Dehydrogenation Polymer−Cell Wall Complexes as a Model for Lignified Grass Walls

John H. Grabber,*,† John Ralph,† Ronald D. Hatfield,† Stéphane Quideau,†,‡ Thomas Kuster,§ and Alice N. Pell||

U.S. Dairy Forage Research Center, Agricultural Research Service, U.S. Department of Agriculture, and Forest Products Laboratory, Madison, Wisconsin 53706, and Animal Science Department, Cornell University, Ithaca, New York 14853

p-Hydroxycinnamyl alcohols were efficiently polymerized into maize walls by wall-bound peroxidases and *in vitro* generated hydrogen peroxide to produce dehydrogenation polymer−cell wall (DHP−CW) complexes. Electron microscopy of $KMnO_4$-stained sections revealed that DHPs were distributed throughout the cell wall matrix. DHP−CW complexes were structurally similar to natural grass lignins according to pyrolysis, thioacidolysis, and ^{13}C-NMR spectroscopy. Nonlignified walls were rapidly degraded by rumen microorganisms and by commercial fungal hydrolases, whereas DHP−CW complexes had a reduced rate and extent of degradation. This system is useful for modeling matrix interactions in lignified walls and for identifying means of improving the utilization of lignocellulosic materials for nutritional and industrial purposes.

Keywords: *Gramineae; Zea mays; cell wall; fiber; peroxidase; p-hydroxycinnamyl alcohols; lignin; dehydrogenation polymer; transmission electron microscopy; pyrolysis; thioacidolysis; NMR; fiber degradability*

INTRODUCTION

Lignification enhances the ability of the plant cell wall to resist mechanical stress and penetration by plant pathogens (Iiyama *et al.*, 1994). Lignin also restricts the degradation of cell walls by hydrolases, limiting the bioconversion of structural polysaccharides into metabolizable energy for livestock or into ethanol for liquid fuel production (Brown, 1985; Jung and Deetz, 1993). Recent evidence suggests that lignified dietary fiber reduces the incidence of gastrointestinal cancer in humans (Kritchevsky, 1991). The effect of lignin on cell wall properties is thought to be mediated by the hydrophobicity of lignin and its incrustation and attachment to structural polysaccharides and proteins. The extent and importance of these interactions in affecting wall properties is poorly understood.

The biosynthesis, structure, and biodegradation of lignin has been modeled *in vitro* by dehydrogenation polymers (DHPs) generated by oxidative polymerization of *p*-hydroxycinnamyl alcohols (Freudenberg, 1968; Kirk *et al.*, 1975; Gagnaire and Robert, 1977; Lewis *et al.*, 1987a). DHPs have also been formed in the presence of carbohydrates, feruloylated oligosaccharides, or protein to investigate how cell wall components are cross-linked to lignin and affect lignin structure (Higuchi *et al.*, 1971; Terashima and Seguchi, 1988; Evans and Himmelsbach, 1991; Ohnishi *et al.*, 1992; Ralph *et al.*, 1992). Synthetic lignins formed under these conditions differ structurally from natural plant lignins (Erdtman, 1957; Lewis *et al.*, 1987b; Tollier *et al.*, 1991), and they do not adequately model the three-dimensional structure of lignified walls. These limitations are overcome in part by forming DHP−cell wall (DHP−CW) complexes using *in situ* peroxidases. Whitmore first used this approach for studying lignin−protein and lignin−carbohydrate interactions in cell walls isolated from callus cultures of *Pinus elliottii* (Whitmore, 1978, 1982). Analysis by IR and UV spectroscopy suggested that DHP−CW complexes were structurally similar to lignified walls in *P. elliottii* seedlings. Our objective was to further develop and evaluate DHP−CW complexes as a model for studying lignification of grass walls.

MATERIALS AND METHODS

Maize cell suspensions (*Zea mays* L. cv. Black Mexican) were grown in 1 L flasks with 300−400 mL of medium (Kieliszewski and Lamport, 1987). Cells were routinely subcultured every 10 days to an initial packed cell volume of 3%. For DHP−CW studies, cultures were grown for 16 days to a packed cell volume of *ca.* 45% (early stationary growth phase). Cells were collected on a nylon mesh (20 μm pore size), resuspended in an equal volume of 25 mM HEPES buffer (pH 7.0 at 4 °C), and ruptured by rapid decompression from a Parr nitrogen bomb maintained at 1500 psi. Cell walls were washed extensively with cold HEPES buffer to remove cytoplasmic debris.

Peroxidases in Maize Walls. Cell walls [*ca.* 1 g dry weight (dw)] were washed with 50 mM NaCl and then extracted overnight in 40 mL of 3 M LiCl at 4 °C. After extraction, cell walls were collected on three layers of miracloth and washed with 3 M LiCl. The filtrate was dialyzed three times against 1000 mL of 10 mM Tris-HCl buffer (pH 7.0 at 4 °C) over a 24 h period. Dialysate was clarified by centrifugation (15000*g*, 20 min) before being concentrated with an ultrafiltration cell (Amicon, 50 mL cell) fitted with a YM 10 filter. Peroxidase isozymes were separated using agarose isoelectric focusing gels (FMC Bioproducts, pH 3−10, 10 × 10 cm) run on an Isobox Flatbed system (Hoefer Scientific). Samples were applied to gels using application wicks and prefocused for 20 min at 1 W of constant power. Wicks were removed and gels focused at constant power (10 W) until the current remained constant (60−90 min). The distribution of

* Author to whom correspondence should be addressed (e-mail jgrabber@facstaff.wisc.edu).

