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Molecular Organization in the Native State of Woody Tissue: Studies of
Tertiary Structure and its Development Using the Raman Microprobe, Solid
State ¹³C NMR, Fluorescence Spectroscopy and Photoconductivity.

Progress Report

for the Period July 1, 1992 to June 30, 1994

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SUMMARY

The studies undertaken over the most recent period represent a search for better understanding of the coupling between the processes of synthesis of the individual constituents of the secondary wall, on the one hand, and the assembly of the integrated matrix that is the secondary wall in fully lignified tissue, on the other. This search is motivated by the realization that native tissues possess collective properties which are characteristic of the constituents when they are associated together in the secondary wall, but which are lost when the individual constituents are isolated. Furthermore, these collective properties appear to be consequences of the manner in which the constituents are assembled during the biogenesis of the secondary walls. Thus, they represent a potential source of valuable information on molecular organization in the native state which may not be available through other avenues of inquiry.

Our pursuit of clearer definition of the couplings that occur during biogenesis is logically divided into three parts not unrelated to the sequence of deposition of the constituents. The first part is focused on understanding the coupling between the deposition of the hemicelluloses and cellulose. The second is directed at exploring the effects of the tertiary structure of the polysaccharide matrix on the structure of lignin that is assembled within it. The third part of our effort is concerned with investigating tissues in the native state to better define their organization at the molecular level, and to explore collective properties that may reflect this organization.

The investigation of coupling between the deposition of hemicelluloses and of cellulose, was carried out through culture of *Acetobacter xylinum* in media to which the hemicelluloses were added. The results provided clear evidence that the hemicelluloses modify the aggregation of cellulose, and that each of the hemicelluloses modifies the aggregation in a different manner. However, in all instances, the pattern of aggregation was found to be closer to that which occurs in higher plants. The experimental results were complemented with molecular modeling studies that shed light on the intermolecular interactions responsible for the associations. The findings, taken together, led us to propose that one key function of the hemicelluloses is the regulation of the aggregation of cellulose in higher plant cell walls.

The coupling between the tertiary structure of the matrix and the structure of lignin formed therein was studied through preparation of DHPs in polysaccharide matrices and examining their structures with respect to the degree of their similarity to the structure of milled wood lignin (MWL). Conditions were found that resulted in DHPs which approximate MWL more closely than any previously reported. Here again, molecular modeling studies provided important insights concerning the dominant intermolecular interactions, and led us to propose that another key role of the hemicelluloses, in association with cellulose, is to provide a template for the assembly of lignin from monomeric and oligomeric precursors.

Investigations of the native state included continuation of our mapping studies, using the Raman microprobe, which show the variability in the relative distribution and orientation of lignin and cellulose within the cell walls. In addition, studies of photoconductivity, together with complementary fluorescence spectral measurements, was developed as a new probe of coherence in the organization of lignin in its native state.

Specific accomplishments during the most recent program period include:

Hemicelluloses and Cellulose:

Demonstration that xylan, mannan, xyloglucan and glucomannan can modify the pattern of aggregation of cellulose, and that each does so in a distinctive manner. The findings led us to propose that:

- (a) One of the functions of the hemicelluloses, in higher plants, is to regulate the aggregation of cellulose in cell walls.
- (b) The action of the hemicelluloses arises from their adsorption on the surfaces of sub-elementary fibrils from individual assembly complexes at the cell membrane; when aggregation to form elementary occurs, the hemicelluloses are integrated into their structure.

Molecular Modeling Studies:

Validation of the use of molecular mechanics to explore intermolecular interactions in cell wall systems through successful prediction of spectral and optical properties of cellobiose and xylobiose in solution.

Computation of the persistence lengths of cellulose and xylan, which provided a rationalization of the relatively low solubility of xylan and related unbranched beta-1,4-linked glycans.

In computational simulation of adsorption on cellulosic surfaces, it was shown that monomeric and oligomeric precursors of lignin are indeed adsorbed quite rapidly from aqueous solutions. The oligomeric precursors were found to adsorb more rapidly than the monomeric ones.

