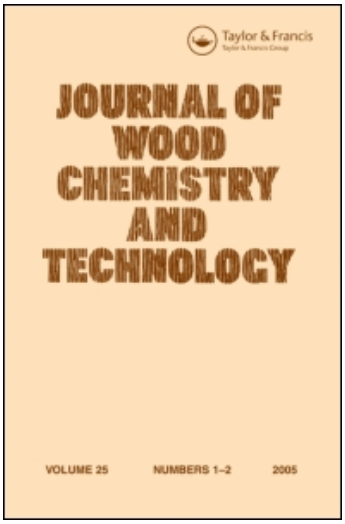

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A CP/MAS ^{13}C NMR STUDY OF RESIDUAL LIGNIN STRUCTURE IN AUTOHYDROLYSIS-EXPLODED WOODS AND BAGASSE

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ABSTRACT

Structural breakdown of the lignin in auto-hydrolysis-exploded Pinus radiata and Eucalyptus regnans wood and in sugarcane bagasse has been examined using cross polarization magic angle spinning (CP/MAS) ^{13}C NMR spectroscopy. The alkali- and acetone-insoluble (i.e. residual) lignins have undergone substantial cleavage of the major (β-O-4) interunit linkage and are most likely mixtures of partly depolymerized and repolymerized lignin fragments. Correlations between residual lignin structure, breakdown of the lignocellulose structure and enzymatic digestibility of the cellulosic carbohydrate are proposed.

INTRODUCTION

Steam or autohydrolysis-explosion processes break down the structure of wood and other lignocellulosic materials by hydrolysing the hemicellulose component, cleaving lignin ether linkages and depolymerizing the lignin component, and defibration.[1,2] This makes the three major components - cellulose, hemicelluloses and

lignin separable and accessible to enzymatic conversion processes. The role of lignin in hindering bio-conversion of the insoluble cellulosic carbohydrate to valuable products is therefore of considerable interest. Recent work suggests that lignin structure and location are more likely than lignin content, to be factors causing hindrance.[3,4] Both the fraction of the lignin soluble in alkali and organic solvents and the insoluble fraction, i.e. the residual lignin, could be involved.

In this paper, the structure of some residual lignins is examined and compared with that of the total lignin (soluble + residual) in the autohydrolysis-exploded lignocellulosic material. This work is a continuation of the cross polarization magic angle spinning (CP/MAS) ^{13}C NMR study of autohydrolysis-exploded woods reported in Reference 4.

Two woods, the softwood, Pinus radiata and the hardwood, Eucalyptus regnans, and sugarcane bagasse were autohydrolysis-exploded in a Siropulper.[5] The products were examined by CP/MAS ^{13}C NMR spectroscopy, both before and after removal of solubilized lignin with (a) alkali and (b) acetone. In the case of Pinus radiata wood, the effect of SO_2 pretreatment prior to autohydrolysis-explosion was examined. This pretreatment improves structural breakdown.[6] Relationships between lignin structure and enzyme access to the insoluble carbohydrate of the treated materials have been explored. Use of a spin-locking pulse to suppress signals from protonated carbon allowed certain lignin signals to be studied without interference.[7]

RESULTS AND DISCUSSION

Measurement of Lignin Contents

The lignin contents given in Table 1 were determined by the NMR method described previously (see

TABLE 1

Lignin Contents, Pulp Yields and Enzymatic Digestibilities of Autohydrolysis-Exploded Wood and Bagasse Substrates.

