Lignin Impact on Fiber Degradation. 3. Reversal of Inhibition of Enzymatic Hydrolysis by Chemical Modification of Lignin and by Additives

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The impact of lignins of various origins on filter paper hydrolysis by fungal cellulase was evaluated. Powdered lignins were added to enzyme incubations, either as isolated or after thorough hydroxypropylation of phenolic sites. Extent of cellulose hydrolysis was reduced by 14−60% by the addition of up to 15% lignin to the substrate. Unmodified lignins were more detrimental to cellulose hydrolysis than hydroxypropylated lignins. The inhibitory effect of lignin addition was only partially overcome by a 10-fold increase in cellulase activity, suggesting inhibitory lignin interactions with both substrate and enzyme. Preincubation of cellulase with underivatized lignins resulted in reduced enzyme activity and soluble protein concentration in the supernatant, suggesting protein precipitation with lignin rather than reduced activity of a lignin−enzyme complex as the inhibitory mechanism. Two further experiments showed that the negative impact of lignin on cellulose hydrolysis can be counteracted by addition of various N compounds and by ammoniation.

Keywords: *Lignin; cellulose degradation; cellulase inhibition; free phenolic group; ammoniation; PEG; PVP; N compounds*

INTRODUCTION

Forage lignins inhibit fiber degradation to an extent that seems to depend on their monomer composition [e.g., Sewalt et al. (1996a), Bernard Vailhé et al. (1996)]. Regeneration of quinone methides from lignin during ruminal digestion was recently proposed as an alternative mechanism by which lignin interferes with fiber digestion (Sewalt et al., 1996a). In addition, extraneously added lignin has a negative impact on microbial degradation of cellulose, which depends upon the phenolic hydroxyl functionality (Sewalt et al., 1996b). It was suggested that the phenolic hydroxyl group mediates binding to the cellulose substrate and/or microbial enzymes but that hydrophobic binding of enzymes by lignin moieties with blocked phenolic sites might also occur.

Few research efforts have addressed the direct impact of lignin on cellulolytic enzymes. Avgerinos and Wang (1983) reported that soluble lignins, obtained by extracting wheat straw in dilute NaOH in 50% ethanol, depressed commercial cellulase activity. More recently, cellulase inhibition by lignin was reported to be due to lignin−enzyme adsorption (Converse et al., 1990), and kinetic analysis has revealed noncompetitive inhibition of xylanases by lignins in pulping liquors (Senior et al., 1991).

The objectives of the four subsequent experiments were to investigate the nature of enzyme inhibition by lignin in relation to lignin functional groups and to evaluate whether the inhibition can be reversed by the addition of various binding compounds, reducing agents, or ammoniation.

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MATERIALS AND METHODS

Lignins. The lignins used were Organosolv pine (*Pinus* spp.) lignin and steam explosion poplar (*Populus* sp.) and barley (*Hordeum vulgare*) straw lignins previously described by Glasser et al. (1983). In addition, an Organosolv mixed hardwood (MHW) lignin [50% maple (*Acer* sp.), 35% birch (*Betula* sp.), and 15% poplar (*Populus* sp.)] was used (Aldrich Chemical Co., Milwaukee, WI). A summary of functional group content, permanganate yield, and molecular weight (M_w) is presented in Table 1 [from Glasser et al. (1983) and Sewalt et al. (1996b)]. These lignin preparations contain at least 85−90% actual lignin (based on UV absorption). Contamination with nonphenolic substances is minor. Carbohydrate contamination averages 3% (Glasser et al., 1983). On the basis of Kjeldahl analysis of the mixed hardwood and its hydroxypropylated counterpart, content of nitrogenous compounds is minimal (0.2−0.7%). Compared with *in situ* lignin, steam explosion lignins are hydrolytically modified, resulting in more free phenolic OH groups, fewer aryl−alkyl ether bonds, and a higher degree of condensation (Glasser et al., 1983).

Hydroxypropyl lignin (HPL) derivatives, previously described by Sewalt et al. (1996b), were prepared according to the method of Jain and Glasser (1993). Lignin hydroxypropylation performed at room temperature and in dilute aqueous alkali results in specific modification of phenolic hydroxyl groups. The removal of free phenolic sites is complete, as indicated by the complete absence of the otherwise prominent phenolic O-acetyl signal in H-NMR spectroscopy lignin (Glasser et al., 1984).