† U.S. Dairy Forage Research Center.

‡ Present address: Chemistry Department, The Pennsylvania State University, University Park, PA 16802.

§ Forest Products Laboratory.

|| Cornell University.

S0021-8561(95)00271-8 CCC: $12.00

peroxidase activity among isozymes was determined by staining gels with diaminobenzidine (Harlow and Lane, 1988).

Formation of DHP−CW Complexes. Cell walls were suspended two times for 10 min in 100 mM $CaCl_2$ and washed with water to remove loosely associated peroxidases. After excess water was drained, subsamples of cell walls were dried at 55 °C to estimate dry matter content. For complex formation, 35 g of cell walls (*ca.* 1 g dw) was suspended in 75 mL of 50 mM PIPES buffer (pH 6.5, plus 2 mM $CaCl_2$) with 1000−1500 units of glucose oxidase (type VII-S, Sigma). Wall suspensions were lignified by adding a PIPES buffer containing 50−200 mg of *p*-hydroxycinnamyl alcohols (Quideau and Ralph, 1992) and glucose (1.25 equiv) with a peristaltic pump over a 24−48 h period. *p*-Hydroxycinnamyl alcohols were polymerized into cell walls by wall-bound peroxidases and hydrogen peroxide generated *in vitro* by glucose oxidase-mediated conversion of glucose to gluconic acid (Tollier *et al.*, 1991). Nonlignified controls were incubated in PIPES buffer without added *p*-hydroxycinnamyl alcohols or glucose. A few drops of toluene were added periodically to inhibit microbial growth. The flasks were kept in the dark and mixed continuously with a magnetic stir bar for 3 days. The activity of peroxidase and glucose oxidase during lignification was monitored by adding a few drops of guaiacol (20 mM in 50 mM PIPES, pH 6.5) and a few grains of glucose to an aliquot of the cell wall suspension. After incubation, cell walls were collected on glass fiber filters (1.2 μm retention), washed with water followed by acetone, oven-dried at 55 °C, and weighed. The lignin content of DHP−CW complexes was estimated according to a modified Klason lignin procedure (Theander and Westerlund, 1986).

The efficiency of complex formation was evaluated by lignifying several batches of maize walls (*ca.* 800 mg dw) with 120 mg of coniferyl alcohol. The DHP−CW complexes were collected on glass fiber filters, washed with water followed by acetone, and dried at 55 °C. Filtrate was evaporated *in vacuo* to remove acetone, acidified (pH 4.5), saturated with NaCl, and extracted with ethyl acetate to isolate dehydrogenation products which were not bound to cell walls. Extracts were dried with anhydrous sodium sulfate, filtered, and evaporated *in vacuo.* Extracts were analyzed by ^{1}H NMR and GC−MS (Ralph *et al.*, 1994b).

Characterization of DHP−CW Complexes. Equimolar concentrations of coniferyl and sinapyl alcohols were polymerized into nonlignified cell walls to form DHP−CW complexes with Klason lignin concentrations of *ca.* 120 mg g^{-1}. Nonlignified walls and DHP−CW complexes were characterized according to the following methods.

Transmission Electron Microscopy. Samples were treated with 1% w/v aqueous potassium permanganate for 1 h at room temperature to stain lignin and other phenolic materials. After washing with water, samples were dehydrated in ethanol, embedded in Quetol 651 resin, and thin sectioned (Nilsson *et al.*, 1989). Sections were viewed and photographed using a Hitachi H-300 transmission electron microscope.

Degradative Analyses of Lignins. Samples were analyzed by pyrolysis−GC−MS (Ralph and Hatfield, 1991). Ether interunit linkages in lignin were cleaved by thioacidolysis, and degradation products were identified by GC−MS and quantified by GC (Lapierre *et al.*, 1985, 1991). For comparative purposes, cell walls from bromegrass stems (Ralph and Hatfield, 1991) and maize rinds (Ralph *et al.*, 1994a) and a DHP were characterized by pyrolysis and thioacidolysis. The DHP was formed in 80% yield from 200 mg of coniferyl alcohol and 230 mg of sinapyl alcohol according to methods described in Ralph *et al.* (1992).

^{13}C NMR of Isolated Lignins. DHP−CW complexes were degraded with crude cellulases (Ralph *et al.*, 1994a), and residues were extracted with 96:4 dioxane/water to isolate lignin which was not covalently bound to cell walls. Residues then were dried, vibratory ball milled for 24 h, and re-extracted with dioxane/water to isolate a wall-bound lignin fraction. This fraction was suspended in 3 mM EDTA (pH 8.0), collected on a nylon membrane (2 μm retention), and washed with water to remove carbohydrate and metal ion contaminants. Proton-decoupled ^{13}C-NMR spectra of DHP−CW and DHP lignins and of lignin isolated from mature bromegrass stems according to the methods of Ralph *et al.* (1994a) were run at 310 K on a Bruker AMX-360 narrow-bore instrument fitted with a 5 mm four-nucleus (QNP) probe. Spectra (50 000−60 000 FIDs) were collected on 25−100 mg samples in 0.4 mL of DMSO-d_6. The central solvent signal was used as internal reference (DMSO: 39.5 ppm). A 70° flip angle was used in all experiments (the 90° pulse angle was 8.2 μs, and the total pulse repetition time was 1.25 s). Exponential apodization, with line broadening of 2 Hz, was applied prior to Fourier transformation.

