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ETHER LINKAGE BETWEEN PHENOLIC ACIDS AND LIGNIN FRACTIONS FROM WHEAT STRAW

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Key Word Index—*Triticum aestivum*; Gramineae; wheat straw; lignin; lignin–carbohydrate complexes; ferulic acid; *p*-coumaric acid.

Abstract—The bonding of the bound-phenolic acids present in three lignin preparations isolated from wheat straw where determined. *p*-Coumaric acid was mainly ester-linked whereas 35–75% of the recovered ferulic acid was ether-linked to milled straw lignin or enzyme lignin. Ferulic acid ethers accounted for 1.1% dry wt of alkali extracted lignin and might explain the high solubility of Gramineae lignins in soda. Isolated lignins were associated to hemicelluloses, principally arabinoglucuronoxylans. The possible existence of ferulic acid cross-links between lignin and arabinoglucuronoxylans is discussed.

INTRODUCTION

Two monomeric phenolic acids are esterified with Gramineae cell wall polymers. Ester-linked *p*-coumaric acid (PC)‡ was first identified by Smith in a lignin preparation from wheat straw [1]. PC is probably exclusively associated with lignin [2–4]. Ferulic acid (FE), on the other hand, is mainly esterified with hemicelluloses [5, 6] and has been identified in many non-lignified tissues [7–9].

Since these phenolic acids are bifunctional, they are able to form ester or ether linkages by reaction of their carboxyl or phenolic groups respectively. The involvment of FE in diaryl [10, 11], alkyl–alkyl [12, 13] and N–acyl bonds with proteins [14] have been reported. These phenolic acids, free or esterified, could copolymerize with lignin, forming alkali resistant bonds, as suggested by *in vitro* peroxidative copolymerization with lignin precursors [3, 15]. The occurrence of phenolic acids with free carboxyl groups has also been suggested in corn stalk lignin by ^{13}C NMR spectroscopy [16]. These results are in agreement with the possible existence of ether linkages between lignin and phenolic acids. We show here the occurrence of such phenolic acid ethers in different fractions of wheat straw lignins by isolation after alkaline and acid hydrolyses and by ^{13}C NMR spectroscopy.

RESULTS AND DISCUSSION

Three lignin preparations were compared: a milled straw lignin, LM; an enzyme lignin, LE, and an alkali-extracted lignin, LA. Phenolic acids bound to the preparations were isolated by three successive hydrolyses: two mild alkaline treatments to hydrolyse exhaustively the phenolic acid esters, then one acid hydrolysis in a dioxane–water mixture under reflux, i.e. 'acidolysis' according to Lundquist [17].

Table 1 shows that at least 93% of PC is alkali labile and thus linked by an ester bond to LM and LE fractions. However in the LA fraction, PC is mainly alkali resistant and only liberated after acidolysis. On the other hand, 35 and 75% of FE is resistant to alkaline hydrolysis in LM and LE fractions respectively, and only solubilized by acidolysis. Furthermore, about 95% of the total FE in the LA fraction is alkali resistant. Thus, it is clear that phenolic acids bound by two different types of linkages coexist in the lignin preparation. Previous studies have shown that some alkyl-aryl-ether lignin model compounds are resistant to mild alkaline hydrolysis but are hydrolysed by acidolysis [18, 19]. These alkali resistant phenolic acids could be present as ethers in the lignin preparations.

The existence of FE ethers was confirmed by ^{13}C NMR spectroscopy. The methyl-esters and 4-methoxy derivatives of PC and FE were chosen for signal assignment of esters and ethers respectively. Signals corresponding to PC esters were identified in LM and LE preparations; they did not appear after alkaline hydrolysis (Fig. 1). Signals of FE esters were not clearly observed due to their low content and to the close proximity of resonance frequencies of other lignin carbons; only the C-6 signal at 122.8 ppm could be assigned. Phenolic acid ether signals were not detected in LM and LE fractions, but after alkaline treatment of LE, three ferulic acid ether signals at 143.8, 167.7 and 122.3 ppm, corresponding to C-α, γ and 6 carbon atoms respectively were clearly visible without any overlap from lignin signals (Fig. 1b). The three signals were also present in the spectrum of alkali extracted lignin LA (not shown). After further acid hydrolysis of alkali treated LE, the two signals of the α and 6 carbons in FE ethers disappeared while the intensity of the γ carbon signal was only partially reduced (Fig. 1c). Persistence of this last signal may suggest the occurrence of carbon–

‡PC, *p*-Coumaric acid; FE, ferulic acid.

