JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Apoplastic pH and Monolignol Addition Rate Effects on Lignin Formation and Cell Wall Degradability in Maize

JOHN H. GRABBER,* RONALD D. HATFIELD, AND JOHN RALPH

U.S. Dairy Forage Research Center, Agricultural Research Service, U.S. Department of Agriculture, 1925 Linden Drive West, Madison, Wisconsin 53706

Monolignol polymerization rate and apoplastic pH and may influence the formation of lignin and its interactions in cell walls. Primary maize walls were artificially lignified by gradual "end-wise" or rapid "bulk" polymerization of coniferyl alcohol at pH 4 or 5.5. Lignification efficiency was greatest for end-wise polymers at pH 5.5 (90−98%), intermediate for bulk polymers formed at either pH (54−82%), and lowest for end-wise polymers at pH 4 (41−53%). End-wise polymers had about 2.2-fold more ether inter-unit linkages and 70% fewer end-groups than bulk polymers. Low pH enhanced the formation of ether linkages in end-wise but not in bulk polymers. Differences in lignin structure did not influence the enzymatic degradability of cell walls, but lowering apoplastic pH from 5.5 to 4.0 during lignification reduced cell wall degradability by 25%. Further studies indicated this pH-dependent depression in degradability was related to cell wall cross-links formed via lignin quinone methide intermediates.

KEYWORDS: ***Zea mays*; cell wall; apoplastic pH; Zulauf bulk polymers; Zutropf end-wise polymers; cross-linking; quinone methide intermediate; lignin-carbohydrate complex; thioacidolysis; enzymatic hydrolysis; degradability**

INTRODUCTION

The enzymatic degradation of fiber in grasses declines during plant maturation due to progressive lignification of the primary and secondary cell walls of xylem and sclerenchyma. As grasses reach physiological maturity, degradation is depressed even further by lignification of primary-walled parenchyma and epidermal tissues (*1*). In most cases, lignified primary walls are less degradable than secondary walls (*2*−*4*). These differences in degradability are partly due to the higher lignin content of primary walls; however, variations in three-dimensional structure of lignin have also been implicated (*5*).

Rapid "bulk" polymerization, as may occur in the middle lamella and primary wall, favors C−C coupling of monolignols into highly branched polymers (*6*−*8*). A branched polymer structure may enhance entrapment of structural polysaccharides by lignin, restricting access of hydrolytic enzymes into primary walls (*5*). In contrast, gradual "end-wise" polymerization, as may occur in secondary walls, favors $\beta-O-4$ coupling of monolignols into relatively linear polymers (*6*−*8*). Linear polymers are thought to permit greater access of hydrolytic enzymes into secondary walls (*5*). Apoplastic pH also influences lignin structure and its cross-linking to matrix components by altering the abundance and reactivity of quinone methide intermediates formed by $\beta-O-4$ coupling of monolignols (**Figure 1**). The pH of the apoplast varies from about 4 to 7,

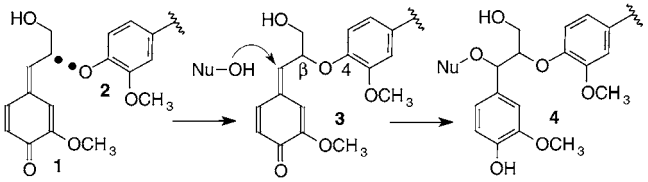

Figure 1. During lignification, monolignol **1** and lignin **2** radicals can undergo β–*O*–4 coupling to form quinone methide intermediates **3**. These intermediates are stabilized by the addition of hydroxyl groups from various nucleophiles (Nu) to form structures **4.** Neutral or slightly acidic conditions (pH > 5) enhance the reaction of quinone methide intermediates with phenols from monolignols or their coupling products to form lignins with a highly branched structure. Acidic conditions (pH < 5) favor the reaction of quinone methide intermediates with water, uronic acids, amino acids, and neutral sugars to form relatively linear lignins substituted with α-hydroxyl groups or cross-linked to matrix components by benzyl α-ester and α-ether linkages (*6, 10, 11, 28–32*).

but most often pH values between 5 and 6.5 are reported (*9*). To our knowledge, no studies have specifically examined the apoplastic pH of lignifying tissues. Studies of lignin structure suggest that the pH of some lignifying tissues may be at or below 4 (*10*).

Oxidative coupling of monolignols in vitro into dehydrogenation polymers (DHPs), in the presence or absence of cell-wall model compounds, have been extremely valuable for modeling the influence of monolignol addition rate and apoplastic pH on lignin structure and lignin-matrixinteractions (*11*−*13*). Unfortunately, DHPs differ structurally from natural plant

* To whom correspondence should be addressed. Tel.: (608) 264 5820. Fax: (608) 264 5147. E-mail jgrabber@wisc.edu.

