A Raman Microprobe Investigation of the Molecular Architecture of Loblolly Pine Tracheids

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ABSTRACT

Our understanding of the molecular architecture of intact, native plant cell walls is very limited. Traditional methods of investigation disturb the tissue to varying degrees and conclusions based on these methods may be intimately related to the technique used. A promising new technique to study native-state organization is polarized Raman spectroscopy. In this present work the macromolecular orientation and compositional variability for both cellulose and lignin in latewood cell walls of loblolly pine tracheids were investigated using Raman spectroscopy. This study has provided direct evidence of molecular organization with respect to cellular morphological features, and suggests that the cell wall components are more highly organized than has generally been recognized.

INTRODUCTION

The use of plant fibers is widespread in our society. The pulp and paper industry and, to a lesser extent, the textile industry derive their livelihood from these materials. The suitability of these various fibers for specific applications is closely related to their development and molecular properties.[1] One area that is lacking in our understanding of the cell wall is the way in which the chemical constituents are structurally organized.

Cellulose, hemicellulose and lignin are the major components of plant cell walls and basically establish the chemical and physical nature of the wall. Cellulose, the b-1,4 linked polymer of anhydroglucose, is the primary component. The principle function of cellulose in the cell wall is to provide a framework while hemicelluloses and lignin serve as matrix materials. The secondary components consists of inorganic materials and extractable compounds, and typically impart certain characteristics to wood, e.g. color and resistance to decay.

The cell wall consists of an intricate intermixing of the various components and is composed of concentric lamallae that are organized in a primary wall and a secondary wall in three distinct layers (S_1, S_2, S_3). The primary wall is laid down in the initial stages of growth. the secondary wall is formed when cell enlargement ceases. the exact physical arrangement of cellulose, hemicellulose and lignin is., for the most part, not known. Most of the investigations have focused on the primary wall; information about the secondary wall is lacking.

Traditional methods of cell wall investigation fall into two major catagories. [2,3] The first provides structural information on the molecular level derived from physico-chemical analyses of isolated cell wall components. Techniques using optical and electron microscopy, which provide descriptions of cell wall morphology, are included in the second category. Although both have been frequently used to elucidate the structure of the cell wall, there are shortcomings associated with each. In the first category, the procedures used to isolate the cell wall components are extremely disruptive of the native-state structures[4] and conclusions drawn from this information are open to question. While the second category has been usefull in describing the morphology of the cell wall, it is limited in the information it can provide about molecular structure.

Polarized Raman microspectroscopy is a promising technique to investigate both orientation and compositional variability of the major cell wall components in undisturbed plant tissue.[5,6] Although the Raman effect was first observed in 1928, it was not until the advent of the intense, monochromatic excitation beam that the laser provided that this vibrational spectroscopy technique advanced. The development of the laser Raman microphobe has opened up new areas of investigation heretofore not possible. 7-9 Information derived from this technique should complement that obtained from traditional methods of investigation.

EXPERIMENTAL

Specimen

Loblolly pine (Pinus taeda L.) was chosen for a number of reasons. It was anatomically optimum: its fibers have thick latewood secondary walls (7-9 microns)[10], and few pits are present.[11] In addition, it has been thoroughly researched with regards to anatomy and deposition of the various components.[10,12] Samples were taken from the main stem of a 60 year old tree grown in South Carolina.

Methods

Radial sections were obtained from never-dried, woody samples and were prepared by microtoming the water-saturated wood. The sections were 30 microns in thickness. After sectioning, the samples were extracted for 24 to 48 hours in an ethanol/toluene mixture (1:2 v/v) at 25°C.[13] (One reason for this was to minimize any contributions extractives might make to the interfering background fluorescence level.[14])

The sections were repeatedly washed in distilled water, prior to mounting in the following manner. A flat-bottomed pyrex beaker was glued to a microscope

slide in a manner that allowed transmitted light to pass through. On the inside bottom surface of the beaker, a circular glass slide was placed. The wet sample was sandwiched between a coverslip through which a 4 mm hole had been drilled and the circular glass slide. A Teflon washer was placed over the coverslip and held in place with a Teflon retaining ring. Water was slowly added into the beaker until the sample was completely immersed. The beaker was mounted onto the stage of the microscope, and the objective was lowered into the H_2O. A thin layer of mineral oil was placed on the surface of the H_2O to prevent evaporation. This sampling procedure provided a heat sink to dissipate energy absorbed by the sample and resulted in high quality spectra due to quenching of the fluorescence.

