CPMAS ^{13}C NMR Study of Lignin Preparations from Wheat Straw Transformed by Five Lignocellulose-Degrading Fungi

Gonzalo Almendros,*,† Angel T. Martínez,‡ Aldo E. González,‡ Francisco J. González-Vila,§ Rudiger Fründ,∥ and Hans-Dietrich Lüdemann∥

Centro de Ciencias Medioambientales (CSIC), Serrano 115 dpdo, E-28006 Madrid, Spain, Centro de Investigaciones Biológicas (CSIC), Velázquez 144, E-28006 Madrid, Spain, Institut für Biophysik und Physikalische Biochemie, P.O. Box 397, D-8400 Regensburg, Germany, and Instituto de Recursos Naturales y Agrobiología (CSIC), P.O. Box 1052, E-41080 Sevilla, Spain

After solid-state fermentation of wheat straw with five lignocellulose-degrading fungi, lignin was extracted and studied by CPMAS ^{13}C NMR. The increase in the intensity of the signals in the 200–164 ppm range is evidence for the oxidative alteration of lignin. The lignins degraded by the white-rot fungi *Ganoderma australe* and *Phanerochaete chrysosporium* showed a decrease in the amounts of aryl carbons, whereas the intensity of the 33 ppm signal from saturated alkyl structures was strongly increased by the latter fungus. The cellulolytic *Chaetomium virescens* and *Trichoderma longibrachiatum* and the ligninolytic *Fomes fomentarius* caused the selective decrease of some signals assigned to lignin–carbohydrate complexes. A low molecular weight fraction was recovered by adsorption on poly(vinylpyrrolidone). The C=O signals of this fraction were strongly increased by the ligninolytic species, whereas the accumulation of aromatic compounds was observed after straw degradation by *P. chrysosporium*.

Considerable efforts have been made in the past decade to elucidate the enzymatic mechanisms of the microbial degradation of lignin as well as to establish the chemical transformations occurring during lignin degradation (Kirk and Farrell, 1987; Buswell and Odier, 1987; Higuchi, 1990; Lewis and Yamamoto, 1990; Martínez et al., 1990). Recent investigations concern the biological upgrading of residual plant biomass leading to delignified materials suitable for paper and feed industries (Kamra and Zadražil, 1988; Eriksson, 1990). Several species of filamentous fungi may cause efficient degradation of plant material under solid-state fermentation conditions. In general, cellulolytic and ligninolytic activities are simultaneously expressed in most species of white-rot fungi. However, the ability for the preferential degradation of lignin, of great interest in processes requiring cellulose-enriched materials, has only been developed by some fungal species (Blanchette et al., 1985; Zadražil et al., 1982). On the other hand, when the lignocellulose is altered by cellulolytic species, the residual lignin may present an increased resistance to biodegradation and is often studied in terms of the formation of soil humus or the composting of agricultural wastes (Wilson, 1987; Almendros et al., 1987).

Studies of the altered lignin polymers have been carried out to obtain information on the different fungal strategies for lignin degradation (Chen and Chang, 1985). In spite of the high resolution of the ^{13}C NMR spectra of lignin solutions, reported in classical studies (Lüdemann and Nimz, 1973; Nimz et al., 1981), the utilization of the CPMAS (cross-polarization and magic-angle spinning) technique leads to diagnostic ^{13}C NMR spectra of solid samples under reasonable acquisition times. This is especially useful not only for insoluble materials, such as most lignin preparations, but also in routine studies of biopolymers by nondestructive methods (Maciel et al., 1981; Gerasimowicz et al., 1984; Fründ and Lüdemann, 1989a; Himmelsbach, 1989).

After the previous screening by Valmaseda et al. (1990) on 45 fungal species, solid-state fermentation of wheat straw with selected ligninolytic and cellulolytic fungi was carried out (Valmaseda et al., 1991), and the fungal alteration of lignin investigated by solid-state NMR.