10.1021/jf030027c
Published on Web 07/17/2003

lignins, and they do not adequately model the three-dimensional structure of lignified walls or provide a means of assessing how lignin-matrix interactions influence cell wall degradability. These limitations are largely overcome by forming DHP−cell wall (DHP−CW) complexes using in situ peroxidase (*14*). These complexes have been extremely valuable for elucidating oxidative coupling reactions of ferulate and its role in cell wall cross-linking and lignin formation in grasses (*15*, *16*). This model system also revealed how variations in ferulate cross-linking and lignin composition affect the enzymatic degradation of cell walls (*17*−*20*). Because DHP−CW complexes are formed with primary walls, our system most accurately models lignification of primary walls, which are more resistant to degradation than secondary walls. The importance of primary walls is also magnified because they act as barriers limiting the enzymatic hydrolysis of adjacent cells or tissues that are not directly exposed to degradative organisms. In this study, DHP−CW complexes were formed by in situ polymerization of coniferyl alcohol into primary maize walls to evaluate how the rate of monolignol addition and apoplastic pH affect the formation of lignin and the enzymatic degradability of cell walls.

MATERIALS AND METHODS

Preparation of DHP−CW Complexes. Cells from suspension cultures of maize (*Zea mays* L. cv Black Mexican) were ruptured by a Parr Nitrogen Bomb and washed sequentially with PIPES buffer (10 mM; pH 6.8), aqueous $CaCl_2$ (50 mM), and water to remove cytoplasmic debris and loosely bound peroxidases (*15*). In Experiment 1, 45 g of wet cell walls (1.3−1.5 g dry weight) were transferred to 500-mL Erlenmeyer flasks and stirred in 100 mL of HOMOPIPES buffer (50 mM; pH 4.0 or 5.5 with 1 mM $CaCl_2$). Cell wall suspensions were then artificially lignified by adding separate solutions of coniferyl alcohol (200 mg; 1.11 mmol) and H_2O_2 (1.33 mmol), each prepared in 70 mL of HOMOPIPES buffer (50 mM; pH 4.0 or 5.5). For bulk polymerizations, coniferyl alcohol solution was poured and stirred with cell wall suspensions for 30 min before H_2O_2 solution was added at a rate of 120 mL/h. For end-wise polymerizations, solutions of coniferyl alcohol and H_2O_2 were gradually added to cell wall suspensions at a rate of 3 mL/h. Nonlignified controls were stirred in Homopipes buffer (pH 4.0 or 5.5). During the course of lignification, the activity of cell wall peroxidase was monitored visually by adding a few drops of guaiacol (20 mM in 50 mM Homopipes, pH 5.5) and a few drops of dilute H_2O_2 to an aliquot of the cell wall suspension. After stirring for a total of 48 h, cell walls were collected on glass-fiber filters (1.2-μm porosity) and washed thoroughly with water to remove nonincorporated monomers and water-soluble oxidative coupling products of coniferyl alcohol. Walls were then washed with acetone to remove DHPs not incorporated into cell walls. In Experiment 2, similar methods were used to prepare end-wise DHP-CWs at pH 4 and 5.5 with coniferyl alcohol added at 25 to 200 mg/g of cell wall. In Experiment 3, similar methods were used to prepare end-wise DHP-CWs at pH 4 and 5.5, with coniferyl alcohol or coniferaldehyde added at 110−140 mg/g of cell wall.

DHP−CW Composition and Degradability. DHP−CW complexes (75 mg) from all experiments were hydrolyzed with H_2SO_4 and insoluble residues were collected to estimate Klason lignin without correction for acid soluble lignin (*21*). The enzymatic degradability of these complexes was estimated by incubating samples (50 mg) in acetate buffer (1% w/v; 20 mM; 40 °C; pH 4.8 with azide) with Celluclast 1.5 L and Viscozyme L (Novo Nordisk), each added at 40 μL/g cell wall (*20*). After 6 and 72 h of incubation, DHP−CWs were pelleted by centrifugation (10 min, 2500*g*) and an aliquot of the supernatant was analyzed by a colorimetric method for total carbohydrate using glucose as a standard (*22*).

One DHP−CW complex from each monolignol addition rate and pH treatment combination in Experiment 1 was further characterized by the following methods: Complexes were saponified at room temperature for 20 h with 2 M aq NaOH to release alkali-labile ferulate and diferulates for analysis by GC-FID (*15*). The quantity of ferulate and diferulates incorporated into lignin was estimated by the difference in alkali-labile acids recovered from nonlignified walls and DHP−CW complexes. Lignins in complexes were degraded by thioacidolysis and monomeric products were identified by GC-MS and quantified by GC-FID (*23*). Acetone filtrates collected after complex formation were evaporated in vacuo to recover DHPs not bound to cell walls. These extracts were surveyed by long-range correlative NMR (HMBC). Spectra were acquired using restricted acquisition in the ^{13}C dimension and a long-range coupling delay of 100 ms. DHP−CWs (150 mg) were hydrolyzed with Celluclast and Viscozyme as described above. Enzyme supernatants were analyzed by a colorimetric method for uronic acids (*24*) and hydrolyzed with TFA for analysis of neutral sugars by HPLC (*25*). Pellets remaining after 72 h of enzymatic hydrolysis were then incubated with a 20-fold greater concentration of Celluclast and Viscozyme for 40 h. After centrifugation (10 min, 2500*g*), wall residues were resuspended in 15 mL of TRIS buffer (200 mM, pH 7, with azide) and incubated at 40 °C with 6 mg of Pronase (Sigma) for 72 h. Indigestible wall residues were then pelleted and washed three times with water by centrifugation (10 min, 2500*g*), frozen, and freeze-dried. Complexes and their indigestible residues were hydrolyzed with H_2SO_4 and insoluble residues were collected to estimate Klason lignin without correction for acid soluble lignin (*21*). Sulfuric acid hydrolysates were analyzed for uronic acids by a colorimetric method (*24*) and for neutral sugars by HPLC (*25*). DHP−CWs and indigestible residues were also analyzed for total nitrogen by a combustion method (Leco 2000, Leco Instruments). All analyses were conducted in duplicate and standard deviations were typically less than 5%.