Raman Microprobe

The Raman microprobe consisted of three basic components: a Spec Triplemate 1877B spectrometer, a modified Zeiss WL research grade microscope coupled to the spectrometer and the Tracor Northern TN6500 Optical Mutichannel Spectroscopy system. The TN6500 system, in turn, consisted of a computer, detector interface and a 1024 element intensified silicon diode array detector.

The 514.5 nm line from a Spectra Physics argon ion laser was used for sample excitation. Laser power levels were 30 mW after the plasma filter.

Spectra were recorded using a Zeiss 100X oil-immersion objective with a numerical aperture of 1.3 that gave a focused spot of approximately 1.5 microns in diameter. Spectral resolution was set to approximately 8 cm^{-1}. The reported spectra were signal averaged. Thirty scans were needed to obtain an acceptable signal-to-noise ratio.

Mapping of the cell wall components requires a well-engineered microscope stage that is capable of making accurate, x-y steps as small as one micron. An LEP custom modified motorized Zeiss stage was used in these studies.

To aid in the cell wall investigations, the microscope was equipped with video enhanced microscopy capabilities. The video signal from a Javelin JE2362 monochrome camera, coupled to the Zeiss microscope, was routed to both a Sony Trinitron monitor for direct viewing and an Imaging Technology PCVision plus frame-grabber for digitization and further processing A Mitsubishi P61U video copy processor was used for obtaining hard copies of the video image.

Spectral Mapping

The Raman band at 1098 cm^{-1} (chain axis stretch) was used to explore the organization of cellulose molecules while the 1595 cm^{-1} aromatic ring breathing band was used to determine lignin organization.

A 10 x 5 grid pattern was used in this study. This grid area included three distinct morphological regions: the latewood secondary cell wall layer (S_2) of one fiber, the compound middle lamella (CML, primary wall + middle lamella) between, and latewood S_2 layer of the adjoining fiber. Ten locations along (parallel to the fiber's longitudinal axis) and two (one for the CML) locations across (perpendicular to the axis) each fiber region were studied. The along fiber spacing between the individual data points was approximately 2.5 microns.

By varying the plane of polarization of the incident laser light relative to the cell wall and observing the changes in the intensities of specific Raman bands, it is possible to argue for or against a particular orientation.[5] Two electric vector orientations were used: parallel to the long axis of the cell wall and perpendicular to it. Because of dichroism in the optics of both the microscope and the spectrometer, the rotation of the electric vector relative to morphological features if usually accomplished by rotating the sample. This was not practical for the mapping studies because of the sensitivity of lignin to laser radiation and the difficulty in accurately positioning the sample after rotation. In lieu of sample rotation, electric vector rotation was accomplished by using a laser-line rotator. In order to minimize dichroic effects, a polarization scrambler was inserted in the optical path just prior to the entrance slit of the spectrometer, effectively making it insensitive to the plane of polarization. The beam splitter in the microscope was another source of dichroism. Here it was necessary to use a toluene standard to correct the spectra for dichroic effects.

due to the sensitivity of lignin to 514.5 nm radiation, the (electric vector) parallel and perpendicular pair of spectra that were used to investigate orientation, could not be collected from the same location. (The initial irradiation modified the area in such a way that the lignin orientation information was lost.) To circumvent this difficulty, it was necessary to collect parallel information from the first location. perpendicular from the next, parallel from the third, and so forth along the fiber. The distance between the orientation pair was approximately 2.5 microns. In the topographic plots, the missing intensities were determined by linear interpolation. For end points where interpolation was not possible, the neighboring (row) intensity value was used.

In order to investigate the compositional variability, the band intensities when the electric vector was oriented parallel and perpendicular to the cell wall were added together for each component to evaluate the total amount of that component in a particular domain.

The general cell wall region used in the mapping investigation was irradiated with a very defocused laser beam for one hour prior to the start of data aquisition. This was done to reduce the level of the interfering background fluorescence.