Substrate and Digestion Conditions. Digestions were performed using small disks of filter paper (Whatman No. 1, Whatman Inc., Fairfield, NY) prepared with an office perforator. In all assays, a commercially available cellulase was used (CEP cellulase, Biovance Technologies, Omaha, NE; specific cellulase activity, 400 FPU/g). In this notation, FPU stands for filter paper units measured as glucose released (μmol/min) from ground filter paper. All incubations were in 0.1 M sodium acetate buffer of pH 4.8 using a temperature-controlled (40

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Table 1. Content of Lignin Functional Groups (Percent),[a] Yield (Percent) of Monomers and Dimers upon Permanganate Oxidation, and Molecular Weight of Four Lignins [after Glasser et al. (1983) and Sewalt et al. (1996b)]

lignin	OCH_3	total OH	phenolic OH	total carbonyl	permanganate yield	M_w
steam explosion						
barley straw[a]	10.3	8.5	5.4	4.7	8.2	1100
poplar[a]	15.0	9.8	4.3	2.9	35.5	3000
Organosolv						
pine[a]	15.1	8.2	6.3	2.3	28.7	1400
mixed hardwood[b]	19.1		4.0			

[a] Glasser et al. (1983). [b] After Sewalt et al. (1996b) (only OCH_3 and phenolic OH contents were determined).

°C) shaking (200 rpm) incubator (Lab-Line Instruments, Melrose Park, IL).

Experiment 1. Filter paper (200 ± 5 mg) was digested in a 20-mL solution of cellulase for 24 h in the presence or absence of the four lignins and their hydroxypropylated counterparts. Treatments were arranged as a 3 × 2 × 4 × 2 factorial with three enzyme concentrations (0, 5, and 50 FPU/g of filter paper), two lignin concentrations (5 and 15% of substrate), and four lignin sources (pine, poplar, barley straw, and MHW lignin), before or after hydroxypropylation of the lignin (unmodified lignin versus HPL). Each treatment combination was incubated in triplicate. Zero lignin controls were included at the three respective enzyme concentrations.

After 24 h of incubation, the solutions were cooled to 4 °C and centrifuged at 500*g*, and the supernatant was retained. Additional buffer was added to the residue, the tubes were vortexed and centrifuged at 500*g*, and the two supernatants were combined and made up to constant volume. The residue was dried overnight at 105 °C and weighed to determine dry matter disappearance. To determine the extent of filter paper digestion, the dried residues were extracted for 1 h with boiling neutral detergent (Van Soest et al., 1991), filtered using a pretared crucible of coarse porosity containing a glass fiber filter (Whatman Grade 934 AH, particle retention 1.5 mm), and dried overnight at 105 °C. For most samples, little or no visible lignin particles remained after neutral detergent extraction. Samples that retained visible lignin particles (originating from the added lignins) were analyzed for Klason lignin to arrive at correct value for filter paper digestion. Ultraviolet absorption at 280 nm was used to determine the concentration of soluble lignins released during enzymatic hydrolysis. Glucose in the supernatants was measured using the glucose oxidase based Trinder kit (Sigma Chemical Co., St. Louis, MO) to estimate the extent of complete cellulose hydrolysis. Data were statistically examined by analysis of variance using the GLM procedure of SAS (1989). The treatments were arranged as a 3 × 2 × 4 × 2 factorial with enzyme concentration, lignin concentration, lignin source, and lignin hydroxypropylation as main effects.

Experiment 2. Triplicate samples of the previously described lignins (10 mg) and samples without lignin addition were preincubated for 3 h at 4 °C with 0.8 FPU of CEP cellulase in 1 mL of digestion buffer followed by centrifugation (10000*g*; 5 min). The supernatant (800 μL) was retained and an aliquot (200 μL) assayed for cellulase activity (in FPU/mL) by incubation with 15 mg of filter paper in 1 mL of buffer at 40 °C for 1 h followed by determination of reducing sugars (Miller, 1959). Soluble protein in the supernatant was determined according to the Bradford method (Bradford, 1976) using a Bio-Rad kit. Data were expressed as percentage of the control and were statistically examined using two-way ANOVA with lignin source and hydroxypropylation as main effects.

