PERGAMON

Phytochemistry 50 (1999) 395–400

PHYTOCHEMISTRY

# Cold alkali can extract phenolic acids that are ether linked to cell wall components in dicotyledonous plants (buckwheat, soybean and flax)

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Received 18 November 1997; revised 26 June 1998

**Abstract**

Experiments were carried out to estimate the amounts and nature of bonding of ferulic acid to cell walls of various dicotyledonous plant materials including soybean heterotrophic and mixotrophic cell suspension cultures, soybean leaves, buckwheat callus, flax phloem and xylem. Isolated cell walls were oxidized with $CuSO_4$-NaOH, with phenol aldehyde products and ferulic acid produced estimated by GC. Ferulic acid content was also analyzed in the cell wall fraction extracted by 1 M alkali at room temperature, which cleaves ester linkages, and in the fraction extracted by hot concentrated alkali, which cleaves ether linkages. Overall, the bulk of the cell wall ferulic acid (60–80%) was found to be ether linked to cell-wall components. Room temperature alkali treatment may release from the cell wall a portion of the ferulic acid that is esterified to cell wall components via saponification. This treatment, however, also extracts a portion of the etherified ferulic acid that is bound to some cell wall components like proteins or glycoproteins that are acid precipitable. Our results demonstrate that hydroxycinnamic acids can form a significant part (0.01–0.19% of cell wall dry weight) of primary cell wall phenolics of dicots and the nature of linkages between ferulic acid and polymers of the primary cell wall varies in different plant materials. 

*Keywords:* *Fagopyrum tataricum*; *Polygonaceae*; *Glycine max*; *Leguminosae*; *Linum usitatissimum*; Linaceae; Dicotyledons; Cell wall; Lignin; Esterified ferulic acid; Etherified ferulic acid

## 1. Introduction

Plant cell walls contain phenolic compounds that are usually divided into two main groups: lignin and hydroxycinnamic acids. Most of the data published on cell wall phenolics was obtained in studies of plant tissues with lignified secondary cell walls. Growing plant cells contain little or no lignin but do have some wall phenolics that could have an important structural significance. Hydroxycinnamic acids can make up a significant proportion of the wall phenolic compounds in non-woody tissues of certain species and may have important functions (Tan, Hoson, Masuda & Kamisaka, 1992; Kato, Yamanouchi, Hinata, Ohsumi & Hayashi, 1994). Hydroxycinnamic acids can be linked by ester or ether bonds to other cell wall components. These linkages can be distinguished by treatment with room temperature alkali (usually 0.1 or 1 M NaOH) that cleaves ester bonds or by hot concentrated alkali (4 M NaOH at 170°C) that also cleaves ether bonds (Lam, Iiyama & Stone, 1990). Esterified hydroxycinnamic acids, such as ferulic and *p*-coumaric acids, have been found in cell walls of many species of vascular plants. Ferulic acid esterified to non-cellulosic polysaccharides forms ether linkages to lignin (Lam et al. 1990; Iiyama, Lam & Stone, 1990). It has been suggested that in non-lignified primary walls the formation of ester and ether linkages between cell wall polysaccharides and feruloyl residues may play an important role in controlling cell wall extensibility (Biggs & Fry, 1987; Kamisaka, Takeda, Takahashi & Shibata, 1990).

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0031-9422/98 $ - see front matter 
PII: S0031-9422(98)00575-5

Arabinoxylans of monocotyledons (Harris & Hartley, 1980; Ahluwalia & Fry, 1986) and the pectic arabinogalactans of spinach and sugar beet (Hartley & Harris, 1981; Fry, 1983; Fry, 1986) and xyloglucans in bamboo (Ishii & Hiroi, 1990) were found to have ferulic acid esterified to specific sugars. Moreover, the saponifiable ferulic acid content of cell walls of grasses always exceeds the *p*-coumaric acid content. A significant fraction of unidentified aromatic material resists saponification and may be involved in cross-linking the primary wall during differentiation through ether bridges (Iiyama, Lam & Stone, 1994).

