

Ferulate Cross-Links Limit the Enzymatic Degradation of Synthetically Lignified Primary Walls of Maize

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Ferulate cross-linking of arabinoxylans to lignin may restrict the enzymatic degradation of structural polysaccharides, limiting the utilization of graminaceous crops and crop residues as feedstuffs and as feedstocks for fuel and chemical production. Maize walls from nonlignified cell suspensions with ca. 5.1 or 17.6 mg g⁻¹ of ferulates were synthetically lignified with coniferyl alcohol and H₂O₂ to form dehydrogenation polymer–cell wall complexes with ca. 4.8 or 15.8 mg g⁻¹ of ferulates incorporated into lignin. Ferulate concentrations in cell walls were reduced from normal levels by growing cell suspensions with 2-aminoindan-2-phosphonic acid or by methylating wall ferulates with diazomethane prior to complex formation. A 70% reduction in ferulate–lignin cross-linking increased carbohydrate solubilization by 24–46% after 6 h and by 0–25% after 72 h of hydrolysis with two fungal enzyme mixtures. Reduced cross-linking enhanced the hydrolysis of xylans and, to a lesser degree, cellulose from walls. The results presented indicate that reduced feruloylation of arabinoxylans will significantly improve the hydrolysis of lignified grass walls.

Keywords: *Gramineae; Zea mays; cell wall; ferulic acid; diferulic acids; lignin; cross-linking; cellulase; degradability*

INTRODUCTION

Ferulic acid is esterified to the C5-hydroxyl of α -L-arabinose moieties of grass xylans (Kato and Nevins, 1985; Mueller-Harvey et al., 1986). During lignification, xylans are cross-linked by oxidative coupling of ferulate monomers into dehydrodimers and by incorporation of ferulates into lignin (Ralph et al., 1994, 1995; Grabber et al., 1995). Ferulate cross-linking of xylans to lignin is thought to limit the enzymatic degradation of grass walls (Hatfield, 1993; Jung and Deetz, 1993), but unambiguous evidence for this is lacking. Digestibility is enhanced when walls are treated with chemicals that cleave ferulate cross-links between xylans and lignin (Fritz et al., 1991; Morrison, 1991; Jung et al., 1992). Unfortunately, the specificity of chemical treatments is poor (Fry, 1986), making it difficult to attribute improvements in digestibility to reductions in ferulate–lignin cross-linking. Studies with normal or mutant germplasm do not show consistent relationships between wall degradation and the quantity of ferulates that are ether-linked to lignin (Jung and Casler, 1991; Jung and Vogel, 1992; Goto et al., 1994; Jung and Buxton, 1994; Argillier et al., 1996; Lam et al., 1996). Correlative studies such as these are limited because ether-linked ferulates represent only a small and variable proportion of the ferulates incorporated into lignin (Grabber et al., 1995, 1998b). In addition, associations between ferulate cross-links and wall degradability are probably masked or confounded by concurrent changes in other wall components that normally occur during plant development (Jung et al., 1998) or that are brought about by genetic manipulation of cell wall biosynthesis.

Recently, we specifically manipulated diferulate cross-linking in walls from maize cell suspensions to demonstrate that these cross-links impede the initial hydrolysis of nonlignified walls (Grabber et al., 1998a). Synthetic lignification of these walls, forming dehydrogenation polymer–cell wall (DHP–CW) complexes, provides a biomimetic means of investigating and manipulating lignin–ferulate cross-linking in walls (Grabber et al., 1996, 1998b). Our objective was to use this model system to more clearly establish whether ferulate cross-links restrict the degradation of lignified walls by fungal hydrolases.

MATERIALS AND METHODS

Preparation of Lignified Walls. Nonlignified walls were isolated from maize cell suspensions grown with 0 or 40 μ M 2-aminoindan-2-phosphonic acid (AIP), a specific inhibitor of phenylalanine ammonia-lyase that reduces cell wall feruloylation (Zon and Amrhein, 1992; Grabber et al., 1995). DHP–CW complexes were formed by the dropwise (40 h) addition of 140–175 mg of coniferyl alcohol and 1.25 equiv of glucose per gram of nonlignified walls stirred in 50 mM PIPES buffer (pH 6.5) with glucose oxidase (2000 units, type VII-S, Sigma). Nonlignified controls were stirred in PIPES buffer and glucose oxidase without added coniferyl alcohol and glucose. Experiments with AIP were replicated three times. Complexes were also formed in a similar manner by adding 50–165 mg of coniferyl alcohol and 1.25 equiv of glucose per gram of walls that had been preincubated with and without diazomethane to methylate the phenolic moiety of ferulates. For methylation, cell walls isolated from normal suspensions were extracted repeatedly with 1,4-dioxane/methanol (9:1) to remove water. Cell walls (~4 g) were then stirred in ice-cold dioxane/methanol (200 mL) with ~16 mmol of diazomethane (40 mL of ethanol-containing ethereal solution) for 10 min. Acetic acid was added to destroy excess diazomethane, and cell walls were collected on a fritted disk (F porosity) and washed with 1,4-dioxane/methanol followed by water prior to complex formation.