Experiment 3. Lignin inhibition of cellulase activity was tested in the presence or absence of six compounds. Pine lignin (2.5 mg) was incubated with CEP cellulase (0.25 FPU in 1 mL of buffer) and filter paper (25 mg) at 40 °C for 24 h, followed by centrifugation (10000*g*; 5 min) and determination of reducing sugars. Prior to incubation, one of the following compounds was added: none; 35 μmol of cysteine hydrochloride; 35 μmol of 2-mercaptoethanol; 2.5 mg of poly(vinylpyrrolidone) (PVP-40; Sigma catalog no. P0930); 2.5 mg of poly(ethylene glycol) (PEG; 3350 MW, Sigma catalog no. P3640); 2.5 mg of ovalbumin (Sigma catalog no. A5503); or 2.5 mg of gelatin (Bio-Rad product no. 170-6537). Cysteine hydrochloride and 2-mercaptoethanol are nucleophilic reducing agents likely to react with quinoid structures (Sewalt et al., 1996a); PVP and PEG are binding agents for phenolics (Garrido et al., 1991); ovalbumin and gelatin are proteins with affinity for phenolics (Petersen and Hill, 1991). The direct effect of each additive on cellulase activity was determined relative to a control containing additive but not lignin. A positive control for inhibition reversal (hydroxypropylated pine lignin, no additives) was included in the experiment. Because of observed interference of S compounds (cysteine hydrochloride, 2-mercaptoethanol) with the reducing sugar assay, a scaled-up experiment was conducted in which 200 mg of filter paper was digested with or without the appropriate amounts of pine lignin and S compounds, followed by filtration and determination of dry residue as in experiment 1. Data from each experiment, expressed as percentage of the control (without lignin or additive), were analyzed by two-way ANOVA with lignin and additive as main effects.

Experiment 4. A final experiment was conducted to evaluate whether ammoniation, a pretreatment used to improve the digestibility of crop residues (Waiss et al., 1972; Creek et al., 1983), results in association of NH_3-N with lignin. A second objective was to investigate the possible role of quinone methide intermediates in this association. Triplicate samples (150 mg) of Organosolv MHW lignin, either unmodified or hydroxypropylated, were suspended in 1.0 mL of 3% aqueous NH_3. After incubation of the lignins in 3% NH_3 at room temperature (25 °C) for 48 h, the mixtures were lyophilyzed, washed with distilled water, oven-dried at 55 °C, and subjected to Kjehldahl analysis to determine N content. The lignins and subsequent treatments were as follows:

(1) MHW lignin, lyophilized after ammoniation, washed with distilled water and filtered using a glass-fiber filter (Whatman Grade 934 AH, particle retention 1.5 μm) in a 30-mL gooch crucible of fine porosity (pore size, 4−5.5 μm).

(2) MHW lignin, after ammoniation acidified with 1 N HCl to pH 1, then lyophilized, washed, and filtered.

(3) As treatment 2 but ammoniated in the presence of 1.43 M 2-mercaptoethanol.

(4) Hydroxypropylated mixed hardwood lignin further processed as treatment 2.

The effect of NH_3-N covalently linked to lignin on inhibition of cellulase activity was determined in triplicate. Fifteen milligrams of filter paper was incubated with 0.25 FPU of CEP cellulase in 1 mL of buffer containing 3 mg of the following lignins: (1) MHW lignin; (2) MHW lignin, ammoniated according to treatment 2 above; (3) MHW lignin, ammoniated in the presence of 2-mercaptoethanol; and (4) hydroxypropylated MHW lignin.

RESULTS AND DISCUSSION

Enzymatic Saccharification of Filter Paper (Experiment 1). Addition of up to 15% lignins to the substrate (equivalent to 1500 μg/mL medium) reduced ($P < 0.001$) the extent of filter paper digestion after 24 h by 14−60% (Table 2) and cellulose saccharification (glucose yield) by 0−70% (Table 3). Unmodified lignins were more ($P < 0.001$) detrimental to cellulose hydrolysis than hydroxypropylated lignins, confirming previous results regarding the inhibitory effect of mixed hardwood lignins on *in vitro* digestion with ruminal fluid (Sewalt et al., 1996b). Lignin inhibition was only partially overcome by increasing cellulase concentration from 5 to 50 FPU/g of substrate, suggesting both enzyme inactivation and substrate binding as modes of inhibition.