EXPERIMENTAL PROCEDURES

Twenty-five grams of wheat straw chopped to 2 cm was moistened with 75 mL of 2.5 mg/L NH_4NO_3 and autoclaved at 120 °C for 1 h. The laboratory-scale solid-state fermentation was carried out in a fermentation device consisting of six 1-L bottles disposed horizontally in a rotary system. The fermentor was kept at 27 °C, the air flow was adjusted to 85 mL/min per bottle, and the rotation was 0.5 rpm. Five bottles were inoculated with four 5 × 5 mm portions of 2-week cultures on malt extract agar, and the sixth bottle was the control for the experiment. The species inoculated were three ligninolytic basidiomycetes, *Phanerochaete chrysosporium* 7754, *Fomes fomentarius* A166, and *Ganoderma australe* A130, and two cellulolytic ascomycetes, *Chaetomium virescens* (=*C. cellulolyticum*) A256 and *Trichoderma longibrachiatum* (=*T. reesei*) A218, conserved in our culture collection (IJFM). Several analytical characteristics of the transformed substrates were determined according to the methods described elsewhere (Valmaseda et al., 1990).

For the extraction of lignin, the transformed straw was dried at 60 °C, successively ground with a refrigerated rotary mill and a ball mill, and processed into Soxhlet extractors with sintered glass cores. Ten grams of wheat straw was extracted with ethanol–benzene 1:2 (v:v) for 24 h. After drying, the cores were extracted for 24 h with dioxane–water 9:1 (v:v) to which HCl was added to a concentration of 0.2 M (Pepper et al., 1959; Monties, 1988). The resulting brown extract was neutralized with $NaHCO_3$, concentrated (×10), and precipitated into 10 g/L Na_2SO_4. The precipitate (HMWL) was centrifuged, dialyzed, and freeze-dried. The soluble yellow supernatant was adsorbed on insoluble poly(vinylpyrrolidone) (Divergan R, BASF), washed with distilled water, and eluted with 0.1 M NaOH. Finally, the sodium was removed by ion-exchange chromatography (Amberlite IR-120 H^+) and the eluent (LMWL) was freeze-dried.

The high molecular weight lignin preparations (HMWL) and the low molecular weight oligomer materials (LMWL) were

† Centro de Ciencias Medioambientales.
‡ Centro de Investigaciones Biológicas.
§ Instituto de Recursos Naturales y Agrobiología.
∥ Institut für Biophysik und Physikalische Biochemie.

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Table I. Analytical Characteristics of Wheat Straw after Fungal Degradation[a]

	wt loss	total N	digestibility	extractives	water-soluble	holocellulose	Klason lignin	HMWL[b]	LMWL[b]
control	0.0	0.67	46.3	9.4	11.8	53.1	15.6	6.3	2.3
C. virescens	28.6	1.06	36.3	4.2	11.8	45.2	21.6	7.0	3.7
T. longibrachiatum	14.0	0.80	38.7	4.1	9.3	54.2	17.8	5.7	4.1
P. chrysosporium	45.9	1.16	51.1	7.9	14.1	39.7	17.1	4.9	3.0
G. australe	30.6	0.92	36.5	7.3	5.2	58.4	14.7	6.9	2.9
F. fomentarius	44.0	1.17	50.5	8.2	9.2	53.1	12.7	3.6	3.1

[a] Weight percentages. [b] HMWL, high molecular weight lignin fraction; LMWL, low molecular weight lignin fraction.

Table II. Characteristics of Lignin Preparations Isolated from Wheat Straw after Fungal Degradation

