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ARTICLE

Isolation of Cellulolytic Enzyme Lignin from Wood Preswollen/Dissolved in Dimethyl Sulfoxide/*N*-Methylimidazole

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Attempts were made to enhance polysaccharide digestibility by crude cellulases in the isolation of cellulolytic enzyme lignin (CEL) by dissolution of ball-milled wood in a dimethyl sulfoxide (DMSO)/*N*-methylimidazole solvent system as a pretreatment step. Wood regenerated from the DMSO/*N*-methylimidazole solution was hydrolyzed with crude cellulases for 48 h, removing 73.7 and 66.9% of the original carbohydrate for basswood and loblolly pine, respectively; only 61.7 and 49.2% were hydrolyzed by the crude cellulases without pretreatment. The yields of CEL isolated from regenerated ball-milled wood samples were therefore higher than those directly from ball-milled wood material, presumably via decreasing crystallinity of cellulose. For basswood, the yields of lignin were 45.8 and 36.5% (based on Klason lignin); for loblolly pine, the yields were 35.3 and 30.5%. The isolated lignins were structurally examined using two-dimensional heteronuclear single-quantum coherence ($^{1}H-^{13}C$ HSQC) NMR methods, which showed that the main structural characteristics of the lignin fractions obtained using these two methods are similar except for slightly higher amounts of carbohydrates in the solvent dissolution product.

KEYWORDS: Lignin; cellulolytic enzyme; cell wall; dissolution; HSQC; isolation method

INTRODUCTION

Lignin, one of the major components of the cell wall of vascular plants, has long been recognized for its negative impact on forage quality, paper manufacturing, and, more recently, cellulosic biofuel production (*1*). As a highly abundant natural polymeric material that is currently underutilized, lignin has attracted increasing interest in wood chemistry, plant biochemistry, and related fields. Good methods to isolate lignin from plant cell walls are valuable for better understanding the detailed structure of lignin. Unlike other natural polymers such as cellulose and proteins, which have only one kind of linkage between units, lignin is a network polymer, connected in a combinatorial fashion, having *β*-*O*-4-, *β*-*β*-, *β*-5-, 5-5-, and 4-*O*-5-linkages (*2*, *3*). Furthermore, lignin may be covalently linked to carbohydrates, forming a lignin−carbohydrate complex. The tight physical binding and chemical linkages between lignin and cell-wall polysaccharides prevent its clean isolation in an unaltered form (*4*).

An established mild method for lignin isolation, proposed by Björkman (*5*, *6*), is based on extensive fine grinding (ball-milling) of plant material followed by extraction with dioxane/water. The lignin preparation obtained by this method is called milled wood lignin (MWL) and has largely been considered as the basic material with which to perform most chemical and biological studies. However, concerns exist over the similarity between MWL and native lignin because of its often low yields (*7*). Another important approach is to subject ball-milled wood to enzymatic treatment with cellulolytic enzymes, generally so-called crude cellulases that also contain hemicellulase activities, to remove most of the polysaccharides before aqueous dioxane extraction of the residual material, producing a lignin preparation called cellulolytic enzyme lignin (CEL) in higher yield (*8*).

Cellulose hydrolysis is the key step for preparation of CEL because the efficiency of cellulose hydrolysis affects the following CEL extraction by aqueous dioxane. It is generally accepted that the accessibility of cellulases to the limited adsorption sites on crystalline cellulose structures play an important role in determining the cellulose hydrolysis rate (*9*). It was shown that cellulase readily hydrolyzes the more accessible amorphous portion of cellulose, whereas the enzymes are not as effective in degrading the less accessible crystalline portions (*10*). Thus, high-crystallinity cellulose will be more resistant to enzymatic hydrolysis. Conversely, decreasing the crystallinity of cellulose increases the digestibility of lignocellulosic materials by cellulases (*11*).