Studies of wall-bound hydroxycinnamic acids and their role in wall polymer cross-linking have been carried out mostly with monocotyledons (Commelinoid) and the Caryophyllales family of dicots (Harris, Kelderman, Kendon & McKenzie, 1997). It is considered that the primary cell walls of the non-commelinoid monocotyledons have a composition similar to that of primary cell walls of most dicotyledons and these cell walls contain no ferulic acid (Henry & Harris, 1997). In the present study esterified (extracted by mild alkaline treatment) and etherified (extracted with concentrated hot alkali) ferulic acid forms were examined in cell walls of several different dicotyledonous plant tissues and tissue cultures.

## 2. Results and discussion

Table 1 shows that the amount and ratio of lignin and ferulic acid in the phenolic products fraction released by cupric hydroxide oxidation from total cell walls of various plant tissues vary widely. Cupric oxide oxidation has been used in our experiments as an oxidative method for chemical characterization of lignin and other wall phenolics since lignin alkaline oxidation (with nitrobenzene or cupric oxide) is a frequently used procedure (reviewed in Monties, 1989; Lewis & Yamamoto, 1990; Chen, 1992). There is data indicating that the yield of cupric oxidation and thioacidolysis are similar, as for example, Zeier and Schreiber Zeier and Schreiber (1997) who reported recently that 'results obtained with cupric oxide oxidation were quantitatively and qualitatively in good agreement with thioacidolysis'. In lignified tissues of flax xylem, the primary products indicate that G/S type lignin, typical of angiosperms, is a predominant aromatic cell wall component. Soybean leaves and cell suspension cultures as well as buckwheat callus and flax phloem do contain a significant proportion of ferulic acid in the phenolic wall oxidation products. Flax xylem and buckwheat callus contain higher levels of the phenolic products than the other tissues analyzed (Table 1).

Table 1
GC analysis of some phenolic products of cell wall oxidation with $CuSO_4$-NaOH

| Plant material | Vanilin | Syringaldehyde | Ferulic acid | Van. + Syr. Ferulic acid |
|---|---|---|---|---|
| | mg/100 g of cell wall DW | | | |
| Flax phloem | 4 | 14 | 19 | < 1 |
| Flax xylem | 432 | 61 | 51 | ~10 |
| SB-H cells | trace | 14 | 10 | ~1 |
| SB-M cells | trace | 18 | 20 | ~1 |
| Soybean leaf | 12 | 23 | 17 | ~2 |
| Buckwheat callus | 278 | 47 | 185 | ~2 |

The commonly used procedure for cell wall phenolic acid analysis is extraction from the cell wall by alkali at room temperature and in many studies this is the only fraction analyzed (for example, Kato et al., 1994; Kauss, Franke, Krause, Conrath, Jeblick, Grimming et al., 1993; Parvez, Wakabayashi, Wakabayashi, Hoson & Kamisaka, 1997). However a portion of the phenolic acids is not extracted and remains in the residue after such treatment. In addition, in order to analyze the phenolic acids cleaved by alkali treatment, the hydrolysate should be acidified to pH 1 and then extracted with an organic solvent such as diethyl ether or ethyl acetate. This acidification often results in the formation of a precipitate that may contain cell wall phenolics linked to polymers by bonds, such as ether linkages, that are not cleaved by the room temperature alkali. To avoid this methodological problem, Lam et al. (1990) estimated esterified phenolics as the compounds extracted by 1 M alkali at room temperature and etherified phenolics as the difference between the products of hot and mild alkaline extraction. Grabber, Hatfield, Ralph, Zon and Amrhein (1995) used another approach: they did not separate the 1 M alkali extract from the remaining cell wall but acidified it first and then treated the total pellet with hot concentrated alkali to estimate cell wall phenolic acids.

In our experiments, ferulic acid content was analyzed in the following cell wall fractions: 1. total cell wall, 2. the fraction extracted by 1 M alkali after acidification both in the supernatant and pellet (extracted with concentrated hot alkali), 3. the fraction extracted by hot concentrated alkali from the residual cell wall pellet obtained after cold alkali treatment.

The amount of *p*-coumaric acid released by alkaline treatment was several times less than the ferulic acid content in soybean leaves and flax xylem and only trace amounts of *p*-coumaric acid were detected in buckwheat callus and flax phloem (data are not shown).

