ABSTRACT

HU, ZHOJIAN. Elucidation of the structure of Cellulolytic Enzyme Lignin from loblolly pine (*pinus taeda*). (Under the direction of Dr. Hou-min Chang).

Milled Wood Lignin (MWL) preparation has long been used as a standard method for studies of lignin structure. However, ball milling reduces the degree of polymerization, creating new free-phenolic hydroxyl groups through cleavage of β-aryl ether linkages as well as increasing α-carbonyl groups via side-chain oxidation. In addition, the maximum yield of MWL from protolignin is about 50%. Therefore, to what extent is milled wood lignin representative of total lignin in situ?

Cellulolytic Enzyme Lignin (CEL) preparation has not been used as widely as MWL in studies of lignin structure. CEL has higher molecular weight, lower condensation and higher β-O-4’ interlinkage compared to MWL. The higher yield of CEL extracted from wood helps us understand the lignin structure as a whole.

To understand the effect of ball milling on lignin structure, CEL and MWL from loblolly pine prepared by three different ball-milling methods were investigated. The structure of CEL at various yields was compared with MWL using wet chemical analyses, FTIR and solution-state NMR techniques. Ball milling of wood degrades β-O-4’ structures in lignin. However, even after extensive ball milling, less than 25% of the β-O-4’ structures are degraded. The extent of degradation is less for softwood than for hardwood lignin. The yield of extractable lignin, either MWL or CEL, is the best way to access the extent and effect of ball milling. CEL is preferred over MWL as it can be isolated in higher yield with less degradation. CEL was isolated with yields ranging from 20% to 86%. Over this range CEL
has similar structures, suggesting that the major fraction of lignin in the secondary wall is uniform in structure. Over 75% extractable yield of CEL, the residual enzyme lignin (REL) is structurally different from CEL and may originate mainly from the middle lamella. In present study, we propose a new procedure for the isolation of lignin for use in studies of lignin structure where the wood is sufficiently milled and successively extracted to produce three lignin fractions representing the total lignin in wood.
ELUCIDATION OF THE STRUCTURE OF CELLULOLYTIC ENZYME LIGNIN FROM LOBLOLLY PINE (Pinus taeda)

by

ZHOUJIAN HU

A thesis submitted to the Graduate Faculty of North Carolina State University
In partial fulfillment of the Requirement for the Degree of Master of Science

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APPROVED BY:

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Chair of Advisory Committee
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1. Introduction to lignin

In 1838, Anselme Payen was first to recognize the composite nature of wood. He found that treatment of wood with nitric acid removed part of the wood substance and left behind a fibrous material, which he named “cellulose”. The removed material had a higher carbon content than the cellulose, which had the same composition as starch. He described this carbon-rich substance, which embedded the cellulose in the wood, as an “encrusting material” (Payen, 1838). The term “lignin”, which was introduced by Schulze in 1865, is derived from the Latin word “lignum” meaning wood. It is now known to be one of the major components of the cell wall of all vascular plants (Sarkanen et al., 1971). In 1907, Klason proposed that lignin is a high molecular weight substance consisting of coniferyl alcohol units joined together by ether linkages. Thus the era of modern lignin chemistry began (Sjöstrom, 1981).

As a part of the cell wall, lignin does not merely act as “encrusting materials”. Rather, lignin performs multiple functions essential for the life of vascular plants. Lignin provides mechanical strength and structural support, particularly in the case of trees, to the growing plant. Lignin prevents the permeation of water across the cell wall, thus facilitating vertical conduction of water, nutrients and metabolites in the xylem tissue. Lignified tissues effectively resist attack by microorganisms by impeding penetration of destructive enzymes into the cell wall (Sarkanen et al., 1971).
1.1 Phenylpropane-the basic unit of lignin macromolecule

Lignin is formed by oxidative coupling of, primarily, 4-hydroxycinnamyl alcohols (Ralph et al., 2004). Generally, lignin includes three primary precursors: trans-\(p\)-coumaryl (A), trans-coniferyl (B), and trans-sinapyl (C) alcohols (Figure 1.1) (Sarkanen et al., 1971b). Hardwood lignin is composed of various proportions of coniferyl alcohol derived subunits and sinapyl alcohol derived subunits. Softwood lignin is derived mostly from coniferyl alcohol monomers. Lignin structure not only consists of subunits derived from these three precursors, in foliage, it also may contain derivatives of hydroxycinnamyl alcohol such as acylated hydroxycinnamyl alcohols and hydroxycinnamate esters (Ralph et al., 1997; Ralph et al., 2000; Ralph et al., 2004). Systematic evaluation or revisions of nomenclature has not been carried out, and the usage of certain terms here is not always consistent with the terms used elsewhere.

Figure 1.1. A) The primary lignin precursors, B) Labeling convention for monolignols
Dual meanings for the same term are common (Table 1.1). The common notation for carbon atoms in phenylpropane units was shown in Figure 1.1. For example, in lignin chemistry the basic name guaiacyl commonly means a 3-methoxy-4-hydroxyphenyl group (e.g. in guaiacylpropane). However, the proper name for “guaiacyl” is only for o-methoxyphenyl groups. This distinction forms a source of confusion to unfamiliar readers.

<table>
<thead>
<tr>
<th>Table 1.1 Examples of Dual meanings in lignin nomenclature^a</th>
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<tbody>
<tr>
<td>Preferable</td>
</tr>
<tr>
<td>Guaiacyl</td>
</tr>
<tr>
<td>Veratryl</td>
</tr>
<tr>
<td>Syringyl</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Coniferyl</td>
</tr>
<tr>
<td>Acetovanillone</td>
</tr>
<tr>
<td>α-ethoxypropiovanillone</td>
</tr>
<tr>
<td>Coniferyl Sulfonic acid</td>
</tr>
<tr>
<td>Vanillyl sulfonic acid</td>
</tr>
</tbody>
</table>

^a (Sarkanen et al., 1971b)

1.2 Lignin biosynthesis

Photosynthesis of glucose is the starting point for lignin precursors biosynthesis. The metabolic pathway from glucose to lignin precursors and the role of participating enzymes have received extensive clarification in recent decades (Baucher et al., 2003). Lignin monomers, or monolignols, are produced intracellularly, then exported to the cell wall, and
subsequently polymerized. From glucose to lignin precursors, biosynthesis follows the Shikimic acid pathway (a primary metabolic pathway) and the monolignol pathway (a secondary metabolic pathway) (Figure 1.2 and Figure 1.3).

The universal and efficient conversion of phenylalanine to lignins has been amply demonstrated by tracer methods (Freudenberg et al., 1958; Brown et al., 1959; Higuchi, 1962). Lignin shares a common precursor, phenylalanine, with many other minor components including flavonoids, coumarins, stilbenes, and lignans (Figure 1.2). Most of the genes involved in monolignol production have been cloned or are present in expressed sequence tag/genomic databases (Christensen et al., 2000; Boerjan et al., 2003; Raes et al., 2003). The lignin biosynthetic pathway has been the subject of many recent reviews (Baucher et al., 1998; Whetten et al., 1998; Grima-Pettenati et al., 1999; Lewis, 1999; Boerjan et al., 2003), but is still revised and updated to incorporate new results. In the pathway of monolignol biosynthesis the first step is the conversion of phenylalanine to cinnamic acid by phenylalanine ammonia-ligase (PAL), followed by a hydroxylation at the para position by cinnamate-4-hydroxylase (C4H) resulting in p-coumaric acid. A series of alternating hydroxylation and methylation steps can convert the p-coumaric acid into the mono- and dimethoxylated analogs, ferulic acid and sinapic acid.

The hydroxylation and methylation reactions ultimately determine the monomeric composition of lignin (because the three monolignols differ only in their degree of methoxylation). Hydroxylation and methylation steps can take place at the level of cinnamic acids, hydroxycinnamoyl-CoAs, and preferentially at the cinnamaldehyde and cinnamyl alcohol levels (Higuchi, 1985; Ye et al., 1994; Li et al., 1997; Inoue et al., 1998; Martz et al., 1998; Maury et al., 1999; Chen et al., 1999; Li et al., 2000; Guo et al., 2002). Figure 1.3
outlines a new proposed biosynthetic pathway, highlighted by blue color, from L-phenylalanine to the main lignin precursors, p-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol (Li et al., 2000; Guo et al., 2002; Baucher et al., 2003).

Figure 1.2 Primary and secondary metabolic pathways leading to the biosynthesis of lignin and other wood components
Figure 1.3 Phenylpropanoid and monolignol biosynthesis pathways. CAD, cinnamyl alcohol dehydrogenase; 4CL, 4-coumarate: CoA ligase; C3H, p-coumarate 3-hydroxylase; C4H, cinnamate 4-hydroxylase; CCoAOMT, caffeoyl-CoA O-methyltransferase; CCR, cinnamoyl-CoA:shikimate/quinate p-hydroxycinnamoyltransferase; F5H, ferulate 5-hydroxylase; PAL, phenylalanine ammonia-lyase; SAD, sinapyl alcohol dehydrogenase. The boxed area represents the most likely pathway to the production of monolignols. All enzymatic reactions shown have been demonstrated by in vitro assays and do not necessarily occur in vivo. F5H? and CCR?, substrate not tested; ?, conversion demonstrated but enzyme unknown; ??, direct conversion not convincingly demonstrated (Baucher et al., 2003).
Monolignols are not abundant in their free form in lignifying tissues, but are found as monolignol glucosides, such as coniferin in gymnosperms (Gross, 1985). Meanwhile, some evidence has been found that glucosidases are attached to cell walls during the onset of lignin biosynthesis, suggested that monolignol glucosides represent the metabolic forms in which monolignols are excreted from the cytoplasm into the lignifying zone (Marcinowski et al., 1978; Sederoff et al., 1991).