It is necessary to decrease the crystallinity for more efficient cellulase hydrolysis of lignocellulosic materials to obtain higher yields of CEL. Although there are a variety of cellulose dissolution solvents that might be considered for disrupting cellulose crystallinity, many of these are not suitable for isolation of relatively intact lignins. Dissolution of cellulose in ionic liquids has been widely documented (*12*−*15*). Most of the reported ionic

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liquids only dissolve purified cellulose, although cellulose in wood was reported to be dissolved partially by 1-*n*-butyl-3-methylimidazolium chloride (*16*) and 1-ethyl-3-methylimidazolium acetate (*17*). Kilpeläinen et al. (*18*) discovered that ionic liquids such as 1-*n*-butyl-3-methylimidazolium chloride and 1-allyl-3-methylimidazolium chloride have good solvating power for Norway spruce sawdust and southern pine TMP fibers, where the crystallinity of the cellulose was eliminated with an ionic liquid dissolution followed by regeneration treatment. However, the elevated temperature required for such dissolution is simply too destructive to be used for isolating lignin preparations useful for structural studies.

A DMSO/*N*-methylimidazole solvent system was recently reported to completely dissolve ball-milled wood at room temperature without degradation of cell wall components (*19*, *20*). When the ball-milled wood (or other plant) sample was suspended in DMSO/*N*-methylimidazole (2:1, v/v) at room temperature for 3 h, a clear solution was formed. Although fine grinding is required for the method, this is a common and required practice for isolating traditional MWL and CEL.

In this work, an improved method for the isolation of lignin from wood is proposed. It is based on preswelling or dissolving ball-milled wood material with DMSO/*N*-methylimidazole, followed by regeneration of the (cellulose-decrystallized) wood by precipitation into ethyl acetate, followed by crude cellulase treatment and aqueous dioxane extraction, the latter steps being similar to the original CEL method. The structures of isolated CELs from the preswollen ball-milled wood materials, which we term SCEL here, were characterized by 2D $^{1}H-^{13}C$-correlative NMR. Comparisons of the yields and resulting lignin composition/structure between SCEL and the traditional CEL method were made to evaluate the effects of the DMSO/*N*-methylimidazole dissolution process and its applicability for producing a useful lignin preparation in higher yield.

MATERIALS AND METHODS

Materials. All solvents used here were of A.R. grade and directly used as purchased without further purification except for 1,4-dioxane. 1,4-Dioxane was distilled prior to being used. All of the chemicals used were purchased from Sigma-Aldrich.

Cellulysin cellulase (EC 3.2.1.4) is a crude cellulase preparation, containing hemicellulase activities, from *Trichoderma viride* supplied by Calbiochem. Its activity was ≥10000 units/g of dry weight.

The Klason lignin contents of basswood and loblolly pine were 16.8 and 27.5%, respectively. Klason lignin was measured by the TAPPI standard. Coarsely ground material (0.5 mm, Wiley milled) was extracted four times with 80% ethanol in an ultrasonic bath for 3 h each time. The air-dried extractive-free wood sample (2.5 g) was then ball-milled using a PM 100 planetary ball-mill (Retsch, Germany) in a 50 mL ZrO_2 bowl with 10 ZrO_2 ball bearings (1 cm diameter) at 600 rpm with 10 min rest after every 10 min of milling. The total actual milling times for the basswood and loblolly samples were 8 and 10 h, respectively.

Dissolution and Regeneration of Ball-Milled Wood. The procedure for dissolution of whole cell walls in DMSO/*N*-methylimidazole was according to the published method (*18*). The ball-milled cell wall sample (1 g) was suspended in DMSO (10 mL) and *N*-methylimidazole (5 mL). The mixture was stirred at room temperature overnight for complete and convenient dissolution. The sample in such clear solution formed was regenerated/precipitated into ethyl acetate (500 mL), and the mixture was kept for 2 h. The precipitated material was recovered by filtration through a nylon membrane (0.2 μm). The product was washed with ethyl acetate and air-dried at room temperature for 1 h.

Isolation of Cellulolytic Enzyme Lignin. CEL or SCEL was then isolated according to the method published by Chang et al. (*8*). The ball-milled material (1 g) or the regenerated material (1 g) was suspended in 20 mM NaOAc buffer (30 mL, pH 5.0). The crude cellulase preparation (40 mg) was added, and the reaction slurry was incubated at 35 °C for 48 h. The solution was centrifuged, and the residues were washed with water and freeze-dried. The residue obtained after crude cellulase treatment was extracted with 96% dioxane (two times, 24 h). After centrifugation, the supernatant was collected and evaporated. The residues were dried under vacuum at room temperature. To preserve all structural features of the isolated CEL, no further purification was performed, although some carbohydrates remained in these preparations. Acetylation of the resulting product was via pyridine (2 mL) and acetic anhydride (1.5 mL); the mixture was kept at room temperature for 24 h. The solvent/reagents were removed by coevaporation at 45 °C with ethanol, several times, using a rotatory evaporator. The resultant acetylated lignin (acetylated CEL, acetylated SCEL) was transferred with 2 mL of acetone into 60 mL of water and freeze-dried.