Sample	Lignin content (%)	Pulp Yield (%)	% Cellulose → Glucose 24h	72h
PR.U	30*(28)‡	100	<1	<1
AHE	40 (37)	79	27	39
AE	37 (35)	69	<1	ND
OSE	37 (ND)	ND	ND	ND
(SO_2)AHE	52 (42)	60	48	62
AE	45 (34)	41	7	15
OSE	40 (ND)	ND	ND	ND
ER.U	26 (31)	100	<1	<1
AHE	36 (38)	70	73	91
AE	11 (14)	53	37	61
OSE	24 (ND)	ND	ND	ND
B.U	23 (24)	100	18	23
AHE	34 (31)	63	81	100
AE	4 (11)	41	29	55
OSE	20 (ND)	ND	ND	ND

PR = Pinus radiata, ER = Eucalyptus regnans, B = bagasse. U = the untreated material, AHE = autohydrolysis-exploded, SO_2 = with SO_2 pretreatment, AE = alkali extracted after AHE, OSE = acetone extracted after AHE. * denotes lignin content by the NMR method of Ref. 4, ‡ Klason plus acid-soluble lignin content. ND = not determined. Enzymatic hydrolysis used 20 FPU cellulase plus 25 units of β-glucosidase per g substrate in pH 5.0 acetate buffer at 50°C.

Experimental). Klason plus acid-soluble lignin contents are shown in parentheses. In most cases the lignin contents determined by the NMR method are within 2-3% of the Klason plus ASL values. The few cases of poor agreement do not affect the interpretation of the results discussed in this paper. The Klason and NMR methods have recently been compared for a range of woods and substrates and the latter is considered to have advantages when comparing the lignin contents of autohydrolysis-exploded substrates.[4,7]

1 Pinus Radiata

The 150ppm regions of the spin-locked spectra of samples of P. radiata wood autohydrolysis-exploded with and without SO_2 pretreatment (samples PR(SO_2)AHE and PR.AHE) are given in Fig. 1. They show a large decrease in intensity in the signal region assigned primarily to C-4 in guaiacyl units etherified at this carbon atom: a result of autohydrolysis-explosion treatment (cf. Ref.4). The decrease is larger for the PR(SO_2)AHE sample and the intensities relative to that in the original, untreated wood are about one-third and one-half respectively. This indicates that extensive cleavage of the major (β-O-4) lignin interunit linkage has taken place in both cases and that it is promoted by SO_2 pretreatment. A corresponding but smaller increase in intensity in the region containing signals from C-4 in non-etherified units is also observed, supporting β-ether cleavage and the formation of free phenolic hydroxy-groups at C-4. However, it should be noted that signals in this region are poorly resolved and therefore changes in intensity are smaller and less easily interpreted. It should also be noted that assignment of signals C-3 and C-4 has not been interchanged as recently proposed,[8] but that interchange should not significantly affect interpretation of the spectra.