RESULTS AND DISCUSSION

Photochemical Effects in Woody Tissue

Certain bands in the Raman spectrum of wet woody tissue have been observed to decrease during exposure to 514.5 nm laser radiation. Figure 1 shows the Raman spectra of a spot lying in the latewood, secondary cell wall of a radial section of black spruce. The upper trace is the singal-averaged spectrum collected during the initial 500 seconds of 514.5 nm irradiation while the lower spectrum is from the same spot after a 13.75 hour exposure.

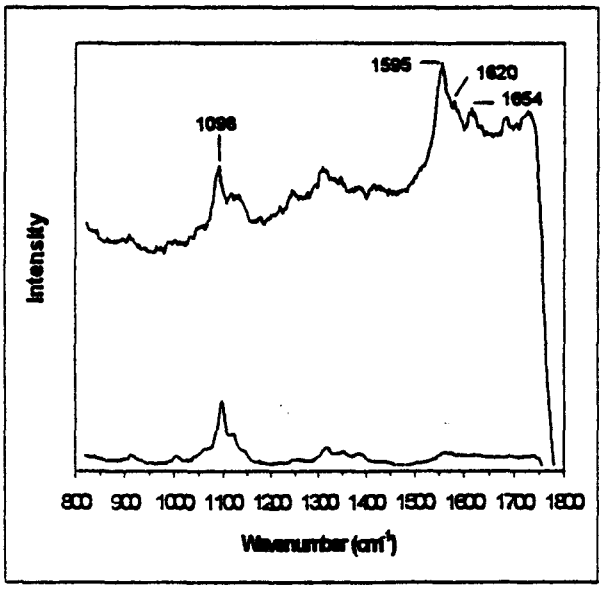

Figure 1. Raman spectra collected from a single location in the latewood, secondary cell wall of black spruce

The 1098 cm^{-1} that has been identified with one of the polarization and orientation-sensitive skeletal modes of the cellulose chain[5] does not undergo an intensity change during irradiation, In contrast to this, the bands at 1595, 1620 (shoulder) and 1654 cm^{-1} have essentially vanished. These bands arise from the lignin macromolecule and have been assigned as follows: 1595 cm^{-1} - aromatic ring stretching vibration; 1620 cm^{-1}-ring-conjugated C=C stretching vibration; and 1654 cm^{-1} - ring-conjugated C=C and C=O stretching vibrations.[5,15,16]

This data suggests that something is happening to the lignin macromolecule during 514.5 nm laser irradiation of wet woody tissue. (This behavior has been seen in all of the woody species investigated in our laboratory.) The almost complete disappearance of the lignin bands after long exposure points to a modification in the macromolecule's structure.

Figure 2 shows a SEM micrograph of an irradiated region on the secondary cell wall (500 seconds irradiation time, 524.5 nm, 100 mW). A roughly circular feature with a concentric ring-like appearance can be seen. Its diameter is roughly four times larger than the laser beam. We believe that woody tissue, viz lignin, undergoes a complex series of photochemical, degradative reactions that are initiated by the absorption of 514.5 nm photons. These reactions resulted in an imprinting of the incident intensity distribution on the secondary cell wall layer. (The feature seen in the SEM micrograph.) During these reactions, the lignin macromolecule appears to become disrupted as evidenced by the almost complete disappearance of its 1595 cm^{-1}, 1620 cm^{-1} and 1654 cm^{-1} Raman bands. Additional evidence not shown here suggested that lignin is being solubilized and the degradation products include quinonoid structures.

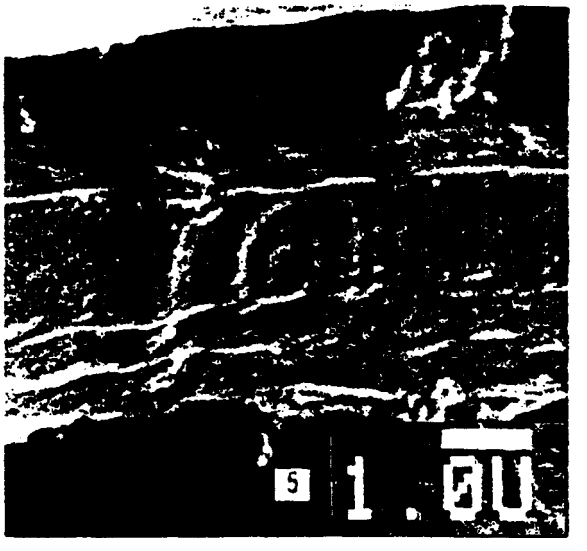

Figure 2 SEM micrograph of black spruce radial wood section showing the S_2 cell wall layer following laser exposure.

