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Coniferyl Ferulate Incorporation into Lignin Enhances the Alkaline Delignification and Enzymatic Degradation of Cell Walls

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Incorporating ester interunit linkages into lignin could facilitate fiber delignification and utilization. In model studies with maize cell walls, we examined how partial substitution of coniferyl alcohol (a normal monolignol) with coniferyl ferulate (an ester conjugate from lignan biosynthesis) alters the formation and alkaline extractability of lignin and the enzymatic hydrolysis of structural polysaccharides. Coniferyl ferulate moderately reduced lignification and cell-wall ferulate copolymerization with monolignols. Incorporation of coniferyl ferulate increased lignin extractability by up to 2-fold in aqueous NaOH, providing an avenue for producing fiber with less noncellulosic and lignin contamination or of delignifying at lower temperatures. Cell walls lignified with coniferyl ferulate were more readily hydrolyzed with fibrolytic enzymes, both with and without alkaline pretreatment. Based on our results, bioengineering of plants to incorporate coniferyl ferulate into lignin should enhance lignocellulosic biomass saccharification and particularly pulping for paper production.

Introduction

Recent discoveries highlighting the metabolic malleability of plant lignification indicate that lignin can be engineered to dramatically diminish its adverse impact on fiber utilization for nutritional and industrial purposes. Perturbing single genes in the monolignol pathway can lead to dramatic shifts in the proportions of normal monolignols (**1**, **2**, **3**; Figure 1) polymerized into lignin or elevated incorporation of pathway intermediates into the polymer.[1] In normal plants, monolignols destined for lignin polymerization can also be extensively preacylated with acetate, *p*-hydroxybenzoate, or *p*-coumarate.[1]

p-Coumarates acylate the γ-position of phenylpropanoid sidechains of mainly syringyl units in lignin.[2,3] Structural and enzymatic studies suggest that syringyl units are enzymatically preacylated with *p*-coumarate prior to their incorporation into lignin,[4] implicating sinapyl *p*-coumarate **5** as the logical precursor. Based on the analysis of isolated lignins and whole cell walls, sinapyl *p*-coumarate could comprise up to 40% of the lignin in some grass tissues.[2,5] *p*-Coumarate esters on lignin form few cross-linked structures mediated by radical coupling reactions and most remain as terminal units with an unsaturated side chain and a free phenolic group.[2]

In contrast to *p*-coumarate, ferulates **4** esterified by simple alcohols, sugars, soluble pectins, or insoluble cell-wall xylans readily undergo diverse radical coupling reactions with each other and with lignin monomers and oligomers to form cross-linked networks.[6−10] Once polymerized into lignin, ferulate cannot be fully released by solvolytic methods.[8] Cleavage of ferulate ester linkages, however, contributes to the unusually high extractability of grass lignin and the dramatically improved enzymatic degradability of grass cell walls following mild alkaline treatments.[11]

Ferulate-monolignol ester conjugates, such as coniferyl ferulate **6** or sinapyl ferulate **7** have not been identified in lignins, but they are naturally produced by some plants as secondary metabolites during, among other things, lignan biosynthesis.[12−15] This raises the exciting possibility that plants could be engineered to produce and transport coniferyl or sinapyl ferulate to the apoplastic space in a manner analogous to sinapyl *p*-coumarate, but with full incorporation of both the ferulate and the monolignol moieties of the conjugate into lignin (Figure 2). We anticipate that incorporation of these conjugates or other related diphenolics would improve lignin extraction during alkaline pulping[16] via one or more of the following mechanisms: (1) cleavage of ester interunit linkages to depolymerize lignin, (2) improved lignin solubility due to ionization of ferulic acid

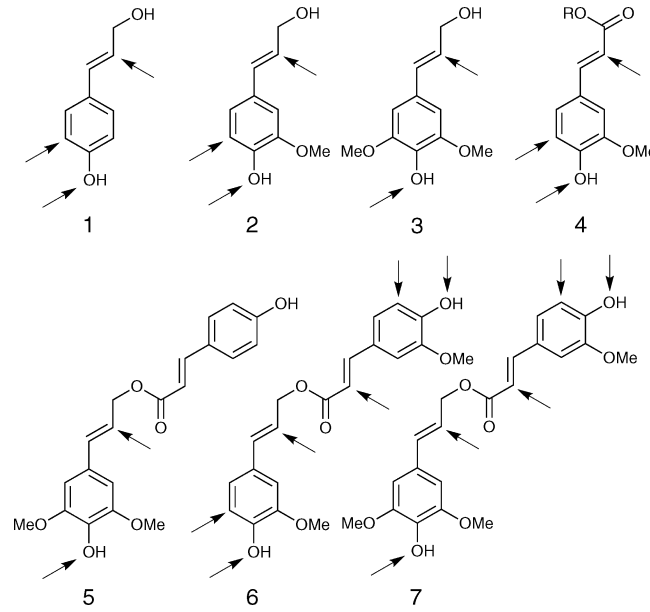

Figure 1. Structures of *p*-coumaryl alcohol **1**, coniferyl alcohol **2**, sinapyl alcohol **3**, ferulate **4**, sinapyl *p*-coumarate **5**, coniferyl ferulate **6**, and sinapyl ferulate **7**. Arrows indicate sites normally involved in radical coupling reactions during lignification.