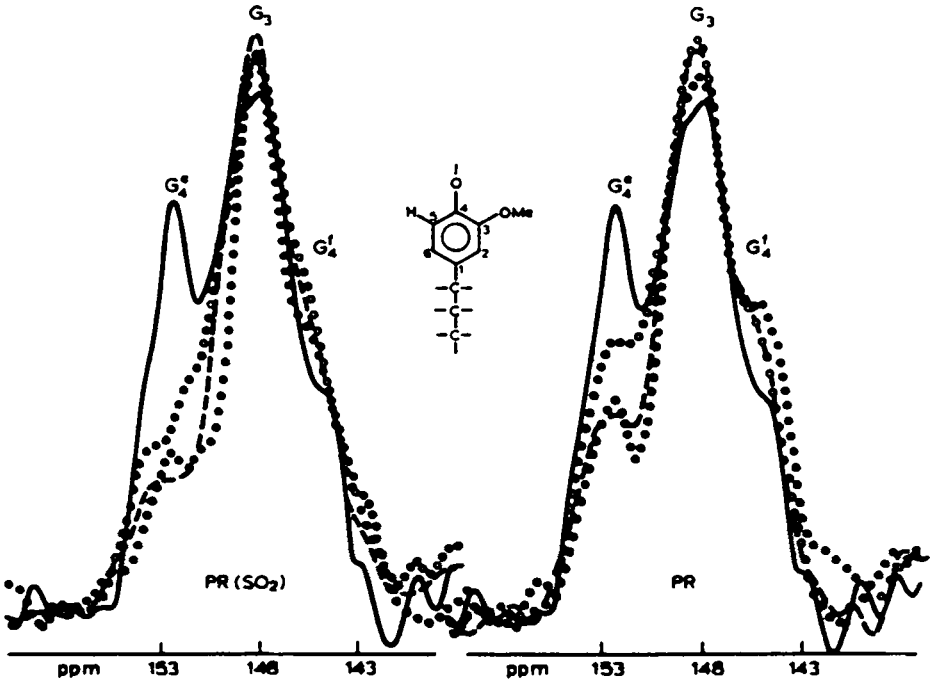

FIGURE 1 Spin-locked NMR spectra of P. radiata with and without SO_2 pretreatment. ——— original, untreated, (U); ----- auto-hydrolysis-exploded, (AHE); 0000 alkali extracted after AHE, (AE); ●●●● acetone extracted after AHE, (OSE).

Extraction (AE) of the alkali-soluble lignin leaves a residual lignin significantly more etherified at C-4 than the total lignin in the AHE sample (Fig. 1), while extraction (OSE) of the acetone-soluble lignin leaves a residual lignin with etherification lying in between that of the lignin in the AHE and AE samples. The lignin contents of the AE and OSE samples are fairly similar (Table 1). These results suggest that there may be a difference in the β-ether content of the lignin removed by the two solvents, or that the AE procedure initiates reactions in the lignin which result in an increased contribution of signals to this region.

Although the residual lignins appear to be more etherified at C-4 than the total lignin and, by inference, the soluble lignins, the extent of β-ether cleavage still appears to be substantial. It is estimated to be in the region of 35% and 45% for the PR.AE and PR.OSE samples and 55% and 60% for the PR(SO_2) AE and OSE samples, i.e. to be not too different from the estimate of about 66% cleavage in soluble lignins obtained from this autohydrolysis-explosion process.[9] Therefore, the residual lignins do not appear to be unreacted lignins arising from inhomogeneous structural breakdown, caused by uneven steam penetration and heating of the wood. Their insolubility may be due in part to repolymerization reactions (which involve formation of carbon-carbon linkages) successfully competing with the cleavage of ether linkages and depolymerization reactions taking place during autohydrolysis-explosion. The relative content of such repolymerized fragments and large, partly depolymerized fragments of the original lignin could in turn affect the location of the residual lignin in the autohydrolysis-exploded wood and the extent to which it hinders enzyme access to the wood carbohydrate. For example, the repolymerized fragments could be more aggregated and therefore cover less of the carbohydrate surface area. Aggregation of lignin has been observed in autohydrolysis-exploded wood samples.[10] It appeared to take place more readily in the hardwood than in the softwood species studied. Lignin repolymerization was also observed.[10]

The signal-to-noise ratios in the 130 and 140 ppm regions of the spectra are too low to detect any change in the content of non-protonated C-5 and C-6 aromatic carbon, arising from repolymerization reactions.

The Effect of SO_2 Pretreatment

For the three pairs of samples, PR and PR(SO_2) AHE, AE and OSE, the intensity of the signal for C-4 in etherified units is considerably higher in the absence of SO_2 pretreatment prior to autohydrolysis-explosion. The intensity of the signal given by the samples which had the pretreatment is 60-80% of that given by the samples not pretreated, which indicates that the extent of β-ether cleavage is greater in both the total lignin and the residual lignin with SO_2 pretreatment. The insolubility of the residual lignin suggests that this difference in β-ether cleavage may reflect a higher content of repolymerized fragments and a lower content of partly depolymerized fragments in the residual lignin of the SO_2 pretreated samples. If this is the case, it may be a factor in the considerably better enzymatic digestibility of the cellulosic carbohydrate of the SO_2 pretreated material, (Table 1), in that enzyme access may be less restricted, as discussed above. It should be noted that the lignin contents of the samples which had SO_2 pretreatment are higher than, or close to, those of the samples which did not (Table 1), i.e., as found previously,[4] there appears to be no correlation between lignin content and enzymatic digestibility of the cellulosic carbohydrate.