RESULTS AND DISCUSSION

Formation and Structure of DHP−CW Complexes. Wall-bound peroxidase and exogenously supplied hydrogen peroxide were used to polymerize coniferyl alcohol into nonlignified primary walls of maize. Previous work has demonstrated that lignins in DHP−CWs are structurally similar to natural lignins formed in grasses (*14*). In Experiment 1, DHP−CWs were formed at pH 4.0 or 5.5 by slow end-wise or rapid bulk polymerization of coniferyl alcohol to determine whether polymerization conditions influence lignin formation and cell wall degradability. Coniferyl alcohol not incorporated into wall-bound polymers was recovered by washing walls with water followed by acetone. Further washing with common lignin solvents such as 96:4 dioxane/water does not remove additional DHPs from walls (*14*). On the basis of mass balance calculations ($n = 3$), the incorporation of coniferyl alcohol into wall-bound bulk polymers ranged from 54 to 69% at pH 4 and from 58 to 82% at pH 5.5. Incorporation of end-wise polymers ranged from 41 to 53% at pH 4 compared to 90−98% at pH 5.5. Most nonincorporated coniferyl alcohol (>80%) was recovered from acetone filtrates as dehydrogenation products with ^{13}C- and ^{1}H NMR spectra characteristic of low molecular weight DHPs. However, following end-wise polymerization at pH 4, only 30% of the nonincorporated coniferyl alcohol was recovered as low molecular weight DHPs due to the apparent inactivation of peroxidase during prolonged lignification under acidic conditions. In contrast, peroxidase activity (based on guaiacol staining) remained high when bulk polymers were rapidly formed at pH 4 or when bulk and end-wise polymers were formed at pH 5.5. Although bulk or end-wise polymers were readily formed, the latter type of polymer incorporated into cell walls much more efficiently. This was particularly evident when cell walls are lignified at pH 5.5 with a 2.5-fold greater concentration of coniferyl alcohol (350 mg/g); incorporation of bulk polymers declined to 45%, while incorporation of end-wise polymers remained above 90% (J. H. Grabber, unpublished). In both cases, ample cell-wall peroxidase activity was detected after coniferyl alcohol addition. Following these high

Table 1. Cross-linked Ferulates,[a] Lignin,[b] and Ether-Linked Guaiacyl Units[c] in Primary Maize Walls Artificially Lignified at pH 4.0 or 5.5 by Rapid Bulk or Gradual End-Wise Polymerization of Coniferyl Alcohol

lignification conditions	cross-linked ferulates	lignin		guaiacyl units		
		Klason	mass	Klason	mass	end-groups
bulk polymerization						
pH 4.0	17.4 (78)	123.8	76.1	342	556	28.8
pH 5.5	17.0 (75)	126.4	81.6	347	537	26.2
end-wise polymerization						
pH 4.0	17.3 (78)	117.3	71.7	881	1441	9.5
pH 5.5	16.4 (86)	122.8	103.9	803	949	8.3

[a] Concentration (mg/g of cell wall) estimated as the difference in alkali–labile ferulates (monomers and dimers) recovered from lignified walls and nonlignified controls. Values in parentheses indicate the overall percentage of ferulates incorporated into lignin. [b] Concentration (mg/g of cell wall) estimated by the Klason lignin procedure or by the difference in mass between lignified walls and nonlignified controls. [c] Yield (μmol/g of lignin) of guaiacyl monomers released by thioacidolysis and proportion (%) of yield derived from end-groups.

rates of monolignol addition, Klason lignin concentrations of DHP−CWs reached only 165 mg/g for bulk polymers, compared to 270 mg/g for endwise polymers. Grass tissues with primary walls can undergo considerable lignification, reaching Klason lignin concentrations of 170 mg/g in primary walled pith parenchyma and 190−220 mg/g in secondarily thickened epidermis, sclerenchyma, and xylem tissues isolated from mature sorghum (*26*). In the latter group of tissues, concentrations of lignin are far greater in primary walls than in secondary walls (*3*). Therefore, if lignification starts as a bulk polymerization process, it must quickly transition to a more end-wise process to permit extensive lignification of cell walls. Extensive lignification of grass cell walls under acidic conditions would also be unlikely unless the secretion or acid tolerance of wall peroxidases exceeds that of our model system. Our preextraction of cell walls with 50 mM $CaCl_2$ prior to lignification may, however, have depleted walls of oxidative activity needed for prolonged and extensive lignification of cell walls under acidic conditions.