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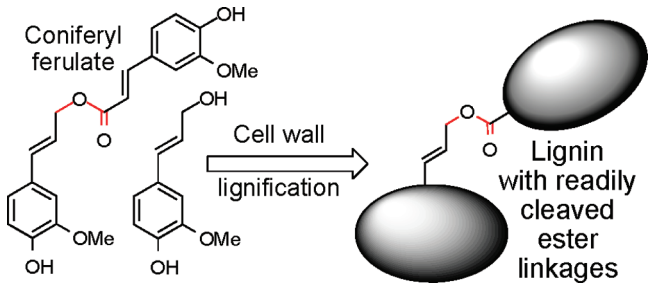

Figure 2. Copolymerization of coniferyl ferulate with monolignols to form lignin with ester-interunit linkages. Such linkages should facilitate lignin depolymerization during alkaline or acidic pretreatment of biomass for saccharification or paper production.

groups, and (3) a smaller inherent size of lignins due to a steady supply of ferulates acting as new initiation sites for polymerization. Such lignins likely would also degrade more readily by acidic or alkaline processes used to pretreat lignocellulosic biomass for saccharification and fermentation to ethanol.[17,18] Because of these perceived benefits, we used a well-developed biomimetic cell-wall model system[19] to test whether bioengineering of plants to incorporate coniferyl ferulate into lignin would enhance the delignification and enzymatic hydrolysis of cell walls.

Materials and Methods

Cell Wall Lignification. Freshly isolated primary cell walls (~1.05 g dry weight) from maize cell suspensions[20] were stirred in 120 mL of HOMOPIPES buffer (25 mM, pH 5.5 with 4 mM $CaCl_2$) and artificially lignified by adding separate solutions of lignin precursors (250 mg in 70 mL of 35% (v/v) dioxane/water) and H_2O_2 (30%, 225 μL in 90 mL water, ~1.4 equiv) at 3 mL/h. Precursor mixtures were comprised of coniferyl alcohol substituted with 0, 20, 40, or 60% (by weight) of coniferyl-ferulate. Precursor treatments were replicated by carrying out two independent runs of the experiment. As in a related study,[21] precursors were prepared in dioxane−water to maintain coniferyl ferulate in stable solutions. Lignin precursors were synthesized as described previously.[22,23] Nonlignified controls were stirred in a solvent mixture similar to the final makeup of the lignification reaction media. Cell wall peroxidase activity during lignification was monitored with guaiacol−H_2O_2 staining.[21] After additions were completed, cell walls were stirred for an additional 72 h before collection on glass-fiber filters (1.2 μm retention) and washed with water followed by acetone to remove nonincorporated lignin precursors. After evaporating off acetone in a hood, cell walls were dried at 55 °C and weighed. Filtrates were evaporated in vacuo to remove acetone and extracted with ethyl acetate to isolate nonincorporated precursors (and their coupling products). Ethyl acetate extracts were dried with anhydrous magnesium sulfate, filtered, evaporated in vacuo, and weighed. Nonincorporated precursors were then dissolved in DMSO and analyzed by ^{1}H NMR. In separate experiments, dehydrogenation polymers for use as analytical standards were formed in high yield (>94%) by slowly adding separate solutions of the aforesaid series of lignin precursors and H_2O_2 to stirred flasks containing HOMOPIPES buffer and horseradish peroxidase.

Cell Wall and Statistical Analyses. The alkaline solubility of lignins was determined by incubating cell walls at 30 °C for 24 h, 100 °C for 2.5 h, or 160 °C for 2.5 h in sealed Teflon vials under N_2 using 0.5 M aqueous NaOH added at 100 mL/g of cell walls. Anthraquinone (0.02 mg/mL) was added to catalyze the hydrolysis of lignin ether interunit linkages at 160 °C.[24] After cooling, alkali-insoluble residues were pelleted (5000 × *g*, 15 min), resuspended in water, neutralized with acetic acid, and then repeatedly pelleted (5000 × *g*, 15 min) and resuspended in water before freeze-drying and weighing. Alkaline hydrolysates recovered from cell walls (and from dehydrogenation polymers subjected to the same series of alkaline treatments) were scanned from 250 to 400 nm with a spectrophotometer. Alkaline hydrolysates were also extracted into ethyl acetate containing 50 mM tricaprylylmethyl ammonium chloride, dried, and dissolved in THF to determine the molecular weight distribution of extracted lignins by SEC-HPLC.[25] After adding 2-hydroxycinnamic acid as an internal standard, alkaline hydrolysates from the 30 °C incubations were acidified with HCl, extracted with ethyl ether, and silylated for GLC-FID analysis of ferulates and diferulates using previously determined response factors.[8] Response factors of ferulate-coniferyl alcohol dimers were assumed to be similar to diferulates.

Cell walls and alkali-insoluble residues were analyzed for lignin by the acetyl bromide method,[26] using dehydrogenation polymers as standards. Cell walls were also analyzed for acid-insoluble lignin by the Klason method.[27] Whole cell walls (~40 mg) were sonicated in 1−2 mL of DMSO-d_6 and subjected to gel-state NMR using a cryoprobe 750 MHz (DMX-750) Bruker Biospin (Rheinstetten, Germany) instrument as described by Kim et al.[28]

Original cell walls and alkali-insoluble residues collected from 30 °C alkaline incubations were suspended (0.5% w/v) in 20 mM MES buffer (pH 5.5, 40 °C) and hydrolyzed with a mixture of Celluclast 1.5 L, Viscozyme L (each added at 80 μL/g cell wall), and Biofeed Beta (added at 80 mg/g cell wall). This mixture of commercial enzymes (Novo Nordisk) was selected to provide a broad complement of cellulase, hemicellulase, and pectinase activities for degrading lignocellulosic material.[29] After 2 and 48 h of enzymatic hydrolysis, residues were pelleted by centrifugation (2 min, 10000 × *g*) and an aliquot of the supernatant was analyzed for uronosyls by a colorimetric method[30,31] and for neutral sugars by a Dionex BioLC.[32]

Data were analyzed according to a randomized complete block design with two replications using PROC GLM.[33] Means were subjected to pairwise comparisons by the LSD procedure when a significant *F*-test was detected at $P < 0.05$. Unless noted otherwise in the text, all reported differences were significant at $P < 0.05$.