Table 2. Extent (Percent) of Filter Paper Digestion (Experiment 1)

lignin source	lignin modification	lignin concn[a]	cellulase loading (FPU[b]/g) 0[c]	5[c]	50[c]	SEM[b]
pine	none	5	−1.8	12.3	46.4	
		15	−6.9	10.3	45.3	
	HPL[b]	5	−4.5	12.3	50.9	
		15	−6.4	13.0	44.1	
mixed hardwood	none	5	−5.6	12.4	48.1	
		15	−6.2	10.3	42.1	
	HPL	5	−1.2	12.3	51.8	
		15	−7.5	17.1	48.2	
poplar	none	5	−3.5	16.9	50.9	
		15	−4.9	13.9	47.4	
	HPL	5	−4.0	12.1	53.2	
		15	−6.1	13.0	51.7	
barley straw	none	5	−2.6	14.4	48.4	
		15	−4.2	13.6	48.2	
	HPL	5	−1.2	16.1	54.2	
		15	−4.7	17.8	49.4	0.84
control[d]	control	0	0.0	25.9	59.3	1.54

[a] Percent of substrate. [b] FPU, filter paper unit; SEM, standard error of the mean; HPL, hydroxypropylated lignin. [c] Negative data in the 0 FPU/g column indicate residual lignin in the sample; the amounts of residual lignin at 0 FPU/g were used to correct filter paper digestion data in the 5 and 50 FPU/g columns. [d] Contrast effects: control vs lignin ($P < 0.001$); lignin source ($P < 0.001$); HPL vs unmodified lignin ($P < 0.001$); lignin concentration ($P > 0.20$); enzyme loading ($P < 0.001$); lignin concentration × enzyme loading interaction ($P < 0.001$).

Table 3. Yield (Milligrams per Gram of Cellulose) of Glucose (Experiment 1)

lignin source	lignin modification	lignin concn[a]	cellulase loading (FPU[b]/g) 5	50	SEM[b]
pine	none	5	149	474	
		15	75	467	
	HPL[b]	5	206	522	
		15	189	491	
mixed hardwood	none	5	145	550	
		15	77	448	
	HPL	5	209	493	
		15	186	481	
barley straw	none	5	163	484	
		15	150	466	
	HPL	5	198	444	
		15	200	510	
poplar	none	5	196	528	
		15	175	465	
	HPL	5	194	517	
		15	215	521	5.6
control[c]		0	250	552	15.4

[a] Percent of substrate. [b] FPU, filter paper unit; SEM, standard error of the mean; HPL, hydroxypropylated lignin. [c] Contrast effects: control vs lignin ($P < 0.01$); lignin source ($P < 0.001$); HPL vs unmodified lignin ($P < 0.001$); lignin concentration ($P < 0.001$); enzyme loading ($P < 0.001$); lignin concentration × enzyme loading interaction ($P < 0.001$).

The extent of filter paper digestion and cellulose saccharification was more inhibited by Organosolv lignins than by steam explosion lignins, especially at low enzyme and high lignin concentrations. The difference in degree of inhibition may be explained by differences in the content of low molecular weight contaminants (i.e., odiferous ethyl ethers and esters giving rise to the peculiar Organosolv lignin smell) or differences in molecular weights or functional groups (Table 1). Both steam explosion lignins have a relatively low content of methoxyl and free phenolic hydroxyl groups (Glasser et al., 1983). In contrast, the hardwood and pine Organosolv lignins are rich in methoxyl and free phenolic hydroxyl groups.

Table 4. Degree of Depolymerization[a] (Experiment 1)

lignin source	lignin modification	lignin concn[b]	cellulase loading (FPU[c]/g) 5	50	SEM[c]
pine	none	5	0.66	0.75	
		15	0.39	0.77	
	HPL[c]	5	0.81	0.79	
		15	0.70	0.80	
mixed hardwood	none	5	0.56	0.82	
		15	0.28	0.76	
	HPL	5	0.87	0.77	
		15	0.68	0.76	
barley straw	none	5	0.62	0.74	
		15	0.58	0.75	
	HPL	5	0.71	0.68	
		15	0.72	0.72	
poplar	none	5	0.74	0.72	
		15	0.54	0.80	
	HPL	5	0.65	0.74	
		15	0.86	0.77	0.036
control[d]		0	0.73	0.77	0.055

[a] Cellulose converted to glucose/solubilized cellulose (w/w). [b] Percent of substrate. [c] FPU, filter paper unit; SEM, standard error of the mean; HPL, hydroxypropylated lignin. [d] Contrast effects: control vs lignin ($P = 0.14$); lignin source ($P > 0.20$); HPL vs unmodified lignin ($P < 0.001$); lignin concentration ($P < 0.01$); enzyme loading ($P < 0.001$); lignin concentration × enzyme loading interaction ($P < 0.001$).