Table 1. *p*-Coumaric and ferulic acid contents of lignin fractions isolated from wheat straw

Treatment	Amount (mg/g lignin)		
	LM fraction	LE fraction	LA fraction
p-Coumaric acid			
1st alkaline hydrolysis	21.4±1.4*	18.2±0.9	1.1±0.1
2nd alkaline hydrolysis	2.3±0.3	4.1±1.3	0.9±0.1
Acidolysis	1.1±0.1	1.5±0.1	3.4±0.1
Ferulic acid			
1st alkaline hydrolysis	2.2±0.1	1.3±0.2	0.09±0.04
2nd alkaline hydrolysis	0.15±0.07	0.22±0.05	0.07±0.02
Acidolysis	1.2±0.1	4.3±0.6	11.3±0.9

*S.d. calculated from four independant determinations.

carbon bonds between ferulic acid and lignin core in addition to acid labile FE ethers.

Polysaccharides associated with the lignin preparations were analysed after trifluoroacetic acid hydrolysis. Total recovered monosaccharides were determined by gas chromatography; they accounted for 7, 17 and 18% of LM, LE and LA preparations respectively. The compositions of LM and LA polysaccharides (Table 2) are very similar to that of 'total wheat straw hemicelluloses' [20]. These hemicelluloses are principally arabinoglucuronoxylans. LE polysaccharides are richer in arabinose, uronic acids and galactose substituents. This is probably due to the cellulase treatment required for its preparation.

In view to establish the existence of ether linkage between FE and lignin itself, different purification methods were applied to LE [21]: a liquid–liquid extraction according to ref. [22], a chromatography on phenyl-sepharose and an enzyme hydrolysis with Driselase. None of these methods allowed the obtention of a fraction with a significantly lower carbohydrate content. However ^{13}C NMR data suggest a direct ether linkage with lignin: after alkaline treatment, sugar signals have been considerably reduced (see xylose signal X on Fig. 1) although signals of FE ethers are clearly visible.

FE ethers might form cross-links between lignin and hemicelluloses by simultaneous esterification of their carboxyl group to arabinose substituents of arabinoglucuronoxylans [5, 6]. This would explain why ^{13}C NMR signals of FE ethers were not observed before alkaline treatment. FE ethers might account, at least in part, for the unidentified alkali labile substituents of arabinose in Gramineae hemicelluloses [23].

Such alkali labile cross-links might explain the high solubility of straw lignins in soda [24] and the effect of alkaline treatments currently used to increase the biodegradability of straws [25]. Such an effect might originate either from alkali cleavage of ferulic acid cross-link between lignin and hemicelluloses and/or from modifications of lignin polyelectrolyte properties induced by free carboxyl groups of phenolic acids ethers.

From a physiological standpoint, these FE ethers might form during lignification by dehydropolymerization or addition of intermediate quinone-methides [26]. A parallel increase in lignin and in PC and FE ethers has been observed in wheat coleoptile cell wall, during the cessation of growth between days 5 and 9 of development [27]. FE esterified to hemicelluloses in young primary cell walls would thus establish cross-links with lignin and in this way, might restrict growth and contribute to the rigidity of the cell wall by a mechanism similar to the peroxidase-mediated dimerization of FE [28, 29].

Table 2. Sugar composition of polysaccharides associated with lignin fractions isolated from wheat straw

Sugar	Wt %*		
	LM fraction	LE fraction	LA fraction
Xylose	78.1	71.5	80.0
Arabinose	8.4	12.5	11.6
Galactose	0.6	1.4	0.6
Uronic acids	4.6	6.3	4.8
Glucose	8.4	8.3	3.1

*S.d. are less than 0.5% (xylose and glucose), 0.3% (arabinose) and 0.2% (galactose and uronic acids).

EXPERIMENTAL

General. ^{13}C NMR: (a) Lignins: 100.6 MHz under total proton-decoupled conditions in a Brucker WM 400 operating in the FT mode. Spectra were recorded at 30° from 300 mg samples dissolved in 1.5 ml DMSO-d_6 after 60000 pulses; (b) Models: 22.6 MHz under total proton-decoupled conditions on a Brucker WH 90 operating in the FT mode. Spectra were recorded at the same temp. from 150 mg samples dissolved in 1.5 ml DMSO-d_6 after 15000 pulses. All chemical shifts are given in ppm downfield from the DMSO central at 39.6 ppm.