Results and Discussion

Cell Wall Lignification. In this study, we polymerized varying proportions of coniferyl alcohol and coniferyl ferulate into nonlignified primary walls of maize via wall-bound peroxidase and exogenously supplied H_2O_2. Previous work has demonstrated that artificial lignins formed by this system are structurally similar to those naturally formed in grasses.[20] Based on mass-balance calculations, the incorporation of precursors into wall-bound lignin declined from 92 to 72% in the first and from 100 to 94% in the second run of the experiment as the proportion of coniferyl ferulate increased from 0 to 60%. While both runs were conducted in an identical manner, guaiacol staining (data not shown) revealed that poorer incorporation of precursors in the first run of the experiment was associated with lower cell wall peroxidase activity at the end of lignification. As noted in a previous study with a structurally related sinapyl *p*-coumarate ester,[21] depressed incorporation of precursors associated with coniferyl ferulate addition was related to an accelerated loss of cell wall peroxidase activity. Because nonbound apoplastic peroxidases were removed prior to artificial lignification,[20] peroxidase inactivation would be more markedly manifested in our model system than in plants.

Based on ^{1}H NMR analysis (data not shown), nonbound precursors recovered after lignification were ~1.4-fold enriched in ferulate compared to the original precursor mixture, indicating that coniferyl ferulate was incorporated somewhat less efficiently than coniferyl alcohol into wall bound lignins. Extensive copolymerization of coniferyl ferulate into cell wall lignins was, however, readily apparent by gel-state 2D NMR of whole cell walls (Figure 3) and from UV spectra of alkali-soluble lignins fully solubilized at 160 °C from cell walls (Figure 4).

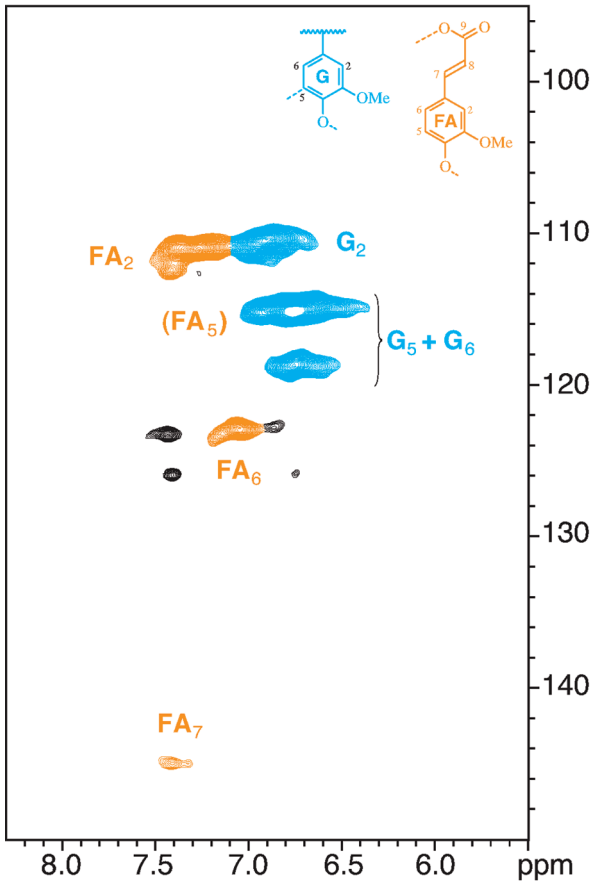

Figure 3. Aromatic $^{13}C-^{1}H$ correlation gel-state 2D NMR spectrum (HSQC) of maize cell walls artificially lignified with 40% coniferyl alcohol and 60% coniferyl ferulate. Correlations from ferulate (FA) units and guaiacyl (G) units (derived from coniferyl alcohol) were assigned using an NMR database.[42]

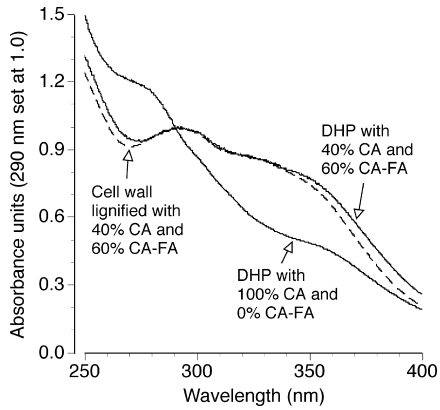

Figure 4. UV spectra of cell wall and dehydrogenation polymer (DHP) lignins prepared with coniferyl alcohol (CA) and coniferyl ferulate (CA-FA) and fully solubilized by 0.5 M aqueous NaOH at 160 °C.

The average mass-balance lignin content of cell walls declined numerically from 186 to 164 mg/g as the proportion of coniferyl ferulate increased (Table 1), but this trend was not statistically significant ($P = 0.21$). Due to the incorporation of matrix components into lignin,[20] cell walls lignified with coniferyl alcohol had higher Klason and acetyl bromide lignin concentrations than that predicted by mass balance calculations. The Klason and acetyl bromide methods also indicated a greater decline in lignin content due to coniferyl ferulate addition. For the acid-insoluble Klason method, this decline may be associated with ester cleavage and loss of free ferulic acid from lignins formed with coniferyl ferulate. The spectrophotometric acetyl-bromide method is sensitive to changes in lignin composition, including the presence of *p*-hydroxycinnamate esters on lignin.[34] To account for shifts in UV absorption coefficients in the acetyl bromide assay, we used dehydrogenation polymers prepared with 0−60% coniferyl ferulate as standards, but this may not fully account for spectral properties of lignin formed in cell walls.