The relative degree of inhibition of cellulose hydrolysis by the different lignins is most clearly represented by their effect on degree of depolymerization (DD; ratio of cellulose converted to glucose to extent of cellulose solubilization), which was reduced at the low enzyme concentration only (Table 4). At the low enzyme level (5 FPU/g), pine lignin and mixed hardwood lignin reduced cellulose DD by 42−50%, compared with 6−29% for steam explosion lignins. The DD obtained at the high (50 FPU/g) enzyme level was not affected by lignin addition. The slight decrease in glucose production at high enzyme levels resulted, therefore, from a reduction in cellulose solubilization or initial hydrolysis, rather than inhibitory effects on subsequent hydrolysis to glucose. Therefore, the extent of the reduction in cellulose hydrolysis at high enzyme levels may be explained mainly by lignin−substrate interactions resulting in reduced enzyme accessibility.

No correlations could be detected between concentration of soluble phenolics (A_{280}) and extent of filter paper digestion, cellulose saccharification, or degree of cellulose depolymerization (data not shown); hence, the inhibition most likely takes place at the solid−liquid interface.

The observed lignin inhibition of enzymatic cellulose digestion is in agreement with the *in vitro* microbial digestibility data of Sewalt et al. (1996b), although the enzyme inhibition was more severe. In that experiment, Organosolv hardwood lignin, added at a rate of 10% of the total substrate (or 1 mg/mL medium), reduced cellulose digestion after 72 h by 60 mg/g (or 18%). When added at a rate of 30% of total substrate (or 3 mg/mL), both unmodified and hydroxypropylated hardwood lignin reduced cellulose digestion, but the reduction by unmodified lignin was more pronounced. Because of the complete and specific removal of free phenolic sites during the hydroxypropylation reaction (Glasser et al., 1984), it is highly unlikely that the reversal in inhibition by hydroxypropylation can be attributed to anything but phenolic OH removal.

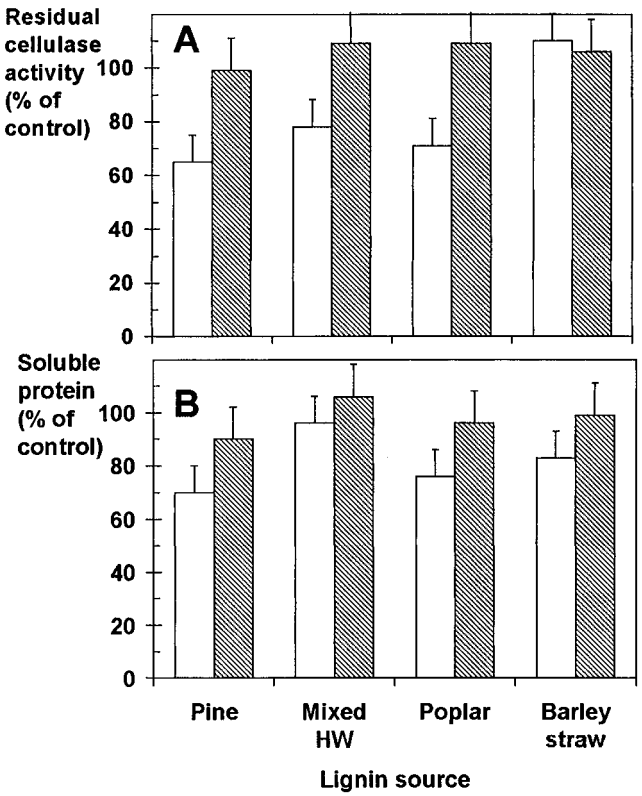

Figure 1. Reduced cellulase activity after preincubation with different industrial lignins (experiment 2). Open bars represent data for unmodified lignins, shaded bars data for hydroxypropylated lignins. (A) Residual filter paper unit activity (percent of control) in supernatant: effects of lignin source ($P = 0.16$), lignin modification ($P < 0.01$), and source × modification interaction ($P = 0.17$). (B) Concentration of soluble protein (percent of control) in supernatant: effects of lignin source ($P < 0.001$), lignin modification ($P < 0.001$), and source × modification interaction ($P < 0.05$).