Plant material. Wheat (*Triticum aestivum* L. cv Champlein) was harvested at full maturity. Air-dried and ground straws (stem and leaves) were exhaustively extracted in a Soxhlet apparatus, with toluene–EtOH (2:1), followed by 96% EtOH and then H_2O.

Lignin preparation. Milled straw lignin (LM) and enzyme lignin (LE) were successively obtained from the same sample of vibratory ball-milled extractive free powder as reported in ref. [30] and modified according to ref. [31]. LM and LE yields were 1.6 and 9.2 mg, respectively, per g extractive free powder. Alkali lignin (LA) was prepared from extractive free powder (25 g) by

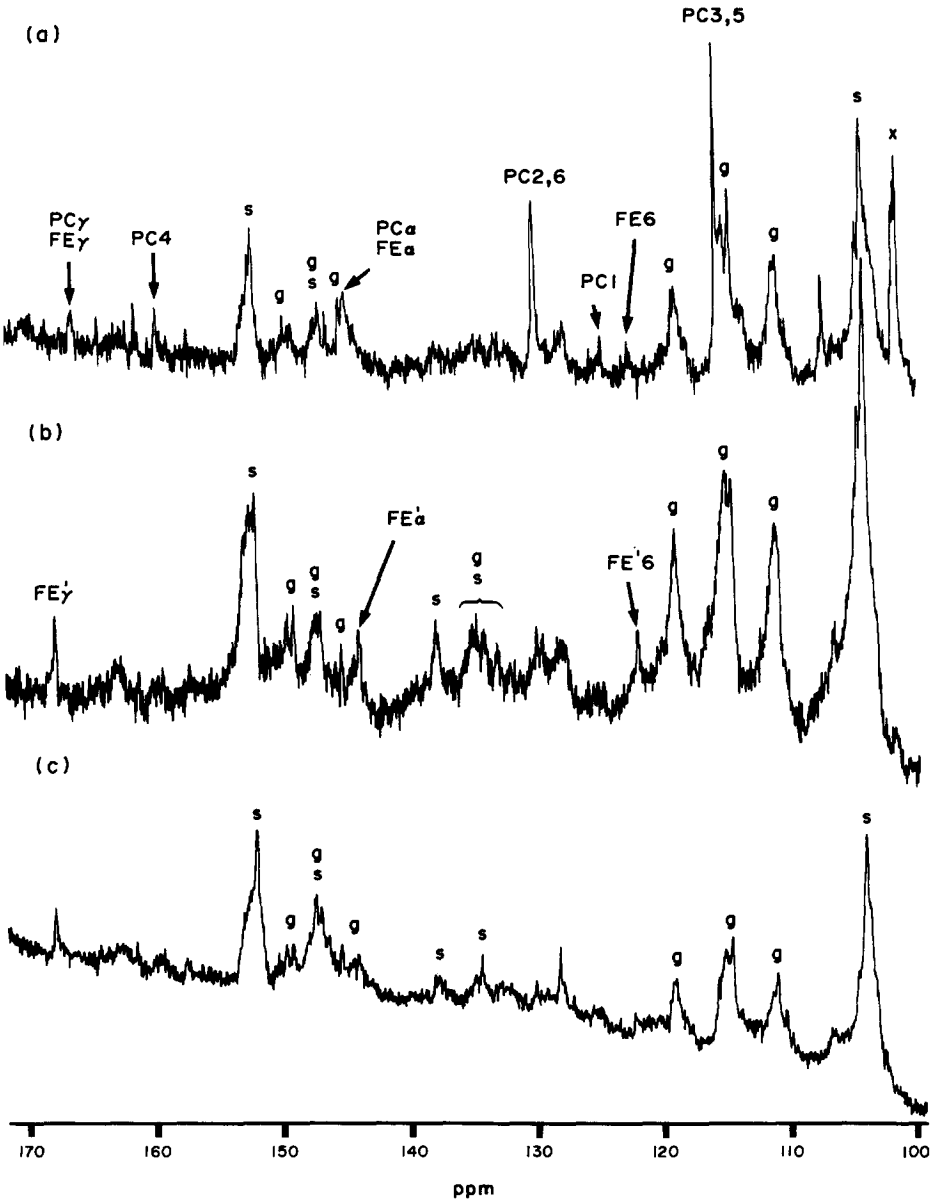

Fig. 1. ^{13}C NMR spectra of wheat lignin fraction LE before and after treatments with alkali or acid. (a) LE fraction; (b) LE residue after alkaline hydrolysis; (c) LE residue after successive alkaline and acid hydrolyses. Abbreviations: g, lignin guaiacyl unit; s, lignin syringyl unit; x, xylose; PC, *p*-coumaric acid ester; FE, ferulic acid ester; FE', ferulic acid ether. Number or letter following corresponds to each carbon according to usual lignin nomenclature [38].

stirring in 1.0 M NaOH (750 ml) under N_2 in a sealed vial for 2 hr at 35°. The soln recovered by filtration, was acidified to pH 1 with HCl and the resultant ppt. recovered by centrifugation. A lignin-rich fraction was extracted with 2 × 50 ml dioxane and then ppted in 1250 ml 1.0 M HOAc. The ppt. was dissolved in 25 ml HOAc and ppted in 2.5 l Et_2O, then freeze-dried. LA yield was 3.4 mg per g extractive free powder.

Determination of phenolic acids. Lignin preparations (100 mg) were dissolved in 2 M NaOH (50 ml). After 1 hr at 35° with stirring under N_2, the soln was acidified to pH 1 with HCl. The insoluble residue was recovered by centrifugation and hydrolysed a second time under the same conditions. After dissolution in 2 ml HOAc, the residue was ppted in 200 ml Et_2O, then freeze-dried. The alkali treated lignin thus recovered was dissolved in 25 ml dioxane–2 M HCl (9:1) and refluxed for 1 hr. After hydrolysis, the residual lignin was precipitated