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Table 1. Chemical Composition (Milligrams per Gram of Cell Wall) of Nonlignified and Synthetically Lignified Walls Prepared from Maize Cell Suspensions Grown with or without AIP, a Specific Inhibitor of Phenylalanine Ammonia-Lyase^a

treatment	ferulates			Klason lignin	arabinose	xylose	glucose	galactose	uronate
	monomers	dimers	total						
nonlignified walls									
0 μ M AIP	8.27	8.66	16.93	10	199	196	336	69	108
40 μ M AIP	1.84	2.84	4.67	8	193	172	356	82	114
lignified walls									
0 μ M AIP	0.30	0.73	1.03	155	173	156	280	67	93
40 μ M AIP	0.07	0.09	0.16	151	159	137	297	68	94
analysis of variance ^b									
AIP	*	*	*	NS	†	*	*	NS	NS
lignin	*	*	*	*	*	*	*	NS	*
AIP \times lignin	*	*	*	NS	NS	NS	NS	NS	NS

^a Synthetically lignified walls were prepared by slowly adding coniferyl alcohol and hydrogen peroxide to nonlignified walls. ^b *, †, NS, significant at the 0.05 and 0.10 levels of probability and not significant, respectively. Data were analyzed according to a split-plot design with AIP as whole plots and lignin as subplots. Variances for *F* tests of treatment effects were pooled according to guidelines provided by Carmer et al. (1969).

Nonmethylated walls were processed in a similar manner except that ether was added in place of the diazomethane solution. Experiments with diazomethane were replicated two times. Additional details of the lignification procedure have been described (Grabber et al., 1996, 1998b).

After mixing in the reaction medium for a total of 60 h, nonlignified controls and DHP-CWs were collected on glass fiber filters (1.2 μ m retention), washed thoroughly with water and acetone, and dried at 55 °C. Filtrates from several DHP-CWs were extracted with ethyl acetate as described previously (Grabber et al., 1996) to isolate dehydrogenation products not bound to cell walls. Nonbound products were partially characterized by ¹H NMR and GC/MS (Grabber et al., 1996).

Cell Wall Analyses. Cell walls were analyzed for neutral sugars (Hatfield and Weimer, 1995), uronic acids (Blumenkrantz and Asboe-Hansen, 1973), and Klason lignin (Hatfield et al., 1994). Alkali-labile hydroxycinnamic acids were determined by GC-FID (Ralph et al., 1994; Grabber et al., 1995). The quantity of ferulates incorporated into lignin was estimated by the difference in alkali-labile acids recovered from nonlignified walls and from DHP-CW complexes. Degradability was assessed by suspending walls (1%, w/v) in 20 mM acetate buffer (pH 4.8, 40 °C) with a mixture of Viscozyme L (Novo) and Celluclast 1.5 L (Novo), each added at 0.04 μ L mg⁻¹ of cell wall. After 6 and 72 h of hydrolysis, wall residues were pelleted by centrifugation (2500g for 10 min) and an aliquot of the supernatant was analyzed for total carbohydrate (Dubois et al., 1956). Degradability was also assessed in a similar manner by incubating walls (1%, w/v) in 20 mM MES buffer (pH 6.0, 40 °C) with a mixture of Celluclast 1.5 L, added at 0.04 μ L mg⁻¹ of cell wall, and Biofeed Beta (Novo, CT form), added at 40 μ g mg⁻¹ of cell wall. Supernatant samples were also analyzed for neutral sugars following trifluoroacetic acid (TFA) hydrolysis (Hatfield and Weimer, 1995).