		atomic ratios		UV[a]		Sephadex G-50 fractions			NMR ratios[b]		
	% N	H/C	O/C	285	350	K_{av} = 0	K_{av} = 0–1	K_{av} > 1	Me/Ar	C=O/Ar	*O*-Al/Ar
						HMWL[c]					
control	1.37	1.03	0.40	0.197	0.068	53.0	0.0	47.0	1.67	0.21	10.38
C. virescens	0.19	1.05	0.39	0.306	0.050	62.0	0.0	38.0	1.38	0.55	8.46
T. longibrachiatum	0.01	1.03	0.40	0.316	0.043	68.2	0.0	31.8	1.38	0.48	9.18
P. chrysosporium	0.01	1.01	0.45	0.269	0.025	73.9	0.0	26.1	1.44	0.65	11.88
G. australe	0.26	1.04	0.44	0.306	0.037	76.1	0.0	23.9	1.67	0.41	11.40
F. fomentarius	0.01	1.05	0.43	0.244	0.022	77.6	0.0	22.4	1.48	0.46	10.44
						LMWL[c]					
control	1.53	1.42	0.67	0.175	0.074	49.2	17.0	33.8	1.11	2.20	12.36
C. virescens	1.27	1.34	0.65	0.203	0.088	55.1	10.2	34.7	1.26	1.67	11.94
T. longibrachiatum	1.45	1.33	0.67	0.171	0.042	23.8	57.8	18.5	1.08	1.79	10.56
P. chrysosporium	1.47	1.07	0.75	0.095	0.032	60.4	27.2	12.4	0.73	2.23	8.58
G. australe	1.12	1.25	0.70	0.104	0.035	69.2	17.2	16.0	1.16	2.48	10.74
F. fomentarius	0.89	1.25	0.77	0.092	0.028	66.7	18.3	12.5	0.78	3.02	12.18

[a] Ultraviolet spectroscopy: intensity of the 280- and 350-nm bands, measured in the second-derivative spectra (UA). [b] From integrals of the spectral regions: C=O = 200–164 ppm; aromatic = 164–102 ppm; *O*-alkyl (*O*-Al) = 102–46 ppm; methoxyl (Me) = 57–52.5 ppm; aryl (Ar) = aromatic/6. [c] HMWL, high molecular weight lignin fraction; LMWL, low molecular weight lignin fraction.

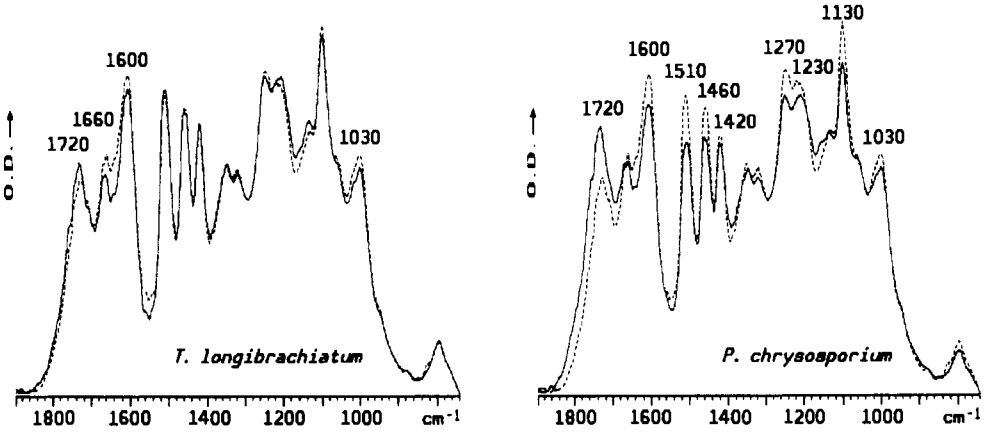

Figure 1. Infrared spectra of dioxane–lignin preparations (HMWL) from wheat straw prior to (dashed line) and after fungal degradation.

characterized by elementary composition (Heraeus CHN-O-Rapid analyzer), gel permeation (Sephadex G-50), IR spectroscopy (300 mg of KBr and 2.0 mg of sample), and UV derivatographic spectroscopy (Shimadzu UV-160) of 20 μg/mL lignin solutions in 0.1 M NaOH. Solid-state ^{13}C NMR spectra were obtained with the CPMAS technique at 75.4 MHz in a Bruker MSL 300 spectrometer (Fründ and Lüdemann, 1989b). The pulse repetition rate was 5 s, and the cross-polarization contact time was 1 ms. The sweep width was 31.25 kHz, the filter width was set to 37.5 kHz, and the acquisition time was 16 ms. Magic-angle spinning was performed at 4 kHz in the commercial Bruker double-bearing probes in phase stabilized zirconium dioxide rotors. For comparative purposes, and because the spectra obtained showed the same quantitative pattern, no attempt was made to suppress spinning sidebands by using the TOSS program. The chemical shift scale was calibrated with glycine, and carbonyl C was adjusted to 173.03 ppm.

