p-Hydroxyphenyl, Guaiacyl, and Syringyl Lignins Have Similar Inhibitory Effects on Wall Degradability

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Studies with normal, mutant, and transgenic plants have not clearly established whether the proportion of *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) units in lignin directly affects the degradability of cell walls by hydrolytic enzymes. Dehydrogenation polymer−cell wall complexes containing varying ratios of H, G, and S lignins were formed by peroxidase/H_2O_2-mediated polymerization of *p*-coumaryl, coniferyl, and sinapyl alcohols into nonlignified walls isolated from cell suspensions of maize (*Zea mays* L). Lignification substantially reduced the degradability of cell walls by fungal hydrolases, but degradability was not affected by lignin composition. On the basis of these results, we propose that improvements in wall degradability, previously attributed to changes in lignin composition, were in fact due to other associated changes in wall chemistry or ultrastructure.

Keywords: *Gramineae; Zea mays; cell wall; brown midrib; transgenic; O-methyltransferase; hydroxycinnamyl alcohols; lignin; cellulase; degradability*

INTRODUCTION

Lignins in angiosperms are formed by peroxidase/H_2O_2-mediated polymerization of *p*-coumaryl, coniferyl, and sinapyl alcohols (Figure 1). At early stages of lignification, coniferyl alcohol and small amounts of *p*-coumaryl alcohol are copolymerized into the primary wall to form mixed G and H lignins. Later, during secondary wall development, coniferyl alcohol and variable amounts of sinapyl alcohol are copolymerized to form mixed G and S lignins (He and Terashima, 1990, 1991). In some cases, however, secondary walls may be lignified before primary walls (Vallet et al., 1996). The S/G ratio of lignin, as estimated by degradative techniques, is not consistently correlated with cell wall degradability across or within normal genotypes (Buxton and Russell, 1988; Jung and Casler, 1991; Jung and Vogel, 1992). Even in mutant and transgenic plants, wall degradability is not consistently associated with changes in the S and G content of lignin (Chabbert et al., 1994; Akin et al., 1986; Thorstensson et al., 1992; Lechtenberg et al., 1972; Bernard Vailhe et al., 1996a; Sewalt et al., 1997). Modifications in lignin composition are generally accompanied by changes in the concentrations of *p*-coumarate esters, ferulate cross-links, lignin, and other wall components (Chabbert et al., 1994; Lam et al., 1996; Thorstensson et al., 1992). The distribution of phenolics in walls, wall thickness, and plant anatomy may also be affected (Morrison et al., 1993; Grenet and Barry, 1991; Goto et al., 1993; Bernard Vailhe et al., 1996b). Due to these and possibly other confounding effects, it has not been demonstrated whether lignin composition *per se* affects the enzymatic degradability of cell walls. Such information would provide a rational basis for directing plant selection or molecular engineering efforts aimed at improving the bioconversion of structural polysaccharides into metabolizable energy for livestock or into ethanol fuels. In this study, dehydrogenation polymer−cell wall (DHP−CW) complexes were used to model how alterations in lignin composition affect wall degradability.

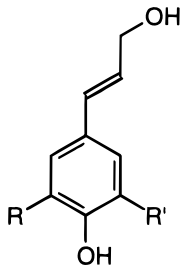

Figure 1. Monolignol precursors of normal angiosperm lignins: *p*-coumaryl alcohol (R = R′ = H); coniferyl alcohol (R = H, R′ = OCH_3); and sinapyl alcohol (R = R′ = OCH_3).