Ferulate Composition of Cell Walls. As is typically done,[35] we incubated cell walls in aqueous NaOH near room temperature for 24 h to cleave and quantify ester-linked *p*-hydroxycinnamates in cell walls. Prior to artificial lignification, alkaline hydrolysis released 0.4 mg/g of *p*-coumarate, 8.4 mg/g of ferulate, and 4.7 mg/g of diferulates from nonlignified cell walls. Due to their extensive copolymerization into lignin by alkali-stable bonds,[8] extremely low levels of alkali-labile ferulate and diferulates were released from xylans in cell walls lignified with only coniferyl alcohol (Table 1). Lignifying cell walls with coniferyl ferulate dramatically increased the amount of ferulate and, to a lesser degree, diferulate released by alkali. Lignifying cell walls with coniferyl ferulate also considerably increased the quantity of alkali-labile ferulate cross-coupled to coniferyl alcohol. As noted previously with ferulate xylan esters,[9] 4-*O*-β cross-coupled dimers predominated over 8−β, and 5−β dimers (data not shown). Assuming similar GC response factors for dimers, it appears that comparable amounts of alkali-releasable ferulate underwent homocoupling into diferulates vs hetero-coupling into cross-coupled ferulate-coniferyl alcohol dimers (Table 1).

Following lignification with coniferyl alcohol, alkali released 9% of the ferulates linked to cell wall xylans as ferulate monomers, diferulates, or cross-product dimers (Table 1). As the quantity of coniferyl-ferulate increased from 20 to 60% of the precursor mixture, the proportion of alkali-labile ferulates derived from cell wall xylans and lignin-incorporated coniferyl ferulate increased from 12 to 18%. Fortuitously, the source of these alkali-labile ferulates can be estimated from (*Z*)-ferulate levels. Nonlignified maize cell walls contained ~1.6 mg/g of alkali-labile (*Z*)-ferulates (as monomers or coupled as (*E*,*Z*)-diferulates) in addition to the predominant (*E*)-ferulate isomers (data not shown). Because we used only the (*E*)-isomer of coniferyl ferulate to lignify cell walls, reductions in the quantity of (*Z*)-ferulates released by alkali can be used as a general indicator of ferulate xylan ester incorporation into lignin via alkali stable bonds. In cell walls lignified with coniferyl alcohol, 92% of (*Z*)-ferulate was incorporated into lignin, which corresponds closely to the 91% overall incorporation of all ferulate monomers and dimers into lignin. As the proportion of coniferyl ferulate increased from 0 to 60%, the incorporation of (*Z*)-ferulate into lignin dropped from 92 to 60%. If this decline is typical, then cell wall xylans contributed roughly 45% of the ferulate monomers, diferulates, and cross-product dimers released by alkali from cell wall lignified with coniferyl ferulate. Thus, adding coniferyl ferulate with monolignols disrupted ferulate xylan ester incorporation into lignin in a manner analogous to that observed with a structurally related conjugate, sinapyl *p*-coumarate.[21] These calculations also indicate that about 90% of the ferulate moieties in coniferyl ferulate were oxidatively coupled to lignin oligomers or polymers. Therefore, ferulate moieties in coniferyl ferulate readily copolymerized into lignin and their addition significantly reduced ferulate xylan cross-linking of cell walls.

Delignification of Cell Walls. Various treatments have been developed to delignify herbaceous or woody biomass for

Table 1. Concentrations (mg/g) of Lignin,[1] Alkali-Labile Ferulates,[2] and Total Ferulates[3] in Cell Walls[4]

	lignin			alkali-labile ferulates				
coniferyl ferulate (%)	mass	klason	AcBr	monomers	dimers	coupled	total	total ferulates
0	186.3	199.4[5,a]	203.0[a]	0.37[d]	0.66[d]	0.14[d]	1.17[d]	13.1[d]
20	165.9	173.5[ab]	183.1[b]	1.72[c]	1.22[c]	1.06[c]	4.00[c]	33.0[c]
40	164.8	168.9[b]	165.2[bc]	4.35[b]	2.57[b]	1.98[b]	8.92[b]	52.1[b]
60	163.9	157.1[b]	160.6[c]	6.57[a]	3.62[a]	2.77[a]	12.98[a]	72.2[a]

[1] Lignin content was estimated from the mass of monolignols polymerized into cell walls and by Klason and acetyl bromide (AcBr) analysis of cell walls. [2] Alkali-labile ferulate released in the form of ferulate monomers, ferulate dimers, and ferulate cross-coupled to coniferyl alcohol. [3] Total quantity of ferulates in lignified cell walls (esterified to cell wall xylans and incorporated into lignin as coniferyl ferulate). [4] From maize cell suspensions artificially lignified with coniferyl alcohol and 0−60% coniferyl ferulate. [5] Means within columns with unlike superscripts differ ($P < 0.05$).

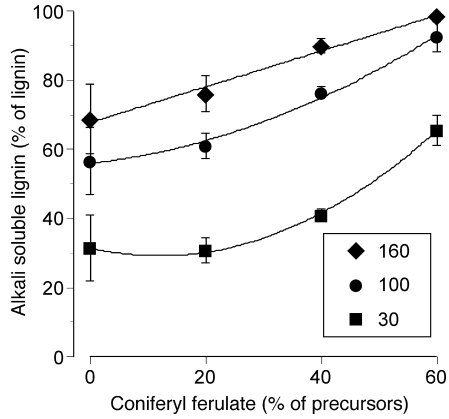

Figure 5. Release of alkali-soluble lignin by aqueous 0.5 M NaOH at 30, 100, or 160 °C from artificially lignified cell walls of maize prepared with 0 to 60% coniferyl ferulate. Bars indicate ± SEM.

fermentative processes or pulp production.[36−38] Because lignins containing ester-interunit linkages should be readily cleaved by alkali, we used 0.5 M aqueous NaOH to study how the incorporation of coniferyl ferulate affects cell-wall delignification. We chose a 30 °C treatment for 24 h (commonly used to quantify ester-linked ferulate in cell walls) to represent a mild alkaline pretreatment of biomass for ethanol fermentation.[36] To represent harsher biomass pretreatments and pulping conditions,[36−38] we used refluxing at 100 °C and cooking at 160 °C for 2.5 h. Refluxing at 100 °C should cleave recalcitrant ester-interunit linkages and fully solubilize nonbound lignins, while cooking at 160 °C for 2.5 h with anthraquinone should solubilize additional lignin by cleaving ether-interunit linkages. In these studies, NaOH concentrations, temperature, time, and anthraquinone levels were not optimized or meant to fully mimic potential commercial practices; conditions were selected to merely illustrate how coniferyl ferulate incorporation into lignin affects the ease of cell-wall delignification by alkali.

