Wood Science and Technology 30 (1996) 99–104 Springer-Verlag 1996

Heterogeneity of lignin concentration in cell corner middle lamella of white birch and black spruce

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Summary Raman microprobe spectroscopy was used to study the concentration of lignocellulosics in the cell corner middle lamella. Spectra obtained from 1.6 µm regions, from 30 cell corner middle lamellae of both birch and spruce, showed the presence of lignin. However, the relative concentration of lignin to cellulose varied considerably. These results corroborate the view expressed in previous reports of the need for caution in using the lignin concentration values of cell corner middle lamella as a internal reference for studying the variation of lignin concentration in other morphological regions of the cell wall, such as secondary cell wall layers.

Introduction

Some of the earliest studies that provided information on the distribution of lignin in the various morphological regions of wood cells of sprucewood were performed using ultra-violet (UV) microscopy (Fergus et al. 1969; Wood and Goring 1971). It was concluded from these studies that the cell corners of sprucewood were highly lignified with values of lignin concentration ranging from 85% to 100%. However, the UV-microscopy technique is dependent on the absorptivity of lignin; therefore, the implicit assumption was made that lignin in various morphological regions had identical absorption (Fukazawa and Imagawa 1983).

With the development of EDXA (electron dispersive x-ray analysis) combined with SEM (scanning electron microscopy) and TEM (transmission electron microscopy), these studies and values were revisited. In one approach, the wood cell samples were brominated to allow detection of lignin in the electron microscopy (EM)-EDXA studies (Saka et al. 1982; Saka and Goring 1988). The conclusion from these studies was that the lignin concentration in secondary walls of sprucewood was similar as measured by both UV and EM-EDXA. However, the EM-EDXA estimation of the lignin concentration in cell corner middle lamella was a factor of 1.7 less than that of the UV-microscopy estimation. This was attributed to the difference of lignin reactivity between the cell

Received 27 June 1994

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corner middle lamella and secondary walls of sprucewood; therefore, 1.7 was proposed as a correction factor. In contrast, studies on lignin from samples of isolated morphological regions of cell wall and center middle lamella showed that the reactivity to bromination was the same, suggesting that the use of a correction factor was not valid (Donaldson and Ryan 1987; Westermark 1985).

Moreover, other problems were related to the bromination technique. Namely, production of hydrobromic acid and pyridynium hydrobromic perbromide when chloroform is used as solvent, both of which cause swelling and are difficult to remove (Westermark et al. 1988). To overcome this limitation, in another EM-EDXA study, mercurization of lignin was utilized to uniformly label lignin (Eriksson et al. 1988; Westermark et al. 1988). Mercurization tags each C_9 unit of lignin with 1 atom of mercury in a manner seemingly insensitive to the structure of lignin. The results from this study showed a similar pattern to that observed in the bromination EM-EDXA study (without using a correction factor).

Therefore, it was concluded that the difference in the values of lignin concentration of corner middle lamella measured by UV microscopy and EM-EDXA was not due to the difference in reactivity of lignin. The values reported for sprucewood cell corner middle lamella lignin concentration using mercurization of bromination EM-EDXA were 50% to 60%, considerably less than the "corrected" UV microscopy results.

A possible explanation for these results may be provided by a recent study of birchwood that utilized chemical and immunological staining combined with TEM-EDXA (Daniel et al. 1991). This study showed that some regions in the cell corner middle lamella were either poorly lignified or nonlignified, characterized by electron-lucent regions in the TEM micrographs. The electron lucent regions were detected consistently, not only in various samples of birch, but also in other angiosperms, such as poplar and European beech. However, not all cell corners were found to contain lignin deficient regions, and some cell corners showed complete signification. This result, to a lesser degree, was also seen in a gymnosperm (pine). These electron lucent regions, representing lignin deficient areas, have been suggested as a cause for the difference in lignin concentration of cell corner middle lamella as measured by UV microscopy and EM-EDXA (Daniel et al. 1991). Whereas, UV microscopy may not be sensitive enough to pick up these variations, the EM-EDXA (bromination/mercurization) is able to delineate these variations. The spatial resolution of the TEM-EDXA studies was 0.04 µm, with the largest electron lucent region in the micrograph measured at about 0.5 µm.

To verify the presence of these less lignified regions in the cell corner middle lamella, a Raman microprobe study was undertaken. Spatial resolution of the order of 1.6 µm can be achieved by this method. The use of Raman microprobe to study compositional characteristics of cell walls has already been demonstrated (Agarwal and Atalla 1986, Bond 1991). In this previous work, the woody tissue was found to be sensitive to the 514.5-nm laser radiation. Subsequently, a method was found to stabilize woody tissue to high intensity laser radiation (Bond et al. [to be published]). The stabilization was brought about by reducing the Υ-carbonyls of the coniferaldehyde units in lignin by a mild and extended sodium borohydride treatment.

Experimental methods

Sample preparation

Cross-sectional samples of gymnosperm woody tissue were microtomed from a black spruce *(Picea mariana* (Miller) B.S.P.) tree, 56 years old, grown in northern Wisconsin.