Biodegradation of Cell Walls. Gas production during *in vitro* fermentation of samples (50 mg) by mixed rumen microorganisms was monitored with pressure transducers (Pell and Schofield, 1993). Samples (100 mg) were also suspended in 10 mL of 20 mM acetate buffer (pH 4.8) and degraded with a mixture of hydrolases from *Trichoderma reesei* (4 μL of Celluclast, NOVO) and *Aspergillus niger* (4 μL of Viscozyme L, NOVO). Degradation by fungal hydrolases was followed by measuring the release of sugars into the buffer solution as described by Ralph *et al.* (1994a).

RESULTS AND DISCUSSION

Evaluation of the Synthetic Lignification System. Suspension cultures of maize (cv. Black Mexican) consist of undifferentiated cells with primary walls that are typical of grasses (Kieliszewski and Lamport, 1988; Grabber *et al.*, 1995). Pyrograms contained small amounts of guaiacol (34), 4-methylguaiacol (51), and vanillin (75) which may be derived from guaiacyl lignin or from other phenolic materials (Figure 1). Thioacidolysis of cell walls produced low yields of guaiacyl-derived products (3 mg g^{-1}), indicating that the cultures underwent limited lignification. Klason lignin concentrations were also very low (3 mg g^{-1}) considering that cell walls from these cultures contain about 10% protein (Kieliszewski and Lamport, 1988) and 85% structural carbohydrates (Grabber *et al.*, 1995). The low lignin values indicate that proteins, nonhydrolyzed carbohydrates, and condensed furans are not major contaminants of Klason residues (Hatfield *et al.*, 1994). Isolated walls from maize cultures contained a range of peroxidase isozymes that were solubilized by 3 M LiCl. Isoelectric focusing of LiCl extracts indicated that major peroxidase isozymes were localized in four acidic bands (pH 4−5), three neutral bands (pH 6−7.5), and two basic bands (pH 8−9). Multiple peroxidase isozymes are present in cell walls of plants, and those of acidic p*I* are probably involved in lignification (McDougall, 1992). Although the functional role of multiple isozymes is not understood, our results indicate that wall-bound peroxidases in maize cultures are representative of those found in other plant systems.

As with other plant species, maize suspensions secrete peroxidases into the culture medium (Grabber and Hatfield, 1994, unpublished results). If lignin precursors were added to maize cultures or isolated cells, secreted peroxidases would probably form DHPs in solution or on the surface of cell walls (Nakashima *et al.*, 1992) rather than within the wall matrix. Therefore, cells were ruptured and washed with 100 mM $CaCl_2$ and water to remove extracellular and loosely bound peroxidases from cell walls. Cell walls were suspended in PIPES buffer (pH 6.5), and wall-bound peroxidases and hydrogen peroxide were used to polymerize exogenously supplied *p*-hydroxycinnamyl alcohols into lignin. The PIPES buffer was selected because it is not oxidized by hydrogen peroxide, it does not precipitate calcium (needed for peroxidase activity and for maintaining ionically bound pectins), and it does not form stable products with lignin quinone methide

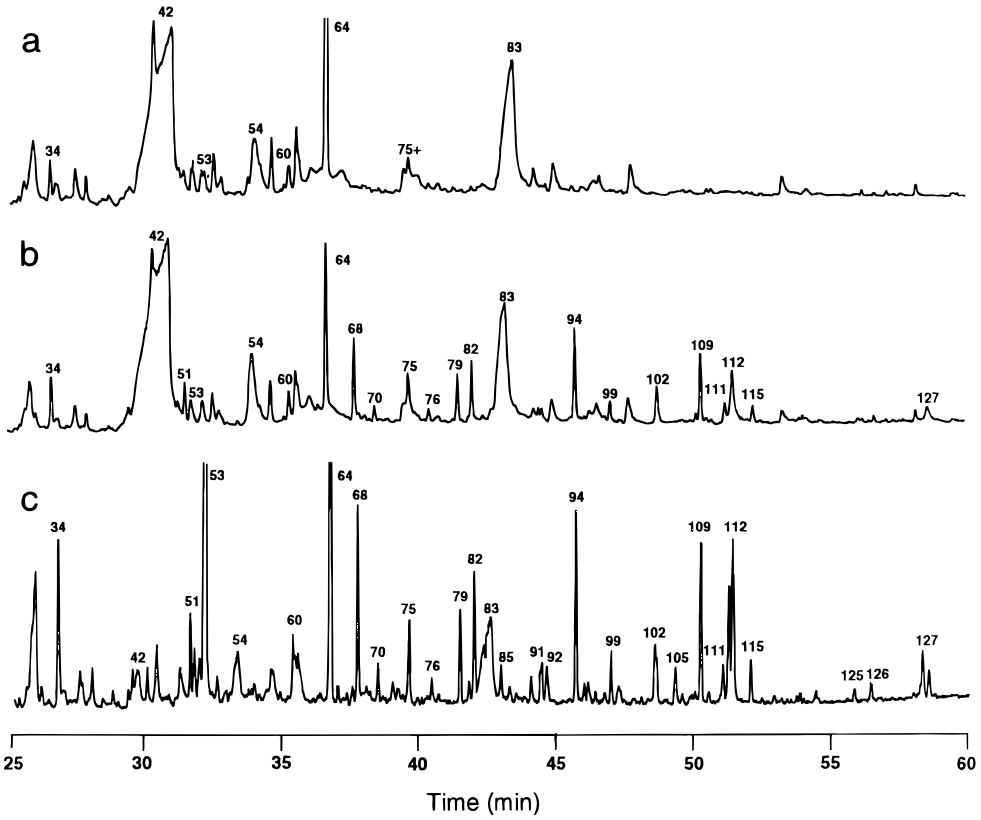

Figure 1. Pyrograms (total ion chromatograms) of cell walls: (a) nonlignified suspension cultures of maize; (b) syringyl/guaiacyl DHP−CW complexes; and (c) bromegrass stems. Peaks are numbered according to the guidelines of Ralph and Hatfield (1991).