Hemicellulose Lignin Interactions:

Synthetic lignins (DHPs) were produced under conditions that simulate the cell wall environment by inclusion of polysaccharide matrices. Two different approaches were used, and both resulted in DHPs with structures that represent closer approximations to milled wood lignin (MWL):

These findings, the molecular modeling studies, and the results concerning organization of lignin given below, led us to propose that:

Another key function of the hemicelluloses is to provide a template for the assembly of lignin from monomeric and oligomeric precursors.

Organization in Cell Wall Lignin:

Pathways for electronic charge transport in lignified woody tissue were demonstrated by measurement of photoconductivity under conditions wherein ionic charge transport was limited. The pathways appear to be regularly organized arrays of adjacent lignin units that give rise to de-localized excited states of relatively low energies. Such pathways are likely to be part of biological control systems that synchronize processes in adjacent cells and tissues.

In the Raman microprobe mapping studies the following advances were made:

1. The processes by which the intensity of certain lignin Raman bands are enhanced were identified. Thus, the interpretation of intensity variations in the spectra can be made with greater confidence.

2. Mapping studies for two species, Black spruce and Loblolly pine, have been carried out. Though the enhancement effects require that quantitative comparisons must remain relative, the results provide some measure of the variability of the constitution of the cell wall as well as some comparisons of adjacent walls.
3. Procedures have been developed to stabilize tissues for long term exposure to laser excitation, and make possible more precise quantitative comparisons of the variability of lignin structures within the cell wall and relative to the cellulose.

OVERVIEW

The work completed in the current program period is reported in 14 publications, some of which have appeared in print, and the rest of which are either in review, or will be by the end of September; five are attached to this report. The reports are conveniently discussed in four categories. The first is concerned with studies of cellulose and of the manner in which the hemicelluloses can influence the aggregation of the cellulose. Thus the focus is the polysaccharide matrix and the couplings that occur between its components. The second category includes the molecular modeling studies. These are new in our program, and cover explorations of the dominant characteristics of the polysaccharides as well as the interactions which can occur between the polysaccharides and the precursors of lignin. The third group of publications address our realization that the polysaccharide matrix may well be the key to understanding the source of organization in native lignins. The fourth set of publications deal with direct observations of organization in native lignin and the characteristic properties which reflect this organization.

Cellulose and the Hemicelluloses

In addition to the primary effort centered on the interactions between the hemicelluloses and cellulose, this group includes two reports focused on native celluloses. The five reports are:

1. "Hemicelluloses as structure regulators in the aggregation of native cellulose", RH Atalla, JM Hackney, I Uhlin and NS Thompson, *Int. J. Biol. Macromol.* 15:109(1993)

This work describes studies of the effects of xylan, mannan, xyloglucan and glucomannan on the structure of bacterial celluloses grown in media containing these hemicelluloses. The effects were monitored by x-ray diffractometry and electron microscopy. The study was based on examination of the effects of hemicelluloses under two very different conditions of culture. The first was under stationary culture conditions, using a strain of Acetobacter xylinum that had been previously well characterized in other laboratories but which is not tolerant of agitation. The second set of conditions was based on availability of an agitation-tolerant strain of Acetobacter xylinum, included mild agitation, and allowed production of larger amounts of the hemicellulose modified celluloses. It was observed, under both sets of conditions, that each of the hemicelluloses influenced the aggregation of cellulose in a different way, though all made it more like higher plant celluloses. These results, taken together, led us to propose that one of the

important functions of the hemicelluloses is regulation of the aggregation of cellulose in higher plant cell walls.

2. "The influence of hemicelluloses on the aggregation patterns of bacterial cellulose", I Uhlin, RH Atalla and NS Thompson, to be submitted to Cellulose by mid August.

This report provides a more detailed account of the studies on the effects of xylan, mannan and xyloglucan on the patterns of aggregation of bacterial celluloses grown under stationary culture conditions. In addition to the primary observations noted in the above publication, this work examines the effects of addition of the hemicelluloses at different levels, and includes chemical analyses, Raman spectral characterization, and the effects of addition at different levels. Another important component of this work was directed at investigating the effects of isolation procedures on the structure of the cellulose. One of the key findings was the observation that the standard procedures for isolation of cellulose from native plant tissues can lead to the induction of a higher level of crystallinity in the isolated celluloses. This observation in turn leads to serious questions about the validity of the two phase model of the polysaccharide matrix implicit in most prevailing models of plant cell walls.