The final steps in the biosynthesis of lignin are the oxidation of the cinnamyl alcohols to the corresponding radicals in the cell wall and their subsequent polymerization. The possible mechanisms of lignin polymerization have been recently reviewed (Sarkanen et al., 1971a; Sederoff et al., 1991; Lewis et al., 1999; Christensen et al., 2000; Boerjan et al., 2003). So far, there are two main models for the final stage of lignin polymerization, a combinatorial coupling model and a dirigent protein model. According to a significant body of research over the past more than sixty years, the currently accepted theory is that lignin macromolecule is formed by combinatorial-like phenolic coupling reactions, via radicals generated by peroxidase-H₂O₂, under simple chemical control where monolignols react endwise with the growing polymer (Erdtman, 1939; Freudenberg, 1965; Sarkanen et al., 1971a; Higuchi, 1985; Sederoff et al., 1991; Boerjan et al., 2003). However, With the discovery of a dirigent protein in Forsythia, capable of catalyzing the stereoselective coupling of two coniferyl alcohol radicals into the lignan pinoresinol (Davin et al., 1997), it has been suggested that monolignol radical coupling during lignin biosynthesis could be also tightly controlled in plants (Gang et al., 1999)-a proposition in direct conflict to the well-established and supported combinatorial coupling model (Hatfield et al., 2001). This controversial suggestion of guided polymerization lacks of some evidence to support it, such
as reverse genetic (Boerjan et al., 2003). Most evidence supports the combinatorial model so far (Baucher et al., 2003; Ralph et al. 2004). A natural heterogeneity and a variation of the content and composition that exist in lignin differ not only among plant taxa, but also between different cell types of a single tissue and even within a single cell wall (Sarkanen et al., 1971a; Saka et al., 1985 Joseleau et al., 1997;). Hence, only facts can end this debate, whether monolignol coupling is a combinatorial or a highly orchestrated process. Figure 1.4 summarizes the mechanisms of radical coupling of the major structure units in lignin of gymnosperms (softwood).

1.3 The structure, classification and distribution of lignin macromolecule

Lignin, the second most common biomass component found on Earth, comprises from 15% to 36% of the total dry weight of wood (Sjöstrom, 1981). The types of subunits in lignin macromolecule are classified based on their monolignol origin, i.e., \( p \)-hydroxyphenyl (H) units arising from primarily \( p \)-coumaryl alcohol, guaiacyl (G) units from coniferyl alcohol, and syringyl (S) units from sinapyl alcohol. The subunit composition of lignin varies with the content of three units. Three types of lignins are well differentiated: gymnosperm lignin contains mainly G-unit with a small amount of H-unit; both S-unit and G-unit are roughly equal components of angiosperm lignin; and significant amounts of all three units are contained in grass lignin (Sarkanen et al., 1971a). The variation of the structure in lignins also comes from the frequency of the interlinkages in lignin macromolecule. Different content of these three units in lignin tremendously changes the frequency of the interlinkages, while the general existing interlinkages in lignin are fairly similar in all plant (Figure 1.4). Hardwood lignin contains relative more \( \beta \)-O-4’ and less 5-5’ and \( \beta \)-5’ interlinkages than
softwood lignin, though generally the most abundant linkage in lignin is β-O-4’. The frequency of a β-O-4’ interlinkage is approximately 45-50% of the phenylpropane units in softwood lignin, while approximately 60-85% phenylpropane units in hardwood lignin (Chen, 1991). Table 1.2 shows an example of the relative differences in distribution of interlinkages between lignins isolated from spruce (softwood) and birch (hardwood).

Figure 1.4. Summary of the radical coupling mechanism and major structure units in lignin macromolecule of gymnosperms. (A: possible monolignol radicals; B: one example of radical coupling reactions; C: Major interlinkages in lignin macromolecule; the bolded and red bonds are the formed in the radical coupling reactions).
Table 1.2 Relative frequencies of linkages per 100 C₉ units in ligninsa.

<table>
<thead>
<tr>
<th></th>
<th>β-O-4'</th>
<th>α-O-4'</th>
<th>β-5'</th>
<th>β-1'</th>
<th>5-5'</th>
<th>4-O-5'</th>
<th>β-β'</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spruce</td>
<td>48</td>
<td>6-8</td>
<td>9-12</td>
<td>7</td>
<td>9.5-11</td>
<td>3.5-4</td>
<td>2</td>
</tr>
<tr>
<td>Birch</td>
<td>60</td>
<td>6-8</td>
<td>6</td>
<td>7</td>
<td>4.5</td>
<td>6.5</td>
<td>3</td>
</tr>
</tbody>
</table>

a (Monties et al., 1985).

Lignin is heterogeneous not only with respect to its macromolecular structure but also morphological location. Non-degradative analytical methods such as UV, IR, Raman, CP/MAS-NMR spectroscopy, SEM- and TEM-EDXA provide useful information on the macromolecular structure and distribution of protolignin in the cell wall (Chen, 1991; Sarkanen et al., 1971a). The cell wall consists of several layers (Figure 1.5). The layers of the cell wall from outer to inner are as follows: middle lamella (ML), the primary wall, the secondary wall (divided into the S1, S2, and S3 layer), and the lumen. A UV-microscopy obtained by Goring and Fergus (Fergus et al., 1969) shows the distribution of lignin within the cell wall (Figure 1.5). The middle lamella and cell corners are highly lignified with the concentration of lignin ranging from 50-60 % in the middle lamella to 85-100 % in the cell corners in softwood, while secondary wall is less lignified with about 20% lignin content. The middle lamella and cell corners only about 6-13% total tissue volume contain only 19-28 % of the total lignin in softwood. The secondary wall, however, contains about 70-80% of the total lignin attributable to its highest tissue volume in the cell wall of softwood (table 1.3).
Figure 1.5 Transverse section of a spruce tracheid photographed in UV light (240nm) (the densitometer tracing has been taken across the tracheid wall along the dotted line.) S, secondary wall; WL, compound middle lamella; CC, cell corner.

Lignification is the final phase of development for wood cells, involving the formation of lignin between the newly formed cells and within their cell walls. Lignification begins in the cell corners and middle lamella and then proceeding to the primary wall. Early and rapid lignification takes place between the cells, designated middle lamella, and primary wall (Wardrop, 1952). Lignification in the secondary wall is a more gradual process, initiated when the middle lamella lignin concentration is approximately 50% of the maximum (Saka et al., 1982) and moves into the secondary wall. Lagging just behind the secondary wall formation. Lignification in the S1 layer begins before cellulose deposition is complete in the S3 layer. Finally, once lignification is complete in the S3 layer, most of cell dies (Thomas, 1991).
In addition, lignins in different morphological regions of the cell wall differ in terms of chemical characteristics. The basic units (H-unit, G-unit, and S-unit) in lignin macromolecule are not evenly distributed across the cell wall among the species (Fergus et al., 1970; Fergus et al., 1970; Terashima et al., 1988; Chen, 1991; Fukushima et al., 1991). Heterogeneity in lignification process has been well studied using microautoradiography with a selective labeling technique of the specific lignin precursors by Fukushima and Terashima. Through this technique, the growing process of the protolignin macromolecule and the distribution of H-unit, G-unit and S-unit in the specific morphological region can be visualized. In the case of gymnosperm (pine), for an instance, they demonstrated that p-hydroxyphenyl (H-unit) lignin is formed mainly in the compound middle lamella and cell corner as a highly condensed form in an early stage of cell wall differentiation. Guaiacyl (G-unit) lignin is deposited as a more condensed form in the middle lamella and as a more non-condensed form in the secondary wall. Syringyl (S-unit) lignin is formed mainly in the inner layer of the secondary wall in a late stage as a minor structural moiety (Terashima et al., 1988). Goring and his colleagues found that gymnosperm lignin in the middle lamella was characterized by lower methoxy content, lower phenolic hydroxy content, higher condensation and difficulty in delignification (Yang et al., 1980; Whiting et al., 1982; Whiting et al., 1982). It suggested

**Table 1.3 Distribution of lignin in Tracheid of Black Spruce (Picea mariana Mill)**

<table>
<thead>
<tr>
<th>Wood</th>
<th>Morphological region&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Tissue volume (%)</th>
<th>% total</th>
<th>Conc. (g/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Earlywood</td>
<td>S</td>
<td>87.4</td>
<td>72.1</td>
<td>0.225</td>
</tr>
<tr>
<td></td>
<td>ML (r, t)</td>
<td>8.7</td>
<td>15.8</td>
<td>0.497</td>
</tr>
<tr>
<td></td>
<td>ML(cc)</td>
<td>3.9</td>
<td>12.1</td>
<td>0.848</td>
</tr>
<tr>
<td>Latewood</td>
<td>S</td>
<td>93.7</td>
<td>81.7</td>
<td>0.222</td>
</tr>
<tr>
<td></td>
<td>ML (r, t)</td>
<td>4.1</td>
<td>9.7</td>
<td>0.60</td>
</tr>
<tr>
<td></td>
<td>ML(cc)</td>
<td>2.2</td>
<td>8.6</td>
<td>1.00</td>
</tr>
</tbody>
</table>

<sup>a</sup> (Fergus et al., 1969);
<sup>b</sup> S= Secondary wall; ML= middle lamella; r = radial; t = tangential; cc = cell corner.
that lignin in the middle lamella contains a substantial proportion of highly condensed p-hydroxyphenylpropane units (H-unit).