intermediates. DHP−CW complexes were also readily formed under more acidic conditions (i.e. pH 4.0−5.5) using HOMOPIPES (Research Organics) or citrate buffers to control pH (Grabber, 1995, unpublished results). Several studies indicate that lignification in plants occurs under acidic conditions (Brunow *et al.*, 1989; Sipila and Brunow, 1991; Quideau and Ralph, 1994). Hydrogen peroxide was slowly generated from glucose by glucose oxidase in the reaction medium. Since gluconic acid also was produced by this reaction, relatively high strength buffers (50 mM) were used to maintain pH. If desired, a dilute solution of hydrogen peroxide (i.e. 0.03%) may be slowly added to the reaction medium in place of *in vitro* generated hydrogen peroxide (Grabber, 1994, unpublished results). Guaiacol staining indicated that peroxidases remained active in cell walls throughout lignification, but some peroxidases gradually leached into the reaction medium. Leaching of peroxidases was greatly reduced if cell walls were extracted overnight with 200 mM $CaCl_2$ prior to *in vitro* lignification (Grabber, 1995, unpublished results).

The efficiency of DHP−CW complex formation with coniferyl alcohol was very high; in several experiments only 9% of the coniferyl alcohol added to the reaction medium was recovered as non-wall-bound DHPs of low molecular weight. In addition, Klason lignin analysis of complexes indicated that coniferyl alcohol was quantitatively incorporated into the wall matrix (Figure 2). Klason values may, however, be slightly elevated by incorporation of ferulates, structural polysaccharides, and proteins to lignin (Evans and Himmelsbach, 1991; Ohnishi *et al.*, 1992; Ralph *et al.*, 1992; Imamura *et al.*, 1994). Complexes were readily generated with mixtures of coniferyl alcohol and *p*-coumaryl or sinapyl alcohols, but yields were occasionally reduced when high proportions (>50 mol %) of sinapyl alcohol were used.

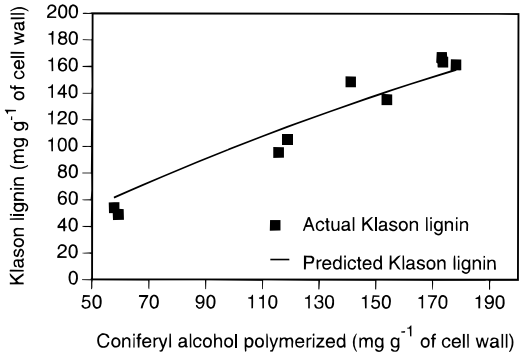

Figure 2. Stoichiometry of guaiacyl DHP−CW complex formation.

Transmission electron microscopy, pyrolysis−GC−MS, thioacidolysis, ^{13}C-NMR spectroscopy, and *in vitro* biodegradability assays were used to evaluate how well syringyl/guaiacyl DHP−CW complexes model lignified grass walls. Transmission electron microscopy of permanganate-stained sections revealed that synthetic lignins in DHP−CW complexes, like natural grass lignins, were distributed throughout the wall matrix (Figure 3). The extent of synthetic lignification, however, appeared to vary among cell walls. Light staining of nonlignified walls was probably due to oxidation of ferulate esters, which comprise about 2% of maize walls (Grabber *et al.*, 1995). Pyrolysis of DHP−CW complexes and bromegrass walls (Figure 1) produced a similar series of compounds derived from guaiacyl units (34, 51, 60, 70, 75, 76, 82, 85, 91, 112) and syringyl units (68, 79, 92, 94, 99,102, 109, 111, 115, 127) in lignin. Phenolic compounds diagnostic of lignin, such as (*E*)-isoeugenol (82), (*E*)-2,6-dimethoxy-4-propenylphenol (109), (*E*)-coniferaldehyde (112), and (*E*)-sinapaldehyde (127), were evident in pyrograms of DHP−CW complexes. Pyro-

Table 1. Yields and Proportions of Syringyl (S) and Guaiacyl (G) Lignin Degradation Products Recovered after Thioacidolysis of DHP−CW Complexes Compared with a DHP and Grass Cell Walls

	monomers		dimers					
	yield (μmol g^{-1} of Klason lignin or DHP)	S/G	yield (μmol g^{-1} of Klason lignin or DHP)	5-5 (%)	β-5 (%)	β-1 (%)	5-*O*-4 (%)	β-β (%)
DHP−CW	727	1.1	80	10	30	27	22	11
DHP	430	0.3	181	15	16	7	15	47
bromegrass stem	1002	1.0	76	10	31	33	13	13
maize rind	593	1.4	64	11	28	20	20	21