RESULTS AND DISCUSSION

Manipulation of Ferulate Cross-Linking in DHP-CW Complexes. Cell walls isolated from maize cell suspensions contained ~18 mg g⁻¹ of total ferulates (monomers plus dimers), 22% of which were ferulate dimers. Incubating these walls with glucose oxidase increased the proportion of diferulates to 51% (Table 1). Ferulate dimerization by wall peroxidase was apparently stimulated by H₂O₂ formed from glucose (released by wall autohydrolysis) via glucose oxidase (Grabber et al., 1995). The Klason procedure indicated that nonlignified controls contained 1% lignin. The actual quantity of lignin is probably ~0.3% because ferulate dimerization slightly elevates Klason lignin concentrations of nonlignified walls (Grabber, unpublished results).

The carbohydrate composition of cell walls (Table 1) was typical of primary walls in grasses (Darvill et al., 1978; Carpita, 1984). On the basis of carbohydrate data from this and other studies of primary maize walls (Carpita, 1984; Kieliszewski and Lamport, 1988; Grabber et al., 1995), we estimate that walls from maize cell suspensions are composed of about 50% hemicelluloses (primarily arabinoxylans with small amounts of mixed-linked glucans and xyloglucans), 25% cellulose, 10% pectin, and 10% protein.

Treatment of cell suspensions with AIP reduced total ferulate concentrations in walls by 72% (Table 1). Although the proportion of diferulates increased from 23 to 61% during incubation of walls with glucose oxidase, the quantity of diferulates formed in these walls was 67% less than in walls prepared from normal cell suspensions. AIP treatment also caused a 5–12% reduction in arabinose and xylose concentrations and a 6% increase in glucose concentration. Although additional work would be needed to confirm this, these changes in carbohydrate composition may reflect structural changes in cell walls made in response to reduced feruloylation and diferulate cross-linking of xylans. Other studies with dicots indicate that structural protein and cellulose deposition are not affected by AIP treatment (Keller et al., 1990; Schmutz et al., 1993).

Wall-bound peroxidases and H₂O₂, generated in situ from glucose by glucose oxidase, were used to synthetically lignify walls with dehydrogenation polymers of coniferyl alcohol. Previous work has demonstrated that this system is an excellent model of lignified grass walls (Grabber et al., 1996, 1998b). Peroxidase activity was detected by guaiacol staining throughout the course of lignification. As a result, walls from both normal and AIP-treated cell suspensions were efficiently lignified; only 6% of the coniferyl alcohol added to the reaction medium was recovered as nonbound dehydrogenation products. Complexes made from normal and AIP treated cell suspensions had similar Klason lignin concentrations (Table 1), but lignin levels were ~15% greater than expected on the basis of the amount of coniferyl alcohol polymerized into walls. Klason lignin values are probably inflated by covalent binding of ferulates and other matrix components to lignin (Evans and Himmelsbach, 1991; Ohnishi et al., 1992; Ralph et al., 1992).

The quantity of total ferulate recovered after saponification of lignified walls was ~5% that of nonlignified controls, indicating that ferulate monomers and dimers

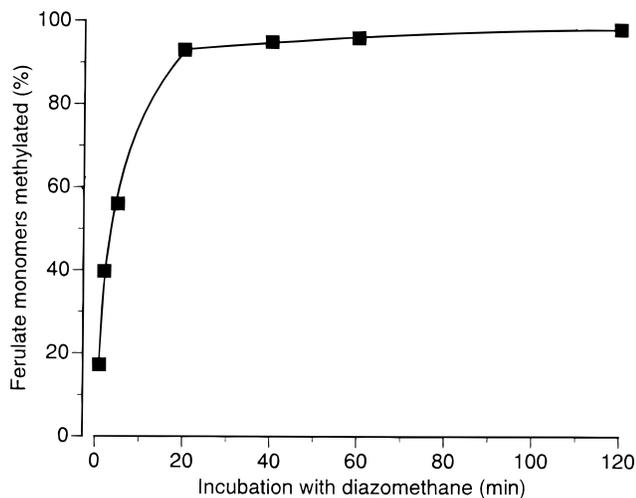


Figure 1. Methylation of ferulate monomers in nonlignified primary walls of maize by diazomethane.

were extensively coupled to lignin by ether and C–C linkages (Table 1). Diferulates, in particular, play a considerable role in cross-linking lignified walls because they not only cross-link xylan chains but also cross-link xyans to lignin. AIP treatment of cell suspensions reduced the quantity of total ferulate incorporated into lignin from 15.9 to 4.5 mg g⁻¹, a 72% reduction in ferulate–lignin cross-linking.