The proportion of lignin solubilized with aqueous 0.5 M NaOH increased with the severity of hydrolysis conditions and with the proportion of coniferyl ferulate used to form lignin (Figure 5). At 30 °C, alkali-soluble lignin, as a proportion of cell-wall lignin increased quadratically from 32 to 66% as coniferyl ferulate increased from 0 to 60% of precursors. At higher temperatures, coniferyl ferulate additions linearly increased alkali-soluble lignin from 57 to 93% at 100 °C, and from 69 to 99% at 160 °C. While alkaline extraction of about 70% of lignin required heating at 160 °C for cell walls lignified with coniferyl alcohol, heating at 100 °C sufficed if coniferyl ferulate comprised about 30% of lignin precursors. Alternatively, coniferyl ferulate addition also permitted more extensive delignification of cell walls at normal 160 °C cooking temperatures. Because coniferyl ferulate reduced the amount of lignin formed in cell walls, it had, however, less effect on the total quantity of alkali-soluble lignin released particularly at 160 °C (Table 2). Thus, in planta benefits of coniferyl ferulate may only be fully realized if enhanced alkaline depolymerization of lignin is combined with a reduced level of cell-wall lignification.

Increased severity of delignification and greater proportions of coniferyl ferulate reduced the molecular weight of lignin released from cell walls. As the severity of delignification conditions increased, the proportion of oligomeric (2.5−17.5 kDa) and polymeric (>17.5 kDa) lignins declined, while the proportion of trimers and smaller fragments (<0.6 kDa) increased (Figure 6). For all delignification treatments, incorporation of coniferyl ferulate into lignin mainly increased the proportion monomers and dimers (<0.4 kDa) and decreased the proportion of oligomeric (2.5−17.5 kDa) and polymeric (>17.5 kDa) lignins released from cell walls (Figure 7). Consequently, incorporation of an ester interunit linkage into lignin via coniferyl ferulate seemed to enhance alkaline depolymerization of lignin, leading to a greater release of lignin from cell walls. Conversely, ferulate's ability to act as an initiation site for lignin polymerization[9,39] could mean that continual coniferyl ferulate addition truncated polymerization to yield more numerous and smaller lignin chains than would be obtained with normal monolignols. In either case, cleavage of ester interunit linkages or a lower inherent size of polymers both would contribute to enhanced solubilization of cell wall lignins formed with coniferyl ferulate.

Due to greater lignin extractability and lower intrinsic lignin levels, the lignin content of alkali-insoluble residue (AIR) at each temperature dropped dramatically as the proportion of coniferyl ferulate increased (Figure 8). Thus, while a 160 °C alkaline hydrolysis of walls lignified with coniferyl alcohol yielded AIR with 123 mg/g of lignin, AIR with comparable lignin levels could be obtained at 100 °C with ∼30% coniferyl ferulate. Alternatively, heating cell walls lignified with ∼30% of coniferyl ferulate at 160 °C could yield AIR with much lower lignin concentrations. As a result, incorporation of coniferyl ferulate into lignin could provide the option of pulping at lower temperatures or pulping at high temperature with reduced cooking time, likely eliminating the need for bleaching.

Yields of alkali-soluble carbohydrate (ASC) increased and AIR decreased, as coniferyl ferulate comprised a greater proportion of lignin. (Table 2). The response of these fractions tended to be most pronounced at low to moderate levels of coniferyl ferulate addition and at higher hydrolysis temperatures. As hydrolysis temperatures increased to 160 °C, the recovery of AIR from nonlignified cell walls and cell walls lignified with ≥40% of coniferyl ferulate leveled off near the cellulose content of cell walls (∼250 mg/g).[29] Because coniferyl ferulate renders lignin more extractable by alkali, it would follow that pectin and hemicellulose extraction would improve as well. Indeed, lignin-degrading pretreatments are often used to improve the extractability of noncellulosic polysaccharides for analysis.[40] Hence, at a given temperature, delignification of cell walls

Table 2. Concentrations (mg/g) of Alkali Soluble Lignin, Alkali-Soluble Carbohydrate, and Alkali-Insoluble Residue[1]

	alkali-soluble lignin			alkali-soluble carbohydrate			alkali-insoluble residue		
coniferyl ferulate (%)	30 °C	100 °C	160 °C	30 °C	100 °C	160 °C	30 °C	100 °C	160 °C
0	66[2,b]	117[b]	141[a]	78[d]	226[d]	361[e]	856[a]	657[a]	497[a]
20	57[b]	113[b]	140[a]	142[c]	355[c]	456[d]	801[b]	532[b]	404[b]
40	68[b]	127[b]	149[a]	184[b]	440[b]	528[c]	749[c]	433[c]	324[bc]
60	106[a]	149[a]	159[a]	178[b]	476[b]	583[b]	716[c]	375[c]	259[c]
NL	ND	ND	ND	354[a]	632[a]	698[a]	646[d]	368[c]	302[c]

[1] Derived from maize cell walls treated with 0.5 M aqueous NaOH at 30, 100, or 160 °C. Nonlignified (NL) cell walls isolated from maize cell suspensions were artificially lignified with coniferyl alcohol and 0 to 60% coniferyl ferulate. ND, not determined. [2] Means within columns with unlike superscripts differ ($P < 0.05$).