by addition of H_2O (25 ml), the dioxane was removed under reduced pressure and the lignin recovered by centrifugation and freeze-dried. Alkaline and acid hydrolysis residues were analysed by ^{13}C NMR spectroscopy. Phenolic acids released by alkaline or by acid hydrolysis were extracted with Et_2O and analysed by HPLC according to ref. [32]. Values were corrected for phenolic acid degradation during hydrolysis. The absence of recondensation reactions during alkaline treatment was checked by hydrolysing mixtures of phenolic acids and lignins under the same conditions. The phenolic acids were recovered in quantitative yields.

Neutral sugar determination. Polysaccharides in lignin preparations were hydrolysed by 2 N TFA (4 ml) at 120° in sealed tubes with *myo*-inositol (0.5 mg) as internal standard. Sugars were analysed by GC as their alditol acetates derivatives [33]. Chromatographic parameters described in ref. [34].

Uronic acid determination. Uronic acids were brought into soln by heating (50°) lignin preparations (25 mg) in 72% H_2SO_4 (0.5 ml) for 10 min. The solns were then diluted with 9.5 ml H_2O and centrifuged. Uronic acids were determined in the supernatant by the 3,5-dimethylphenol colorimetric method [35].

^{13}C NMR of phenolic acid derivatives. Methyl esters of phenolic acids were prepared according to ref. [36]. δ assignments were made after refs [16, 37]. Carbons are named after the usual nomenclature of phenylpropane units in lignin chemistry [38]. Methyl ester of *p*-coumaric acid: δ166.6 (C-γ), 159.5 (C-4), 144.4 (C-α), 130.0 (C-2, C-6), 124.8 (C-1), 115.6 (C-3, C-5), 113.7 (C-β). Methyl ester of ferulic acid: δ166.7 (C-γ), 149.0 (C-4), 147.6 (C-3), 144.7 (C-α), 125.3 (C-1), 122.8 (C-6), 115.3 and 114.0 (C-β and C-5), 111.1 (C-2). 4-Methoxycinnamic acid: δ167.5 (C-γ), 160.6 (C-4), 143.5 (C-α), 129.6 (C-2, C-6), 126.6 (C-1), 116.3 (C-β), 114.1 (C-3, C-5). 3,4-Dimethoxycinnamic acid: δ167.5 (C-γ), 150.4 (C-4), 148.7 (C-3), 143.8 (C-α), 126.8 (C-1), 122.3 (C-6), 116.5 (C-β), 111.4 (C-5), 110.2 (C-2).

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