Several chemical approaches were considered for selectively protecting phenolic hydroxyl groups on ferulates prior to complex formation, thereby blocking oxidative coupling of ferulate esters with lignin. This modification would also prevent ferulate cross-linking by the addition of ferulate phenolic hydroxyls to lignin quinone methide intermediates. *N*-Acetylimidazole, commonly used to “selectively” acetylate tyrosine phenols (Riordan et al., 1965), readily acetylated ethyl ferulate in neutral aqueous media. Further work with this reagent was abandoned because <50% of the ferulate monomers in nonlignified walls could be acetylated even after a 1 h treatment with a 500 molar excess of *N*-acetylimidazole (Grabber and Ralph, unpublished results). In contrast, ferulate monomers in nonlignified walls were rapidly methylated by a 40 molar excess of diazomethane (Figure 1), but peroxidase activity was severely reduced by extended reaction times. Therefore, a brief (10 min) methylation was used in subsequent work to help maintain peroxidase activity. The 10 min diazomethane treatment reduced the total quantity of ferulate monomers and dimers with free phenolic hydroxyls by 70% (Table 2). The quantity of fully methylated ferulates is subject to some uncertainty since both phenolic moieties of 5–5 and 8–8 coupled dehydrodimers must be protected to block their incorporation into lignin. Partial methylation of these dehydrodimers, however, was inconsequential because they comprised only ~6% of the total ferulate in nonlignified walls. As noted earlier, incubation of nonlignified walls with glucose oxidase leads to extensive diferulate formation; diferulates comprised 48% of total ferulates in unmethylated walls and 64% of total ferulates in diazomethane treated walls.

Unfortunately, even after a brief 10 min methylation, peroxidase activity was exhausted when walls were lignified with high levels of coniferyl alcohol. As a result, lignification of diazomethane-treated walls was limited to ~100 mg g⁻¹ (Figure 2a). Coniferyl alcohol

not polymerized into wall-bound lignin was recovered as β -O-4, β -5, and β - β coupled coniferyl alcohols and oligomeric dehydrogenation products (data not shown). Ferulates were extensively incorporated into lignin, even at low levels of lignification (Figure 2b; Table 2). Diazomethane methylation reduced the quantity of ferulates incorporated into lignin from 15.7 to 5.2 mg g⁻¹, a 67% reduction in ferulate–lignin cross-linking.

A potential shortcoming of the diazomethane treatment is that several other wall components, in addition to ferulate, may be methylated. Although other phenolic constituents such as tyrosine and lignin are probably methylated, these are minor components in walls isolated from maize cell suspensions (Kieliszewski and Lamport, 1988; Grabber et al., 1995). Diazomethane also methylates carboxylic acids such as galacturonic and glucuronic acids, which together comprised ~11% of nonlignified maize walls. Saponification–methanol analysis of walls indicated that methyl esterification of uronic acids was increased from about 50 to 65% by the 10 min diazomethane treatment (Grabber, unpublished results). Since glutamate and aspartate also comprise ~1–2% of these walls (Kieliszewski and Lamport, 1988), a portion of the additional methanol released by saponification may have originated from these acidic amino acids. Methyl esterification blocks the addition of uronic acids to lignin quinone methide intermediates, thereby reducing the formation of benzyl ester cross-links in walls. Fortunately, diazomethane methylation of acidic wall components should not greatly affect our results because stable benzyl uronate esters apparently form only under acidic conditions and not at the neutral pH we used to lignify walls (Sipilä and Brunow, 1991). Other wall components (neutral polysaccharides, neutral or basic amino acids, etc.) are not methylated by diazomethane. Overall, it appears that AIP treatment of cell suspensions and, to a lesser degree, diazomethane treatment of walls provide a relatively specific and independent means of determining whether cell wall degradability is enhanced by reductions in ferulate–lignin cross-linking.

Effect of Ferulate Cross-Linking on Cell Wall Degradability. Cell walls were degraded with a mixture of Viscozyme L and Celluclast 1.5 L or Biofeed Beta and Celluclast 1.5 L, commercial enzyme preparations selected to provide pectinase, xylanase, and cellulase activities necessary for degrading cell walls. In previous work, both enzyme mixtures were extremely effective for degrading nonlignified maize walls (Grabber et al., 1998a). Viscozyme L is marketed as a mixed-linked β -glucanase preparation from *Aspergillus* sp. that contains pectinase, cellulase, and xylanase activities. Celluclast is a crude cellulase preparation from *Trichoderma reesei* that contains xylanase, mixed-linked β -glucanase, protease, and small amounts of pectinase activities (Massiot et al., 1989). Biofeed Beta is marketed as a xylanase, mixed β -glucanase, and amylase preparation from *Humicola insolens* and *Bacillus amyloliquefaciens*. Of the three enzyme preparations, only Biofeed contained feruloyl esterase activity (Grabber et al., 1998a). Biofeed and Celluclast appeared to release carbohydrate from maize walls more rapidly and extensively than Viscozyme and Celluclast (Tables 2–4). These comparisons are somewhat tenuous, however, because the enzyme mixtures were not used concurrently to evaluate cell wall degradability. Fortunately,