NMR Analysis. The NMR spectra were acquired on a Bruker Avance 500 MHz spectrometer fitted with a cryogenically cooled 5 mm gradient probe with inverse geometry (proton coils closest to the sample). Acetylated lignin preparations (about 60 mg) were dissolved in 0.5 mL of $CDCl_3$. The central chloroform solvent peak at δ_C/δ_H 77.0/7.26 ppm was used as internal reference. The standard Bruker implementations of one- and two-dimensional (gradient-selected, ^{1}H-detected HSQC) NMR experiments were used for structural characterization and assignment authentication.

RESULTS AND DISCUSSION

CEL preparation is one of the most used methods to isolate wood lignins for structural studies. The method uses cellulolytic enzyme mixtures (containing cellulases and hemicellulases) to remove most of the carbohydrate fraction prior to lignin extraction with aqueous dioxane (*4*, *8*, *21*); the removal of carbohydrates using crude cellulases increases the yields of lignin in the following aqueous dioxane extraction step. Because the cellulose in plant cell walls is partly crystalline, with a high degree of polymerization, and also embedded in a matrix of wood polysaccharides and lignin, it is highly inaccessible to enzymatic attack (*22*). Ball-milling helps, but dissolution or swelling of cellulose by solvents was considered to be a more facile method for disrupting the crystalline structure to increase its accessibility to cellulases. Thus, hydrolysis of cellulose can be improved dramatically (*23*).

In this study, the solvent system DMSO/*N*-methylimidazole was used for dissolution of the ball-milled wood. Basswood and loblolly pine were chosen as hardwood and softwood experimental materials. The ball-milled wood material was suspended in DMSO/*N*-methylimidazole solvent and stirred until the solution became clear. Usually this process takes hours depending on the materials used. However, simply stirring overnight works well for most lignocellulosic samples. The wood sample could be completely regenerated, by rapid precipitation into ethyl acetate, as a porous and amorphous mixture of its original components. After treatment with crude cellulase, the mass losses for regenerated ball-milled basswood and regenerated ball-milled loblolly pine were 73.7 and 66.9%, respectively. However, for ball-milled basswood and ball-milled loblolly pine, enzymatic hydrolysis treatment with crude cellulases resulted in only 61.7 and 49.2% mass losses. These results showed that the digestibility of cellulose (and other polysaccharides) by the cellulases was significantly improved after dissolution in, and regeneration from, the DMSO/*N*-methylimidazole solvent system. The significant hydrolysis enhancement of cellulose was likely mainly the result of cellulose decrystallization.

The residues remaining after the cellulase hydrolysis were then extracted with 96% dioxane to isolate lignins. For basswood, the yield of isolated SCEL was 45.8% (based on the Klason lignin content of the original wood) from DMSO/*N*-methylimidazole-treated material, whereas the yield of CEL was only 36.5% from untreated material. The results for loblolly pine were similar, that is, 35.3% from DMSO/*N*-methylimidazole-treated material and