3. "Modification of crystallinity and crystalline structure of Acetobacter xylinum cellulose in the presence of water-soluble beta-1,4-linked polysaccharides: ¹³C NMR Evidence", JM Hackney, DL VanderHart and RH Atalla, Int. J. Biol. Macromol. - In press. [Copy attached to report]

The studies described in this report were made possible by availability of the agitation tolerant strain of Acetobacter xylinum, which allowed the preparation of the modified celluloses in amounts sufficient for measurement of the Solid State ¹³C NMR. These measurements confirmed the effects described in the earlier publication. They demonstrated that both xyloglucan and glucomannan result in celluloses that are much more like higher plant celluloses than pure bacterial cellulose. Furthermore, the Solid State ¹³C NMR spectra detected a distinct difference between the effects of the xyloglucan and that of the glucomannan. The effect of the xyloglucan, which is of particular interest because of its presence in primary walls, was to convert the I/alpha cellulose to I/beta cellulose, with little reduction in crystallinity. The glucomannan also reduced the I/alpha content, but in this instance it was balanced by increased disorder rather than conversion to I/beta. One of the key components in this report is the proposal of a mechanism for the by which the hemicelluloses act to transform the cellulose, and in the context of the presentation of the mechanism we also propose a model for the formation of the two forms of native cellulose. This model is in sharp contrast to one proposed by other investigators on the basis of electron diffraction studies of algal celluloses; the matter is dealt with in greater detail in the attached copy of this publication, currently at the galley stage, but likely to be in print within weeks of issuance of this report.

4. "Influence of hydrodynamic environment on composition and macromolecular organization of structural polysaccharides in Egria menziesii cell walls", JM Hackney, GP Kraemer, RH Atalla, DL VanderHart and DJ Chapman, Planta 192:461(1994).

This report describes a collaboration wherein we characterized celluloses and other structural polysaccharide components in cell walls of an

alga which had been grown under conditions that differed with respect to the nature and intensity of mechanical perturbations to which the sample was exposed during growth. Raman spectroscopy, x-ray diffractometry and Solid State ^{13}C NMR were used to characterize the celluloses isolated. The key observations were that mild translational deformation (wave action) resulted in subtle but measurable differences in the levels of order in the native cellulose, while tensile loading resulted in more significant changes, including the formation of some cellulose II. These observations are consistent with the view that even in primitive organisms, modifications in the structure of the cellulose are an integral part of adaptive responses to external perturbations, particularly those of a mechanical nature.

5. "The structures of Native Celluloses", RH Atalla, in "Trichoderma reesei cellulases and other hydrolases", P.Suominen and T.Reinikainen, eds., Foundation for Biotechnical and Industrial Fermentation Research, Volume 8, 1993, p 25-39.

This report provides an overview of our current understanding of the variability of celluloses from different native sources, and the issues that arise when the levels of order in these celluloses are to be quantified. The analysis presented was designed to meet the needs of investigators who are examining the multiplicity and diversity of the cellulases produced in nature, and the degree to which they can be rationalized as consequences of elicitation of different hydrolases in response to subtle differences in the cellulosic substrates.

Molecular Modeling Studies:

The work in this area is new in our program and is directed at using available molecular modeling methods to help us gain insight into the nature of the intermolecular interactions which dominate the organization of the cell wall. The first two reports center on studies of cellulose and xylan and their primary oligomers. These were carried out to help us assess the plausibility of conclusions based on the computational methods. The third moves into the area of current primary focus for our program, that is, the interactions of lignin precursors with the polysaccharide matrix.

6. "Cellobiose and xylobiose in water: a theoretical analysis of conformational variation", C Houtman and RH Atalla, submitted to Carbohydrate Research.