Table 2. Ferulate Composition and Carbohydrate Degradability (Milligrams per Gram of Cell Wall) of Nonlignified and Synthetically Lignified Walls Prepared with Normal or Diazomethane-Methylated Walls Isolated from Maize Cell Suspensions^a

treatment	ferulates			VC		BC	
	monomers	dimers	total	6 h	72 h	6 h	72 h
nonlignified walls ^b							
unmethylated	9.57 ± 0.68	8.74 ± 0.69	18.31 ± 0.02	398 ± 6	708 ± 4	592 ± 0	766 ± 3
methylated	1.96 ± 0.02	3.49 ± 0.11	5.44 ± 0.13	421 ± 7	731 ± 12	584 ± 6	773 ± 7
lignified walls ^c							
unmethylated	0.46	2.10	2.56	214	459	312	578
methylated	0.11	0.16	0.27	265	573	387	647
<i>P</i> level	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01

^a Synthetically lignified walls were prepared by slowly adding coniferyl alcohol and hydrogen peroxide to nonlignified walls. Walls were hydrolyzed with a mixture of Viscozyme and Celluclast (VC) or Biofeed and Celluclast (BC). ^b Mean ± SE (*n* = 2). ^c Least-squares means, adjusted to a Klason lignin concentration of 103 mg g⁻¹, were compared using the PDIFF option of the GLM procedure (SAS, 1990).

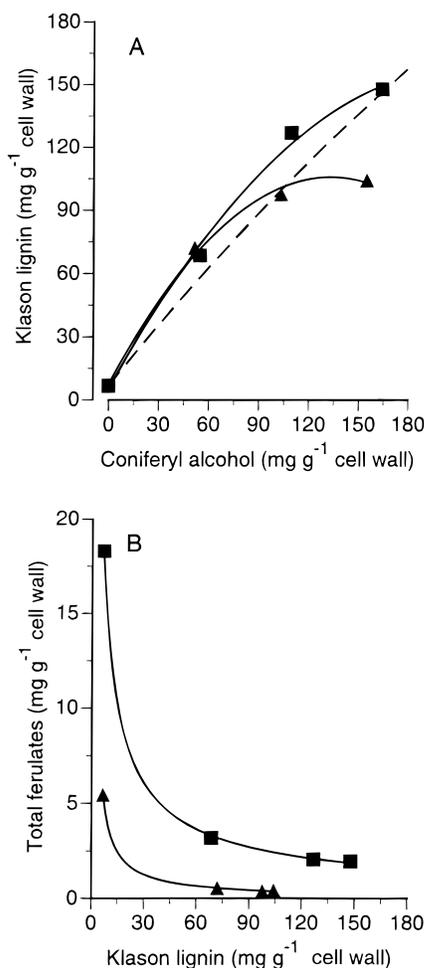


Figure 2. Formation of synthetically lignified walls with un-methylated (■) and diazomethane-methylated (▲) primary walls of maize. (A) Stoichiometry of cell wall lignification with coniferyl alcohol. The dashed line indicates the predicted lignin content of walls with complete polymerization of coniferyl alcohol into wall-bound lignin. (B) Incorporation of total ferulates (monomers plus dimers) into synthetic maize wall lignin.

both enzyme mixtures were used concurrently to evaluate the release of individual neutral sugars from maize walls (Tables 3 and 4), thereby permitting a direct comparison of hydrolytic activities. Averaged over all treatments, Biofeed and Celluclast released 236% more xylose after 6 h and 28% more xylose after 72 h of hydrolysis than Viscozyme and Celluclast. Because

~90% of the xylose in primary maize walls is derived from xylans (Carpita, 1984), this suggests that the Biofeed and Celluclast mixture had greater xylanase activity. Although feruloyl esterase activity in Biofeed may enhance the degradation of soluble xylans, the activity of these enzymes on cell walls is low (Coughlan and Hazlewood, 1993; McCrae et al., 1994). More importantly, these enzymes are not capable of hydrolyzing most types of diferulate esters in soluble xylan substrates, and their action on diferulates (and probably ferulate–lignin cross-links) in cell walls is probably nil (Bartolome et al., 1997; Grabber et al., 1998a). Therefore, it is unlikely that their action would enhance the solubilization of feruloylated xylans or the degradation of maize walls. Biofeed and Celluclast may also contain greater cellulase and possibly mixed-linked β -glucanase activities because they released 18% more glucose after 6 h and 9% more glucose after 72 h than Viscozyme and Celluclast. Viscozyme and Celluclast, however, release pectic sugars from maize walls at a ~2-fold greater rate than Biofeed and Celluclast (Grabber, unpublished results).