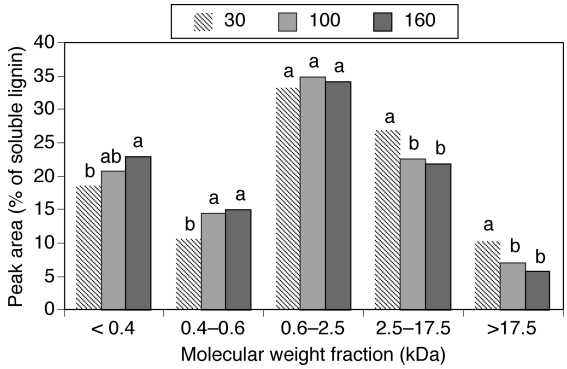

Figure 6. Molecular weight distribution of alkali-soluble lignins released from maize cell walls by aqueous 0.5 M NaOH at 30, 100, or 160 °C. Data are averaged over cell walls artificially lignified with 0−60% coniferyl ferulate. Means within a molecular weight group with unlike letters differ ($P < 0.05$).

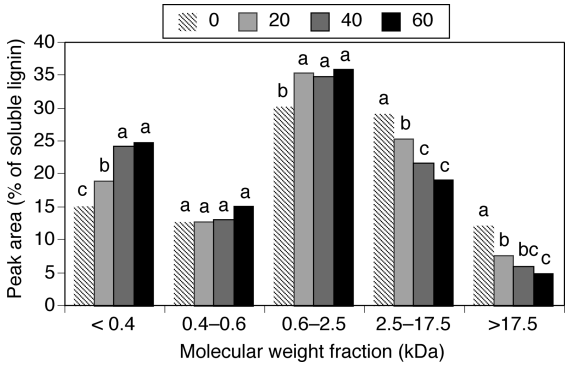

Figure 7. Molecular weight distribution of alkali-soluble lignins from artificially lignified cell walls of maize prepared with 0−60% coniferyl ferulate. Data are averaged over 0.5 M NaOH treatments at 30, 100, and 160 °C. Means within a molecular weight group with unlike letters differ ($P < 0.05$).

containing coniferyl ferulate yields AIR with less noncellulosic and, as mentioned above, less lignin contamination. Alternatively, coniferyl ferulate provides the option of delignifying cell walls under milder conditions to increase total fiber yields. For example, cell walls lignified with coniferyl alcohol yielded 497 mg/g of AIR at 160 °C compared to 749 mg/g of AIR at 30 °C for cell wall lignified with 40% coniferyl ferulate (Table 2); both types of AIR contained similar amounts of lignin (~130 mg/g, Figure 8).

Enzymatic Hydrolysis of Cell Walls and Alkali Insoluble Residues. Cell walls and alkali-insoluble residues were incubated with high loadings of fibrolytic enzymes to assess whether coniferyl ferulate incorporation into lignin enhances the rate, and above all, the extent of structural polysaccharide hydrolysis. The release of all sugars (i.e., glucose, arabinose, xylose, galactose, uronosyls) responded similarly to coniferyl ferulate incorporation into lignin; therefore, only total carbohydrate

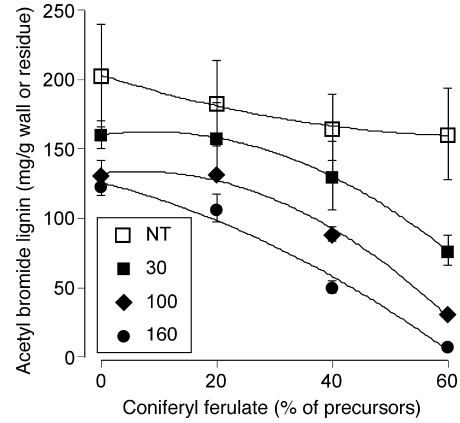

Figure 8. Impact of forming lignins with 0−60% coniferyl ferulate on the lignin content of nontreated (NT) cell walls and of alkali-insoluble residues recovered following hydrolysis at 30, 100, or 160 °C. Bars indicate ± SEM.

yields are reported and discussed below. Incorporation of coniferyl ferulate into lignin improved carbohydrate yields from both cell walls and AIR recovered following treatment with aqueous NaOH at 30 °C (Table 3). Prior to alkaline pretreatment, yields of carbohydrates from artificially lignified and nonlignified cell walls linearly increased as lignin content declined after both 2 and 48 h of enzymatic hydrolysis (Figure 9). Thus, coniferyl ferulate enhanced carbohydrate yields primarily by reducing the lignin content of cell walls. Even so, reduced cross-linking of lignin to feruloylated xylans with coniferyl ferulate additions could also play a role in enhancing cell wall hydrolysis.[29]

Pretreatment with NaOH dramatically improved the enzymatic hydrolysis of carbohydrates from AIR derived from all types of lignified cell walls (Table 3). The dramatic degradability response of grass cell walls to alkaline pretreatments has been mainly attributed to cleavage of cross-links between lignin and feruloylated xylans and to lignin extraction.[11] Coniferyl ferulate improved lignin extractability and carbohydrate yields from AIR, but yields quickly plateaued even as lignin levels continued to decline (Figure 9). Indeed, after both 2 and 48 h of enzymatic hydrolysis, maximal carbohydrate yields from lignified AIR were >100 mg/g lower than nonlignified AIR, indicating lignin content per se was not the only factor limiting cell wall hydrolysis. While cleavage of ferulate-lignin cross-links by alkali precludes their role in this phenomenon, alkali-resistant benzyl ether cross-links between lignin to polysaccharides could contribute to incomplete hydrolysis of AIR.[41]

On a cell wall basis, hydrolytic enzymes released a fairly constant proportion of carbohydrate from AIR derived from nonlignified and lignified cell walls (Table 3). If ASC are included, then total fermentable carbohydrate yields following alkaline pretreatment were greatest from nonlignified cell walls,

Table 3. Carbohydrate (mg/g) Released from Maize Cell Walls (CW) and Alkali-Insoluble Residues (AIR) by Enzymatic Hydrolysis, and Carbohydrate (mg/g) Released Enzymatically from AIR Plus Alkali-Soluble Carbohydrate (AIR+ASC)[1]