Selected DHP−CWs from Experiment 1 were subjected to a more detailed analysis of lignin and ferulates. Compared to mass balance calculations, the Klason procedure overestimated the lignin content of DHP−CWs by 18 to 62% (**Table 1**). Because cell contents were removed from walls prior to complex formation, Klason lignin levels were probably inflated by the incorporation of cell wall ferulates, structural polysaccharides, and proteins to artificial lignin (*15*).

The structure of lignins in these DHP−CWs was assessed by cleaving $\beta-O-4$ and $\alpha-O-4$ ether inter-unit linkages by thioacidolysis; the yield of monomeric degradation products indicates the frequency of these linkages in lignin. Yields of guaiacyl monomers recovered after thioacidolysis of end-wise polymers were about 2.3 times greater than those of bulk polymers, indicating that the former had a much greater proportion of $\beta-O-4$ and $\alpha-O-4$ ether linkages (**Table 1**). Under end-wise polymerization conditions, lignin would tend to grow by repeated $\beta-O-4'$ coupling of coniferyl alcohol (*27*), leading to high yields of thioacidolysis products. By contrast, bulk polymerization mainly involves monolignol dimerization and coupling to the growing lignin polymer by C−C linkages, yielding a low proportion of ether inter-unit linkages and low yields of thioacidolysis products (*27*). Expressing thioacidolysis data on a Klason or mass-balance lignin basis affected absolute yields, but not relative differences between bulk and end-wise polymerization treatments. As with in vitro DHP studies (*11*, *12*, *33*), the formation of ether inter-unit linkages was enhanced under acidic lignification conditions but only in DHP−CWs formed by end-wise polymerization. In HMQC NMR experiments, noncyclic $\alpha-O-4$ benzyl aryl ethers formed by the addition of coniferyl alcohol to quinone methide intermediates (*6*) were readily apparent in bulk but not in end-wise DHPs washed out of walls following lignification at pH 5.5 (data not shown). As expected, these structures were absent in bulk and end-wise polymers recovered after lignification at pH 4.0. Therefore the contribution of $\alpha-O-4$ ether-linked structures to thioacidolysis yields was probably minimal for all DHP−CWs except for bulk polymers formed at pH 5.5.

As with naturally formed lignins (*12*, *33*), end-wise DHP−CWs (**Table 1**) had a much lower proportion of ether-linked coniferyl alcohol end-groups (~9%) compared to bulk DHP−CWs (~27%). End-groups are abundant under bulk polymerization conditions, which favor coniferyl alcohol dimerization rather than end-wise addition of coniferyl alcohol to a growing lignin polymer. In contrast, DHPs formed in solution contain a high proportion of end groups (15−40%) regardless of whether they are formed under bulk or end-wise polymerization conditions (*12*, *33*). The markedly differing levels of end-groups, in addition to substantial differences in thioacidolysis yields, provides compelling evidence that end-wise and bulk polymers in DHP−CWs had vastly different structures. These results further support our claim (*14*) that DHP−CW complexes are the best system for modeling the lignification process within plant cell walls.

The concentration of ferulates (monomers plus dimers) in DHP−CWs selected from Experiment 1 ranged from 19.1 to 22.7 mg/g and their overall incorporation into lignin ranged from 75 to 86% (**Table 1**). Copolymerization of feruloylated xylans with monolignols leads to cross-linking of xylans to lignin (*15*). The quantity of ferulate cross-links formed in DHP−CWs was not markedly influenced by pH or the rate of monolignol addition. Oxidative coupling of wall ferulate and diferulates into lignin is initially very rapid but declines dramatically once their incorporation exceeds 70% (*15*). Depletion of readily coupled wall ferulate and diferulates may be one reason lignification was less efficient under bulk than under end-wise polymerization. At the initial stages of lignification, bulk and end-wise DHPs are probably anchored to cell walls only via oxidative cross-coupling with ferulate and diferulate xylan esters (*16*). Once readily available ferulate and diferulates are incorporated, further lignification of walls under bulk polymerization conditions would essentially stop, because oxidative coupling of newly formed dilignols or oligomers to wall-bound polymers is very inefficient (*27*). In the case of end-wise polymerization, cell wall lignification continues even after ferulate incorporation is complete, because monolignols readily couple to growing lignin polymers (*27*). Once attached to the wall matrix by ferulate, bulk and end-wise polymers probably become further anchored to cell walls by benzyl ester and ether cross-links (*28*−*32*) or other mechanisms, since alkaline hydrolysis of ferulate esters releases only a portion the lignin in cell walls (*18*).