As will be discussed further below, it appears that the extent of β-ether cleavage in residual lignins, estimated from intensity changes in the signal for C-4 in etherified units, could be used as an indicator of the extent of breakdown of the wood structure. This is because the autohydrolysis-explosion process depolymerizes both the hemicellulose and the lignin components. Therefore, there should be a correlation between their respective breakdowns and consequently, between the breakdown of one and that of the wood

structure as a whole. If so, in the case of P. radiata, an estimated cleavage in excess of 50% seems to be necessary for enzyme access to the cellulosic carbohydrate to be significantly improved.

2. Eucalyptus Regnans

In the case of hardwoods, signals for C-3 and C-5 in syringyl units etherified and non-etherified at C-4 are well resolved and occur at 153 and 148 ppm respectively. Therefore, although they overlap the broad band for C-3 and C-4 in guaiacyl units, they show changes in etherification quite clearly. In contrast, the C-4 signals for syringyl units occur in a broad band at 133-139 ppm, which also contains C-1 signals.[4]

The content of units non-etherified at C-4 is low in E. regnans wood, as is the content of guaiacyl units. This is shown by the weak shoulder on the intense signal for C-3,5 in etherified units, given by these units in the 148 ppm region of the spectrum (Fig. 2).

Autohydrolysis-explosion results in the production of an intense signal at 148 ppm for C-3,5 in non-etherified units, indirectly showing the formation of free phenolic hydroxy-groups. The intensity of this signal is about 1.6 times that of the signal for C-3,5 in etherified units, and cleavage of about 50% of the β-O-4 interunit linkages is estimated to have taken place.

As observed in the case of the softwood, removal of the alkali-soluble and acetone-soluble lignin leaves residual lignins which are more etherified than the total lignin in the autohydrolysis-exploded wood. However, ether cleavage is still extensive and the changes in the intensity of the C-3,5 signals indicate cleavage of about 40% of β-O-4 linkages. The residual lignin in the acetone-extracted sample (ER.OSE) appears

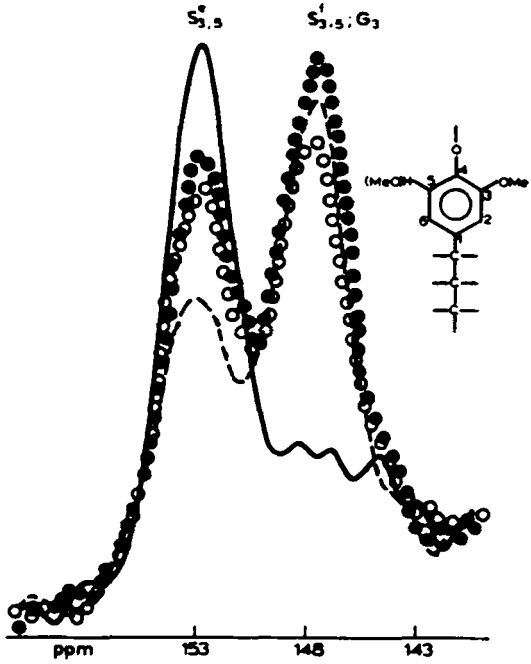

FIGURE 2 Spin-locked NMR spectra of E. regnans. ——— (U), ------ (AHE), 0000 (AE), ●●●●● (OSE).

to be only slightly less etherified than that in the alkali-extracted sample (ER.AE), but the lignin content of the OSE sample is considerably higher than that of the AE sample (Table 1).

Therefore, as proposed for P. radiata, the insolubility of the residual lignins is likely to be due to their being a mixture of repolymerized and large, partly depolymerized fragments. In addition, the high enzymatic digestibility of the cellulosic carbohydrate of this hardwood (Table 1) suggests that, in this case, a residual lignin with a β-ether content about 60% of that of the lignin of the untreated wood

does not significantly hinder enzyme access. Also, as proposed in the case of the softwood, it should be possible to use the estimated extent of β-ether cleavage in residual lignins as an indicator of the extent of structural breakdown of the wood as a whole, and to further correlate it with facilitation of enzyme access to the wood carbohydrate. These correlations would apply only within a given wood species and the dichotomy of the structurally quite different softwoods and hardwoods is shown quite well by the higher estimated β-ether cleavage of the residual lignins of the PR(SO_2) samples, cf. that of the ER samples, but the significantly lower enzymatic digestibility of the softwood's cellulosic carbohydrate (Table 1). There are, of course, also other factors involved in the facilitation and hindrance of digestion.