MATERIALS AND METHODS

DHP−CW complexes were formed by adding dilute H_2O_2 and varying proportions of *p*-coumaryl, coniferyl, and sinapyl alcohols to nonlignified walls isolated from cell suspensions of maize (Grabber et al., 1996). Monolignols were prepared according to the methods of Quideau and Ralph (1992). After complex formation, cell walls were thoroughly washed with water followed by acetone to remove unreacted monolignols and nonbound DHPs. Complexes were analyzed for Klason lignin without correction for acid-soluble lignin (Hatfield et al., 1994). Nonlignified walls and selected complexes were analyzed for lignin composition by pyrolysis GC−MS (Ralph and Hatfield, 1991) and for alkali-labile ferulates and lignin following saponification at room temperature for 20 h with 2 M aqueous NaOH. Ferulates were analyzed by GC−FID (Ralph et al., 1994). The quantity of lignin in alkaline extracts was estimated by comparing the absorbance (280 nm) of diluted and acidified (pH 2) extracts to that of G and mixed S−G DHPs (Grabber et al., 1996). Cell walls (100 mg in 10 mL of 20 mM acetate buffer, pH 4.8, 39 °C) were degraded with hydrolases from *Trichoderma reesei* (4 μL of Celluclast, NOVO) and *Aspergillus niger* (4 μL of Viscozyme L, NOVO). The quantity of carbohydrate released from complexes by

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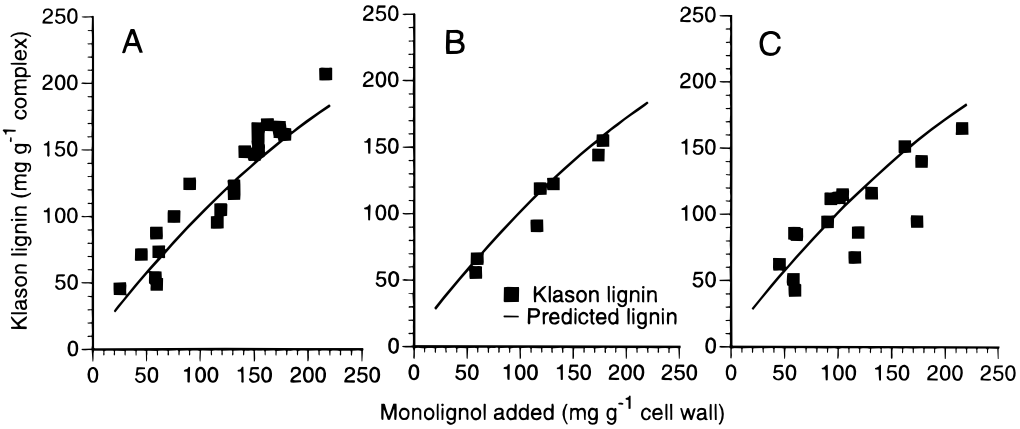

Figure 2. Stoichiometry of DHP−CW complex formation with coniferyl alcohol (A), an equimolar mixture of coniferyl and *p*-coumaryl alcohols (B), and an equimolar mixture of coniferyl and sinapyl alcohols (C).

hydrolases was estimated by a colorimetric technique (Dubois et al., 1956).

RESULTS AND DISCUSSION

Cell walls from maize cell suspensions are composed of about 0.3% G lignin, 1.8% alkali-labile ferulates, 10% protein, 10% pectin, 50% hemicellulose (primarily glucuronoarabinoxylans with small amounts of mixed-linked glucans), and 25% cellulose (Grabber et al., 1995, 1996). Overall, the composition of these cell walls are typical of nonlignified primary walls in grasses.

Wall-bound peroxidases and exogenously supplied hydrogen peroxide were used to polymerize monolignols into cell walls to form DHP−CW complexes; Klason lignin concentrations ranged from 50 to 220 mg g^{-1}. Previous work has demonstrated that lignins formed within these complexes are structurally similar to natural grass lignins (Grabber et al., 1996). Coniferyl alcohol and mixtures of coniferyl alcohol and *p*-coumaryl alcohol were consistently and efficiently polymerized into wall-bound DHPs (Figure 2). In many cases, Klason lignin concentrations were greater than predicted, probably due to inclusion of some covalently bound ferulates, protein, or carbohydrate with lignin (Grabber et al., 1996). Lignification of walls with mixtures of sinapyl and coniferyl alcohols gave somewhat more variable results. In cases where lignification efficiency was high, the relative abundance of pyrolysis products derived from S and G units was proportional to the ratio of sinapyl and coniferyl alcohol used to form complexes. Degradative analysis of S−G complexes by thioacidolysis also gave similar results (Grabber et al., 1996). These results indicate that the desired S−G lignins had been formed. Oxidation of sinapyl alcohol by maize peroxidases (for both cell suspensions and plants) is much less efficient than that of coniferyl alcohol (Hatfield, 1996, unpublished results). This probably accounts for the variable polymerization of sinapyl alcohol into complexes. We are currently conducting experiments to determine if ferulate polysaccharide esters and *p*-coumaroylated lignin precursors enhance the formation of S lignins in maize. Ferulate or *p*-coumarate esters may enhance the formation of S lignins by peroxidases having a low affinity for sinapyl alcohol (Takahama et al., 1996).