Prior studies on carbohydrates had used equilibrium conformational calculations. For our purposes, particularly in the context of lignin cellulose interactions, we felt it more appropriate to use a molecular dynamics approach to the theoretical analysis. The first study in this series was designed to test the consistency of this approach by seeking to establish how well it can predict experimentally observed molecular properties. This was accomplished by applying the method to analysis of the dynamics of the glycosidic linkage in cellobiose and xylobiose when they are in aqueous solutions. It was demonstrated that through use of molecular dynamics it is possible to calculate averages for spectral and optical rotation data that represent satisfactory agreement with experimentally observed values. Thus we felt confident that the computational methods represent sufficiently good approximations of reality to be useful in our further analyses.

7. "Factors that control chain conformations of beta-1,4-linked polysaccharides", C Houtman and RH Atalla, to be submitted to Carbohydrate Research by mid August.

This computation was an extension of the studies of the dynamics of the glycosidic linkage in cellobiose and xylobiose, to consideration of the persistence lengths of cellulose and xylan. The values calculated for cellulose were close to those reported on the basis of experimental studies, but perhaps the most significant result was the finding that xylan also has a very high persistence length, in spite of the lack of hard sphere hindrance to rotation about the linkage bonds. This finding provides, for the first time, an adequate rationalization of the the very low solubility of unbranched beta-1,4-linked glycans in aqueous media. In the past, the low solubility of the hexosans had been understood in terms of the restriction of rotation about the glycosidic linkage imposed by the position of C6, but the low solubility of the pentosans had not been rationalized because the glycosidic linkage appears unrestricted with respect to rotation. Our finding reflects the presence of an intramolecular hydrogen bond that, though too short lived to be detected in spectral measurements, nevertheless provides sufficient restriction to result in a high persistence length. The latter, in turn, limits the entropy change upon dissolution and, hence, the solubility.

8. "Cellulose-Lignin Interactions: a computational study", C Houtman and RH Atalla, to be submitted to Plant Physiology by mid August. [Copy attached to report]

This report describes the application of the molecular dynamics computational methods to studies of the adsorption of lignin monomers and oligomers on a cellulosic surface. In particular, analyses were carried out for the case of coniferyl alcohol and for the case of the beta-O4-linked trimer. The results confirm that, at the nanoscale level, the interactions between lignin and the polysaccharides cannot be interpreted on the basis of extrapolation of macroscopic notions of hydrophobicity of the former and hydrophilicity of the latter. The computations show that when the lignin precursors are in solution in an aqueous environment above a cellulose surface, the precursors move towards the surface and are adsorbed on it. Furthermore, it becomes clear that the larger the precursor molecule, the more rapidly it adsorbs on the surface. These results provide, for the first time, a sound rationalization for the observed effects of polysaccharide matrices on the structures of DHPs formed within them. They also point to the potential role of hemicellulose branch points as selective binding sites for specific lignin precursors.

Hemicellulose Lignin Interactions

As evidence accumulated pointing to strong interactions, at the nanoscale level, between lignin precursors and the cell wall polysaccharides, we have considered the different roles that the hemicelluloses might play in the assembly of the cell wall and in imparting to it its distinctive properties. Out of these have arisen the proposed model set forth in the 9th report and the collaborative studies with Professor N. Terashima described in the 10th report.

9. "The hemicelluloses and the assembly of lignin in higher plant cell walls", RH Atalla, submitted to Plant Physiology. [Copy attached to the report]

This manuscript was prepared as a "Viewpoint" contribution proposing that another key role for the hemicelluloses in the assembly of secondary cell walls may well be the provision of a template for the assembly of lignin. It is envisioned to occur in a manner that simulates, to a limited degree, the role of messenger RNA in the assembly of proteins. This proposal is based on the results reported in (8) above, the well known effect of polysaccharide matrices on the structures of DHPs assembled within them, and on the finding by NG Lewis and coworkers that oligomers of lignin are indeed formed by membrane bound enzyme systems. Another consideration was the view that an assembly process governed entirely by the kinetics of free radical coupling reactions, is not likely to provide the degree of control of the primary structure typical of biological systems; such a process would be far more susceptible to perturbation by thermal and environmental variables. In consequence, it could not possibly account for the degree of consistency in the structures of lignins within individual species. In the search for a higher level of organizing influences, the hemicelluloses, given their equally consistent variation with species, seemed the logical complement to lignin, at least within the context of our overall program on secondary cell wall structure.