The total lignin content of the highly digestible E. regnans autohydrolysis-exploded wood is similar to that of the poorly digestible sample of P. radiata autohydrolysis-exploded wood, (see Table 1), which is further support for the absence of a correlation between total lignin content and hindrance of enzymatic digestion.

3. Sugarcane Bagasse

The spectrum of sugarcane bagasse is similar to that of the hardwood E. regnans, in that the signal for C-3,5 in syringyl units etherified at C-4 predominates. However, in contrast to the hardwood, signals in the 148 ppm region of the spectrum are much more intense (Fig. 3), most likely reflecting a higher guaiacyl unit content in the grass lignin.

Autohydrolysis-explosion results in a spectrum similar to that of the ER.AHE sample, in that the

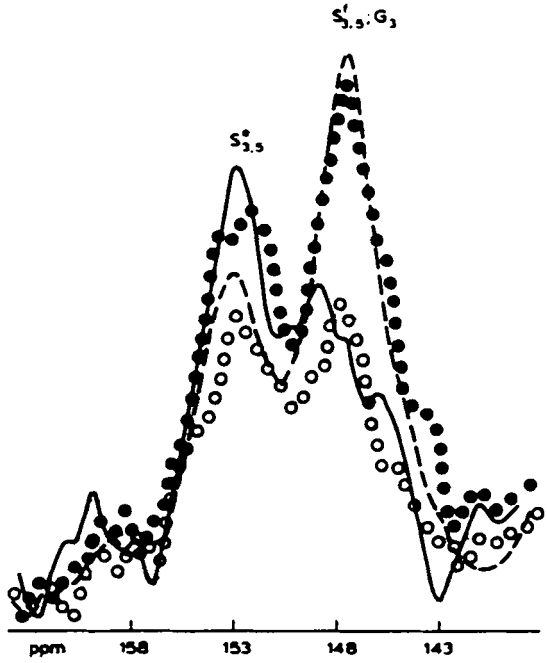

FIGURE 3 Spin-locked NMR spectra of bagasse. _____ (U), ------ (AHE), 0000 (AE), ●●●● (OSE).

relative intensities of the two signals at 148 and 153 ppm respectively are in the region of 3:2. With this substrate, the estimated extent of cleavage of β-ether linkages is about 35%.

As found with the wood samples, the residual lignins in the extracted samples are more etherified at C-4 than the total lignin in the autohydrolysis-exploded sample. However, in contrast to the E. regnans samples, the B.AE sample shows considerably less β-ether cleavage than the B.OSE sample. The lignin content of the B.AE sample is also much lower than that of the B.OSE sample (Table 1), so it appears that alkali extraction is removing more of the material contributing signals to the 148 ppm region

of the spectrum, i.e. material with a high free phenolic hydroxy-group content at C-4 and a high guaiacyl unit content.

Grass lignins contain cinnamic acids, which may be incorporated into the lignin and linked to both lignin and carbohydrate.[11,12] Bagasse lignin is reported to have a high content of cinnamic acids, particularly of p-coumaric.[11] Their presence and perhaps cleavage of additional linkages by the alkaline extraction procedure may be a reason for the greater solubility of this autohydrolysis-explosion lignin in alkali.

This autohydrolysis-exploded substrate is highly digestible (Table 1). Therefore, a β-ether content in the residual lignin of the AE sample comparable to (or in the case of the OSE sample, about 75% of) the content of guaiacyl and non-etherified syringyl units indicates that the extent of structural breakdown is adequate for enzyme access. It should also be noted that, in contrast to the untreated woods, untreated bagasse is partly digestible.