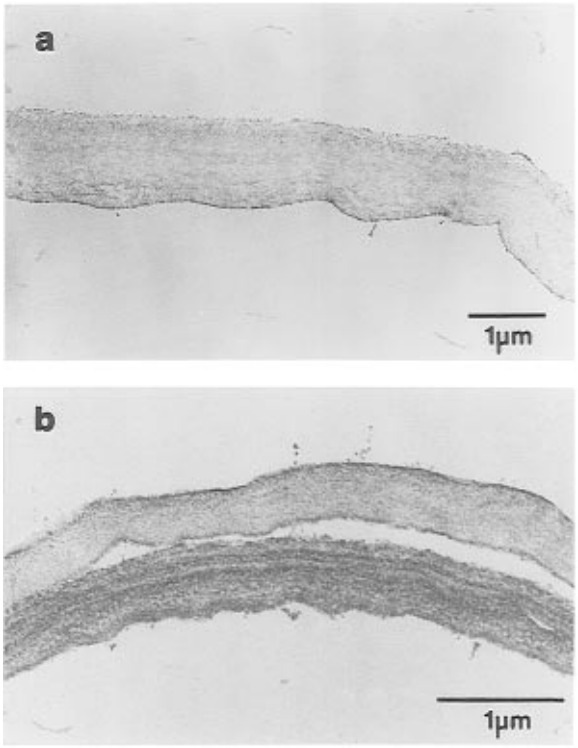

Figure 3. Electron micrographs of cell walls from maize stained with $KMnO_4$: (a) nonlignified suspension cultures of maize; and (b) syringyl/guaiacyl DHP−CW complexes.

grams of bromegrass were dominated by 4-vinylguaiacol (64) derived from ferulate esters on arabinoxylans and by 4-vinylphenol (53) derived primarily from *p*-coumarate esters on lignin (Ralph and Hatfield, 1991). In pyrograms of nonlignified maize walls, 4-vinylguaiacol was much more abundant than 4-vinylphenol, reflecting the preponderance of ferulate esters over *p*-coumarate esters on arabinoxylans (Mueller-Harvey *et al.*, 1986). Since *p*-coumarate precursors were not used to synthetically lignify maize walls, 4-vinylphenol was a minor pyrolysis product from DHP−CW complexes. Synthetic lignification of maize walls reduced the yield of 4-vinylguaiacol (64), presumably because a high proportion of ferulate esters became covalently attached to lignin (Grabber *et al.*, 1995). Yields of products derived from xylose (54) and glucose (83) were comparable for cell walls from maize cultures and bromegrass. Peak 42 from arabinose was extremely large in pyrograms of nonlignified maize walls and DHP−CW complexes, reflecting the abundance of arabinose in primary walls (Grabber *et al.*, 1995) and the high pyrolytic efficiency of arabinose relative to that of xylose or glucose (Ralph and Hatfield, 1991). Thioacidolysis cleaves β-*O*-4 and α-*O*-4 interunit linkages in lignin (Lapierre *et al.*, 1985); the yield of degradation products indicates the frequency of these linkages in lignin. Analysis of dimeric degradation products released by thioacidolysis provides information on "condensed" structures linked by C−C or diaryl ether bonds (Lapierre *et al.*, 1991). Yields of degradation products and the proportions of dimeric products from DHP−CW complexes were intermediate to those from maize rind and bromegrass stems (Table 1), suggesting that synthetic wall lignins were structurally similar to natural grass lignins. Lignins in DHP−CW complexes and maize rinds were unusual in having a high proportion of 5-*O*-4 structures compared to bromegrass stems or the DHP. The DHP differed considerably from DHP−CW and grass lignins in product yields and in the proportions of dimeric products. As is characteristic of DHPs (Tollier *et al.*, 1991), the dimeric fraction had low proportions of β-1 and high proportions of β-β structures compared to lignins formed within a wall matrix. The ratio of syringyl to guaiacyl units for the DHP was only 0.3 compared to 1.0 for the DHP−CW complex, even though both synthetic lignins were made with equimolar amounts of coniferyl and sinapyl alcohols. The low ratio for the DHP may be due to inefficient polymerization of sinapyl alcohol or high incorporation of syringyl units into β-β linked structures. Lignins were not extracted from cellulase-degraded DHP−CW complexes by 96:4 dioxane/water. About 10% of the lignin (on a Klason lignin basis) was extractable after ball-milling, indicating that it was tightly bound to the wall matrix. Lignin isolated from DHP−CW complexes had a ^{13}C-NMR spectrum comparable to that of a syringyl/guaiacyl lignin isolated from bromegrass stems (Figure 4). DHP−CW lignin had more β-*O*-4 structures (59.9, 62.6, 72.0, and 86.1 ppm) and fewer endgroups (61.7 ppm) and β-β structures (53.7, 71.3, and 85.0 ppm) than the DHP but more of the latter structures than lignin isolated from bromegrass stems. Prominent signals at 115.9 and 130.4 ppm from bromegrass lignin, due to *p*-coumaroyl units (Ralph *et al.*, 1994a), were not observed with DHP−CW lignin because *p*-coumarate precursors and *p*-coumaryl alcohols were not used to synthetically lignify walls.

Nonlignified maize walls were rapidly and extensively degraded by rumen microorganisms and by extracellular fungal hydrolases (Figure 5). When nonlignified walls were complexed with DHPs, the extent of fermentation and release of total sugars were reduced by about 25%. A similar depression of cell wall degradation has been associated with lignification of maize plants (Jung and Buxton, 1994), suggesting that the degradation characteristics of DHP−CW complexes are similar to those of naturally lignified grass walls. Although many groups have attempted to produce DHPs which are representative of *in vivo* lignins, our findings suggest that DHP−CW complexes may be the best system for modeling the lignification and degradation of grass fiber.