Spectral Mapping Studies

The decay of lignin was found to be a multifaceted problem. Reduction, oxidation and substitution reactions of the lignin macromolecule, as well as the use of fluorescence quenchers and radical scavengers failed to stop lignin band decay. Because a method could not be found to adequately stabilized lignin to laser radiation, a methodology that was thought to minimize the photomodification of lignin was followed in the spectral mapping studies. This involved using a low laser power level (30 mW), keeping acquisition time to a minimum and treating each acquisition location identically.

Figure 3b shows the cell wall area used in the mapping studies after 90 seconds of irradiation (bottom center of micrograph). The wide feature that runs from left-to-right just above the center of the micrograph is a

lumen. A disruption caused by the laser irradiation is readily apparent (dark circular areas).

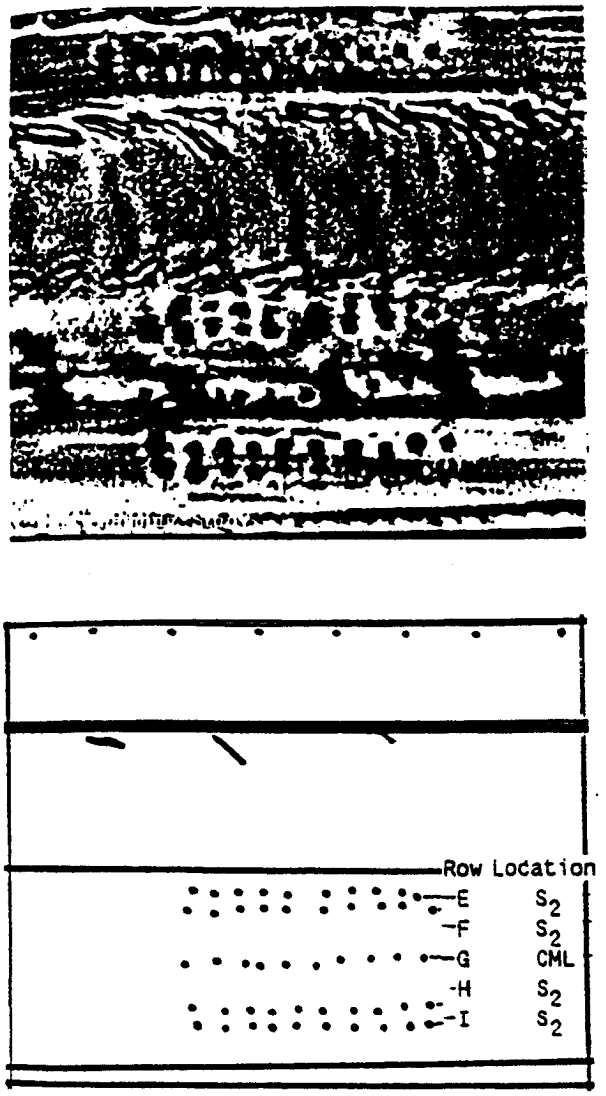

Figure 3. video enhanced print showing the area used in the mapping study of loblolly pine after irradiation (upper). The grid pattern (lower), Radial section.

The approximate location of the data points is shown in Figures 3 (lower) and Figure 4. A total of 50 data points were used in the analysis (10 x 5 grid).

Cellulose

Figure 5 is a topographic plot of cellulose concentration versus cell wall location. It can be seen that the cellulose concentration is not uniform but varies over the different morphological cell wall regions. The concentration appears to decline across the cell wall in going from the inner S_2 (row E or I) to the CML (row G). This trend is fairly universal over the region studied.

The cellulose 1098 cm^{-1} band intensity row averages were analyzed to more clearly see the trends in organization between the various morphological regions (Figure 6). It can be seen that, on the average, the parallel intensity values were greater than the perpendicular values for all of the secondary wall locations. This most likely reflects the preferential orientation of the chain axis of cellulose in planes parallel to the cell wall.