1.4 Isolation of lignin

Isolation of lignin from wood in a chemically unaltered form is one of barriers to elucidation of the structure of lignin. Lignins in plant tissues are not extractable by organic solvents and isolation of lignin free from carbohydrates is impossible. Separation of lignin from most carbohydrates was difficult unless some chemical or physical treatment before lignin extraction. The acid lignin preparations were exclusively used in the chemical characterization (Björkman, 1954). It, however, realized that that lignin differ from the original lignin as a higher condensation (Lai et al., 1971). Brauns lignin, Milled Wood Lignin (MWL) and Cellulolytic Enzyme Lignin (CEL) were thought to be the preparations with less chemical modification than acid preparations (Lai et al., 1971).

1.4.1 Brauns lignin

The original procedure by Brauns is based on thorough extraction of finely ground wood (100-150 mesh) with 95% ethanol and precipitation of the ethanol solution by water. The final purification was carried out by precipitating a solution of the crude product in dioxane into ethyl ether. Originally designated as “native lignin”, though “ soluble lignin” or “Brauns lignin” is preferable because extremely low yields of this lignin can be obtained from plants with a variation among 0.2% to 3.2% of dry weight of plant materials. Without removing extractives from plant materials before ethanol extraction, Brauns lignin consists of some
impurities, such as carbohydrates and extractive components (Lai et al., 1971). Compared to other preparations, such as Milled Wood lignin, Brauns lignin from conifers is characterized by similar elemental compositions, low molecular weight lignin, large amounts of ester groups and higher phenolic hydroxyl content (Lai et al., 1971).

1.4.2 Milled Wood Lignin

Staudinger and his coworkers made the first observations on the effect of milling on the solubility of wood (Lai et al., 1971). Björkman developed an isolation procedure to extract a larger proportion of lignin from wood. According to Björkman, when extractive-free wood meal of a woody species is ground for 48 hr or more in a vibratory ball mill under nitrogen atmosphere either with a non-swelling embedding liquid, such as toluene, or dry, the wood tissues disintegrate, and about 30-50% of lignin in the wood become extractable with aqueous dioxane, usually 90-96% (Björkman, 1954; Björkman, 1956; Björkman, 1957b). With some purification, the resulting lignin called Milled Wood Lignin (MWL) is a cream-colored powder free of ash and contains a few percent of carbohydrates for either gymnosperm or angiosperm.

Ball milling effects on the yield and chemical structure of MWL have been extensively studied. Figure 1.6 shows that the yield of extractable MWL from conifer wood as a function of ball milling time follows an S-shaped curve leveling off at about 50% of the total lignin (Lai et al., 1971).
1.4.3 Origin of Milled Wood Lignin

Lignin is present in the middle lamella of the wood cell wall at a high concentration and in the secondary cell wall at a low concentration in cell wall of softwood. Lignin in secondary wall has a greater chance to have covalent or hydrogen bonds with carbohydrate. After Milled Wood Lignin extraction, Lignin-Carbohydrate Complex (LCC) can be extracted from the residue by dimethylformamide, dimethyl sulfoxide, or aqueous acetic acid (1:1) (Lai et al., 1971). Björkman demonstrated that the main portion of MWL derives from compound middle lamella, whereas LCC comes from the secondary wall based on the residual carbohydrate composition of MWL (Björkman, 1957a). However, in Figure 1.6 also suggests that a part of isolated LCC is derived from intermediates in the formation of MWL.
The amount of LCC reaches a maximum during an early stage of milling and declines when the release of MWL (10%) becomes rapid (Lai et al., 1971). A decreasing in the yield of LCC may be due to a large proportion of lignin extracted out from the secondary wall. This interpretation also can be supported by UV-microscopic studies of ball-milled spruce and birch wood, which demonstrate that MWL extracted from the secondary wall after ball milling (Maurer et al., 1992a; Maurer et al., 1992b). Whiting and Goring also argued that the origin of the main portion of MWL is deriving from the secondary wall according to the structural studies of dioxane-extracted lignin from compound middle lamella and secondary wall of spruce (Whiting et al., 1981; Whiting et al., 1982a). Using radiotracer method, Terashima (Terashima et al., 1992) strongly suggested that MWL of ginkgo originates from the secondary wall instead of the middle lamella. Recently studies through the use of antibody probes to examine the distribution of MWL also indicated that MWL from softwood is morphologically from the secondary wall (Kim et al., 1997). So far, summarizing all studies on the morphological origin of milled wood lignin in softwood and hardwood, especially in softwood, we understand that there were some errors in our old assumptions and some new insights. The content of sugar residues in MWL and vanillin yield of nitrobenzene oxidation is insufficient to deduce the morphological origin and supports the Björkman’s demonstration that MWL is mainly from the middle lamella. However, evidences from UV-microscopy, antibody probes and radiotracer studies strongly support that origin of MWL is from secondary wall and not from the middle lamella in softwood.
1.4.4 Cellulolytic Enzyme Lignin

Milled Wood Lignin has long been used as an ordinary lignin preparation from wood to study the chemical structure. The obvious disadvantage, however, was that no more than one-fourth of the lignin can be recovered as essentially carbohydrate-free milled wood lignin. In order to obtain lignin from milled wood as a whole, Pew (Pew, 1957) showed that around 95% carbohydrates in woods can be removed by a glucosidase treatment of finely ground wood meal, which was ground 5 to 8 hours in a vibratory ball mill in a dry milling state. Chang (Chang et al., 1975) ground spruce wood in a vibratory ball mill for 48 hours in the presence of toluene. The milled wood was extracted with 96 % (v/v) dioxane to obtain MWL and then subjected to treatment with a commercial cellulase enzyme. Subsequent 96 % (v/v) dioxane extraction yielded an additional portion of lignin called Cellulolytic Enzyme Lignin (CEL-96). The insoluble material was then extracted with 50 % (v/v) dioxane yielding CEL-50 for a total yield of 70-85 % of protolignin. MWL and CEL-96 were very similar based on elemental analysis and molecular weight distribution. While, the yield of oxidation products, ether nitrobenzene oxidation or permanganate oxidation, was a little bit lower in MWL than CEL. Holtman (Holtman et al., 2004), who studied the similarities between MWL and CEL from loblolly pine, also found the same evidence (Table 1.4). In view of those findings, Holtman suggested that more lignin from the middle lamella was obtained during MWL preparation than CEL preparation (Holtman et al., 2004).
### Table 1.4 Structural characterization of MWL and CEL

<table>
<thead>
<tr>
<th>Lignin</th>
<th>C wt.%</th>
<th>H wt.%</th>
<th>OCH$_3$ mol/100C$_9$</th>
<th>Ph-OH mol/100C$_9$</th>
<th>C=O mol/100C$_9$</th>
<th>Vanillin$^a$ mol/100C$_9$</th>
<th>Ester$^c$ mol/100C$_9$</th>
<th>DFRC$^d$ mol/100C$_9$</th>
<th>Thioacidolysis$^d$ mol/100C$_9$</th>
</tr>
</thead>
<tbody>
<tr>
<td>SpMWL$^e$</td>
<td>62.7</td>
<td>5.7</td>
<td>95</td>
<td>19.5</td>
<td>11</td>
<td>32.1$^b$</td>
<td>13.6</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>SpCEL$^e$</td>
<td>61.2</td>
<td>5.5</td>
<td>96</td>
<td>19.9</td>
<td>8</td>
<td>36.9$^b$</td>
<td>12.8</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>LpMWL$^f$</td>
<td>63.2</td>
<td>5.4</td>
<td>96</td>
<td>17</td>
<td>12</td>
<td>31.8</td>
<td>---</td>
<td>14.5</td>
<td>18.1</td>
</tr>
<tr>
<td>LpCEL$^f$</td>
<td>62.8</td>
<td>5.8</td>
<td>94</td>
<td>19</td>
<td>8</td>
<td>32.3</td>
<td>---</td>
<td>14.5</td>
<td>20.3</td>
</tr>
</tbody>
</table>

a. Vanillin yield from nitrobenzene oxidation.
b. Calculation based on a monomer unit weight of 196 for spruce
c. Total ester yield from permanganate oxidation without Kraft cooking.
d. Monomer yield
e. MWL and CEL from Spruce
f. MWL and CEL from Loblolly pine
1.5 Characterization of the structure of lignin by degradation methods

Wet chemistry methods can be differentiated by functional group analysis (OMe, total OH, phenolic OH, carbonyl group, carboxyl groups) and degradation methods. The major advantage of degradation techniques is possibility of analyzing lignin without its isolation. The relative accuracy of degradation techniques is pretty high. The common degradation methods include Nitrobenzen Oxidation (NBO), Thioacidolysis (TA), Derivative Followed by Reductive Cleavage (DFRC), Permanganate Oxidation (PO) and Ozonolysis (Sarkanen et al., 1971a; Adler, 1977; Sakakibara, 1991; Chen, 1991; Lin et al., 1992).