RESULTS

Characteristics of the Decayed Substrates and the Lignin Preparations. Some analytical characteristics of the wheat straw after fungal transformation are shown in Table I. The weight losses accounted for 14–46%, but the changes in the relative amounts of holocellulose and lignin were small and slightly correlated with the in vitro digestibility. The yield of the lignin extraction (HMWL) ranged between 30 and 50% of the Klason lignin content of the sample. This agrees with the results reported by Monties (1988) and represents a high lignin recovery when compared with that of other extraction procedures. The LMWL fraction amounted to between 2 and 4% of the straw weight.

The N content of the lignin preparations was strongly reduced after fungal degradation, mainly in the HMWL, showing the utilization of lignin-associated protein (Dill et al., 1984) during the fungal growth (Table II). The atomic H/C ratios of the HMWL preparation showed small variations, and the ligninolytic species caused some increase in the atomic O/C ratio. The formation of oxygen-containing functional groups after fungal alteration was more conspicuous in the LMWL fractions, where the decreased H/C ratios suggested accumulation of some aromatic material after the decay with ligninolytic species.

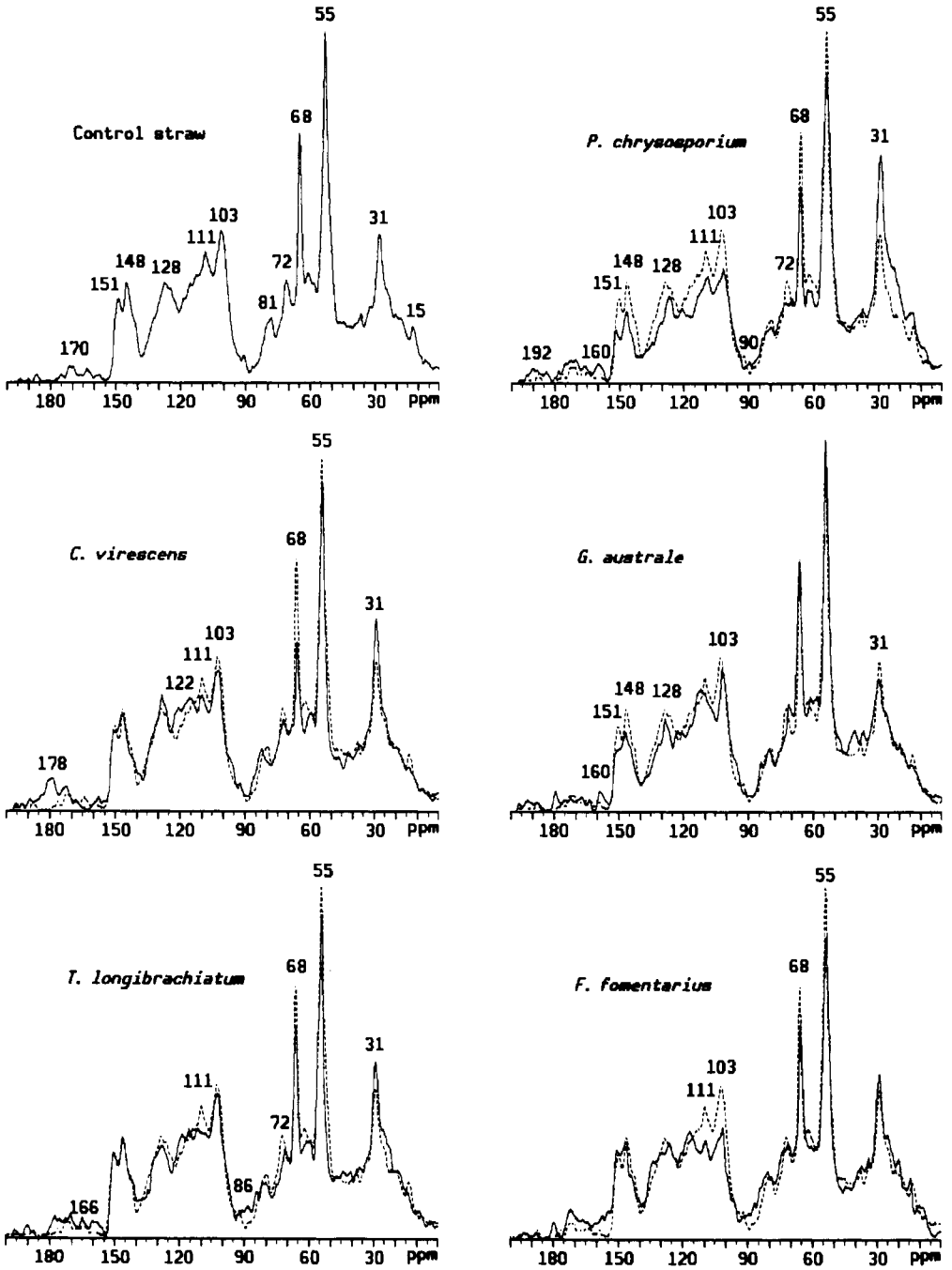

Figure 2. Equal-area normalized CPMAS ^{13}C NMR spectra of dioxane–lignin preparations (high molecular fraction, HMWL) from wheat straw prior to (dashed line) and after fungal degradation.