If the assumption is made that cellulose orientation does not vary significantly over a 2.5 micron distance, then the parallel + perpendicular data indicate that the peak cellulose concentration occurs in the inner S_2 layer and declines to a minimum near the CML. This trend was seen for both fibers. These data parallel the generally accepted trend for cellulose concentration in conifer cell walls.[11]

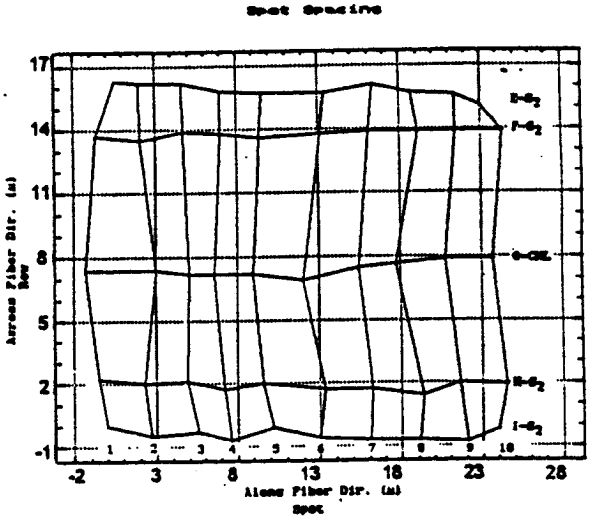

Figure 4. The position and spacing of the analysis points used in the spectral mapping study of loblolly pine cell wall organization.

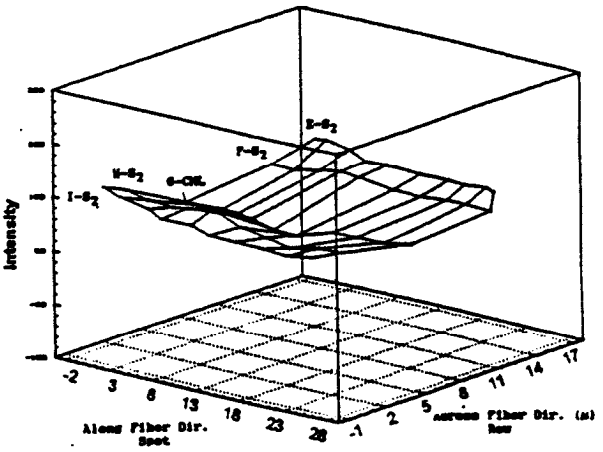

Figure 5. Topographic plot of cellulose concentration vs. cell wall location. Electric vector parallel + perpendicular 1098 cm^{-1} band intensities.

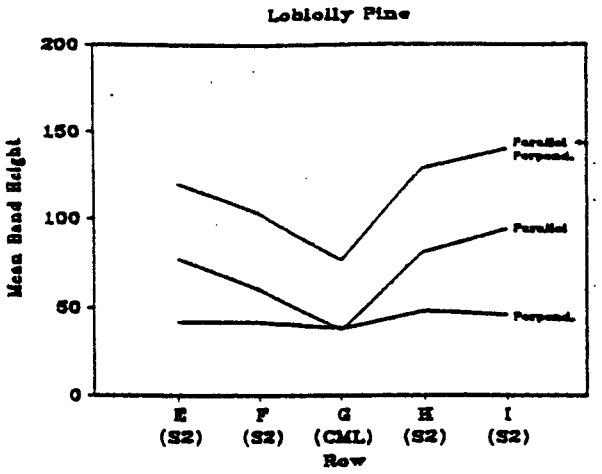

Figure 6. The variation of cellulose's 1098 cm-l band intensity over different morphological regions in the latewood cell walls of loblolly pine tracheids.

Lignin

In Figure 7 it can be seen that the lignin concentration varies over the different morphological regions. The concentration appears to decline from the inner S_2 and then increase in the CML region. While this trend appears to be universal, the specific location concentrations vary. This indicates that there is a variation in concentration not only between the different cell wall regions but also within a single region.