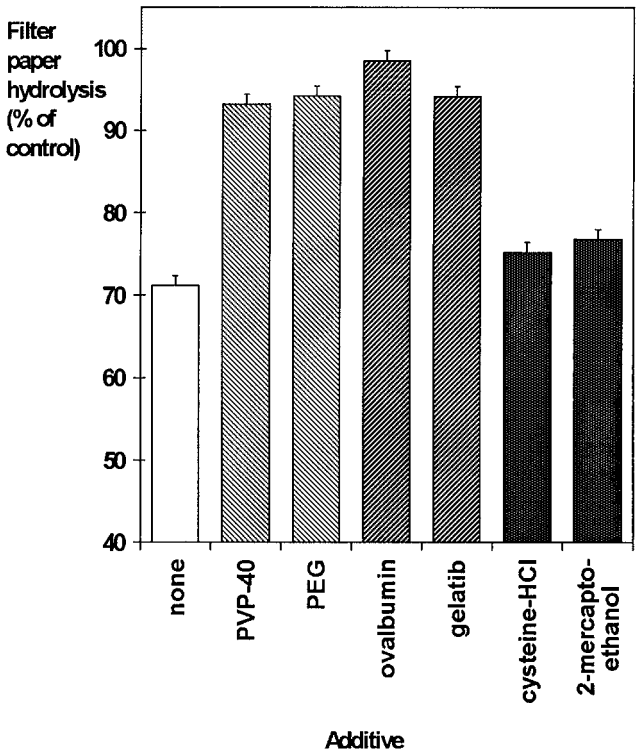

Figure 2. Reversal of inhibition of filter paper hydrolysis by pine lignin by addition of various N compounds and reducing agents (experiment 3): effects of lignin addition ($P < 0.001$), choice of additive ($P < 0.001$), and lignin × additive interaction ($P < 0.001$). Additives did not directly affect ($P > 0.25$) cellulase activity. Filter paper hydrolysis in the presence of HPL was 95.8%.

The impact of soluble lignin on enzymatic cellulose hydrolysis is related to its adsorption characteristics, which, in turn, are related to (phenolic) functionality. Because the isolated lignins contain more free phenolic hydroxyl groups than *in situ* lignins, the magnitude of the observed inhibition may be lower during ruminal degradation of forage cell walls than in our model system. Although the inhibitory effects of isolated lignins may differ from the effect of *in situ* lignins, our results can be extrapolated to digestion of plant cell walls, because lignins are released from the cell wall matrix during microbial digestion in the rumen as lignin−carbohydrate complexes (LCC). The release of LCC may exert a negative effect on rumen digestion by copolymerization with cell wall components (Sewalt et al., 1996a) or by binding other digesta components (proteins, microbial enzymes) as suggested by our current findings.

Cellulase Activity after Co-incubation with Lignins (Experiment 2). Cellulase activity was determined in the soluble phase after preincubation with lignins to (1) quantify the direct lignin inhibition of cellulase enzyme and (2) to differentiate between enzyme precipitation with lignin and possibly reduced activity of a soluble lignin−cellulase complex. After centrifugation, cellulase activity remaining in the supernatant was reduced by co-incubation with all unmodified lignins ($P < 0.05$) except that isolated from steam-exploded barley straw (Figure 1). Hydroxypropylated lignins, which lack phenolic hydroxyl groups and are therefore not able to regenerate quinone methide intermediates (Sewalt et al., 1996b), did not affect cellulase activity.

Levels of soluble protein also decreased ($P < 0.001$) after preincubation with lignins with the exception of preincubation with mixed hardwood lignin (Figure 1). When expressed as a percentage of control, no difference could be detected, using a paired *t*-test, between levels of reducing sugars and soluble protein. Thus, the reduction in cellulase activity results almost exclusively from protein precipitation with the lignins. Our data confirm those of Kawamoto et al. (1992), who first demonstrated the protein adsorption capacity of lignins. That study suggested the degree of protein adsorption by lignins to be dependent upon phenolic hydroxyl content and the method of lignin preparation, which affects distribution pattern and flexibility of phenolic hydroxyl groups.