The heartwood sample was taken from a location in the tree that showed no evidence of the presence of compression wood. The microtomed samples were approximately 1.0 by 0.5 cm, with a thickness of 30 µ. Similarly, cross-sectional samples of angiosperm wood were microtomed from a white birch *(Betula papyrifera)* tree from northern Wisconsin. Microtomed sections were repeatedly washed with distilled water. The sections were washed with 95% ethanol and stored in methanol. The mounting of the sample for the microprobe was described by Agarwal and Atalla (1986).

Stabilization using borohydride

The two sets of samples corresponding to the two species selected were treated in this stage. The samples, 10 sections from each species, were immersed in 100 ml distilled water and 1 g of $NaBH_4$ was added to each sample container. For each of the following 5 days, an additional 0.5 g of $NaBH_4$ was added. Thus, the total amount of $NaBH_4$ added over 6 days was 3.5 g. Both sets of samples were taken out of the containers, washed with deionized water, solvent exchanged into methanol, and stored separately in methanol.

Raman microprobe

The system consists of three major components: a Raman spectrometer, a modified microscope, and an automated system for data handling. The spectrometer used was a SPEX Triplemate 1877B system (from SPEX Industries, Edison, New Jersey) using a 514.5-nm line of a Coherent Radiation (Palo Alto, California) Innova 90 argon ion. The system is equipped with a Tracer Northern (Middleton, Wisconsin) TN-6500 optical multichannel detector system containing a photodiode array detector. The system can be programmed to obtain multiple spectra. The microprobe consists of a Zeiss WL (SPEX Industries) microscope that was adapted to allow coupling of the laser into the optics. The microscope is designed to deliver the laser beam to the sample, collect radiation scattered from the sample, and focus it on the entrance slit of the spectrometer (see Agarwal and Atalla 1986 for details). The sample holder specifically designed for use in the present study was described by Agarwal and Atalla (1986).

Spectra were recorded using a 1OO× objective with a numerical aperture of 1.3, which corresponds to a 1.6 µ spot on the sample. The power level of the incident laser beam was kept constant at 100 mW, which translates to approximately 5 mW on the sample. The entrance slits of the monochromator were set at 400 µ. The spectrometer used had a holographic grating at 1,200 grooves/mm. The spectral resolution that can be obtained from the aforementioned monochromator setting is 8 cm^{-1}. The TN-6500 detector system consists of a computer, detector interface, and a 1,024 channel stationary grating silicon diode array detector. The array detector permits simultaneous recordings of the intensity of scattered radiation over an interval of 1000 cm^{-1}. Therefore, the rate at which a spectrum can be obtained is an order of magnitude faster than conventional spectrometers based on photomultiplier tube (PMT) technology. Spectra reported were obtained in the multiple scanning mode that were averaged for 60 scans, and each scan was characterized by a dwell time of 20 s. Hence, the total time to acquire a spectrum was 1,200 s. Most spectra were obtained in this fashion, although total time for some was 1,800 s.

Results and discussion

In the Raman spectra of woody tissue, Agarwal and Atalla (1986) showed that the 1098 cm^{-1} band is indicative of cellulose, and the 1595 cm^{-1} is representative of lignin (Atalla and Agarwal 1985). The ratio of the areas under the lignin and cellulose bands can be used as an indicator of the lignin-to-cellulose ratio. Table 1 shows ratios of lignin

and cellulose bands for several white birch cell corner middle lamella regions. Although significant variation in lignin-to-cellulose ratio existed (as much as 92%, Table 1), lignin was present in the spots from which spectra were obtained. Table 2 shows lignin band height values obtained from earlywood and latewood corner middle lamella regions of black spruce. Here, the cellulose peak was very small; therefore, it was decided to report only the lignin band height. Moreover, as experimental conditions remained constant during data acquisition, lignin band height values indicated concentration of lignin; i.e., the greater the band height, the greater the lignin concentration. In addition, the band height of lignin in both earlywood and latewood corner middle lamella regions was similar, indicating that the lignin concentration in these regions is similar.

Table 1. Lignin/cellulose ratio of white birch cell corner middle lamella

Number	Lignin/cellulose ratio	Number	Lignin/cellulose ratio
1	1.03	16	1.02
2	1.25	17	1.20
3	0.98	18	1.76
4	1.22	19	1.43
5	1.75	20	1.45
6	1.35	21	1.19
7	1.02	22	1.26
8	1.36	23	1.00
9	1.44	24	1.13
10	1.30	25	1.46
11	1.31	26	0.91
12	1.17	27	1.00
13	1.22	28	1.00
14	1.13	29	1.42
15	1.00	30	1.30

Table 2. 1595 cm^{-1} band height (absolute) in the specimen of black spruce cell corner middle lamella