Some changes occurred in the molecular size of the extracted lignins. The gel permeation profiles showed increased percentages of the excluded fractions ($K_{av} = 0$, MW > 10^4), most pronounced for the ligninolytic species. This could be interpreted as the result of preferential degradation of low molecular size fractions during the solid-state fermentation.

The IR spectra of the HMWL preparations showed several changes in the peak intensities. Only two representative lignins are shown in Figure 1, since the patterns observed were the same for either the cellulolytic or ligninolytic species.

With the ligninolytic species an increase in the intensity of the C=O band (1720 cm^{-1}) was appreciable, and the decrease in the intensities of the peaks at 1510 and 1460 cm^{-1} suggests the depletion of aryl and side-chain lignin structures. In the cellulolytic species the changes were small, showing a relative decrease of intensities of the 1660-cm^{-1} peak, affected by C=O groups conjugated to the aromatic ring (Fengel and Wegener, 1984).

The second-derivative UV spectra of the lignins were used for quantitative estimations (Table II). A decrease of the peak at about 350 nm, corresponding to conjugated C=O groups, was observed after fungal degradation, mainly by the ligninolytic species. The contrary tendency was observed for the 285-nm peak, pointing to the increase in the relative amount of phenolic groups during lignin degradation by fungi.

CPMAS ^{13}C NMR Study of Lignins. Figures 2 and 3 show the equal-area normalized CPMAS ^{13}C NMR spectra of the extractive lignin fractions after fungal decay, superimposed to those from the control straw. The assignments of the most prominent NMR signals (Lüdemann and Nimz, 1973; Maciel et al., 1981; Nimz et al., 1981; Scalbert et al., 1986; Robert and Chen, 1989; Martínez et al., 1991) are presented in Table III.