10. "New preparations of synthetic lignins under conditions that approximate cell wall lignification", N Terashima, RH Atalla, C Lapierre, B Monties, S Ralph and LL Landucci, submitted to *Holzforschung*. [Copy attached to the report]

This study describes the preparation of synthetic lignins (DHPs) under conditions that attempt to simulate the cell wall environment in different ways. Two different approaches were taken towards closer approximation of cell wall environments. The first was based on use of coniferin in conjunction with a beta-glucosidase as the source of lignin precursors, together with a glucose oxidase to produce hydrogen peroxide in equimolar proportion, and a peroxidase as the ultimate free radical generator. This was also carried out with the addition of konjac mannan as a polysaccharide matrix medium. The second approach was based on the use of coniferyl alcohol, but in the presence of pectin as the polysaccharide environment. Each of the polysaccharide containing preparative procedures produced a DHP with a structure that represents a closer approximation to the structure of milled wood lignin (MWL), than was the structure of the corresponding DHP prepared in the absence of the polysaccharide. However, the two novel preparations moved towards approximating the structure of MWL along different paths.

Organization in Cell Wall Lignin

We have continued to pursue more detailed characterization of the organization of lignin in the native state. In addition to continuation of our program using the Raman microprobe and Raman spectroscopy to assess and map the distribution and orientation of lignin in the cell walls, we have initiated a program based on measurements of photoconductivity and fluorescence spectroscopy applied to native woody tissue. The latter relies on probing the electronic excited states in native tissue as a source of information on the organization of lignin.

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11. "Photoconductivity in wood: evidence for electron transport pathways in native lignin", RH Atalla and B Abosharkh, to be submitted to *Plant Physiology*. [Copy attached to report].

This manuscript describes the discovery of the phenomenon of photoconductivity in native woody tissue and our understanding of the molecular level processes involved in it. The existence of pathways for electronic charge transport in lignified woody tissue was demonstrated by measurement of photoconductivity under conditions wherein ionic charge transport was limited. The source of mobile electrons was identified as a photoexcited state of coniferyl alcohol-like structures; this was on the basis of the coincidence of the photoconductivity peak with an absorption band of coniferyl alcohol. In preliminary studies of hardwoods, the photoconductivity peak appears shifted slightly towards the corresponding peak of sinapyl alcohol. The pathways for electron transport appear to be regularly organized arrays of adjacent lignin units that give rise to de-localized excited (exciton) states of relatively low energies. Such pathways allow the rationalization of the influence of oxidative enzymes in domains that are not accessible to them. These pathways are also likely to be part of biological control systems that synchronize processes in adjacent cells and tissues. The phenomenon of photoconductivity has, so far, been observed in three coniferous species and two angiosperms, and its response to chemical modification is consistent with the model put forth for its interpretation.

The following three papers, in abbreviated form, have been included in the proceedings of the International Symposium on Raman Spectroscopy. They will be submitted to *Plant Physiology* by the end of September after revision to make them accessible to the readership of the journal. This submission has been discussed with the Editor-in-Chief for the journal.

12. "The contribution of preresonance Raman and conjugation effects to the Raman spectrum of native lignin", JS Bond, UP Agarwal and RH Atalla.

The first in the series deals with enhancement mechanisms which influence the intensity of key bands in the Raman spectrum of lignin. Once these effects were identified and understood it was possible to move forward with the mapping work that is the primary objective of the Raman microprobe studies.

13. "Raman microprobe mapping of the patterns of molecular order in the secondary walls of black spruce and loblolly pine", JS Bond, UP Agarwal and RH Atalla.

The second in the series describes some of the first mapping studies for two species, black spruce and loblolly pine. Though the enhancement effects and some transient component in them made it necessary that quantitative comparisons must remain relative, the results provide some insight into the range of variability of the constitution of the cell wall, as well as some comparisons of adjacent walls.

14. "Treatment of woody tissue to enhance stability for long term exposure to visible laser excitation of micro-Raman spectra", JS Bond, UP Agarwal, VC Tirumalai and RH Atalla.

The third in the series describes the procedures which we have used to stabilize tissues to allow for long term exposure and make possible more precise quantitative comparisons of the variability of lignin structures within the cell wall and relative to the cellulose.