1.5.1 Nitrobenzene oxidation (NBO)

Freudenberg and co-workers (Freudenberg, 1939) introduced nitrobenzene oxidation of lignin in 1939 to obtain further evidence for the aromatic nature of lignin. The optimum conditions for the nitrobenzene oxidation of lignin have been described by Leopold (Leopold, 1952) as a result of model compound experiments. The highest yields of phenolic aldehydes were obtained in a 2M sodium hydroxide solution with nitrobenzene at 170-180°C for 2-3 hrs. Extensive modifications have been made in the qualitative and quantitative determination of the oxidation products by means of chromatography, high-performance liquid chromatography (HPLC), gas chromatography (GC) and $^1$H-NMR spectroscopy (Stone et al., 1951; Chen, 1988; Katahira et al., 2001). Under alkaline nitrobenzene oxidation, the sidechains of uncondensed moieties of lignin yield primarily vanillin, syringaldehyde, and $p$-hydroxybenzaldehyde from G-, S-, and H-units, respectively (Figure 1.7). Total yields of these three products may be as much as 25% by weight of the lignin. Nitrobenzene oxidation of lignin can indicate the type of lignin and the quantity of uncondensed phenylpropane units...
presents in the lignin. It also can provide a relative measurement of the extent of condensation in the aromatic moieties of lignin. However, the detailed information about the structure of lignin by nitrobenzene oxidation is limited.

![Figure 1.7 Major products from nitrobenzene oxidation](image)

1.5.2 Thioacidolysis (TA) and Derivative Followed by Reductive Cleavage (DFRC)

Both thioacidolysis and DFRC share the same ideal to analyze the structure of lignin through cleavage of β-aryl ether bonds, the most abundant interlinkage in lignin. Lapierre and co-workers developed thioacidolysis to facilitate analysis of lignin structure by decreasing condensation reactions and hydrolysis products and increasing monomer yields from acidolysis (Lapierre et al., 1986). The thioacidolysis method utilizes nucleophilic ethanethiol, which displaces the α-hydroxyl or α-alkoxyl group by a SN1 mechanism under catalytic influence of boron trifluoride etherate by the way of the corresponding conjugate acid intermediate. The final products can be quantitatively determined by gas chromatography (GC) after silylation or directly by high-performance liquid chromatography (HPLC). A modified method, coupling methylation and thioacidolysis (Figure 1.8), has also been used for the structural analysis of lignin (Lapierre et al., 1988; Holtman et al., 2003). As a result, the monomers obtained from free phenolic endgroups can be differentiated from those
derived from consecutive $\beta$-O-4’ linkages. To change the malodorous chemicals, Lu and Ralph (Lu et al., 1997; Lu et al., 1998a; Lu et al., 1998b; Lu et al., 1999; Ralph et al., 1998) introduced a new selective $\alpha$, $\beta$-aryl ether cleavage protocol, derivertization followed by reductive cleavage (DFRC). DFRC uses mild conditions and consists of three simple steps: bromination of the benzylic position and concomitant acetylation of free hydroxyl groups by acetyl bromide; reductive cleavage of the $\beta$-aryl ether bonds via zinc metal coordination; acetylation of newly generated phenolic hydroxyl groups for quantification by gas chromatography. Like modified thioacidolysis, modified DFRC combines methylation, DFRC and propylation, which allows the quantitative determination of three different monomers in lignin: the uncondensed phenolic $\beta$-O-4’ (unit A), the uncondensed $\alpha$-O-4’ (unit B), and the uncondensed etherified $\beta$-O-4’ (unit C) structures (Figure 1.8). Although DFRC can’t completely cleave $\beta$-aryl ether linkage in lignin and gives a lower monomer yield compared to thioacidolysis, DFRC is still useful method for specific structural elucidation (Holtman et al., 2003).

1.5.3 Ozonolysis

Ozone is one of the strongest oxidizing agents. It is exceeded in electronegative oxidation potential only by F$_2$, F$_2$O, and oxygen atom (Eckert et al., 1980). Ozone has long been known to cleave unsaturated bonds via 1,3-dipolar-cycle addition and the products are often useful in elucidating the structure of the parent compound. The first ozonation of wood and lignin
Figure 1.8 Schematic of the modified thioacidolysis (A) and DFRC (B)

was carried out by Doree and Cunningham (Doree et al., 1913). Lignins were highly reactive with ozone even at room temperature, while polysaccharides were much more resistant. Studies with model compounds (Kaneko et al., 1983; Eriksson et al., 1985) indicate that the rates of ozonation decrease in the order: stibenes > styrenes > phenolic structures > muconic acid intermediates > nonphenolic structures > aroyl structures > carbohydrates. In most studies, ozonation of the isolated lignins has been performed in aqueous organic solvents such as acetic acid, methanol, dioxane, and methylene chloride (Kaneko et al., 1983; Matsumoto et al., 1984; Matsumoto et al., 1986; Habu et al., 1990; Tsutsumi et al., 1990). Matsumoto (Matsumoto et al., 1986) discovered that extensive ozonation and subsequent alkali treatment of lignin in acid completely destroyed the aromatic moieties while leaving stereo structures of the original side chains, which could be recovered in the form of identifiable mono- and
dicarboxylic acids. Therefore, the configurational structures (e.g., erythro and threo) of the side chain of lignin can be evaluated from the ozonation products. This method can be extended to evaluate the configurational structures (e.g. erythro and threo in β-O-4’) and quantity of different types of interlinkages, such as β-O-4’, β-5’, β-1’, LCC, etc. (Matsumoto et al., 1984; Matsumoto et al., 1986; Habu et al., 1990; Karlsson et al., 2000; Akiyama et al., 2002; Karlsson et al., 2004) (Figure 1.9).

![Figure 1.9](image)

**Figure 1.9 Summary of the mechanism of ozonolysis.** A, overall reaction for β-O-4’, β-1’ and β-5’ interlinkage; B, a mechanism of β-O-4’ by ozonolysis.
1.6 Ball milling effect on the structure of protolignin and isolated lignin

Extracting lignin from finely ball-milled wood with aqueous dioxane has long been used as a standard method for the structure studies of lignin. Does this portion of lignin from wood represent protolignin? If yes, how does this portion of lignin represent the heterogeneous character of lignin in the woody cell wall? Does chemical modification exist during ball milling? If yes, what kinds of structural change during ball milling? Ball milling reduces the degree of polymerization, creating new free-phenolic hydroxyl groups through cleavage of β-aryl ether linkages as well as increasing α-carbonyl groups via side-chain oxidation (Lai et al., 1971; Chang et al., 1975; Gellerstedt et al., 1989; Ikeda et al., 2002; Fujimoto et al., 2005). Based on nitrobenzene oxidation of lignin in the whole wood meal, both Fujimoto and Ikeda found out the total yield of aldehydes from hardwood or softwood is independent of the intensity of ball milling (Ikeda et al., 2002; Fujimoto et al., 2005). Thus, there was no modification of the aromatic ring during ball milling. On the other hand, the results of modified DFRC, an aryl-ether cleavage method, a significant correlation (Figure 1.10) can be found between etherified β-O-4’ linkage and phenolic β-O-4’ linkage (Ikeda et al., 2002). Strongly suggesting that the main effect of ball milling is cleavage of β-O-4’ ether bond. Fujimoto also observed a steady decrease in β-O-4’ linkages with increasing ball milling intensity (Fujimoto et al., 2005). In other words, β-O-4’ ether bond cleavage of lignin is the major structural change during the ball milling process.
Figure 1.10 correlation between phenolic β-O-4’ and etherifed β-O-4’ (Ikeda et al., 2002)

1.7 Reference


2. Objective

There were three objectives in this study. The first is to further understand the ball milling effect on the lignin structure in loblolly pine. The second is to understand the morphological origin and chemical structure of isolated Cellulolytic Enzyme Lignin. The third is to suggest a new method of isolation of lignin.
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(2) Laboratory of Wood Chemistry, Department of Biomaterial Sciences, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan
(3) Department of Wood Science, University of British Columbia, Vancouver, B.C. V6T 1Z4, Canada
3.1 Abstract

Cellulolytic Enzyme Lignin (CEL) and Milled Wood Lignin (MWL) were prepared by three different ball-milling methods. The structure of CEL at various yields was elucidated and compared with MWL using wet chemical analysis, FTIR and solution-state NMR techniques. Results show that ball-milling of wood degrades β-O-4’ structures in lignin. However, even after an extensive ball milling, less than 25% of the β-O-4’ structures are degraded. The extent of degradation is less for softwood than for hardwood lignin. Extractable lignin yield, either MWL or CEL, is the best way to access the extent and effect of ball milling. CEL is preferred over MWL as it can be isolated in higher yield with less degradation. CEL was isolated with yields ranging from 20-86%. Over this range the CEL has similar structures, suggesting that lignin in the secondary wall is uniform in structure. The residual enzyme lignin (REL) is structurally different from CEL and may originate mainly from the middle lamella. In this paper we propose a new procedure for the isolation of lignin for use in structural studies wherein the wood is sufficiently milled and successively extracted to produce three lignin fractions representing the total lignin in wood.