The proportion of the total wall bound ferulic acid found in the fractions obtained by such treatments varied with the different plant material used (Table 2). The bulk of cell wall ferulic acid (60–80%) was found

Table 2
Ferulic acid content of cell wall fractions of different tissues and species

| Cell wall fractions | Flax phloem | Flax xylem | SB-H cells | SB-M cells | SB leaf | Buckwheat callus |
|---|---|---|---|---|---|---|
| | mg/100 g of cell wall DW | | | | | |
| Cell wall oxidized | 19 | 51 | 10 | 20 | 17 | 185 |
| Acidified | | | | | | |
| 1 M NaOH room temperature extract | | | | | | |
| (A)-Supernatant | 5 | 11 | 4 | 7 | 3 | 49 |
| (B)-Pellet treated with hot 4 M NaOH | 9 | 39 | 3 | 6 | 6 | 79 |
| (C)-Residual pellet after (B) treated with $CuSO_4$-NaOH | 0 | 0 | 0 | 0 | 0 | 27 |
| 4M NaOH extract (170°C) of the pellet after 1 M NaOH treatment | 5 | 5 | 3 | 8 | 9 | 36 |

to be etherified to cell wall components since it remained in the cell wall or in the pellet after acidification of the 1 M NaOH room temperature extract. It was noteworthy that the extract obtained by room temperature alkali treatment included a significant portion of the ether-bound ferulic acid (acid-precipitable) and part of the ferulic acid remained ether-linked to the cell wall after mild alkali hydrolysis (Table 2). Etherified ferulic acid was obtained by hot concentrated alkali treatment of the pellet formed by acidification of the alkali extract.

Based on these results it can be concluded that room temperature alkali treatment releases the portion of the ferulic acid that is esterified to cell wall components as a result of cleavage of the ester bond (Lam et al., 1990). This treatment may also extract a portion of the etherified ferulic acid without cleavage of the ether bond by solubilization of the wall polymers linked to ferulic acid.

There could be several types of ether linkages between ferulic acid and the cell wall polymers. Ferulic acid is known to be etherified to lignin in monocotyledonous plants but this has not been reported with dicotyledonous plants. It was shown earlier for Gramineae that all of the etherified ferulic acid in the dioxane-$H_2O$-soluble fractions of walls of wheat and *Phalaris aquatica* internodes is also ester-linked (Lam, Iiyama & Stone, 1992). It was calculated in this study that there are 9 to 10 ferulic acid ester–ether bridges for every 100 C6-C3 lignin monomers.

Lam et al. (1992) concluded, based on theoretical considerations and indirect evidence obtained in studies with monocots, that the ferulic acid ether linkages would be more likely to be with lignin than with carbohydrates.

In our experiments the pellet obtained after acidification of the room temperature alkali extract contained much lower amounts of presumed monolignol derivatives than ferulic acid (Table 3). For example in buckwheat callus the sum of all monolignol derivatives in all fractions analyzed in the pellet formed after acidification of 1 M alkali extracts was less than one-third the ferulic acid content on a dry matter basis (monolignol derivatives 32 and ferulic acid 106 mg/100 g cell wall). In cell suspension cultures (SB-M) the proportion of monolignols/ferulic acid in the pellet formed after acidification of the alkali-soluble fraction was 1/6 (syringaldehyde 1 mg and ferulic acid 6 mg per 100 g cell wall dry weight).

We can consider that some ferulic acid units esterified to the cell wall can also be ether linked through their phenolic oxygen to a cell wall structural protein or glycoprotein (with participation of a novel enzyme) and this bond cannot be broken by room temperature dilute alkali. In our experiments, significant amounts of cell wall protein were extracted by mild alkali treatment, and the main proportion of that protein was found in the pellet after acidification of the alkaline extract (Table 4). The presence of cell wall protein along with ferulic acid in the same wall extract allows

Table 3
Monolignol derivatives and ferulic acid content of fractions obtained from the precipitate formed as a result of acidification of the 1 M NaOH extract of the buckwheat callus cell wall

| Fraction | Vanilin | Acetovanilin | Syringaldehyde | Aceto-syringone | Ferulic acid |
|---|---|---|---|---|---|
| | mg/100 g of cell wall DW | | | | |
| Pellet treated with hot 4 M NaOH | trace | 15 | 2 | 6 | 79 |
| Residual pellet oxidized with CuSO4-NaOH | trace | 3 | 3 | 2 | 27 |

* Hot 4 M NaOH extracted a portion of the phenolics from the pellet, but after acidification of the extract the precipitated material was solubilized completely by $Cu(OH)_2$ treatment.