	2 h enzymatic hydrolysis				48 h enzymatic hydrolysis			
coniferyl ferulate (%)	CW	AIR	AIR[2]	AIR+ASC[2]	CW	AIR	AIR[2]	AIR+ASC[2]
0	$244^{a,c}$	460^{c}	393^{a}	471^{c}	521^{c}	671^{d}	574^{a}	652^{d}
20	294^{bc}	573^{bc}	459^{a}	602^{b}	551^{bc}	703^{cd}	563^{a}	706^{c}
40	325^{b}	663^{ab}	497^{a}	675^{b}	578^{b}	777^{bc}	582^{a}	766^{b}
60	351^{b}	692^{ab}	497^{a}	681^{b}	587^{b}	799^{b}	571^{a}	749^{bc}
NL	664^{a}	838^{a}	541^{a}	895^{a}	777^{a}	914^{a}	590^{a}	944^{a}

[1] Nonlignified (NL) cell walls isolated from maize cell suspensions were artificially lignified with coniferyl alcohol and 0−60% coniferyl ferulate. Cell walls and AIR prepared with aqueous 0.5 M NaOH at 30 °C were treated with commercial enzymes containing cellulase, hemicellulase, and pectinase activities. [2] Carbohydrate released on a whole cell wall basis. [3] Means within columns with unlike superscripts differ ($P < 0.05$).

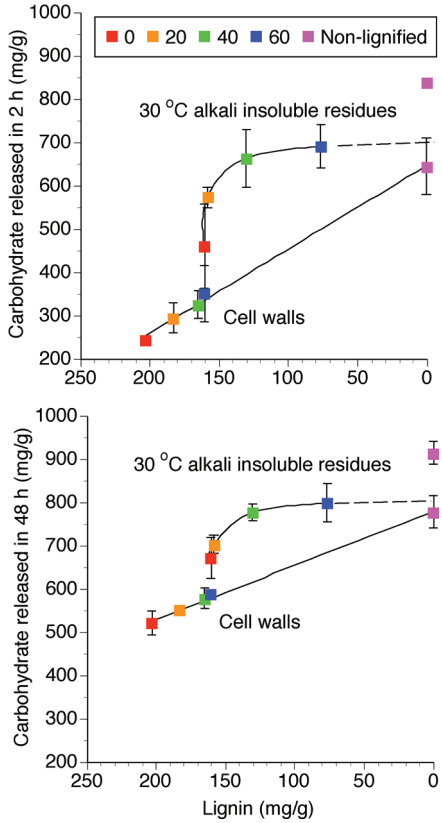

Figure 9. Relationship between lignin content and carbohydrate released after a 2 or 48 h enzymatic hydrolysis of cell walls (lignified with 0−60% coniferyl ferulate or nonlignified) and their alkali-insoluble residues prepared with 0.5 M NaOH at 30 °C. Bars indicate ± SEM.

intermediate from cell walls lignified with coniferyl ferulate, and lowest from cell walls lignified with coniferyl alcohol. Thus, the benefits of alkaline pretreatment and incorporation of alkali-labile coniferyl ferulate into grass lignins will only be fully realized if noncellulosic ASC are recovered and utilized for fermentation. This also indicates shifts in lignin alkaline solubility mainly alters the proportion of nondegradable carbohydrate versus ASC in cell walls without markedly changing the size of the degradable AIR fraction in cell walls. While not examined here, coniferyl ferulate incorporation into lignin could also reduce biomass conversion costs if lower enzyme loadings could be used for saccharification.

Conclusions

Based on our model studies, incorporation of coniferyl ferulate into graminaceous feedstocks should reduce lignification and permit more efficient delignification and enzymatic hydrolysis of cell walls. This in turn would reduce inputs for energy, pressure vessel construction, and bleaching during papermaking or lessen pretreatment or enzyme costs associated with biomass conversion. Comparable or greater benefits are anticipated for hardwoods, softwoods, and herbaceous dicots that have lower inherent lignin extractability. In preliminary studies, adding sinapyl ferulate with coniferyl and sinapyl alcohols had comparable effects on lignin formation, lignin extractability, and cell wall degradability. Therefore, we anticipate genetic engineering of plants to incorporate coniferyl ferulate into guaiacyl lignins in softwoods or coniferyl and sinapyl ferulates into mixed syringyl-guaiacyl-type lignins in hardwoods and herbaceous plants would greatly enhance the utilization of plant cell walls. Such plant engineering efforts are currently underway in our laboratories.

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References and Notes

(1) Ralph, J.; Lundquist, K.; Brunow, G.; Lu, F.; Kim, H.; Schatz, P. F.; Marita, J. M.; Hatfield, R. D.; Ralph, S. A.; Christensen, J. H.; Boerjan, W. *Phytochem. Rev.* **2004**, *3*, 29–60.

(2) Ralph, J.; Hatfield, R. D.; Quideau, S.; Helm, R. F.; Grabber, J. H.; Jung, H.-J. G. *J. Am. Chem. Soc.* **1994**, *116*, 9448–9456.

(3) Grabber, J. H.; Quideau, S.; Ralph, J. *Phytochemistry* **1996**, *43*, 1189–1194.

(4) Lu, F.; Ralph, J. 13th International Symposium on Wood, Fiber, and Pulping Chemistry, Auckland, New Zealand, May 16−19, 2005, APPITA: Auckland, New Zealand, 2005; pp 233−237.

(5) Hatfield, R. D.; Wilson, J. R.; Mertens, D. R. *J. Sci. Food Agric.* **1999**, *79*, 891–899.

(6) Oosterveld, A.; Grabber, J. H.; Beldman, G.; Ralph, J.; Voragen, A. G. *J. Carbohydr. Res.* **1997**, *300*, 179–181.

(7) Grabber, J. H.; Hatfield, R. D.; Ralph, J.; Zon, J.; Amrhein, N. *Phytochemistry* **1995**, *40*, 1077–1082.