**KEYWORDS:** Lignin; Cellulolytic Enzyme Lignin (CEL); Effect of Ball Milling; Ozonation; Method of Lignin Isolation; Quantitative $^{13}$C NMR spectroscopy

3.2 Introduction

Lignin is arguably the second most abundant biopolymer. It is formed in the cell wall of vascular plants through oxidative coupling of 4-hydroxyphenylpropanoid compounds. The structure and biosynthetic pathway of lignin have been studied for more than a century,
however its structure and biosynthesis pathway have not yet to be completely elucidated (Ralph et al., 2004; Boerjan et al., 2003). A primary problem in the elucidation of the structure of native lignin is that it cannot be isolated in a chemically unaltered form. The first major advance towards isolating lignin in a relatively unaltered state was made by Björkman (Björkman, 1954), extracting lignin from finely ball-milled wood with aqueous dioxane. The resulting milled wood lignin (MWL) is considered to be a representative source of native lignin and has been extensively used in the elucidation of native lignin structure. However, concerns exist over the similarity between MWL and native lignin based on the low yields (25-50% of protolignin) and structural alteration due to ball milling. Ball milling reduces the degree of polymerization, creating new free-phenolic hydroxyl groups through cleavage of β-aryl ether linkages as well as increasing α-carbonyl groups via side-chain oxidation (Lai et al., 1971; Chang et al., 1975; Ikeda et al., 2002; Fujimoto et al., 2005; Gellerstedt et al., 1989). Recently, Ikeda et al. investigated the effects of several balling-milling methods on the cleavage of β-aryl ether linkages in MWL using a modified DFRC (Derivatization Followed by Reductive Cleavage) method in combination with nitrobenzene oxidation (Ikeda et al., 2002). It was found that the phenolic β-O-4’ structures increased continuously at the expense of the etherified β-O-4’ structures during ball-milling. Based on the increase in α-aryl ether groups, it was suggested that some condensation reactions may occur during the ball milling process, especially if the ball-milling is conducted in a vibratory ball mill without toluene (dry ball-milling). Ball milling primarily affects the side-chain structure of the C₉ phenyl-propane units in lignin (Ikeda et al., 2002; Fujimoto et al., 2004). The β-O-4’ inter-unit linkage is the main inter-unit linkage in lignin accounting for ~48% of the inter-unit linkages in gymnosperms and ~ 60% in angiosperms (Adler, 1977). The β-O-4’
inter-unit linkage exists in two isomers, erythro and threo (Lundquist et al., 1980; Matsumoto et al., 1986). These isomers can be quantitatively analyzed by ozonation analysis (Matsumoto et al., 1986; Akiyama et al., 2002). The ratio of erythronic and threonic acids (E/T ratio), two final products of ozonation, indicates the erythro/threo ratio of β-O-4’ structure while the total yield of erythronic and threonic acids (E+T) gives information about the relative content of this structure in lignin. The ratios of erythro and threo diastereomers were determined to be about 1:1 in guaiacyl ethers and 3:1 in syringyl ethers (Ralph et al., 2004). Recently, Fujimoto et al. (Fujimoto et al., 2005) used ozonation to quantitatively analyze the change of β-O-4’ linkages in sweetgum lignin of the whole wood meal under different ball-milling conditions. They observed a steady decrease in β-O-4’ linkages with increasing ball milling intensity. The ratio of erythronic and threonic acids (E/T) and the total yield of the two acids (E+T) decrease linearly with extractable MWL yield, suggesting that extractable MWL yield is a better indication of the extent of ball milling than milling time.

Further improvements in the yield of lignin isolated from ball-milled wood have come through the use of cellulolytic enzymes (Chang et al., 1975; Ikeda et al., 2002; Pew, 1957; Holtman et al., 2004). Chang et al utilized cellulolytic enzymes to remove carbohydrates prior to aqueous dioxane extraction of ball-milled wood meal (Chang et al., 1975). The cellulolytic enzyme lignin (CEL) was found to be structurally similar to MWL (Chang et al., 1975; Pew, 1957), but was obtained in higher yield with less degradation and hence more representative of the total lignin in wood (Chang et al., 1975). More recently Chang and co-workers (Ikeda et al., 2002; Holtman et al., 2004) isolated lignin by extracting MWL first, and then treating the residue with cellulolytic enzymes followed by 96% aqueous dioxane extraction to isolate CEL. Comparison of the chemical structure of MWL and CEL using wet
chemistry and modern NMR spectroscopy revealed that the MWL is slightly more condensed than the CEL, suggesting MWL may contain a higher proportion of lignin from the middle lamella (Ikeda et al., 2002; Holtman et al., 2004). In this paper we further explore the extent to which isolated cellulolytic enzyme lignin may be more representative of the total lignin in wood than MWL. Using three different milling methods MWL and CEL were isolated from sapwood of loblolly pine and characterized structurally using ozonation, elemental and methoxyl analyses, and advanced spectroscopic techniques including infrared (FTIR) spectroscopy, quantitative $^{13}$C NMR spectroscopy and two-dimensional HMQC NMR spectroscopy.

3.3 Materials and Methods

3.3.1 Materials

DMSO-d$_6$ was purchased from Cambridge Isotope Laboratories and used as received. 1, 4-Dioxane was purchased from Fisher Scientific and distilled over NaBH$_4$ prior to use. Cation exchange resin, Dowex 50WX8-100, was purchased from Alfa Aesar. All other chemicals were purchased from Sigma-Aldrich and used as received. Loblolly pine (Pinus taeda L.) sapwood (18 years old) was ground to pass a 20-mesh screen in a Wiley mill and Soxhlet-extracted with acetone for 48 hrs (Wiley wood) (Yokoyama et al., 2002). The Wiley wood was air-dried and stored in a desiccator under vacuum over P$_2$O$_5$. 
3.3.2 Rotary ball-milling method

Wiley wood meal (3 x 100g) was ground for 1 week, 6 weeks and 8 weeks in a 1-gallon porcelain jar using glass balls under a nitrogen atmosphere. The resulting rotary ball-milled woods are labeled; rotary-ball-milled-wood-1-week (RB1), rotary-ball-milled-wood-6-week (RB6) and rotary-milled-wood-8-week (RB8), respectively.

3.3.3 Vibratory ball-milling method

Rotary-ball-milled-wood-1-week (2 x 10g) was ground in a vibratory ball-mill (Siebtechnik GMBH, 433 Mulheim 011380, Germany) with stainless steel balls in the presence of toluene for 48 hrs and 72 hrs to obtain vibratory-ball-milled-wood-48-hour (VB48) and vibratory-ball-milled-wood-72-hour (VB72), respectively.

3.3.4 Planetary ball milling method

Wiley wood (2.5g) was ground in a planetary micromill (Fritsch, Idar-Oberstein, Germany) under an argon atmosphere. A 45-ml zirconium dioxide bowl with 18 zirconium dioxide balls (1cm diameter) was used in a planetary micromill. Milling frequency was 650 rpm, and a 15-min pause was used every 30 min in the milling processing to prevent the bowl from overheating. Five batches of Wiley wood were ground for 4 hours, 8 hours and 15 hours (excluding pause time) to obtain planetary-ball-milled-wood-4-hour (PB04), planetary-ball-milled-wood-8-hour (PB08), and planetary-ball-milled-wood-15-hour (PB15), respectively.
3.3.5 Lignin isolation method

Milled wood lignins (MWLs) were isolated according to the method of Björkman (Bjorkman, 1954), wherein vibratory-ball-milled-wood (VB48) 20g was extracted 2 times with dioxane/water (2 x 200 ml; 96:4, v/v) for 24 hours under a nitrogen atmosphere. The supernatant was collected by centrifugation after each extraction, combined, concentrated and freeze-dried to obtain crude MWL. The crude MWL (4g) (only VB48 sample) was purified according to the method of Björkman (Bjorkman, 1954).

Cellulolytic enzyme lignin (CEL) was isolated according to Chang, et al (Chang et al., 1975). Specifically, milled wood (10g) was suspended in acetate buffer (100ml, pH 4.9) and 1 ml of Dyadic Fibrezyme ACL (Dyadic international Inc., Jupiter, FL) was added and incubated for 24 hours at 50°C. The reaction system was centrifuged, the supernatant was removed and the residue was again suspended in acetate buffer (50 mL, pH 4.9) and treated with Fibrezyme (0.5 ml) for an additional 24 hours at 50°C. The residue was again collected by centrifugation, washed with distilled water (200ml), centrifuged and freeze-dried. The freeze-dried residue was extracted twice (2×24hrs) with 100 ml of dioxane/water (96:4, v/v) under a nitrogen atmosphere. After each extraction, the supernatant was collected, combined and poured into a 250 ml volumetric flask. The residue from the second extraction was washed with 80 ml of 96% aqueous dioxane, centrifuged, and the supernatant added to the volumetric flask. The extract was diluted to 250 ml with fresh 96% aqueous dioxane and an aliquot removed for the determination of extractable CEL (described in the following section). The remaining solution was concentrated, precipitated in water, and freeze-dried. No-further purification was attempted. The MWL and CEL isolation procedure is summarized in Figure 3.1.
3.3.6 Determination of lignin content, extractable yields of MWL and CEL

Klason lignin method was used to determine the lignin content (including acid soluble lignin) in the Willy wood and isolated MWL and CEL (Dence, 1992). Extractable MWL and CEL were determined for all ball-milled-wood samples using a UV method modified from that of Fujimoto et al (Fujimoto et al., 2005) (described below). Both extractable MWL and CEL yields were calculated as weight % based on the Klason lignin content of the Willy wood. Extractable MWL yield was determined by extracting ball-milled-wood (40 mg) with 10 ml of freshly distilled 96% aqueous dioxane in a capped centrifuge tube under constant shaking
for 48 hours. The reactant was then centrifuged and 2 ml (VB72, PB08, PB15, RB6 and RB8) or 5 mL (VB48, PB04 and RB1) of the supernatant was carefully transferred into a 25 ml volumetric flask. One ml of alkaline sodium borohydride solution (1 mg sodium borohydride dissolved in one ml of 0.05 M sodium hydroxide) was added to the flask and kept overnight at room temperature. The solution was neutralized with 1 ml of glacial acetic acid and diluted to 25 ml with 96% aqueous dioxane. The lignin content was calculated from the absorbance at 280 nm using a UV spectrophotometer and gram absorptivity of 15.4 cm l/g (Chang et al., 1975).