Enzyme activity was depressed, in descending order, by pine, poplar, and mixed hardwood lignin. This effect corresponded to relatively high contents of free phenolic hydroxyl groups (pine), high molecular weight (poplar), or high methoxyl group content (mixed hardwood). The barley straw lignin, which did not inhibit cellulase activity, is of low molecular weight with low contents of methoxyl and free phenolic hydroxyl groups.

Reversal of Inhibition by Additives (Experiment 3). Extent of cellulose saccharification from filter paper was reduced by 29% by pine lignin added at 10% of the substrate (w/w). The N compounds (PVP, PEG, ovalbumin, and gelatin), added with lignin in a 1:1 ratio (w/w), reverted the inhibition, resulting in about 95% of potential saccharification. This level was similar to that obtained using the pine HPL positive control (Figure 2). The PEG, PVP, gelatin, and ovalbumin have been

used previously to counteract inhibitory effects of tannins on protein digestion (Garrido et al., 1991; Petersen and Hill, 1991). None of these additives appeared to affect saccharification in control (no lignin) incubations (data not shown). Addition of the reducing agents, cysteine hydrochloride and 2-mercaptoethanol, did not improve saccharification. At this relatively low level of enzyme activity (10 FPU/g of filter paper), lignin−enzyme interactions might contribute more to the reduced filter paper digestion than lignin−substrate interactions. The latter would be ameliorated by addition of nucleophilic agents such as cysteine hydrochloride and 2-mercaptoethanol. Alternatively, the affinity of the pine lignin for proteins may be much higher than for reducing agents.

Covalent Lignin−Ammonia Association and Reversal of Inhibition (Experiment 4). It is generally accepted that lignin−carbohydrate linkages in cell walls are inhibitory to forage digestion [e.g., Jung (1989, 1990)]. Improvements in fiber digestibility with alkali treatment such as ammoniation are presumably caused by saponification of ester linkages involved in lignin−carbohydrate complexes (Chesson, 1988; Van Soest et al., 1984). However, the effectiveness of ammonia in saponifying esters is questionable due to the buffering capacity of many crop residues (Van Soest et al., 1984; Sewalt et al., 1996c). An additional mechanism was recently proposed (Sewalt et al., 1996c) to explain the accumulation of N with Klason lignin from corn stover treated with aqueous ammonia. The N accumulation with lignin was hypothesized to be caused by nucleophilic addition reactions of NH_3 to quinone methides regenerated under alkali conditions. A covalent lignin−ammonia interaction could prevent addition to lignin by other nucleophilic groups, such as carboxyl and hydroxyl groups in carbohydrates, lignin, and other phenolics. The lignin−ammonia association could prevent further cross-linking of cell wall constituents and, possibly, lignin−protein or lignin−enzyme interactions during feed storage and digestion in the rumen (Sewalt et al., 1996c). This hypothesis was examined in our enzyme system using one of the available lignins and its hydroxypropylated counterpart.

Nitrogen content of the mixed hardwood lignin before ammoniation was 1.2 mg/g for unmodified lignin and 0.4 mg/g for HPL. After ammoniation, the lignins contained 9.2 and 0.8 mg/g, respectively (see Figure 3A). Given these N concentrations, it was calculated that 1 in every 10 monomeric C_9 units of the unmodified lignin retained an NH_3-N, whereas HPL retained only 1 N in 100 monomeric C_{12} units. Acidification of the ammoniated lignins, a treatment added to distinguish between covalently and ionically bound N, did not affect ($P > 0.15$) their N content. The combination of NH_3 and 2-mercaptoethanol, a treatment added to reduce quinone structures, decreased ($P < 0.05$) the N incorporation by 57%, supporting the hypothesis of involvement of quinoid structures in the NH_3-N binding to lignin.

The modifying effect of 2-mercaptoethanol and the low inherent N binding capacity of hydroxypropylated lignin suggest that the phenolic hydroxyl group mediates N binding by allowing addition reactions to regenerated quinone methide intermediates. The original hypothesis (Sewalt et al., 1996c) of ammonia addition to quinone structures in lignin was confirmed by this experiment.