(8) Grabber, J. H.; Ralph, J.; Hatfield, R. D. *J. Agric Food. Chem.* **2000**, *48*, 6106–6113.

(9) Grabber, J. H.; Ralph, J.; Hatfield, R. D. *J. Agric. Food Chem.* **2002**, *50*, 6008–6016.

(10) Ralph, J.; Helm, R. F.; Quideau, S.; Hatfield, R. D. *J. Chem. Soc., Perkin Trans. 1* **1992**, 2961–2969.

(11) Fahey, G. C., Jr.; Bourquin, L. D.; Titgemeyer, E. C.; Atwell, D. G. In *Forage Cell Wall Structure and Digestibility*; Jung, H. G., Buxton, D. R., Hatfield, R. D., Ralph, J., Eds.; Am. Soc. Agronomy: Madison, WI, 1993; pp 715−766.

(12) Paula, V. F.; Barbosa, L. C. A.; Howarth, O. W.; Demuner, A. J.; Cass, Q. B.; Vieira, I. J. C. *Tetrahedron* **1995**, *51*, 12453–12462.

(13) Seca, A. M. L.; Silva, A. M. S.; Silvestre, A. J. D.; Cavaleiro, J. A. S.; Domingues, F. M. J.; Pascoal-Neto, C. *Phytochemistry* **2001**, *56*, 759–767.
(14) Hsiao, J. J.; Chiang, H. C. *Phytochemistry* **1995**, *39*, 899–902.
(15) Li, S. L.; Lin, G.; Tam, Y. K. *Planta Med.* **2005**, *72*, 278–280.
(16) Baucher, M.; Halpin, C.; Petit-Conil, M.; Boerjan, W. *Crit. Rev. Biochem. Mol. Biol.* **2003**, *38*, 305–350.
(17) Dien, B. S.; Jung, H. J. G.; Vogel, K. P.; Casler, M. D.; Lamb, J. F. S.; Iten, L.; Mitchell, R. B.; Sarath, G. *Biomass Bioenergy* **2006**, *30*, 880–891.
(18) Murnen, H. K.; Balan, V.; Chundawat, S. P. S.; Bals, B.; Sousa, L. D.; Dale, B. E. *Biotechnol. Prog.* **2007**, *23*, 846–850.
(19) Grabber, J. H. *Crop Sci.* **2005**, *45*, 820–831.
(20) Grabber, J. H.; Ralph, J.; Hatfield, R. D.; Quideau, S.; Kuster, T.; Pell, A. N. *J. Agric. Food Chem.* **1996**, *44*, 1453–1459.
(21) Grabber, J. H.; Lu, F. *Planta* **2007**, *226*, 741–751.
(22) Lu, F.; Ralph, J. *J. Agric. Food Chem.* **1998**, *46*, 2911–2913.
(23) Lu, F.; Ralph, J. *J. Agric. Food Chem.* **1998**, *46*, 1794–1796.
(24) Kubes, G. J.; B. I., F.; MacLeod, J. M.; Bolker, H. I. *Wood Sci. Technol.* **1980**, *14*, 207–228.
(25) Majcherczyk, A.; Huttermann, A. *J. Chromatogr., A* **1997**, *764*, 183–191.
(26) Hatfield, R. D.; Grabber, J. H.; Ralph, J.; Brei, K. *J. Agric. Food Chem.* **1999**, *47*, 628–632.
(27) Hatfield, R. D.; Jung, H. G.; Ralph, J.; Buxton, D. R.; Weimer, P. J. *J. Sci. Food Agric.* **1994**, *65*, 51–58.
(28) Kim, H.; Ralph, J.; Akiyama, T., *Bioenerg. Res.* **2008**, *1*, 56–66.
(29) Grabber, J. H.; Ralph, J.; Hatfield, R. D. *J. Agric. Food Chem.* **1998**, *46*, 2609–2614.
(30) Blumenkrantz, N.; Asboe-Hansen, G. *Anal. Biochem.* **1973**, *54*, 484–489.
(31) Shea, E. M.; Hatfield, R. D. *J. Agric. Food Chem.* **1993**, *41*, 380–387.
(32) Hatfield, R. D.; Weimer, P. J. *J. Sci. Food Agric.* **1995**, *69*, 185–196.
(33) *SAS*, PC Windows Version 9.1.3; SAS Institute Inc.: Cary, NC, 2003.
(34) Fukushima, R. S.; Hatfield, R. D. *J. Agric. Food Chem.* **2004**, *52*, 3713–3720.
(35) Hartley, R. D.; Morrison III, W. H. *J. Sci. Food Agric.* **1991**, *55*, 365–375.
(36) Dien, B. S.; Iten, L. B.; Skory, C. D. *Handbook of Industrial Biocatalysis*; CRC Press LLC: Boca Raton, FL, 2005; pp 1−11.
(37) Shatalov, A. A.; Pereira, H. *Bioresour. Biotechnol.* **2005**, *96*, 865–872.
(38) Gratzl, J. S.; Chen, C. L. *Lignin: Historical, Biological, and Materials Perspectives*; ACS Symposium Series; American Chemical Society: Washington, DC, 2000; Vol. 742, pp 392−421.
(39) Ralph, J.; Grabber, J. H.; Hatfield, R. D. *Carbohydr. Res.* **1995**, *275*, 167–178.
(40) Selvendran, R. R.; Stevens, B. J. H.; O'Neill, M. A. In *Biochemistry of Plant Cell Walls*; Brett, C. T., Hillman, J. R., Eds.; Cambridge University Press: Cambridge, 1985; pp 39−78.
(41) Grabber, J. H.; Hatfield, R. D.; Ralph, J. *J. Agric. Food Chem.* **2003**, *51*, 4984–4989.
(42) Ralph, S. A.; Landucci, L. L.; Ralph, J. http://ars.usda.gov/Services/docs.htm?docid=10429, 2005.

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