Number Earlywood	Absolute peak height of 1595 cm^{-1} band	Number Latewood	Absolute peak height of 1595 cm^{-1} band
1	30	15	26
2	35	16	29
3	29	17	34
4	32	18	26
5	32	19	26
6	31	20	33
7	32	21	33
8	28	22	34
9	21	23	34
10	20	24	30
11	26	25	34
12	27	26	30
13	32	27	33
14	32	28	23
		29	30
		30	33
		31	33.5

As the lignin-to-cellulose ratio varied over a wide range (Table 1), the presence of the poorly lignified regions reported (Daniel et al. 1991) is apparently verified. By obtaining a series of spectra from the same location, it was noted that the lignin-to-cellulose ratio was constant (within experimental error of about 10%). Therefore, the variation observed here is not an experimental artifact but a real variation of lignin-to-cellulose ratio. Moreover, the fact that lignin content varies can be noted from the spectra shown in Fig. 1, where spectra from two adjacent corner middle lamella of white birch are shown. The variation may not be obvious in the figure but performing the relative band height calculation (SPECTRACALC program from Galactic Industries, Salem, New Hampshire) shows the variation. These spectra were obtained under identical experimental conditions; therefore, spectral differences were not due to changes in experimental variables. The difference in lignin-to-celhdose ratio was due to difference in lignin concentration. However, the presence of lignin bands in all spectra indicated that the nonlignified regions reported (Daniel et al. 1991) must be less than 1.6 µm in diameter. In addition, the presence of a strong cellulose band in all white birch spectra suggests that cell corner middle lamella cellulose was present in greater quantities than previously reported (Fergus et al. 1969) (where corner middle lamella lignin was assumed to be close to 100%).

Raman spectroscopy is not well suited for absolute quantitative analysis. The theory of spectral band intensities is not as well developed as infra-red (IR) spectroscopy. Only a few factors that affect band intensity of lignin are known. Nevertheless, relative quantification is straightforward when the experimental parameters for the acquisition of spectra are kept constant. The relative quantification technique used here (ratio of lignin to cellulose band heights) shows that the nonlignified regions, as evidenced by the TEM-EDXA technique, were not present. Some spots in the TEM-EDXA study (Daniel et al. 1991) showed presence of nonlignified regions of about 0.5 µm in diameter. Such 'holes' were not observed in the present study as a result of the limitation of Raman microprobe spatial resolution (1.6 µm). All spectra obtained had band heights in the same order of magnitude. At the most, the ratio differed by a factor of 2 in the case of white birch, and lignin band height differed anywhere from 1.48 (latewood) to 1.75 (earlywood) for black spruce (Table 2). Therefore, the variation observed may be due both to the variation in lignin concentration and the presence of lignin deficient regions. However, note that the mercurization combined with TEM-EDXA is a technique that relies on secondary analysis. That is, the results of the TEM-EDXA are dependent on the efficacy of the tagging reaction in its labeling of the lignin. In addition, the TEM-EDXA technique uses x-rays of 100 keV that can cause removal of the woody

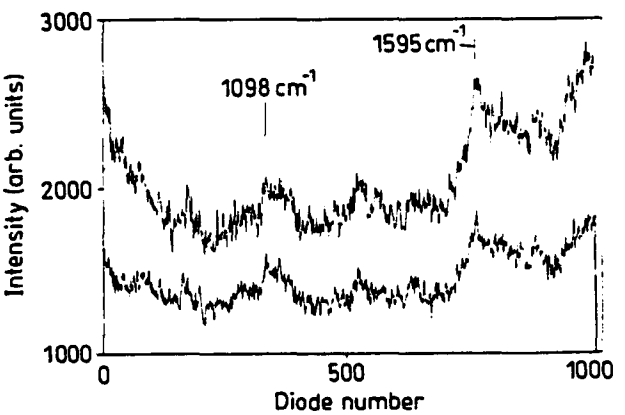

Fig. 1. Raman spectra of two adjacent cell corner middle lamella of white birch: (a) and (b) are numbers 18 and 17, respectively, (Table 1) showing difference in 1595 cm^{-1} band height

tissue under observation. The stability of the woody tissue to TEM-EDXA needs to be investigated further. Whereas, the Raman microspectroscopy used in the present study provides information on the presence (or absence) of lignin without chemical derivatization. The chemical treatment (borohydride reduction) of the woody sections render the sample resistant to photodegradation; the treatment does not modify the phenyl rings of lignin. Therefore, the presence of the 1595 cm^{-1} band indicates the presence of phenyl rings, which in turn indicate the presence of lignin.

Conclusions

Raman microprobe spectroscopy was utilized to study the heterogeneity of lignin content in the cell corner middle lamella of white birch (angiosperm) and black spruce (gymnosperm). All corner middle lamella regions showed presence of lignin. However, the ratio of the lignin-to-cellulose bands varied greatly, indicating that the lignin distribution in the cell corner middle lamella is not uniform. This reinforces the argument presented previously by Daniel et al. (1991), which stated that the use of cell corner middle lamella lignin values for subsequent quantitative studies of lignin distribution in the cell walls should be used with caution.

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