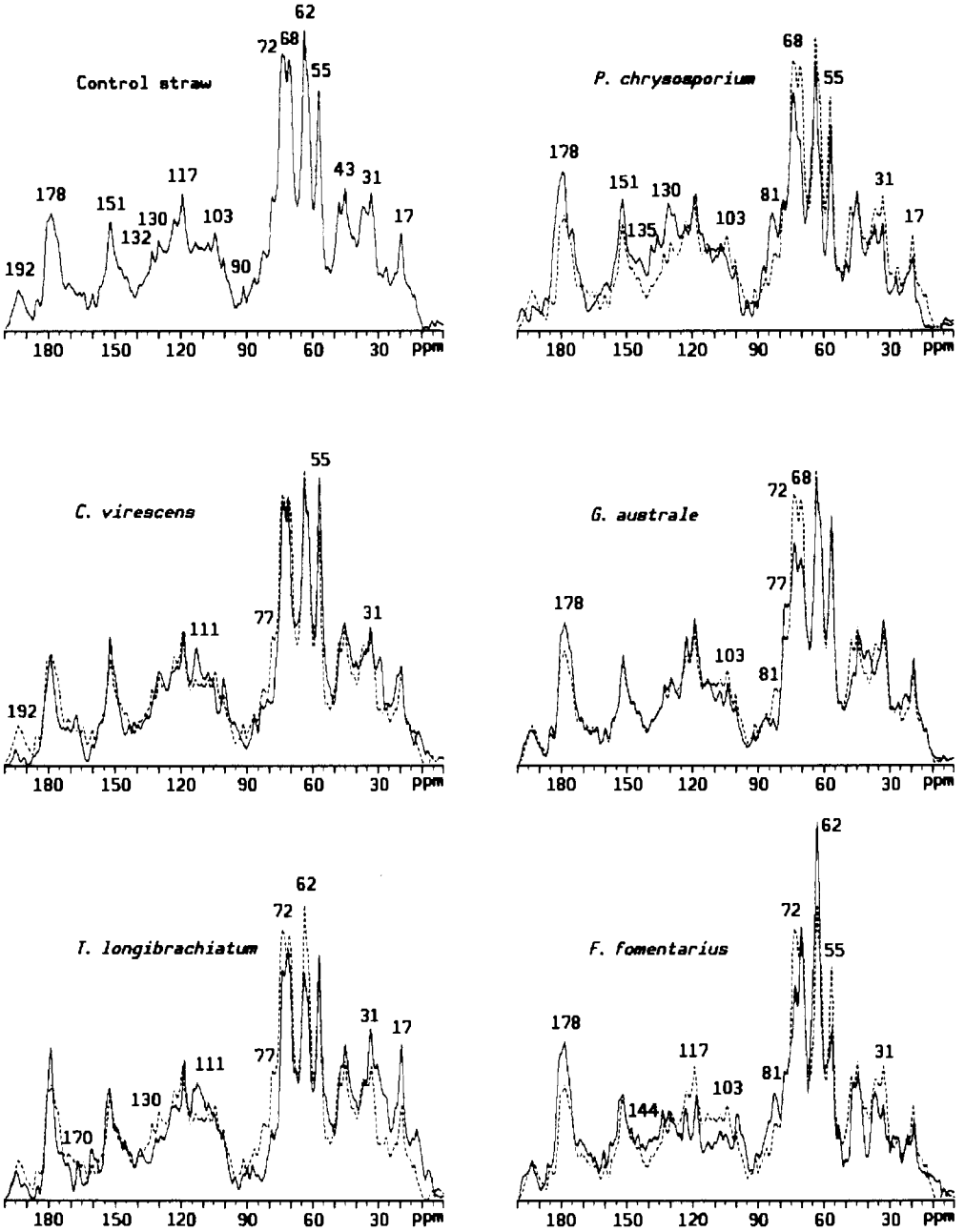

Figure 3. Equal-area normalized CPMAS ^{13}C NMR spectra of acid-soluble dioxane–lignin preparations (low molecular weight lignin, LMWL) from wheat straw prior to (dashed line) and after fungal degradation.

The decrease in the intensity of some signals in the 164–102 ppm region of the HMWL spectra, especially those at 151 and 148 ppm, shows the comparatively reduced amounts of lignin aromatic structures by the ligninolytic *P. chrysosporium* and *G. australe*. On the other hand, the percentage of protonated aryl carbons with chemical shift around 111 ppm decreased after degradation with each of the species studied, and the lowest intensities were found in the straw treated with *P. chrysosporium* and *F. fomentarius*. A possible contribution of other straw residual polymers (such as polysaccharides and proteins) to the signal at 111 ppm is suggested from the solid-state NMR spectra of straw lignin and lignin–carbohydrate complexes reported by Himmelsbach (1989).