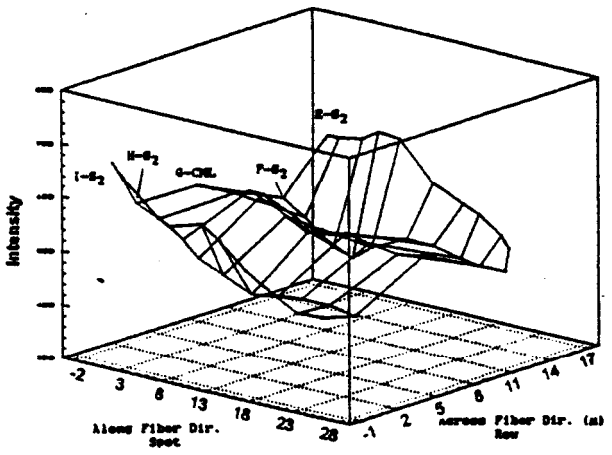

Figure 7. Topographic plot of lignin concentration vs. cell wall location. Electric vector parallel + perpendicular 1595 cm^{-1} band intensities.

The row averages for the 1595 cm^{-1} band intensity were analyzed to investigate the general trends in organization across the various morphological cell wall regions (Figure 8). It can be seen that the electric vector parallel intensity values are consistently greater than the perpendicular values. This suggests that, on the average, the aromatic rings are preferentially oriented in planes parallel to the cell wall.

The parallel + perpendicular data indicate that the lignin concentration decreases from a high value near the lumen to a minimum in the outer S_2, and then increases in the CML region. This trend in lignin concentration parallels that found by Heazel[17] using sulfonated pine latewood chips and agrees with the generally accepted lignin distribution in conifer cell walls.[11]

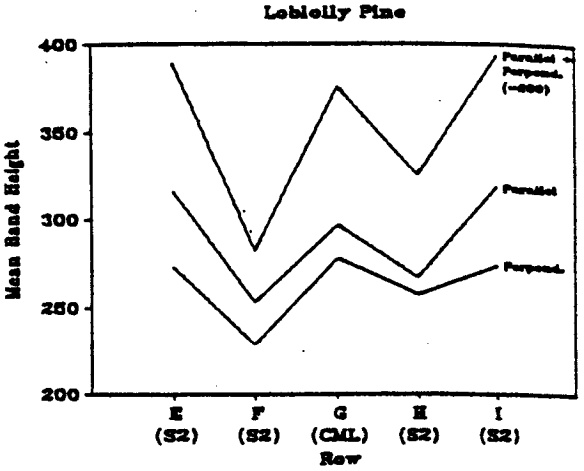

Figure 8. The variation of lignin's 1595 cm-1 band intensity over different morphological regions in the latewood cell wall of loblolly pine tracheids.

CONCLUSION

Raman microprobe studies of native woody tissue provided an opportunity for measurements of the macromolecular organization of cellulose and lignin across cell walls. These measurements were complicated by photo-induced changes in the lignin macromolecule that occurred during laser irradiation of the wet tissue. A methodology that was thought to minimize the photomodification of lignin was followed in the spectral mapping studies of cellulose and lignin.

Analysis of the spectral mapping data confirmed that in loblolly pine, on the average, cellulose was not uniformly distributed in the three morphological regions: S_2, CML, S_2. The peak cellulose concentration occurred in the inner S_2 cell wall layer and declined to a minimum near the CML. This trend was also seen in the adjoining fiber, although the peak concentration differed slightly. The data also suggested that cellulose is more oriented parallel to the cell wall in the inner S_2 layer and this became less pronounced nearer the CML.

Lignin also showed a non-uniform distribution. The 1595 cm^{-1} lignin peak intensity decreased from a high value near the lumen to a minimum in the outer S_2, and then increased in the CML region. This was paralleled

in the adjoining fiber. The data also suggested that, on the average, that the aromatic rings of the phenylpropane units were slightly more oriented parallel to the cell wall in the inner S_2 region and thus, paralleled the trend seen for cellulose. These data taken together support the idea that during biogenesis, cellulose may act as a template for lignin organization.

In addition to these average trends across the three morphological regions, the molecular organization of cellulose and lignin was found to vary along these regions as well. This may be indicative of the occurrence of nodes in the organization of these components.

This present study lays the foundation for future systematic spectral mapping studies of the two major biopolymers in woody plant cell walls. A hurtle that has yet to be overcome is the photostabilization of lignin to laser radiation. Studies are currently underway in our laboratory to address this.

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