The quantity of ferulates released by saponification was reduced 90−95% when walls were lignified to a Klason lignin content of *ca.* 120 mg g^{-1} with all monolignol mixtures. Ferulate polysaccharide esters are oxidatively coupled to lignin by alkali-stable bonds

Table 1. Lignin Concentration and Fungal Hydrolase Degradability of DHP−CW Complexes Formed with Coniferyl, *p*-Coumaryl, and Sinapyl Alcohols (n = 3)[a]

monolignol	Klason lignin	carbohydrate released 6 h	carbohydrate released 72 h
coniferyl alcohol	111	221	530
coniferyl + *p*-coumaryl alcohol (1:1 ratio)	111	212	509
coniferyl + sinapyl alcohol (1:1 ratio)	102	221	528
CV%	5.2	5.7	7.1

[a] Means within each column were similar ($P > 0.05$). Concentrations and degradability are given in units of mg per g of DHP−CW complex.

Table 2. Lignin Concentration and Fungal Hydrolase Degradability of DHP−CW Complexes Formed with Coniferyl Alcohol (CA) and Sinapyl Alcohol (SA, n = 2)[a]

ratio of CA:SA	Klason lignin	carbohydrate released[b] 6 h	carbohydrate released[b] 72 h
100:0	80	230	623
80:20	74	224	638
60:40	80	224	627
40:60	85	230	627
CV%	10.8	0.8	1.6

[a] Means within each column were similar ($P > 0.05$). Concentrations and degradability are given in units of mg per g of DHP−CW complex. [b] Klason lignin concentration was used as a covariate to adjust means and to increase precision.

(Grabber et al., 1995). Therefore, the low recovery of alkali-labile ferulates indicates that all complexes had a similar, high degree of ferulate−lignin cross-linking. Lignin composition of complexes also did not affect the quantity of alkali-soluble lignin; about one-half of lignin (on a Klason lignin basis) was solubilized by 2 M aqueous NaOH. These analyses, although limited in scope, suggest that lignin composition does not appreciably alter interactions between lignin and other matrix components.

Nonlignified walls were rapidly and extensively degraded by fungal enzymes with 395 mg g^{-1} of total sugars released after 6 h and 735 mg g^{-1} of total sugars released after 72 h of incubation. Due to variation in lignification efficiency (particularly for complexes formed with sinapyl alcohol), only replicates having high incorporation rates of monolignols into wall-bound DHPs were used for degradability studies. Cell-wall degradability was reduced to a similar degree by several monolignol mixtures (Table 1). Varying the proportions

of coniferyl and sinapyl alcohol used to form complexes also did not affect degradability (Table 2). In addition, studies with DHP−CW complexes and *Arabidopsis thaliana* mutants indicate that lignin composition has no effect on the degradation of cell walls by mixed rumen microorganisms (Grabber et al., 1992; Jung and Chapple, 1996). Overall, these results suggest that lignin composition does not directly affect the degradability of cell walls by fungal enzymes or by rumen microorganisms. We propose that improvements in fiber degradability, previously attributed to modifications in lignin composition of mutant and transgenic germplasm, are in fact due to other associated changes in wall chemistry or ultrastructure.

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