4. The Roles of Extractable and Residual Lignin

In all cases, the removal of soluble lignin decreases the enzymatic digestibility of the cellulosic carbohydrate, (Table 1), while total (residual + soluble) lignin content appears to be an unimportant factor in hindering digestion. These observations suggest that the soluble lignin in situ is not a hindrance to digestion. They may also be (albeit rather indirect) support for the aggregation of soluble and repolymerized lignin and for any lignin hindrance of enzyme access being due more to the content and location of large, partly depolymerized, residual lignin fragments, rather than to condensed or repolymerized fragments formed from solubilized lignin.

There is also no evidence for a relationship between residual lignin content and digestibility within a species, although gross differences between species may have some effect. Conversely, there is no evidence against, and some for, the structure and hence properties and location of residual lignin having a role in digestibility differences both within and between species. Recent work indicates that decreases in digestibility are brought about by decreases in pore size and accessible surface area of the cellulosic substrate.[3] Decreases in these could be brought about by the extraction procedures for removal of soluble lignin and concomitant changes in the location and interactions of residual lignin.

Summary

1. With all the species studied, the softwood, the hardwood and the grass, it appears that the residual lignin is comprised of partly depolymerized plus partly repolymerized lignin, rather than of unreacted lignin, and it is suggested that the partly depolymerized fragments are likely to be a greater hindrance to enzyme access to the cellulosic carbohydrate than the repolymerized fragments, which are likely to be more aggregated.
2. It is also proposed that, for each species, changes in the content of units etherified at the C-4 carbon atom, i.e. essentially, in the content of β-O-4 ether linkages, can be correlated with the extent of structural breakdown of the lignocellulosic material and with facilitation of enzymatic digestion of the cellulosic carbohydrate.
3. There is no evidence for correlations between enzymatic digestibility and lignin content, total, soluble or residual, but some support for the structure,

properties and location of residual lignins having a role in digestibility differences.

EXPERIMENTAL

Pinus radiata and *Eucalyptus regnans* sawdust, and sugarcane bagasse were pretreated by autohydrolysis-explosion in a "Siropulper" as described elsewhere.[2,5] Autohydrolysis was carried out at 200°C and 6.9 MPa applied N_2 gas pressure for 5 min. with bagasse and *E. regnans* sawdust, and for 10 min. with *P. radiata* sawdust. Heating was by direct steam injection, which took about 6 min. to reach 200°C. Following autohydrolysis, the "Siropulper" contents were rapidly discharged through a multiple-bar nozzle, designed to achieve maximum defibration.[2,5] The exploded material was pressed to remove the autohydrolysis liquor and the pulp was washed thoroughly with water.

P. radiata sawdust was treated with SO_2 by passing SO_2 gas through a 1 kg sample of the sawdust at ambient temperature and pressure for 10 min. The amount of gas absorbed (4.44%(w/w)) was determined by weight. The sawdust was then transferred to the "Siropulper" and autohydrolyzed for 10 min., as described above.

The alkali-extracted samples were prepared by treating the autohydrolysis-exploded material with 0.125 M aqueous sodium hydroxide at 70°C for 2 h. The acetone-extracted samples were prepared by extracting the AHE material with 90% acetone/H_2O (3x), removing the solvent by filtration and washing the residue with water.

Enzymatic digestions were performed as described previously.[2] The saccharification of each sample was evaluated using *Trichoderma reesei* C-30 cellulase (20 FPU), 25 β-glucosidase (*Aspergillus niger*) units and 10% (w/v) substrate concentrations, at 50°C (pH 5.0) for 24

and 72 h. Glucose was determined by the glucose oxidase method.[2]

CP/MAS ^{13}C NMR spectra were run on a Varian XL-200 spectrometer operating at 50.3 MHz as described previously,[4] while the use of a spin-locking pulse sequence to suppress signals from protonated carbon is described in Reference 7, as is the resolution enhancement carried out on the spin-locked spectra of P. radiata samples. Lignin contents were determined by the NMR method described previously, with minor corrections added for weak spinning sidebands falling in the 150 ppm region.[4,7] Spectra for this method were run without either resolution enhancement or spin-locking. Klason plus acid-soluble lignin contents were also determined as described previously.[2]

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