Concerning the *O*-alkyl structures, the predominant decrease in the HMWL signals at 68 and 72 ppm by *P. chrysosporium*, *F. fomentarius*, *T. longibrachiatum*, and *C. virescens* suggests breakdown of lignin–carbohydrate bonds. The increase in digestibility by the above ligninolytic species may be related to the fungal breakdown of these linkages (Himmelsbach and Barton, 1980). The calculation of the methoxyl/aryl ratio showed that more or less intense demethoxylation (55 ppm signal) occurred with both the cellulolytic and ligninolytic species (Table II). Another generalized change was the comparatively reduced proportion of carbons contributing to the intensity of the signal at around 103 ppm, probably produced from the overlapping of the resonances of C_2 and C_6 carbons in syringyl units with those from the anomeric carbons of residual sugars in lignin–carbohydrate complexes.

The intensity of the signals in the 0–46 ppm spectral region, mainly in the case of *P. chrysosporium*, suggests the accumulation of saturated alkyl structures in the altered lignins. It has also been considered that some of this material could correspond alternatively to fungal-derived nonextractable lipid material (Chua et al., 1982; Himmelsbach, 1989).

One of the most important signal decreases in the LMWL fraction after degradation by the ligninolytic species corresponded to *O*-alkyl resonances at 72 ppm,

Table III. CPMAS ^{13}C NMR Chemical Shift Assignments for Figures 2–4

^{13}C shift, ppm	probable assignment
192	C=O in aromatic aldehyde
178	C=O in aliphatic acid
170	C=O in aliphatic ester
166	C=O in aromatic acid
160	C_4 in *p*-hydroxyphenyl
151	C_3 and C_5 in etherified syringyl
148	C_3 and C_4 in etherified guaiacyl
144	C_4 and C_4' in etherified 5–5′ units and C_α in cinnamic acid and ester
135	C_1 in etherified syringil and guaiacyl
132	C_5 and C_5' in etherified 5–5′ guaiacyl and C_1 in nonetherified guaiacyl and syringyl
130	C_α and C_β in cinnamyl alcohol and aldehyde
128	C_2 and C_6 in *p*-hydroxyphenyl
122	C_6 in guaiacyl
117	C_5 in guaiacyl and C_3 and C_5 in *p*-hydroxyphenyl
111	C_2 in guaiacyl
103	C_1 in carbohydrate and C_2 and C_6 in syringyl
90	C_1 in xylose reducing end (α-anomer) and C_α in phenylcoumaran
86	C_β in β-O-4-linked unit (threo)
81	C_β in β-O-4-linked unit (erythro) and C_4 in 4-*O*-methylglucuronic acid
77	C_3 in xylose nonreducing end
72	C_2 in xylose internal unit and C_α in β-O-4-linked unit
68	C_4 in xylose nonreducing end and C_γ in β-O-4-linked unit with C_α=O
62	C_5 in xylose internal unit and C_γ in β-O-4-linked unit
55	aromatic methoxy group
43	C_α methyne with aliphatic substitution
35	C_α in arylpropanol
31	alkyl CH_2
20	CH_3 group in acetylated xylan
15–17	terminal CH_3 group

assigned to OH-substituted carbons in lignin side chains. However, the decreases of the 72 and 68 ppm signals could also be caused by the depletion of residual pentosans involved in lignin–hemicellulose bonds. In the case of the two cellulolytic species the degradation patterns were characterized by the decreased contribution to the total spectral area of the region with maximum at 77–81 ppm and the increased one for the 111 ppm signal. The most conspicuous increase in some LMWL signals with the ligninolytic species occurred in the C=O resonances between 200 and 164 ppm (Table II), showing approximately two C=O groups per aromatic unit. This is reflected by the increase of the C=O/Ar ratio by the ligninolytic species which shows the release of oxidized compounds after the fungal alteration of lignin.