Lignification reduced the degradability of maize walls in all experiments; each percentage point of lignin polymerized into walls caused roughly a two percentage point decline in carbohydrate solubilization after 6 and 72 h of hydrolysis. Reductions in ferulate cross-linking generally enhanced cell wall hydrolysis, but the response varied somewhat depending on how cross-linking was reduced and which enzyme mixture was used to degrade walls. Reductions in ferulate cross-linking, via AIP inhibition of ferulate biosynthesis, increased carbohydrate solubilization from nonlignified and lignified walls by 97 mg g⁻¹ after 6 h and by 68 mg g⁻¹ after 72 h of hydrolysis with Viscozyme and Celluclast (Tables 3 and 4). AIP treatment enhanced the release of all major neutral sugars, particularly that of xylose. The results differed somewhat when walls were degraded with Biofeed and Celluclast. In this case, AIP-mediated reductions in ferulate cross-linking had little effect on hydrolysis of nonlignified walls; only the release of xylose was slightly enhanced after 6 h of hydrolysis. For lignified walls, AIP treatment improved carbohydrate release by 57 mg g⁻¹ after 6 h but differences were not significant after 72 h of hydrolysis. AIP treatment enhanced the release of all major neutral sugars, particularly xylose, and the increase was most pronounced after 6 h of hydrolysis. Overall, AIP-mediated reductions in ferulate cross-linking most consistently improved the initial hydrolysis of lignified walls. Reductions in ferulate–lignin cross-linking had a greater

Table 3. Carbohydrate (Milligrams per Gram of Cell Wall) and Neutral Sugars (Percent) Released from Nonlignified and Synthetically Lignified Walls by a 6 h Hydrolysis with Viscozyme and Celluclast (VC) or Biofeed and Celluclast (BC)^a

treatment	carbohydrate		arabinose		xylose		glucose	
	VC	BC	VC	BC	VC	BC	VC	BC
nonlignified walls								
0 μ M AIP	353	546	37.4	73.8	28.7	75.0	50.2	62.2
40 μ M AIP	467	523	50.8	74.8	39.6	82.1	58.4	60.4
lignified walls								
0 μ M AIP	171	199	17.1	34.5	10.9	33.4	19.7	29.5
40 μ M AIP	250	256	29.7	44.0	22.6	49.8	30.5	35.9
analysis of variance ^b								
AIP	*	*	*	*	*	*	*	†
lignin	*	*	*	*	*	*	*	*
AIP \times lignin	NS	*	NS	†	NS	*	NS	*

^a Treatments, chemical composition data, and statistical methods were described in Table 1. ^b *, †, NS, significant at the 0.05 and 0.10 levels of probability and not significant, respectively.

Table 4. Carbohydrate (Milligrams per Gram of Cell Wall) and Neutral Sugars (Percent) Released from Nonlignified and Synthetically Lignified Walls by a 72 h Hydrolysis with Viscozyme and Celluclast (VC) or Biofeed and Celluclast (BC)^a

treatment	carbohydrate		arabinose		xylose		glucose	
	VC	BC	VC	BC	VC	BC	VC	BC
nonlignified walls								
0 μ M AIP	690	759	76.9	91.4	72.4	90.6	82.2	88.9
40 μ M AIP	745	745	81.2	90.8	78.8	91.2	83.3	86.4
lignified walls								
0 μ M AIP	416	475	55.3	73.4	41.5	64.8	51.4	62.4
40 μ M AIP	498	499	68.8	79.7	59.2	76.1	64.8	68.7
analysis of variance ^b								
AIP	*	NS	*	NS	*	*	*	†
lignin	*	*	*	*	*	*	*	*
AIP \times lignin	NS	NS	*	†	*	*	*	*

^a Treatments, chemical composition data, and statistical methods were described in Table 1. ^b *, †, NS, significant at the 0.05 and 0.10 levels of probability and not significant, respectively.

effect on the release of sugars derived from xylans than cellulose, which is expected since ferulates are esterified to xylans. In related studies, AIP-mediated reductions in ferulate–lignin cross-linking dramatically enhanced the rate, but the not extent, of maize wall fermentation by mixed rumen microorganisms (Grabber, Mertens, and Weimer, unpublished results).