Extractable CEL yield was determined using 0.5 ml of the CEL extract solution collected after dioxane/water extraction of the enzyme-treated ball-milled wood as described in the previous section. The CEL extract was transferred to a 50 ml (VB 48, PB04, PB08 and RB1) or 100 ml (VB72, and 250 ml for PB15, RB6 and RB8) volumetric flask and reduced with 1mg sodium borohydride in 1ml of 0.05M NaOH at room temperature for 24 hrs. As per the MWL, the reactions were neutralized with 1ml of glacial acetic acid, diluted to volume with 96% aqueous dioxane, and lignin content determined spectrophotometrically. All extractable lignin determinations were conducted in duplicate.

3.3.7 Ozonation

Ozonation was performed according to the procedure reported by Akiyama et al. (Akiyama et al., 2002), using 3% ozone in oxygen produced using a Polymetrics ozone generator (Polymetrics, Inc., U.S.A). Ozonation of milled wood (50 mg) and extracted lignin (15mg) were performed in duplicate. Ozonation products were quantitatively determined by Gas Chromatography (GC) (Hewlett-Packard 6890) using an HP-1 (Hewlett-Packard) 30 m ×
0.32 mm column (length × inside diameter). The carrier gas was helium with a flow rate of 2.0 ml/min. The injection temperature was 250 °C with a split ratio of 30:1 and the FID detector temperature was 280 °C. The oven temperature was held isothermally for 5 min at 120 °C, raised at 4 °C/min to 170 °C, thereafter raised at 10 °C/min to 280 °C and finally held for 5 min. The amounts of erythronic acid and threonic acid were determined relative to erythritol as an internal standard using response factors derived from pure compounds.

3.3.8 NMR analysis

NMR spectra were recorded on a Bruker Avance 300MHz spectrometer at 300K using a 5 mm Symmetrical NMR Microtube (Shigemi, Inc.) and DMSO-d₆ as a solvent. Chemical shifts were referenced to TMS (0.0 ppm). Quantitative ¹³C-NMR was performed using a ca. 20% lignin solution and QNP ¹H/¹³C probe. Chromium (III) acetylacetonate (0.01M) was added to the lignin solution to decrease acquisition time. Conditions used included a 90° pulse width, a 1.2 s acquisition time, and a 1.7 s relaxation delay. Quantitative ¹H-NMR analysis of acetylated lignin was recorded at a lignin concentration of 5% in DMSO-d₆, using a 90° pulse width and a 1.7 s acquisition time. A 2 s relaxation delay was used and a total of 128 scans were collected. ¹H -¹³C correlation 2-D HMQC NMR analysis was performed using a 5% lignin solution. The experimental conditions used were a 90° pulse width, a 0.15 s acquisition time, a 1.4 s relaxation delay, and ¹J_C-H of 147 Hz.

3.3.9 FTIR Spectra

KBr pellets for FT-IR spectroscopy in absorbance module were prepared using a Perkin-Elmer pellet die (2 mg lignin samples in 200 mg KBr). The spectra were recorded with a
NEXUS 670 Spectrometer (Thermo Nicolet Corporation, Madison, WI) at a resolution of 8 cm\(^{-1}\) with a total of 64 scans per measurement.

### 3.3.10 Sugar composition, elemental and methoxyl analysis

Carbohydrate composition of the lignin samples was determined by a modified alditol-acetate gas chromatographic method (Blakeney et al., 1983). Gas chromatography (GC) (Hewlett-Packard 5890) was performed using an RTX-225 (Hewlett-Packard) 15 m × 0.32 mm (length × inside diameter) column. The carrier gas was helium with a flow rate of 2.0 ml/min. The injection temperature was 220 °C with a split ratio of 30:1, and the FID detector temperature was 240 °C. The oven temperature was raised from an initial 210 °C to 230 °C at 2 °C/min. The concentration of sugar monomers was determined relative to inositol as an internal standard using response factors derived from pure compounds.

Elemental analysis (C, H and N) was performed by the Analytical Service Laboratory, Department of Soil Science, North Carolina State University. Oxygen content was determined by difference, 100-(C+H+N), assuming no other elements were present. Methoxyl content was carried out according to the modified method of Zakis (Zakis, 1994).

### 3.4 Results and Discussion

#### 3.4.1 Effect of Ball-milling on Extractable Yield of MWL and CEL

Three different ball mills (rotary ball mill, vibratory ball mill and planetary ball mill) were used to produce ball-milled wood. The effects of milling time on the extractable yields of MWL and CEL by different ball mills are given in Table 3.1. Included are the Klason lignin
contents of the CEL and some MWL preparations. It should be noted that the extractable yields of MWL and CEL are based on UV method and indicated a pure lignin yield. As can be seen in Table 3.1, regardless of the ball milling method, at a given extent of ball-milling the extractable yield of CEL is much higher than that of MWL. Even without purification, the Klason lignin content of all CEL samples is higher than 80%.

Table 3.1. Yield and Klason lignin content of MWL and CEL

<table>
<thead>
<tr>
<th>Sample name</th>
<th>Extractable lignin yield</th>
<th>Klason lignin content (%±1.7)%</th>
<th>MWL</th>
<th>CEL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Orig. wood</td>
<td>----</td>
<td>----</td>
<td>---</td>
<td>29.8b</td>
</tr>
<tr>
<td>RB1</td>
<td>6</td>
<td>12</td>
<td>---</td>
<td>81.9</td>
</tr>
<tr>
<td>RB6</td>
<td>35</td>
<td>65</td>
<td>---</td>
<td>86.9</td>
</tr>
<tr>
<td>RB8</td>
<td>42</td>
<td>75</td>
<td>72.5</td>
<td>85.2</td>
</tr>
<tr>
<td>VB48</td>
<td>22</td>
<td>34</td>
<td>87.1c</td>
<td>81.1</td>
</tr>
<tr>
<td>VB72</td>
<td>36</td>
<td>53</td>
<td>---</td>
<td>86.5</td>
</tr>
<tr>
<td>PB04</td>
<td>10</td>
<td>23</td>
<td>86.6</td>
<td>81.3</td>
</tr>
<tr>
<td>PB08</td>
<td>30</td>
<td>56</td>
<td>---</td>
<td>80.2</td>
</tr>
<tr>
<td>PB15</td>
<td>46</td>
<td>86</td>
<td>---</td>
<td>83.5</td>
</tr>
</tbody>
</table>

a: pooled standard error
b: Klason lignin content in original wood
c: MWLVB48 was purified lignin

3.4.2 Effect of Ball-milling on β-O-4’ Structure

Ozonation was performed on all ball-milled wood samples. Figures 3.2 show the E/T and E+T values as functions of extractable MWL and CEL yields. Both E+T and E/T decrease linearly with increasing extractable yields of MWL and CEL, irrespective of the ball milling methods employed. This confirms that β-O-4’ structures are degraded during the ball milling process and that the erythro isomer is preferentially degraded. These results are in full agreement with those of Fujimoto et al on sweetgum (Fujimoto et al., 2005) and confirm that
extractable lignin yield, whether MWL or CEL, is a valid way to compare the effect of ball milling on the lignin structure for both softwoods and hardwoods (Fujimoto et al., 2005).

Figure 3.2 Dependence of the structure and yield of β-O-4’ linkages in milled wood on the extractable yield of Milled Wood Lignin (MWL) and Cellulolytic Enzyme Lignin (CEL) under different milling conditions

a) Erythro/Threo ratio (E/T ratio) of β-O-4’ structures of lignin in milled wood. Equation with extractable CEL yield: \( y = -0.00087x + 1.002 \) \( R^2 = 0.89 \). Equation with extractable MWL yield: \( y = -0.00154x + 1.002 \) \( R^2 = 0.88 \). Pooled Standard Error: ±0.007.

b) Total E+T yield (sum of Erythronic and Threonic acid) of β-O-4’ linkages in milled wood. Equation with extractable CEL yield: \( y = -0.0441x + 17.9 \), \( R^2 = 0.85 \). Equation with extractable MWL yield: \( y = -0.0796x + 18.0 \), \( R^2 = 0.88 \). Pooled Standard Error: ±0.3%. C\(_{900}\) unit molecular weight: 18200.
It is noteworthy that the amount of β-O-4’ structures affected during the ball milling process is small: E+T yield decreased less than 25% (18.4% to 14.5-15.0%) with extensive ball milling (CEL yield ~ 75% and 86 % for CELRB8 and CELPB15, respectively). This is consistent with our previous work in which we observed a small decrease in etherified β-O-4’ structures with a small increase in phenolic β-O-4’ during various ball-milling methods using a modified DFRC technique (Ikeda et al., 2002). While the present results are in total agreement with those of Fujimoto et al on sweetgum (Fujimoto et al., 2005; Fujimoto et al., 2004), the β-O-4’ structures in sweetgum appear to be degraded to a greater extent than in loblolly pine. At an extractable MWL yield of 25%, 22% of the β-O-4’ structures in sweetgum are degraded (Fujimoto et al., 2004), whereas only ~ 12% of the β-O-4’ structures in loblolly pine are degraded (Figure 3.2). This may be due to the facts that hardwood lignin has (i) a higher amount of β-O-4’ linkages than softwood lignin and (ii) hardwood lignin has a much higher E/T than softwood lignin, wherein the E isomer is preferentially degraded (Fujimoto et al., 2004).