Effect of Lignin Polymerization Conditions on DHP−CW Degradability. DHP−CWs were degraded with a mixture of Viscozyme L and Celluclast 1.5 L, commercial enzyme preparations selected to provide a full complement of pectinase, xylanase, and cellulase activities for degrading cell walls (*20*). Due to greater cell wall incorporation of coniferyl alcohol, lignin concentrations in Experiment 1 were greatest following end-wise polymerization at pH 5.5, but in all treatments, cell wall

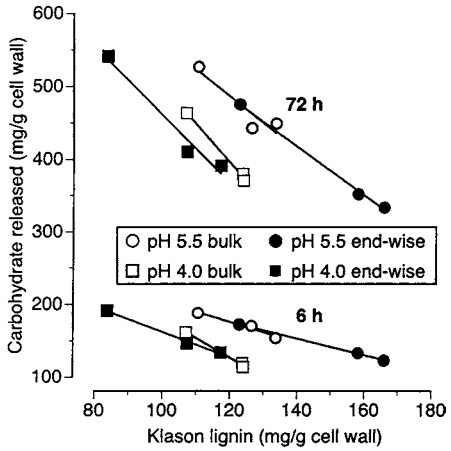

Figure 2. Carbohydrate released after 6 and 72 h of hydrolysis by Celluclast and Viscozyme (1:1) from primary maize walls artificially lignified at pH 4.0 or 5.5 by rapid bulk or gradual end-wise polymerization of coniferyl alcohol, added at 130 to 150 mg/g of cell wall.

Table 2. Carbohydrate Released[a] after 6 and 72 h of Hydrolysis by Celluclast and Viscozyme (1:1) from Primary Maize Walls Artificially Lignified at pH 4.0 or 5.5 by Rapid Bulk or Gradual End-Wise Polymerization of Coniferyl Alcohol

treatment	6 h	72 h
bulk polymerization		
pH 4.0	128	385
pH 5.5	172	473
end-wise polymerization		
pH 4.0	128	367
pH 5.5	181	487
analysis of variance[b]		
polymerization type	NS	NS
pH	*	*
polymerization type X pH	NS	NS
CV (%)	5.4	4.0

[a] Least-squares means (mg/g cell wall) adjusted to an average Klason lignin concentration of 120 mg/g of cell wall ($n = 3$). [b] *, NS = significant at the 0.05 level of probability and not significant, respectively.

degradability was negatively associated with lignin content (**Figure 2**). Statistical analysis of data, using Klason lignin concentration as a covariant, revealed that DHP−CW degradability was influenced by lignification pH but not by the mode of coniferyl alcohol polymerization (**Table 2**). Adjusting degradability data to account for differences in lignin content revealed that forming complexes at a pH of 4 instead of 5.5 reduced the release of sugars by 49 mg/g after 6 h and 104 mg/g after 72 h of enzymatic hydrolysis. In contrast, lignins of divergent structure, formed by bulk and end-wise polymerization, had similar effects on DHP−CW degradability. The latter findings are consistent with our earlier work demonstrating that artificial lignins formed with varying ratios of *p*-coumaryl, coniferyl and sinapyl alcohols have a similar impact on cell wall degradability (*17*), even though coniferyl and possibly *p*-coumaryl alcohols form more highly branched lignins than sinapyl alcohol (*13*).

As noted in previous studies with lignified primary walls from grasses (*19*, *26*), the release of xylose during enzymatic hydrolysis was much less than other cell wall sugars (**Table 3**). Poor hydrolysis of xylose-containing polysaccharides is due in part to ferulate and diferulate cross-linking of xylans to lignin (*19*). While variations in ferulate and diferulate cross-linking primarily affect xylan hydrolysis, acidic lignification pH uniformly depressed the release of all neutral sugars and uronic acids by hydrolytic enzymes. This suggests that the mechanism limiting hydrolysis was general in nature, not involving interactions with specific structural polysaccharides or variations in ferulate and diferulate cross-linking. It is also unlikely that ferulate cross-linking contributed to the differential effect of lignification pH on degradability, because all treatments had comparable amounts of ferulate monomers and dimers incorporated into lignin (**Table 1**). After exhaustive enzymatic hydrolysis, degradability differences between the pH 4 and 5.5 treatments were very small (ca. 25 mg/g), suggesting that the mechanism limiting hydrolysis directly involved a very small proportion of each cell wall sugar. As noted in other studies (*34*, *35*), a substantial proportion of lignin was solubolized during cell wall degradation.

Additional studies were pursued in an attempt to discover the mechanism by which lignification pH influences cell wall degradability. When cell walls were lignified over a wide range of coniferyl alcohol concentrations (Experiment 2), acidic lignification pH depressed wall degradability only as cell walls become hydrophobic with at least moderate amounts of lignin (**Figure 3**). Degradability differences between the pH 4 and 5.5 treatments were most consistently observed at an early stage (6 h) of cell wall hydrolysis. In Experiment 3, it was observed