In the final enzyme incubation, the extent of filter paper saccharification in the presence of MHW lignin amounted to 55% of that of the no-lignin control (Figure 3B). This inhibition was almost completely overcome by the ammoniation pretreatment, similar to the effect of hydroxypropylation. Combined pretreatment with NH_3 and 2-mercaptoethanol resulted in slightly lower production of reducing sugars, indicating possible interference with the reducing sugar assay as previously observed (experiment 3).

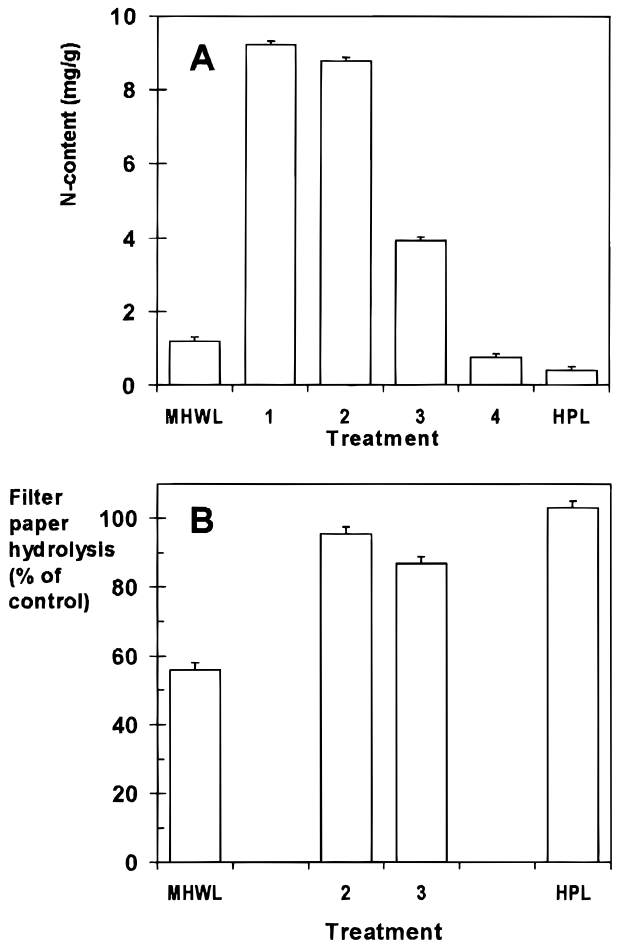

Figure 3. Nitrogen content and reversal of cellulase inhibition for mixed hardwood lignins (MHWL) and hydroxypropylated lignins (HPL) subjected to various ammoniation treatments (experiment 4): (1) MHWL, lyophilized after ammoniation, washed with distilled water, and filtered; (2) MHWL, acidified to pH 1 after ammoniation, then filtered; (3) as (2) but ammoniated in the presence of 1.43 M 2-mercaptoethanol; (4) HPL as treatment 2. Data are means of three replicates. Error bars indicate standard errors. (A) Nitrogen content before and after ammoniation. Treatments 3 and 4 differ ($P < 0.05$) from treatments 1 and 2. Treatment 4 differs ($P < 0.05$) from treatment 3. (B) Percent inhibition of enzymatic filter paper saccharification by MHWL, HPL, and mixed hardwood lignins subjected to treatments 2 and 3 above. Treatments 2 and 3 differ ($P < 0.001$) from MHW control. Treatment 3 differs ($P < 0.01$) from treatment 2.

Conclusions. This study of the inhibitory effect of added lignins on enzymatic filter paper digestion provides evidence that a pre-established lignin−carbohydrate bond is not a prerequisite for inhibition of fiber digestion. Both lignin−carbohydrate and lignin−enzyme (protein) interactions apparently occur after addition of extraneous lignins. Similar inhibitory effects may occur during large-scale bioconversion of lignocellulosics and ruminal digestion of forages after partial disruption of the fiber matrix and solubilization of lignin fragments or lignin−carbohydrate complexes. The reversal of inhibition with PEG and other compounds

illustrates that the binding properties of lignin are not unlike those of tannins, although tannins have higher affinity for proteins rather than cell wall constituents. Finally, the reversal of inhibition by the demonstrated lignin−ammonia association occurring during ammoniation provides an alternative mechanism of digestibility improvement via this pretreatment.

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