The E/T and E+T of all extracted CEL as well as 3 MWL samples are shown in Figure 3.3. It shows that the E/T ratios of the extracted CEL are lower than those of the original ball-milled wood from which they are extracted. Furthermore, the E/T ratios remain constant over the wide range of extractable CEL yields. By comparison the E/T ratios of the three MWL samples are even lower than the CEL, but also remain constant. As E/T ratios are an indication of the extent of cleavage of β-O-4’ structures (Fujimoto et al., 2005), these results indicate that both CEL and MWL are lignin fractions that have undergone degradation during ball milling. In addition to the extracted lignin, it also includes E/T ratios for the residual lignin (REL) remaining after extraction with 96% dioxane. By contrast to the MWL and
CEL, the E/T ratios of the REL remain essentially constant at the same values as the original wood, E/T = ~ 1, indicating the β-O-4’ structures of REL are minimally degraded. Surprisingly, the E+T yields of CEL as well as MWL are high. Figure 3.3 shows the E+T yields of CEL are higher than the corresponding ball-milled wood. In fact, with exception of the two very low yield MWL and CEL, the E+T yields of CEL up to ~60% extractable yield are about the same as that of the original Willy wood. Beyond 60% extractable CEL yield the E+T yields decrease with increasing intensity of ball milling and eventually approach those of the ball-milled wood. As expected, The E+T yields of the REL are much lower than the corresponding CEL and ball-milled wood. Of particular interest is the RELPB15, the residual lignin left after the extraction of CELPB15 at 86% extractable lignin yield. RELPB15 showed a very low yield of E+T, indicating that the β-O-4’ content of RELPB15 is much lower than the CELPB15.

### 3.4.3 Effect of Ball Milling on the Structure of MWL, CEL and REL

It is clear that ball milling affects the structure of lignin, cleaving up to 25% of the β-O-4’ linkages in softwood lignin, and facilitates the release of lignin fragments extractable by 96% dioxane. MWL and CEL have higher β-O-4’ contents and presumably less condensed structure than the corresponding residual lignin (REL). Therefore, as the majority of structural research on native lignin using MWL, the question remains as to how representative of the original lignin are MWL and/or CEL? Compounded on this is the fact
Figure 3.3 Dependence of the structure and yield of $\beta$-O-4’ linkages in Milled Wood Lignin (MWL), Cellulolytic Enzyme Lignin (CEL), Residue lignin, and Residue Lignin (REL) on the extractable yield of MWL and CEL under different milling condition

a) E/T ratio (the ratio of erythronic acid to threonic acid) of $\beta$-O-4’ structures of lignin in MWL, CEL, and REL. Pooled Standard Error: ±0.008

b) Total E+T yield (sum of Erythronic and Threonic acid) of $\beta$-O-4’ linkages in MWL, CEL, and REL. Equation of REL with extractable yield of lignin (MWL and CEL): $y = -0.1232x + 18.735$ R² = 0.89. Pooled Standard Error: ±0.3%. C₉₀₀ unit molecular weight: 18200.

that conclusions have been drawn and comparisons made using MWL extracted with various milling procedures and MWL yields. In the following section we compare the structure of MWL (MWLVB48) to CEL (CELPB4) at the same yield (~ 22%), as well as look at the
effect of CEL yield (CELPB4 – 22% and CELPB15 – 86%) on both the structure of CEL and the corresponding REL (RELPB15). RELPB15 is of special interest since it is the residue lignin of CELPB15 (86% yield), representing the last 14% of lignin in wood. The reference MWLVB48, on the other hand, was isolated according to the standard procedure of Björkman involving all purification steps (Björkman, 1954).

### Table 3.2 Elemental analysis of MWL and CEL

<table>
<thead>
<tr>
<th>Sample name</th>
<th>Sugar content %±1.7%&lt;sup&gt;a&lt;/sup&gt;</th>
<th>100 C9 formula&lt;sup&gt;c&lt;/sup&gt;</th>
<th>OCH&lt;sub&gt;3&lt;/sub&gt; %±0.2%&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Elemental composition (%±0.2%)&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>MWLVB48&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.8</td>
<td>C&lt;sub&gt;900&lt;/sub&gt;H&lt;sub&gt;780&lt;/sub&gt;O&lt;sub&gt;241&lt;/sub&gt;(OCH&lt;sub&gt;3&lt;/sub&gt;)&lt;sub&gt;95&lt;/sub&gt;</td>
<td>16.0</td>
<td>65.0</td>
</tr>
<tr>
<td>CELPB04</td>
<td>16.6</td>
<td>C&lt;sub&gt;900&lt;/sub&gt;H&lt;sub&gt;802&lt;/sub&gt;O&lt;sub&gt;282&lt;/sub&gt;(OCH&lt;sub&gt;3&lt;/sub&gt;)&lt;sub&gt;90&lt;/sub&gt;</td>
<td>14.7</td>
<td>62.8</td>
</tr>
<tr>
<td>CELPB15</td>
<td>14.1</td>
<td>C&lt;sub&gt;900&lt;/sub&gt;H&lt;sub&gt;781&lt;/sub&gt;O&lt;sub&gt;275&lt;/sub&gt;(OCH&lt;sub&gt;3&lt;/sub&gt;)&lt;sub&gt;86&lt;/sub&gt;</td>
<td>14.3</td>
<td>63.5</td>
</tr>
</tbody>
</table>

<sup>a</sup>: pooled standard error  
<sup>b</sup>: purified lignin  
<sup>c</sup>: sugar content considered in the C900 calculation

### 3.4.4 Elemental and Methoxyl Compositions of CEL and MWL

The results obtained from elemental, methoxyl and sugar analysis of three lignin samples are shown in Table 3.2. Nitrogen analysis (< 0.02% nitrogen) indicates there is little to no contamination from the cellulolytic enzymes. However, as expected the two CEL samples isolated without purification have much higher sugar contents than the purified MWL sample. (The sugar contents presented are based on the sum of glucose, mannose, galactose, xylose and arabinose without taking into consideration 4-O-methyl-glucuronic acid and other minor sugars. Thus, the actual difference in sugar contents between MWL and CEL may be slightly larger than that shown in Table 3.2.). Both CEL samples have a slightly lower methoxyl and slightly higher oxygen content than MWL. The elemental and methoxyl data have been corrected for sugar content, and these differences may be the result of errors.
associated with the sugar content determination. In fact, NMR analysis (described later) shows little difference in methoxyl content between MWL and CEL. It is quite surprising how similar the three lignin samples are in light of the fact that the isolation procedure is quite different between MWL and CEL, and the large difference in yield between the two CEL samples (23% vs. 86%).

Figure 3.4 shows the quantitative $^{13}$C-NMR spectra for the non-acetylated MWL and two CEL preparations. Surprisingly, the three spectra appear quite similar. HMQC as well as FTIR analysis (data not shown) also indicate very little difference between the three lignin preparations. These observations are quite surprising considering the significant difference in

3.4.5 Spectroscopic Analyses of MWL and CEL

Figure 3.4 shows the quantitative $^{13}$C-NMR spectra for the non-acetylated MWL and two CEL preparations. Surprisingly, the three spectra appear quite similar. HMQC as well as FTIR analysis (data not shown) also indicate very little difference between the three lignin preparations. These observations are quite surprising considering the significant difference in
yield between the two CEL preparations, and the different isolation procedure used for MWL as compared to CEL. The close resemblance of the FTIR and $^{13}$C-NMR spectra is remarkable, indicating that the lignin fraction released as a result of ball-milling process is uniform in its structure.

Quantitative analysis of the $^{13}$C-NMR data (Capanema et al., 2004) reveals little subtle differences between the lignin preparations. **Figure 3.5** and **Table 3.3** summarize the estimated amounts of various inter-unit linkages and function groups. With exception of the $\beta$-O-4’ linkages very little difference is observed in the amounts of the major inter-unit linkages among these three lignin preparations. As expected, CELPB15 has a slightly lower amount of $\beta$-O-4’ linkages than the other two lignin preparations, consistent with the more extensive ball-milling.