Applications and Limitations of Synthetically Lignified Cell Walls. Because DHP−CW complexes are formed with primary walls, our system most accurately models lignification of cell types such as parenchyma which have only primary cell walls. Other cell types, like xylem and sclerenchyma, have thick secondary walls in addition to primary walls. Primary and secondary walls both contain noncellulosic polysac-

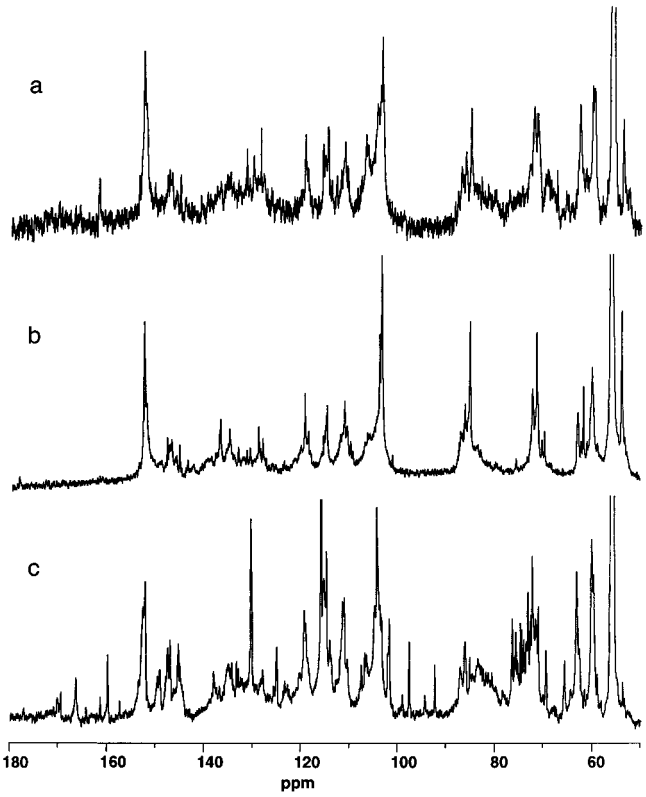

Figure 4. ^{13}C-NMR spectra of lignins: (a) lignin isolated from syringyl/guaiacyl DHP−CW complexes; (b) syringyl/guaiacyl DHP; and (c) lignin isolated from bromegrass stems.

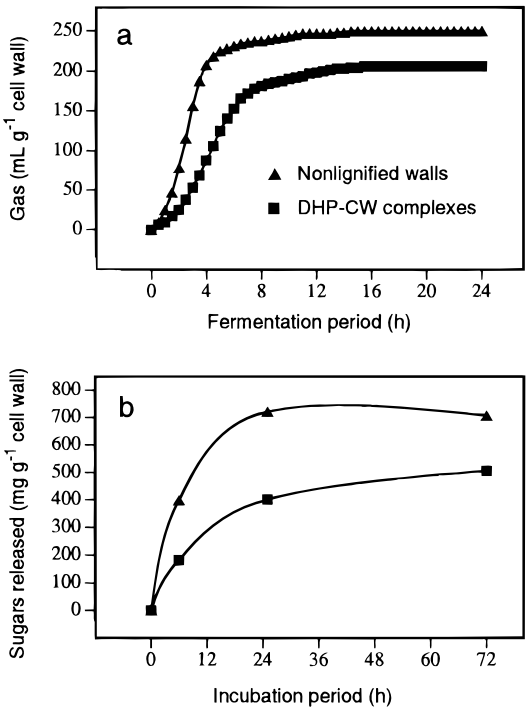

Figure 5. Degradation curves of nonlignified walls isolated from suspension cultures of maize and syringyl/guaiacyl DHP−CW complexes: (a) gas production during *in vitro* degradation with rumen microorganisms; and (b) release of sugars during *in vitro* degradation with hydrolases from *Trichoderma* and *Aspergillus* species.

charides, cellulose, structural proteins, hydroxycinnamic acids, and lignin (Ye and Varner, 1991; Hatfield, 1993; Iiyama *et al.*, 1993; Terashima *et al.*, 1993), but in differing proportions. Even in these tissues, primary walls are more heavily lignified and more resistant to degradation than secondary walls (Chesson *et al.*, 1986), and they limit penetration of degradative organisms and enzymes to secondary walls (Wilson, 1993). Since lignification occurs concurrently with secondary wall formation (Terashima *et al.*, 1993), it is not possible to adapt our system to cell walls with secondary thickenings because cell walls must be isolated prior to complex formation.

Secondary cell wall formation and lignification can be stimulated in suspension or callus cultures by judicious use of phytohormones or other elicitors (Hosel *et al.*, 1982; Ingold *et al.*, 1988). Although these systems are valuable for studying the biosynthesis of lignin, manipulation of lignin composition or lignin−matrix interactions in these cultures is limited, requiring reduced expression of a large number of specific enzymes. Developing enzyme inhibitors or genetically altered cultures to manipulate lignin composition and interactions is time-consuming and problematic. In contrast, synthetic lignification of cell walls gives us tremendous flexibility in manipulating lignin−matrix interactions. For example, incorporation of ferulate esters into lignin and its effect on fiber degradability can be evaluated by using cell walls from cultures grown with various amounts of 2-aminoindan-2-phosphonic acid (AIP) (Grabber *et al.*, 1995), a specific inhibitor of phenylalanine ammonia-lyase (Zon and Amrhein, 1992). Since these cultures do not synthesize lignin, blocking phenylpropanoid metabolism with AIP specifically reduces ferulate ester deposition into cell walls and hence incorporation of ferulate esters into synthetic lignin. Other lignin−matrix interactions, such as those involving structural proteins and structural polysaccharides, can also be evaluated with this system. Chemical synthesis of various lignin precursors also allows us to study how lignin concentration and composition affect wall properties. Because of the importance of primary cell walls in controlling wall properties, we believe that our system adequately models many types of lignin−matrix interactions in graminaceous plants.