Table 4
Protein content of 1 M NaOH extract of cell wall before and after acidification

| Plant material | 1 M NaOH room temperature extract | Supernatant after acidification of 1 M NaOH extract |
|---|---|---|
| | g/100 g of cell wall DW | |
| Flax phloem | 0.3 | 0 |
| Flax xylem | 0.4 | 0 |
| SB-H | 5.9 | 0.8 |
| SB-M | 9.7 | 1.9 |
| Soybean leaf | 10.8 | 2.2 |
| Buckwheat callus | 1.1 | 0 |

one to assume the possibility of the existence of ether links between ferulic acid and wall proteins in the room temperature alkali extractable fraction. This possibility needs further study however.

Involvement of proteins in covalent cross-linking in unlignified cell walls was discussed extensively by Iiyama et al. (1994) and Wallace and Fry (1994). Covalent cross-links between hydroxycinnamic acids esterified to wall polysaccharides and Tyr or Cys residues of wall proteins through dehydrogenative dimerization was postulated (see Bacic, Harris & Stone, 1988 for references).

The existence of ether-linked ferulic acid amides in the cell walls of normal and wounded periderm of potato tuber was reported recently (Negrel, Pollet & Lapierre, 1996) and this possibility could also be considered. It is also possible that some suberin-like substance can be extracted by room temperature alkali treatment and this could contain ether-linked ferulic acid.

Thus our results demonstrate that ferulic acids could be a significant constituent of the primary cell wall phenolics of dicotyledons and the nature of the linkages between ferulic acid and cell wall polymers varies in different plant materials. The amount of ferulic acid found in the different dicots analyzed in our experiments varied from 0.01 to 0.19% of the cell wall which is considerably lower than that reported for Gramineae: from 0.02–0.16% (rice suspension, Negrel et al., 1996) to 1.77% (maize suspension culture, Grabber et al., 1995) or 1.8% (wheat aleurone cells, Bacic & Stone, 1981) of the cell wall. The walls of graminaceous monocots typically contain larger amounts of hydroxycinnamic acids than dicotyledons (Ishii, 1997). Although cell wall linked ferulic acid is usually detected as a minor wall component in most dicots, it could be of significant importance for plants. Thus recently, a 10-fold increase was reported in the amount of ferulic acid released by mild alkaline hydrolysis from transgenic tobacco plants that were down-regulated from cinnamoyl-CoA reductase compared to control plants (Piquemal, Lapierre, Myton, O'Connell, Schuch, Grima-Pettenati et al., 1998). In plant material analyzed in our experiments more than half of the ferulic acid extracted was ether-linked to cell wall components. It is noteworthy that saponification may release ferulic acid that is ether-linked to some cell wall compound(s). The possible modes of incorporation of ferulic acid into the cell wall remains to be elucidated. Recently since cell wall phenolics have become the targets for biotechnology, a significant revision in the mechanisms of regulation of the wall phenolic biosynthetic pathways have occurred. Unusual phenolic compounds linked to the cell wall were detected in transgenic plants (Piquemal et al., 1998), indicating the lack of our knowledge about the chemical plasticity of wall phenolics and their functional significance. Our results provide evidence for the existence in dicotyledonous plants of ferulic acid ether-linked to yet unidentified wall components different from lignin. Further study is needed to elucidate these components.

## 3. Experimental

### 3.1. Plant material

Soybean (*Glycine max* (L.) Merr. cv. Corsoy) suspension culture (Horn, Sherrard & Widholm, 1983) was grown mixotrophically (SB-M) with 1% sucrose in KN1 medium with ambient $CO_2$. Cell suspensions were grown under continuous fluorescent light (about 250 $\mu Em^{-2} s^{-1}$) at 28 to 30°C and were subcultured every 21 d. Cultures were used in the experiments 3 d after inoculation, which corresponded to the time of cell division. The heterotrophic soybean cultures were initiated from cotyledons of germinating Williams 82 seedlings on MS solid basal medium (Murashige & Skoog, 1962) with 0.4 mg/l 2,4-D and 3% sucrose (denoted MX medium). The callus was placed in liquid MX medium and the suspension cultures transferred to fresh medium every two weeks.