DISCUSSION

The CPMAS ^{13}C NMR spectra of the wheat straw lignins studied here differed from those of wood lignin preparations analyzed by the same technique (Maciel et al., 1981; Gerasimowicz et al., 1984; Leary et al., 1986; Hatfield et al.; 1987; Martínez et al., 1991). The former spectra showed several carbohydrate bands (e.g., those at 103, 72, and 68 ppm) and relatively high aliphatic carbon signals (as shown by the *O*-Al/Ar ratio in Table II), which could be explained by the release of lignin–polysaccharide complexes. In fact, very similar NMR spectra of grass lignin have been obtained by Himmelsbach (1989).

The increase of the C=O signals at 192, 178, and 170 ppm, often attributed to the formation of aldehydes, acids, and aroxyacetic structures (Robert and Chen, 1989), showed the oxidative alteration of lignin side chains. This suggests oxidative degradation of C_α–C_β bonds, as reported in *P. chrysosporium* and other fungi (Chua et al., 1982; Robert and Chen, 1989), but the present study points also to preferential depletion of aryl carbons by this species and by *G. australe*. After integration of the CPMAS ^{13}C NMR spectra (Table II), the above changes are also evident in the 2–3-fold increase of the C=O/aryl ratio in the HMWL fraction and the increased carboxylation of the LMWL fraction by the ligninolytic species.

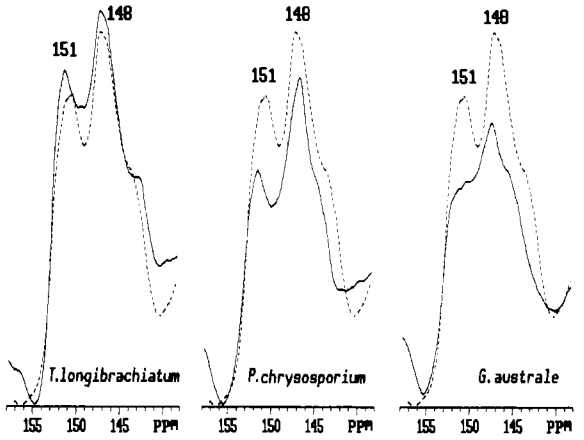

Figure 4. Detail of the aromatic carbon region of the CPMAS ^{13}C NMR spectra of dioxane–lignin (HMWL) from wheat straw prior to (dashed line) and after fungal degradation.

One of the most significant changes in lignin composition concerns the decrease of the syringyl/guaiacyl (S/G) ratio after fungal decay of wheat straw. The quantitative estimation of the lignin S/G ratio according to the method proposed by Manders (1987) for obtaining the S and G aromatic components of hardwood lignin is difficult to apply because of the complexity of the straw lignin NMR spectrum in the 140–100 ppm region. However, the S/G ratio decrease is evident in the changes of the relative intensities of the 151 and 148 ppm signals (Figure 4), mainly produced by the oxygen-linked carbons in etherified S and G units (Table III). The most pronounced decrease of the 151 ppm signal in the straw lignin spectra was produced by *G. australe*, whereas no change in this spectral region was induced by the cellulolytic *T. longibrachiatum* (Figure 4). This decrease of the lignin S/G ratio was previously reported by Martínez et al. (1990) in decayed straw and reflects the comparatively lower condensation and resistance toward biodegradation of the syringyl–lignin.

Besides the carboxylation and the C_α–C_β breakdown characteristic of LMWL degradation by the ligninolytic species, only slight changes in this fraction were caused by *G. australe*. Finally, the results obtained with the cellulolytic *C. virescens* and *T. longibrachiatum* did not show a substantial decrease of the aromatic content in the LMWL fraction. However, they produce changes in *O*-alkyl spectral region from the breakdown of lignin–carbohydrate bonds.

The changes in lignin composition are probably not solely the consequence of the selective degradation of lignin units but also result from its partial depolymerization into oligomer structures. In fact, several changes in the LMWL fraction, especially after degradation with *P. chrysosporium*, are contrary to those observed in the HMWL fraction. This could reflect partial accumulation of the material removed from HMWL in the LMWL fraction. Thus, the decrease of the *O*-alkyl/aryl ratio in the LMWL suggests a tendency for the enrichment of aromatic structures in this fraction after straw degradation by fungi.

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