LITERATURE CITED

Brown, A. Review of lignin in biomass. *J. Appl. Biochem.* **1985**, *7*, 371−387.

Brunow, G.; Sipila, J.; Makela, T. On the mechanism of formation of noncyclic benzyl ethers during lignin biosynthesis. Part 1. The reactivity of *β-O*-4 quinone methides with phenols and alcohols. *Holzforschung* **1989**, *43*, 55−59.

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. Appl. Bacteriol.* **1986**, *60*, 327−336.

Erdtman, H. Outstanding problems in lignin chemistry. *Ind. Eng. Chem.* **1957**, *49*, 1385−1386.

Evans, J. J.; Himmelsbach, D. S. Incorporation of peroxidase into synthetic lignin. *J. Agric. Food Chem.* **1991**, *39*, 830−832.

Freudenberg, K. In *Constitution and Biosynthesis of Lignin*; Freudenberg, K., Neish, A. C., Eds.; Springer-Verlag: Berlin, 1968; pp 47−116.

Gagnaire, D.; Robert, D. A polymer model of lignin (D. H. P.) carbon-13 selectively labelled at the benzylic positions: synthesis and NMR study. *Makromol. Chem.* **1977**, *178*, 1477−1495.

Grabber, J. H.; Hatfield, R. D.; Ralph, J.; Zon, J.; Amrhein, N. Ferulate cross-linking in cell walls isolated from maize cell suspensions. *Phytochemistry* **1995**, *40*, 1077−1082.

Harlow, E.; Lane, D. *Antibodies: A Laboratory Manual*; Cold Spring Harbor Laboratory: Cold Spring Harbor, NY, 1988; p 508.

Hatfield, R. D. Cell wall polysacchride interactions and degradability. In *Forage Cell Wall Structure and Digestibility*; Jung, H. J., Buxton, D. R., Hatfield, R. D., Ralph, J., Eds.; ASA-CSSA-SSSA: Madison, WI, 1993; pp 285−313.

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.

Higuchi, T.; Ogino, K.; Tanahashi, M. Effect of polysaccharides on dehydropolymerization of coniferyl alcohol. *Wood Res.* **1971**, *51*, 1−11.

Hosel, W.; Fiedler-Preiss, A.; Borgmann, E. Relationship of coniferin β-glucosidase to lignification in various plant cell suspension cultures. *Plant Cell, Tissue Organ Cult.* **1982**, *1*, 137−148.

Iiyama, K.; Lam, T. B. T.; Meikle, P. J.; Ng, K.; Rhodes, D.; Stone, B. A. Cell wall biosynthesis and its regulation. 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 621−683.

Iiyama, K.; Lam, T. B. T.; Stone, B. Covalent cross-links in the cell wall. *Plant Physiol.* **1994**, *104*, 315−320.

Imamura, T.; Watanabe, T.; Kuwahara, M.; Koshijima, T. Ester linkages between lignin and glucuronic acid in lignin-carbohydrate complexes from *Fagus crenata*. *Phytochemistry* **1994**, *37*, 1165−1173.

Ingold, E.; Sugiyama, M.; Komamine, A. Secondary cell wall formation: changes in cell wall constituents during the differentiation of isolated mesophyll cells of *Zinnia elegans* tracheary elements. *Plant Cell Physiol.* **1988**, *29*, 295−303.

Jung, H.-J. G.; Buxton, D. R. Forage quality variation among maize inbreds: relationships of cell-wall composition and in-vitro degradability for stem internodes. *J. Sci. Food Agric.* **1994**, *66*, 313−322.

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.

Kieliszewski, M.; Lamport, D. T. A. Purification and partial characterization of a hydroxyproline-rich glycoprotein in a graminaceous monocot, *Zea mays*. *Plant Physiol.* **1987**, *85*, 823−827.

Kieliszewski, M.; Lamport, D. T. A. Tying the knots in the extensin network. In *Self-Assembling Architecture, Proceedings of the 46th Symposium of the Society for Developmental Biology*; Varner, J. E., Ed.; St. Paul, MN, 1988; pp 61−77.

Kirk, T. K.; Connors, W. J.; Bleam, W. D.; Hackett, W. F., Zeikus, J. G. Preparation and microbial decomposition of synthetic (^{14}C) lignins. *Proc. Natl. Acad. Sci. U.S.A.* **1975**, *72*, 2513−2519.

Kritchevsky, D. *Evaluation of Publicly Available Scientific Evidence Regarding Certain Nutrient-Disease Relationships: Dietary Fiber and Cancer No. 5*; Center for Food Safety and Applied Nutrition, Food and Drug Administration. Department of Health and Human Services: Washington, DC, 1991.

Lapierre, C.; Monties, B.; Rolando, C. Thioacidolysis of lignin: comparison with acidolysis. *J. Wood Chem. Technol.* **1985**, *5*, 277−292.

Lapierre, C.; Pollet, B.; Monties, B.; Rolando, C. Thioacidolysis of spruce lignin: GC-MS analysis of the main dimers recovered after Raney nickel desulphuration. *Holzforschung* **1991**, *45*, 61−68.

Lewis, N. G.; Newman, J.; Just, G.; Ripmeister, J. Determination of bonding patterns of carbon-13 specifically enriched dehydrogenatively polymerized lignin in solution and solid state. *Macromolecules* **1987a**, *20*, 1752−1756.