Buckwheat (*Fagopyrum tataricum*, Gaertn) callus was initiated from immature embryos and cultured on RX medium (Rumyantseva, Sergeeva, Khakimova,

Salnikov, Gumerova & Lozovaya, 1989; Lozovaya, Gorshkova, Yablokova, Zabotina, Ageeva, Rumyantseva et al., 1996). The regenerable callus was identified by morphology, color and texture as described (Rumyantseva et al., 1989). Regeneration was via both embryogenesis and organogenesis and this ability was confirmed periodically.

Flax plants (*Linum usitatissimum* L., cv. Novotorzhskii) were grown in the greenhouse and phloem was analyzed at the end of the fast growth stage and xylem at the yellow ripeness stage. Xylem can be easily separated from other tissues of flax stems (McDougall & Morrison, 1995). In our experiments the flax stem was divided into xylem tissues and fiber-rich phloem peels. This separation can be easily done, beginning at the base of the stem and proceeding to a 'snap' point, i.e., a point about 10 cm from the apex (Gorshkova, Wyatt, Salnikov, Gibeaut, Ibragimov, Lozovaya et al., 1996). We analyzed the purity of tissues obtained by fluorescence microscopy in combination with Cellufluor staining. This procedure allows one to distinguish the tissues: xylem is yellow due to autofluorescence of lignin, bast fibers are deeply stained by Cellufluor which results in blue fluorescence, and parenchyma produces red fluorescence due to the presence of chlorophyll. Our observations indicate that the fiber-enriched strips never contain any xylem contaminates while the xylem tissues may rarely contain a single fiber cell. The tissues were frozen in liquid nitrogen and freeze-dried separately.

### 3.2. Cell wall isolation and fractionation

Cell walls were isolated from homogenized plant material by a series of intracellular content extractions with phosphate buffer (pH 6.0), ethanol, water and acetone as described (Talmadge, Keegstra, Bauer & Albersheim, 1973). A portion of the cell wall sample was oxidized with $CuSO_4$-NaOH as described previously (Murashige & Skoog, 1962) while a similar sample (50–100 mg) was fractionated by extraction with 8 ml of 1 M NaOH for 24 h at room temperature. The supernatant was collected after centrifugation at 2500*g* for 10 min, transferred to another tube and acidified with concentrated HCl to pH 1. The precipitate formed after acidification was pelleted by centrifugation (2500*g* for 10 min), washed twice with 3% HCl, dried and treated with 4 M NaOH at 170°C for 2 h. The extract was acidified to pH 1 with concentrated HCl. If a precipitate formed during acidification it was treated similarly to the previous one and was subjected to $CuSO_4$-NaOH oxidation. Cell wall residue after 1 M NaOH extraction was washed twice with water, dried and extracted with 4 M NaOH at 170°C for 2 h in stainless steel vessels. All acidified supernatants were extracted with diethyl ether and the organic phase was used for GC analysis. Samples were dried and stored overnight under vacuum over $CaCl_2$. From 30 to 100 µl of Sigma-Sil-A (Sigma, USA) were added for 2 h and silyated derivatives were immediately applied to the GC (Chrom 5, Laboratorni Pristroje, Praha) glass column (250 × 3 mm) filled with 4% CE52 on Chromosorb W (100–120 mesh). The column temperature was programmed as follows: 145°C–10 min, from 145 to 190°C at 2° min, from 190°C to 235°C at 5° min and then constant at 235°C with a detector temperature (flame-ionization) of 240°C, injector temperature 170°C and helium gas flow of 30 ml/min.

There were 2 to 4 GC analyses done for replicated samples. The deviations between samples were less than 5%.

### 3.3. Protein determination

Protein in cell wall fractions was determined using the Bradford reagent (Bio-Rad) with bovine serum albumin (Sigma) as standard.

## Acknowledgements

This work was supported in part by funds from the Illinois Agricultural Experiment Station, U.S. National Academy of Sciences, Russian Academy of Sciences and Illinois Soybean Program Operating Board.

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