Reductions in diferulate cross-linking, due to diazomethane methylation of ferulate phenolic hydroxyl groups, appeared to have little or no effect on the hydrolysis of nonlignified walls (Table 2). Diazomethane treatment, however, significantly improved carbohydrate solubilization from lignified walls by 51–75 mg g⁻¹ after 6 h and by 68–114 mg g⁻¹ after 72 h of hydrolysis with both enzyme mixtures providing additional, although less specific, evidence that reductions in ferulate–lignin cross-linking enhance cell wall degradability.

In conclusion, two dissimilar but relatively specific methods for limiting ferulate incorporation into primary wall lignins have provided good evidence that reductions in ferulate–lignin cross-linking will significantly improve the rate, and to a lesser degree, the extent of cell wall degradation in grasses. Some caution, however, must be used in extrapolating our results to plants because the abundance of ferulate–lignin cross-links and their effects on degradability may differ in tissues with secondary cell walls. However, on the basis of our findings, we propose that selection or genetic engineering of grasses for low feruloylation of arabinoxylans will significantly enhance the enzymatic hydrolysis and subsequent utilization of structural polysaccharides for nutritional and industrial purposes.

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LITERATURE CITED

- Argillier, O.; Barriere, Y.; Lila, M.; Jeanneteau, F.; Gelinet, K.; Menanteau, V. Genotypic variation in phenolic components of cell-walls in relation to the digestibility of maize stalks. *Agronomie* **1996**, *16*, 123–130.
- Bartolome, B.; Faulds, C. B.; Kroon, P. A.; Waldron, K.; Gilbert, H. J.; Hazlewood, G.; Williamson, G. An *Aspergillus niger* esterase (ferulic acid esterase III) and a recombinant *Pseudomonas fluorescens* subsp. *cellulosa* esterase (XylD) release a 5-5' ferulic dehydrodimer (diferulic acid) from barley and wheat cell walls. *Appl. Environ. Microbiol.* **1997**, *63*, 208–212.
- Blumenkrantz, N.; Asboe-Hansen, G. New method for quantitative determination of uronic acids. *Anal. Biochem.* **1973**, *54*, 484–489.
- Carmer, S. G.; Walker, W. M.; Seif, R. D. Practical suggestions on pooling variances for F tests of treatment effects. *Agron. J.* **1969**, *61*, 334–336.
- Carpita, N. C. Cell wall development in maize coleoptiles. *Plant Physiol.* **1984**, *76*, 205–212.
- Coughlan, M. P.; Hazlewood, G. P. β -1,4-D-Xylan degrading enzyme systems: biochemistry, molecular biology and applications. *Biotechnol. Appl. Biochem.* **1993**, *17*, 259–289.
- Darvill, A. G.; Smith, C. J.; Hall, M. A. Cell wall structure and elongation growth in *Zea mays* coleoptile tissue. *New Phytol.* **1978**, *80*, 503–516.
- 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.