Lewis, N. G.; Yamamoto, E.; Wooten, J. B.; Just, G.; Ohashi, H.; Towers, G. H. N. Monitoring biosynthesis of wheat cell-wall phenylpropanoids in situ. *Science* **1987b**, *237*, 1344−1346.

McDougall, G. J. Changes in cell wall-associated peroxidases during the lignification of flax fibres. *Phytochemistry* **1992**, *31*, 3385−3389.

Mueller-Harvey, I.; Hartley, R. D.; Harris, P. J.; Curzon, E. H. Linkage of *p*-coumaryl and feruloyl groups to cell wall polysaccharides of barley straw. *Carbohydr. Res.* **1986**, *148*, 71−85.

Nakashima, J.; Takabe, K.; Saiki, H. Artificial lignification within unlignified cell walls of *Populus koreana*. *Mokuzai Gakkaishi* **1992**, *38*, 1136−1142.

Nilsson, T.; Daniel, G.; Kirk, T. K.; Obst, J. R. Chemistry and microscopy of wood decay by some higher ascomycetes. *Holzforschung* **1989**, *43*, 11−17.

Ohnishi, J.; Watanabe, T.; Koshijima, T. Synthesis of dehydrogenation polymer-polyose complexes by peroxidase. *Phytochemistry* **1992**, *31*, 1185−1190.

Pell, A. N.; Schofield, P. Computerized monitoring of gas production to measure forage digestion in vitro. *J. Dairy Sci.* **1993**, *76*, 1063−1073.

Quideau, S.; Ralph, J. Facile large-scale synthesis of coniferyl, sinapyl, and *p*-coumaryl alcohol. *J. Agric. Food Chem.* **1992**, *40*, 1108−1110.

Quideau, S.; Ralph, J. A biomimetic route to lignin model compounds *via* silver (I) oxide oxidation 1. Synthesis of dilignols and non-cyclic benzyl aryl ethers. *Holzforschung* **1994**, *48*, 12−22.

Ralph, J.; Hatfield, R. D. Pyrolysis-GC-MS characterization of forage materials. *J. Agric. Food Chem.* **1991**, *39*, 1426−1437.

Ralph, J.; Helm, R. F.; Quideau, S.; Hatfield, R. D. Lignin-feruloyl ester cross-links in grasses. Part 1. Incorporation of feruloyl esters into coniferyl alcohol dehydrogenation polymers. *J. Chem. Soc., Perkin Trans. 1* **1992**, 2961−2969.

Ralph, J.; Hatfield, R. D.; Quideau, S.; Helm, R. F.; Grabber, J.; Jung, H. G. Pathway of *p*-coumaric acid incorporation into maize lignin as revealed by NMR. *J. Am. Chem. Soc.* **1994a**, *116*, 9448−9456.

Ralph, J.; Quideau, S.; Grabber, J. H.; Hatfield, R. D. Identification and synthesis of new ferulic acid dehydrodimers present in grass cell walls. *J. Chem. Soc., Perkin Trans. 1* **1994b**, 3485−3498.

Sipila, J.; Brunow, G. On the mechanism of formation of non-cyclic benzyl ethers during lignin biosynthesis. Part 2. The effect of pH on the reaction between a β-*O*-4 type quinone methide and vanillyl alcohol in water-dioxane solutions. The stability of non-cyclic benzyl aryl ethers during lignin biosynthesis. *Holzforschung* **1991**, *45*, 275−278.

Terashima, N.; Seguchi, Y. Heterogeneity in formation of lignin. IX. Factors influencing the formation of condensed structures in lignins. *Cellulose Chem. Technol.* **1988**, *22*, 147−154.

Terashima, N.; Fukushima, K.; He, L.-F.; Takabe, K. Comprehensive model of the lignified plant cell wall. 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 247−270.

Theander, O.; Westerlund, E. A. Studies on dietary fiber. 3. Improved procedures for analysis of dietary fiber. *J. Agric. Food Chem.* **1986**, *34*, 330−336.

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*; Australian Pulp and Paper Industries Technical Association: Melbourne, Australia, 1991; Vol. 2, pp 35−41.

Whitmore, F. W. Lignin-carbohydrate complex formed in isolated cell walls of callus. *Phytochemistry* **1978**, *17*, 421−425.

Whitmore, F. W. Lignin-protein complex in cell walls of *Pinus ellliottii*: amino acid constituents. *Phytochemistry* **1982**, *21*, 315−318.

Wilson, J. R. Organization of forage plant tissues. 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 1−27.

Ye, Z.-H.; Varner, J. E. Tissue-specific expression of cell wall proteins in developing soybean tissues. *Plant Cell* **1991**, *3*, 23−37.

Zon, J.; Amrhein, N. Inhibitors of phenylalanine ammonia lyase: 2-aminoindan-2-phosphonic acid and related compounds. *Justus Liebigs Ann. Chem.* **1992**, 625−628.

Received for review May 4, 1995. Revised manuscript received September 12, 1995. Accepted April 22, 1996.[⊗] Supported in part by the USDA-ARS Administrator Funded Post Doc program, USDA-NRI Competitive Grant 92-37304-8057 (Plant Growth and Development Section), and by the W. H. Miner Agricultural Research Institute, Chazy, NY. Mention of trade name, proprietary product, or specific equipment does not constitute a guarantee of the product by the USDA and does not imply its approval to the exclusion of other products that may also be suitable.

JF9502717

[⊗] Abstract published in *Advance ACS Abstracts,* May 15, 1996.