFA (benzofuran form).

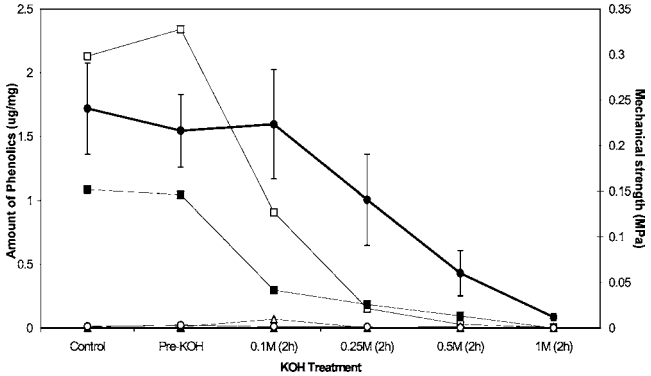

Figure 9. Wall-bound ester-linked phenolics remaining after each stage of sequential extraction series A of canned CWC tissue: (□) total FA; (■) total diferulates; (◊) *p*-coumaric acid; (♦) *p*-hydroxybenzoic acid; (△) vanillic acid; (▲) *p*-hydroxybenzaldehyde; (○) vanillin; (●) mechanical strength.

Canned CWC. **Figure 9** shows the wall-bound ester-linked phenolics remaining after each stage of sequential extraction B. As in fresh CWC, the levels of esterified phenolic acids in the AIR of canned CWC strips decreased considerably (>90%) after the 0.25 M KOH treatment for 2 h when compared with the pre-KOH-treated samples. Only 7.2% of the original FA monomeric components and 17.0% of the total diferulic acid moieties remained. The level of 8,8′-DiFA (AT form) dehydrodimer began to decrease after extraction with 0.5 M KOH (**Figure 10**). Decreases in the mechanical strength of the canned CWC tissue after the 0.5 M KOH treatment coincided only with a decrease in the level of the 8,8′-DiFA (AT form) dehydrodimer.

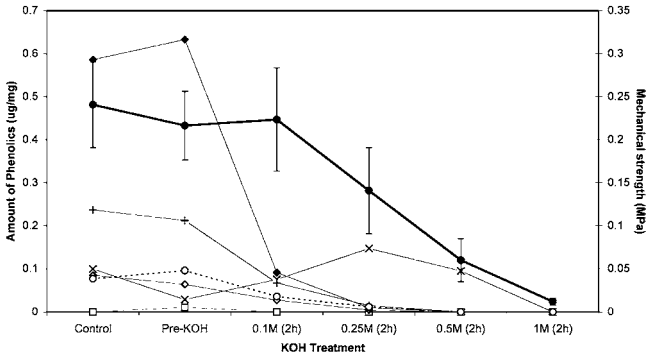

Figure 10. FA dehydrodimers remaining after each stage of sequential extraction series A of canned CWC tissue: (×) 8,8′-DiFA (aryltetralin form); (□) 8,8′-DiFA; (◊) 8,5′-DiFA; (+) 5,5′-DiFA; (♦) 8-*O*-4′-DiFA; (○) 8,5′-DiFA (benzofuran form).

DISCUSSION

In this study, strips of CWC tissue have been subjected to two sequential extraction procedures designed to progressively cleave cell-wall chemical bonds. During this process, the tensile strength and strain have been measured in conjunction with phenolic analysis and identification of the mode of tissue failure. Treatment of strips of fresh or canned CWC with 0.5 M KOH for increasing lengths of time causes a decrease in the tensile strength of the tissue. This tissue softening is due to a reduction in cell adhesion and occurs at a stage after the majority of cell-wall phenolics and FA dehydrodimers are solubilized. However, it coincides with the loss of 8,8′-DiFA (AT).

As discussed above, previous studies (*3*, *9*−*12*) have indicated that FA cross-linking appears to be responsible for thermal stability of texture in CWC. This functionality has been supported by studies (*17*, *20*) on beet root tissues: Ng et al. (*20*) reported that the rate of thermal softening of beet root tissue could be reduced by treatment with H_2O_2. This was associated with the increased oxidative cross-linking of FA-esterified pectic polysaccharides, which become less soluble in water as a result. Chufa tissues, which also demonstrate thermal stability of texture, have been shown to contain wall-esterified FA and its dehydrodimers (*21*).

The current study raises the prospect that cell adhesion in CWC may be particularly dependent on the formation of 8,8′-DiFA (AT form) cross-links at the edges of the cell faces (*12*). The solubilization of the other five dehydrodimers before cell separation occurs does not preclude their involvement in cell adhesion. However, it does indicate that the 8,8′-DiFA (AT form) plays a fundamental role in adhesion. Understanding the role of peroxidases in the formation of this dehydrodimer and the synthesis of the polymers that it cross-links may provide more precise targets for controlling thermal stability of cell adhesion than is offered by controlling cell-wall FA levels overall.

CONCLUSIONS

(1) The mechanical strength of both fresh and canned CWC tissue decreased after 0.5 M KOH treatment of the sequential extraction. This decrease in mechanical strength was accompanied by an increase in cell separation.

(2) All phenolic moieties, including all but one of the dehydrodiferulates, were extracted from fresh and canned CWC cell wall prior to the main changes in mechanical properties. This indicates that the bulk of the ferulic acid and diferulic acid components are not the final limiting factors in cell adhesion during the sequential extraction of CWC tissue in increasing strengths of alkali. However, this does not preclude their involvement in the adhesion process.

(3) The reduction in tissue strength during sequential extraction is closely associated with the loss of 8,8′-DiFA (AT form) from the cell walls and cell separation, indicating that this dehydrodimer probably plays a prominent role in cell adhesion.

ABBREVIATIONS USED

CWC, Chinese water chestnut; FA, ferulic acid; 8,8′-DiFA-(AT), 8,8′-diferulic acid (aryltetralin form); VICS, vortex-induced cell separation; PDA, pH-dependent autofluorescence; CDTA, cyclohexane-*trans*-1,2-diamine-*N*,*N*,*N*′,*N*′-tetraacetate; TA, texture analyzer; AIR, alcohol insoluble residue; HPLC-DAD, high-performance liquid chromatography diode array detector.

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