Table 3. Chemical Composition[a] and Loss of Constituents[b] during Enzymatic Hydrolysis of Primary Maize Walls Artificially Lignified with Coniferyl Alcohol

	protein	arabinose	galactose	glucose	xylose	uronosyls	lignin	total
				chemical composition				
cell wall	30.1 ± 1.8	167.8 ± 2.1	74.4 ± 0.9	287.4 ± 0.2	136.9 ± 3.2	91.0 ± 1.0	122.6 ± 2.0	904.5 ± 2.2
				loss of constituents				
6-h hydrolysis								
lignified at pH 4.0	ND	19.8 ± 1.7	19.1 ± 1.7	19.0 ± 1.8	10.5 ± 1.1	26.5 ± 0.1	ND	ND
lignified at pH 5.5	ND	23.2 ± 0.4	22.6 ± 0.2	26.5 ± 1.5	15.1 ± 1.3	35.3 ± 3.3	ND	ND
72-h hydrolysis								
lignified at pH 4.0	ND	58.1 ± 0.7	58.5 ± 1.0	51.6 ± 1.9	36.1 ± 1.4	80.3 ± 0.1	ND	ND
lignified at pH 5.5	ND	71.8 ± 3.8	73.8 ± 0.3	66.1 ± 0.7	50.0 ± 1.8	82.9 ± 4.5	ND	ND
exhaustive hydrolysis								
lignified at pH 4.0	86.9 ± 0.1	76.2 ± 0.4	83.6 ± 0.6	87.3 ± 1.6	61.1 ± 0.9	91.6 ± 1.6	44.9 ± 1.2	75.8 ± 1.1
lignified at pH 5.5	82.6 ± 0.1	79.6 ± 0.8	85.8 ± 1.1	90.7 ± 1.1	63.6 ± 1.2	93.3 ± 0.3	42.1 ± 4.1	77.4 ± 0.3

[a] Average composition (mg g^{-1} ± SD) of cell walls lignified by bulk and end-wise polymerization at pH 4 and 5.5. [b] Sugars released (% ± SD) from cell walls after 6 and 72 h of incubation with Celluclast and Viscozyme (1:1). Wall residues were then exhaustively degraded with a 20-fold greater concentration of Celluclast and Viscozyme (40 h) followed by Pronase (72 h). Data were averaged over the bulk and end-wise polymerization treatments.

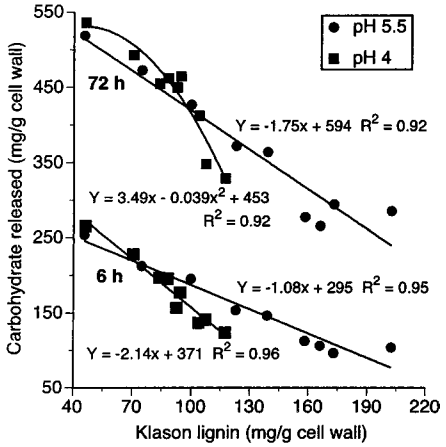

Figure 3. Carbohydrate released after 6 and 72 h of hydrolysis by Celluclast and Viscozyme (1:1) from primary maize walls artificially lignified at pH 4.0 or 5.5 by gradual end-wise polymerization of coniferyl alcohol, added at 25 to 200 mg/g of cell wall.

Table 4. Carbohydrate Released[a] after 6 and 72 h of Hydrolysis by Celluclast and Viscozyme (1:1) from Primary Maize Walls Artificially Lignified at pH 4.0 or 5.5 by Gradual End-Wise Polymerization of Coniferyl Alcohol or Coniferaldehyde

pH and monolignol	6 h	72 h
coniferyl alcohol		
pH 4.0	169	504
pH 5.5	259	520
coniferaldehyde		
pH 4.0	155	395
pH 5.5	161	377
	analysis of variance[b]	
monolignol	*	*
pH	NS	NS
monolignol X pH	*	NS
CV (%)	6.6	2.5

[a] Least-squares means (mg/g of cell wall) adjusted to an average Klason lignin concentration of 120 mg/g of cell wall ($n = 2$). [b] *, NS = significant at the 0.05 level of probability and not significant, respectively.

that acidic lignification pH depressed the degradability of DHP−CWs formed with coniferyl alcohol but not with coniferaldehyde, a structurally related monolignol precursor (**Table 4**). As in a previous study (*18*), the degradability of DHP−CWs formed with coniferaldehyde were lower than those formed with coniferyl alcohol, due to its greater hydrophobicity. Although coniferaldehyde undergoes similar types of coupling reactions as coniferyl alcohol, $\beta-O-4$ coupling of coniferaldehyde does not permit cross-linking of matrix components via nucleophilic addition to quinone−methide intermediates (*36*−*38*). Cross-links of this type are most readily formed under hydrophobic and acidic lignification conditions (*28*−*32*)—the same conditions that depressed the degradability of DHP−CWs formed with coniferyl alcohol. These results indicate that poor degradability of DHP−CWs formed under low pH is related to reactions of lignin quinone methide intermediates, possibly involving benzyl α-ester cross-links with acid groups on uronosyl and benzyl α-ether cross-links with hydroxyl groups on neutral sugars. Additional studies are, however, needed to confirm the existence of these cross-links in our model system and to assess whether acidic pH increases their abundance in cell walls.

LITERATURE CITED

(1) Akin, D. E. Histological and physical factors affecting digestibility of forages. *Agron. J.* **1989**, *81*, 17−24.