![Figure 3.5 Calculated Moieties/100 Aromatic Ring in Lignin by Quantitative $^{13}$C- NMR](image)

<table>
<thead>
<tr>
<th>Linkage</th>
<th>MWL</th>
<th>CELPB04</th>
<th>CELPB15</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\beta$-O-4’</td>
<td>46</td>
<td>43</td>
<td>39</td>
</tr>
<tr>
<td>$\beta$-5’/$\alpha$-O-4’</td>
<td>11</td>
<td>12</td>
<td>13</td>
</tr>
<tr>
<td>$\beta$-$\beta$’</td>
<td>2</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>Spirodienone</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>5-5’</td>
<td>17</td>
<td>15</td>
<td>16</td>
</tr>
<tr>
<td>$\beta$-1’</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Dibenzodioxocin</td>
<td>6</td>
<td>5</td>
<td>6</td>
</tr>
</tbody>
</table>
Table 3.3 Quantification of spectral region of the $^{13}$C NMR spectra

<table>
<thead>
<tr>
<th>Spectral region</th>
<th>MWLVB48</th>
<th>CELPB04</th>
<th>CELPB15</th>
<th>RELPB15</th>
</tr>
</thead>
<tbody>
<tr>
<td>Degree of condensation</td>
<td>40</td>
<td>37</td>
<td>42</td>
<td>--</td>
</tr>
<tr>
<td>Aromatic methine carbons</td>
<td>262</td>
<td>251</td>
<td>249</td>
<td>--</td>
</tr>
<tr>
<td>Aromatic carbon-carbon</td>
<td>171</td>
<td>160</td>
<td>160</td>
<td>--</td>
</tr>
<tr>
<td>Oxygenated aromatic carbons</td>
<td>218</td>
<td>202</td>
<td>207</td>
<td>--</td>
</tr>
<tr>
<td>Carbon from carbonyl type</td>
<td>11</td>
<td>9</td>
<td>7</td>
<td>--</td>
</tr>
<tr>
<td>OMe</td>
<td>95</td>
<td>97</td>
<td>97</td>
<td>72</td>
</tr>
<tr>
<td>Phenolic OH</td>
<td>29</td>
<td>30</td>
<td>31</td>
<td>--</td>
</tr>
<tr>
<td>p-hydroxyphenyl(H-unit)</td>
<td>5</td>
<td>5</td>
<td>6</td>
<td>20</td>
</tr>
</tbody>
</table>

3.4.6 Comparison of CELPB15 and RELPB15

CELPB15 was isolated from planetary ball-milled wood after 15 hours of milling. The extractable lignin yield was 86%, leaving the residual lignin (RELPB15) as insoluble residue after 96% dioxane extraction. Thus, RELPB15 represents only 14% of the original lignin in wood. The structure of RELPB15 is of particular interest since ozonation suggests unusually low $\beta$-O-4’ linkages (cf. Fig. 3.3 as indicated by very low E+T yield) as compared to CELPB15. Although insoluble in most solvents, RELPB15 became completely soluble in DMSO after acetylation. NMR analyses (spectrum not shown) clearly showed much lower methoxyl content and a much higher $p$-hydroxyphenyl-propane (H) unit content as compared to the corresponding CEL (Table 3.3). Likewise, clear differences are evident in the FTIR spectrum of RELPB15 as compared with that of CELPB15 as shown in Figure 3.6. The RELPB15 spectrum has lower absorption at 1467 cm$^{-1}$ and a completely different absorption pattern at 800-900 cm$^{-1}$ region. The latter indicates a shift from a typical guaiacyl-propane aromatic substitution pattern to a more complicated one. These results, together with those of ozonation, clearly indicate that CELPB15 and RELPB15 are different in their chemical
structures. Similar differences in FTIR spectra features were also observed between CELRB8
(extractable CEL yield 75%) and RELRB8 but not in other pairs with lower extractable CEL
yield. These results indicate that structure of CEL is different from REL once the extractable
CEL yields exceed 75%.

Figure 3.6. FT-IR spectra of extracted lignin and residue lignin

3.4.7 Morphological Origin of MWL, CEL and REL

It is well known that lignin in the secondary wall and lignin in the compound middle lamella
are different in chemical structure (Yang et al., 1980; Whiting et al., 1982). Lignin in the
compound middle lamella has lower methoxyl content, is richer in H units (Whiting et al.,
1982), and has lower phenolic hydroxyl content (Yang et al., 1980) than lignin the in
secondary wall. In lignin biogenesis studies using autoradiography, lignin in the compound
middle lamella was found to have a higher degree of condensation and contain much higher
H units than lignin in the secondary wall (Fukushima et al., 1991; Terashima et al., 1988). Using ultraviolet microscope, the majority of lignin in spruce (72% in earlywood and 81% in latewood) was found in the secondary wall (Scott et al., 1969). Loblolly pine has thicker cell walls than spruce and hence should have even higher proportion of lignin in the secondary wall. Taking into consideration these earlier findings and our current results it can be deduced that RELPB15 as well as RELRB8 likely originate mainly from the compound middle lamella. In addition, at extractable CEL yields of below 75%, the corresponding REL is a mixture of lignin from the middle lamella and secondary wall in varying proportions.

Initially during ball milling only a small amount of lignin is extractable as MWL (Lai et al., 1971). Even after grinding in a rotary ball mill (glass balls) for 10 days, less that 5% of lignin is extractable as MWL, whereas the extractable lignin carbohydrate complex (LCC) reaches a maximum and decreases thereafter upon further grinding (Lai et al., 1971). It is likely that lignin in the secondary wall have more linkages with carbohydrates and only becomes extractable as MWL after the carbohydrates are degraded sufficiently by the ball milling process. Thus, lignin isolated as MWL during the early part of ball milling (low yield MWL) may originate mainly from lignin in the middle lamella. This is supported by the FTIR spectra of CELRB1 and MWLRB1 (Figure 3.6). After ball milling for a week in a rotary ball mill, crude MWL (MWLRB1) and crude CEL (CELRB1) were obtained in 6% and 11% yield, respectively. The FTIR spectrum of CELRB1 is very similar to that of RELPB15 and is different from those of the other CELs. On the other hand, the FTIR spectrum of MWLRB1 is different, suggesting the possibility that the lignin released during the early part of ball milling may be structurally different from the rest of the lignin. The nature of the lignin released early during ball milling needs to be further studied in view of the following
two recent findings. First, in the present study, CEL and MWL at extractable yields below 20% showed lower content of β-O-4’ structure (E+T) than the CEL at higher yields (cf. Figure 3.3). Second, MWL gives lower yields of thioacidolysis products than the milled wood from which it is extracted (Onnerud et al., 2003; Holtman et al., 2003). In both studies, the yield of MWL was below 20%. Thus, it is suggested that CEL and MWL originate mainly from the secondary wall. However, at low yield, they may be contaminated by lignin from the compound middle lamella and/or from lignin that differs from the rest of lignin. The effect of contamination diminished as the yield increases.

3.4.8 A Suggested Lignin Isolation Procedure for the Structural Study of Lignin

In light of the results discussed above, a new cellulolytic enzyme lignin isolation procedure for the structural study of native lignin is suggested as shown in Figure 3.7. The procedure involves ball-milling extractive-free wood meal, using any ball-milling device, until an extractable CEL yield of 75-80% is achieved. The ball-milled wood is treated with cellulolytic enzymes prior to extraction with 96% aqueous dioxane (2*24 hours). After centrifugation, the combined supernatants are freeze-dried to obtain crude CEL. The crude CEL is dissolved in 90% acetic acid and the solution is added drop-wise into stirring water to obtain purified CEL as a precipitate. The CEL is collected by centrifugation, washed with water and freeze-dried. The CEL is then further purified by dissolving in 1,2-dichloroethane/ethanol according to the method of Björkmman (Björkmman, 1954). The supernatants from the acetic acid / water precipitation and centrifugation steps can be combined, concentrated under reduced pressure to remove some acetic acid, and freeze-dried to obtain a water soluble LCC preparation. This carbohydrate-rich LCC can be utilized for
the study of lignin carbohydrate bonds and the structure of lignin in LCC (Capanema et al., 2005; Balakshin et al., 2006). Likewise, the lignin residue from the dioxane extraction can be collected, washed with water and freeze-dried to obtain REL. Thus, three lignin fractions are isolated, representing the total lignin in wood; CEL and LCC are largely representative of lignin in the secondary wall whereas REL represents lignin in the compound middle lamella. This procedure requires that researchers determine the extent of ball milling needed to achieve 75-80% extractable CEL yield for a given ball-milling device. The protocol described in the experimental section of this paper can be used to determine the extractable CEL yield after ball milling.
Ball milling of wood degrades $\beta$-O-4’ structures in lignin. The effect is less for softwood than for hardwood lignin. However, even after extensive ball milling, less than 25% of the $\beta$-
O-4’ structures are degraded. Extractable lignin yield, either as MWL or CEL, is the best way to measure the extent of ball milling. CEL is preferred over MWL since CEL can be isolated at higher yield with less degradation. It appears that CEL isolated with yields ranging from 20-86% have similar structures, suggesting that the lignin in the secondary wall is uniform in structure. The REL is structurally different from CEL and may originate mainly from the middle lamella. Based on these results, we propose a new procedure for the isolation of lignin for use in structural studies wherein the wood is sufficiently milled to obtain CEL in 75-80% yield, and successively extracted to produce three lignin fractions representing the total lignin in wood.

3.6 Acknowledgements

We acknowledge Drs. E. A. Capanema and M. Y. Balakshin for valuable discussion of the NMR results and Dr. T. Yamada, for helping in methoxyl analysis. The authors are grateful to US Dept. of Agriculture for a research grant in support of this research (CSREES grant No. 2001-52104-11224).

3.7 References

4. Future work

In the present study, only softwood (loblolly pine) was used for the method development. The same idea can be used for other different species. Isolation of Cellulolytic Enzyme Lignin from hardwood will be an interesting topic for the future studies.

In the present study, the author observed that early extracted lignin (yield less than 10%) and the residue lignin (after extract 86% of lignin) was abnormal on the lignin structure compared to others. In order to better understand the morphological origin and the chemical structure of residue lignin after Enzymatic treatment, further studies have to consider these residue parts of lignin after enzymatic treatment.

Molecular distribution of CEL is also another research topic to finalize the utilization of CEL preparation as a routine isolation procedure for the structural studies of lignin.