- Evans, J. J.; Himmelsbach, D. S. Incorporation of peroxidase into synthetic lignin. *J. Agric. Food Chem.* **1991**, *39*, 830–832.
- Fritz, J. O.; Moore, K. J.; Vogel, K. P. Ammonia-labile bonds in high- and low-digestibility strains of switchgrass. *Crop Sci.* **1991**, *31*, 1566–1570.
- Fry, S. C. Cross-linking of matrix polymers in the growing cell walls of angiosperms. *Annu. Rev. Plant Physiol.* **1986**, *37*, 165–186.
- Goto, M.; Matsuoka, J.; Sato, T.; Ehara, H.; Morita, O. Brown midrib mutant maize with reduced levels of phenolic acids ether-linked to the cell walls. *Anim. Feed Sci. Technol.* **1994**, *48*, 27–38.
- 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.
- 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.
- Grabber, J. H.; Hatfield, R. D.; Ralph, J. Diferulate cross-links impede the enzymatic degradation of nonlignified maize walls. *J. Sci. Food Agric.* **1998a**, in press.
- Grabber, J. H.; Ralph, J.; Hatfield, R. D. Modeling lignification in grasses with monolignol dehydropolymerisate-cell wall complexes. In *Lignin and Lignan Biosynthesis*; Lewis, N. G., Sarkanen, S., Eds.; ACS Symposium Series 697; American Chemical Society: Washington, DC, 1998b, 163–171.
- Hatfield, R. D. Cell wall polysaccharide 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.; 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.
- 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.
- Jung, H. 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.; Casler, M. D. Relationship of lignin and esterified phenolics to fermentation of smooth bromegrass fibre. *Anim. Feed Sci. Technol.* **1991**, *32*, 63–68.
- 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.
- Jung, H. G.; Vogel, K. P. Lignification of switchgrass (*Panicum virgatum*) and big bluestem (*Andropogon gerardii*) plant parts during maturation and its effect on fibre degradability. *J. Sci. Food Agric.* **1992**, *59*, 169–176.
- Jung, H. G.; Valdez, F. R.; Hatfield, R. D.; Blanchette, R. A. Cell wall composition and degradability of forage stems following chemical and biological delignification. *J. Sci. Food Agric.* **1992**, *58*, 347–355.
- Jung, H. G.; Morrison, T. A.; Buxton, D. R. Degradability of cell-wall polysaccharides in maize internodes during stalk development. *Crop Sci.* **1998**, in press.
- Kato, Y.; Nevins, D. J. Isolation and identification of *O*-(5-*O*-feruloyl- α -L-arabinofuranosyl)-(1-3)-*O*- β -D-xylopyranosyl-(1-4)-D-xylose as a component of *Zea* shoot cell-walls. *Carbohydr. Res.* **1985**, *137*, 139–150.
- Keller, B.; Nierhaus-Wunderwald, D.; Amrhein, N. Deposition of glycine-rich structural protein in xylem cell walls of french bean seedlings is independent of lignification. *J. Struct. Biol.* **1990**, *104*, 144–149.
- Kieliszewski, M.; Lamport, D. T. A. Tying the knots in the extensin network. In *Self-Assembling Architecture. 46th Symposium of the Society for Developmental Biology*; Varner, J. E., Ed.; St. Paul, MN, 1988; pp 61–77.
- Lam, T. B. T.; Iiyama, K.; Stone, B. Lignin and hydroxycinnamic acids in walls of brown midrib mutants of sorghum, pearl millet and maize stems. *J. Sci. Food Agric.* **1996**, *71*, 174–178.
- Massiot, P.; Thibault, J. F.; Rouau, X. Degradation of carrot (*Daucus carota*) with cell-wall polysaccharide-degrading enzymes. *J. Sci. Food Agric.* **1989**, *49*, 45–57.
- McCrae, S. I.; Leith, K. M.; Gordon, A. H.; Wood, T. M. Xylan-degrading enzyme system produced by the fungus *Aspergillus awamori*: isolation and characterization of a feruloyl esterase and a *p*-coumaroyl esterase. *Enzyme Microb. Technol.* **1994**, *16*, 826–834.
- Morrison, I. M. Changes in the biodegradability of ryegrass and legume fibers by chemical and biological pretreatments. *J. Sci. Food Agric.* **1991**, *54*, 521–533.
- Mueller-Harvey, I.; Hartley, R. D.; Harris, P. J.; Curzon, E. H. Linkage of *p*-coumaroyl and feruloyl groups to cell wall polysaccharides of barley straw. *Carbohydr. Res.* **1986**, *148*, 71–85.
- Ohnishi, J.; Watanabe, T.; Koshijima, T. Synthesis of dehydrogenation polymer-polyose complexes by peroxidase. *Phytochemistry* **1992**, *31*, 1185–1190.
- 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.; 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* **1994**, 3485–3498.
- Ralph, J.; Grabber, J. H.; Hatfield, R. D. Lignin-ferulate cross-links in grasses: active incorporation of ferulate polysaccharide esters into ryegrass lignins. *Carbohydr. Res.* **1995**, *275*, 167–178.
- Riordan, J. F.; Wacker, W. E. C.; Vallee, B. L. N-Acetylimidazole: a reagent for determination of "free" tyrosyl residues of proteins. *Biochemistry* **1965**, *4*, 1758–1765.
- Schmutz, A.; Jenny, T.; Amrhein, N.; Ryser, U. Caffeic acid and glycerol are constituents of the suberin layers in green cotton fibres. *Planta* **1993**, *189*, 453–460.
- Sipilä, J.; Brunow, G. On the mechanism of formation of noncyclic benzyl ethers during lignin biosynthesis. Part 4. The reactions of a β -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.
- Zon, J.; Amrhein, N. Inhibitors of phenylalanine ammonia lyase: 2-aminoindan-2-phosphonic acid and related compounds. *Liebigs Ann. Chem.* **1992**, 625–628.

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