(2) Chesson, A.; Stewart, C. S.; Dalgarno, K.; King, T. P. Degradation of isolated grass mesophyll, epidermis, and fibre cell walls in the rumen and by celluloytic rumen bacteria in axenic culture. *J. Applied Bacteriol.* **1986**, *60*, 327−336.

(3) Engels, F. M.; Schuurmans, J. L. L. Relationship between structural development of cell walls and degradation of tissues in maize stems. *J. Sci. Food Agric.* **1992**, *59*, 45−51.

(4) Wilson, J.; Hatfield, R. Structural and chemical changes of cell wall types during development: Consequences for fibre degradation by rumen microflora. *Aust. J. Agric. Res.* **1997**, *48*, 165−180.

(5) Jung, H. G.; Deetz, D. A. Cell wall lignification and degradability. In *Forage Cell Wall Structure and Digestibility*; Jung, H. G., Buxton, D. R., Hatfield, R. D., Ralph, J., Eds.; ASA-CSSA-SSSA: Madison, WI, 1993; pp 315−346.

(6) Quideau, S.; Ralph, J. A Biomimetic route to lignin model compounds via silver (i) oxide oxidation. 1. Synthesis of dilignols and noncyclic benzyl aryl ethers. *Holzforschung* **1994**, *48*, 12−22.

(7) Burlat, V.; Ambert, K.; Ruel, K.; Joseleau, J. P. Relationship between the nature of lignin and the morphology of degradation performed by white-rot fungi. *Plant Physiol. Biochem.* **1997**, *35*, 645−654.

(8) Jurasek, L. Experimenting with virtual lignins. In *Lignin and Lignan Biosynthesis*; Lewis, N. G., Sarkanen, S., Eds.; American Chemical Society: Washington, DC, 1998; pp 276−293.

(9) Grignon, C.; Sentenac, H. pH and ionic conditions in the apoplast. In *Annual Review of Plant Physiology and Plant Molecular Biology*; Briggs, W. R., Jones, R. L., Walbot, V., Eds.; Annual Reviews Inc.: Palo Alto, CA, 1991; pp 103−128.

(10) Brunow, G.; Sipilä, J.; Mäkelä, T. On the mechanism of formation of noncyclic benzyl ethers during lignin biosynthesis. Part 1: The reactivity of $\beta-0-4$ quinone methides with phenols and alcohols. *Holzforschung* **1989**, *43*, 55−59.

(11) Terashima, N.; Atalla, R. H. *Formation and structure of lignified plant cell wall-factors controlling lignin structure during its formation.* KCL.; Vol. I, 1995. p 69−76.

(12) Terashima, N.; Atalla, R. H.; Ralph, S. A.; Landucci, L. L.; Lapierre, C.; Monties, B. New preparations of lignin polymer models under conditions that approximate cell wall lignification. II. Structural characterization of the models by thioacidolysis. *Holzforschung* **1996**, *50*, 9−14.

(13) Saake, B.; Argyropoulos, D. S.; Beinhoff, O.; Faix, O. A comparison of lignin polymer models (DHPs) and lignins by ^{31}P NMR spectroscopy. *Phytochemistry* **1996**, *43*, 499−507.

(14) Grabber, J. H.; Ralph, J.; Hatfield, R. D.; Quideau, S.; Kuster, T.; Pell, A. N. Dehydrogenation polymer-cell wall complexes as a model for lignified grass walls. *J. Agric. Food Chem.* **1996**, *44*, 1453−1459.

(15) Grabber, J. H.; Ralph, J.; Hatfield, R. D. Cross-linking of maize walls by ferulate dimerization and incorporation into lignin. *J. Agric Food. Chem.* **2000**, *48*, 6106−6113.

(16) Grabber, J. H.; Ralph, J.; Hatfield, R. D. Model studies of ferulate-coniferyl alcohol cross-product formation in primary maize walls: Implications for lignification in grasses. *J. Agric. Food Chem.* **2002**, *50*, 6008−6016.

(17) Grabber, J. H.; Ralph, J.; Hatfield, R. D.; Quideau, S. *p*-Hydroxyphenyl, guaiacyl, and syringyl lignins have similar inhibitory effects on wall degradability. *J. Agric. Food Chem.* **1997**, *45*, 2530−2532.

(18) Grabber, J. H.; Ralph, J.; Hatfield, R. D. Severe inhibition of maize wall degradation by synthetic lignins formed with coniferaldehyde. *J. Sci. Food Agric.* **1998**, *78*, 81−87.

(19) Grabber, J. H.; Ralph, J.; Hatfield, R. D. Ferulate cross-links limit the enzymatic degradation of synthetically lignified primary walls of maize. *J. Agric. Food Chem.* **1998**, *46*, 2609−2614.

(20) Grabber, J. H.; Hatfield, R. D.; Ralph, J. Diferulate cross-links impede the enzymatic degradation of nonlignified maize walls. *J. Sci. Food Agric.* **1998**, *77*, 193−200.

(21) Hatfield, R. D.; Jung, H. G.; Ralph, J.; Buxton, D. R.; Weimer, P. J. A comparison of the insoluble residues produced by the Klason lignin and acid detergent lignin procedures. *J. Sci. Food Agric.* **1994**, *65*, 51−58.
(22) Dubois, M.; Giles, K. A.; Hamilton, J. K.; Rebers, P. A.; Smith, F. Colorimetric method for determination of sugars and related substances. *Anal. Chem.* **1956**, *28*, 350−356.
(23) Lapierre, C. Application of new methods for the investigation of lignin structure. In *Forage Cell Wall Structure and Digestibility*; Jung, H. G., Buxton, D. R., Hatfield, R. D., Ralph, J., Eds.; ASA-CSSA-SSSA: Madison, WI, 1993; pp 133−166.
(24) Blumenkrantz, N.; Asboe-Hansen, G. New method for quantitative determination of uronic acids. *Anal. Biochem.* **1973**, *54*, 484−489.
(25) Hatfield, R. D.; Weimer, P. J. Degradation characteristics of isolated and in situ cell wall lucerne pectic polysaccharides by mixed ruminal microbes. *J. Sci. Food Agric.* **1995**, *69*, 185−196.
(26) Hatfield, R. D.; Wilson, J. R.; Mertens, D. R. Composition of cell walls isolated from cell types of grain sorghum stems. *J. Sci. Food Agric.* **1999**, *79*, 891−899.
(27) Sarkanen, K. V. Precursors and their polymerization. In *Lignins, Occurrence, Formation, Structure, and Reactions*; Sarkanen, K. V., Ludwig, C. H., Eds.; Wiley-Interscience: New York, 1971; pp 95−163.
(28) Ralph, J.; Young, R. A. Stereochemical aspects of addition reactions involving lignin model quinone methides. *J. Wood Chem. Technol.* **1983**, *3*, 161−81.
(29) Sipilä, J.; Brunow, G. On the mechanism of formation of noncyclic benzyl ethers during lignin biosynthesis. Part 4. The reactions of a $\beta-O-4$-type quinone methide with carboxylic acids in the presence of phenols. The formation and the stability of benzyl esters between lignin and carbohydrates. *Holzforschung* **1991**, *45*, 9−14.
(30) Toikka, M.; Sipilä, J.; Teleman, A.; Brunow, G. Lignin-carbohydrate model compounds. Formation of lignin-methyl arabinoside and lignin-methyl galactoside benzyl ethers via quinone methide intermediates. *J. Chem. Soc., Perkin Trans. 1* **1998**, 3813−3818.
(31) Toikka, M.; Brunow, G. Lignin-carbohydrate model compounds. Reactivity of methyl 3-*O*-(α-l-arabinofuranosyl)-β-D-xylopyranoside and methyl β-D-xylopyranoside towards a $\beta-O-4-$ quinone methide. *J. Chem. Soc., Perkin Trans. 1* **1999**, 1877−1883.
(32) Kishimoto, T.; Ikeda, T.; Karlsson, O.; Magara, K.; Hosoya, S. Reactivity of secondary hydroxyl groups in methyl β-D-xylopyranoside toward a $\beta-O-4$-type quinone methide. *J. Wood Sci.* **2002**, *48*, 32−37.
(33) Tollier, M.-T.; Lapierre, C.; Monties, B.; Francesch, C.; Rolando, C. *Structural variations in synthetic lignins (DHPs) according to the conditions of their preparation;* Proceedings of the Sixth International Symposium of Wood and Pulping Chemistry, Melbourne, Australia. 1991; Vol 2, p 35−41.
(34) Conchie, J.; Hay, A. J.; Lomax, J. A. Soluble lignin-carbohydrate complexes from sheep rumen fluid: Their composition and structural features. *Carbohydr. Res.* **1988**, *177*, 127−151.
(35) Grabber, J. H.; Jung, G. A. In-vitro disappearance of carbohydrates, phenolic acids, and lignin from parenchyma and sclerenchyma cell walls isolated from cocksfoot. *J. Sci. Food Agric.* **1991**, *57*, 315−323.
(36) Connors, W. J.; Chen, C.-L.; Pew, J. C. Enzymic dehydrogenation of the lignin model coniferaldehyde. *J. Org. Chem.* **1970**, *35*, 1920−1924.
(37) Higuchi, T.; Ito, T.; Umezawa, T.; Hibino, T.; Shibata, D. Red-brown color of lignified tissues of transgenic plants with antisense CAD gene: Wine-red lignin from coniferyl aldehyde. *J. Biotechnol.* **1994**, *37*, 151−158.
(38) Kim, H.; Ralph, J.; Lu, F.; Ralph, S. A.; Boudet, A. M.; MacKay, J. J.; Sederoff, R. R.; Ito, T.; Kawai, S.; Ohashi, H.; Higuchi, T. NMR analysis of lignins in CAD-deficient plants. 1. Incorporation of hydroxycinnamyl and hydroxybenzyl aldehydes into lignins. *Org. Biomol. Chem.* **2003**, *1*, 1−15.

Received for review January 15, 2003. Revised manuscript received May 19, 2003. Accepted May 20, 2003. Mention of a trademark or proprietary product does not constitute a guarantee or warranty of the product by the USDA and does not imply its approval to the exclusion of other products that may also be suitable.

JF030027C