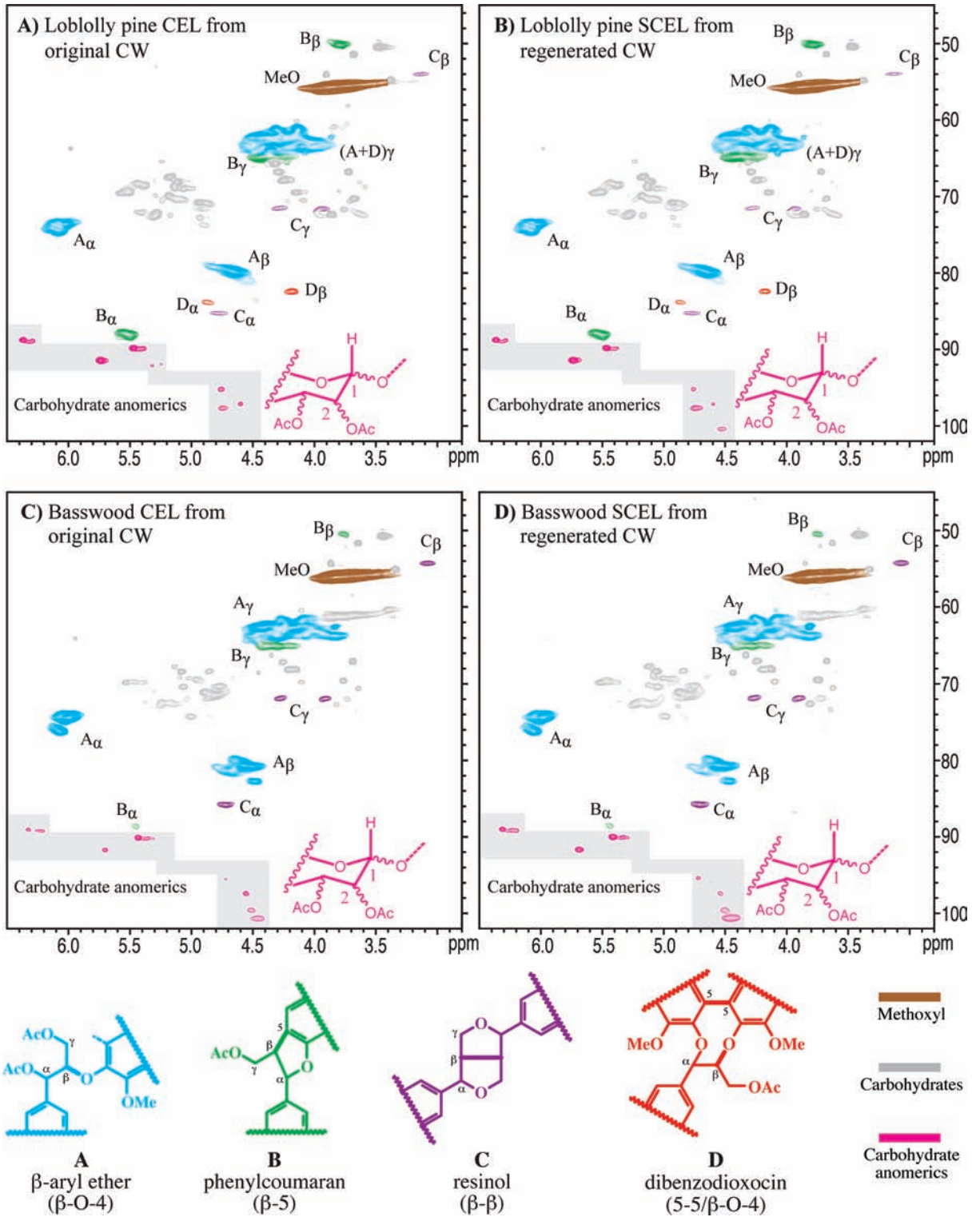

Figure 1. 2D HSQC NMR spectra of acetylated CEL and SCEL lignins isolated from loblolly pine (**A** and **B**) and basswood (**C** and **D**). Only the dialogistic side chain (aliphatic) regions are shown. The major substructures **A**−**D** with various interunit linkages are drawn in the same colors as the C−H correlations in the spectra corresponding to their side chains. The general carbohydrate structures showing their anomeric C−H correlations are colored magenta.

only 30.5% from the untreated material. These results showed that higher yields of lignin were indeed obtained from hardwood and softwood predissolved with DMSO/*N*-methylimidazole due to the improved enzymatic hydrolysis of carbohydrates.

To study the composition/structure of lignin obtained, the acetylated lignin was analyzed with 2D $^{1}H-^{13}C$ HSQC NMR (**Figures 1** and **2**). The main structural characteristics of the lignins, including various units linked by ether and C−C bonds, can be observed in the HSQC spectra. The diagnostic lignin side-chain regions are shown in **Figure 1**. From these well-resolved NMR spectra, C−H correlations from all major units, representing the various linkages between lignin units, can be readily assigned. Thus, C−H correlation signals at δ_C/δ_H ~73−75/6.0−6.2, 79−83/4.4−4.7, and 61−63/3.9−4.5 belong to the $C_\alpha-H_\alpha$, $C_\beta-H_\beta$, and $C_\gamma-H_\gamma$ correlations of the *β-O*-4-ether substructures **A** colored in cyan. The green-colored correlations

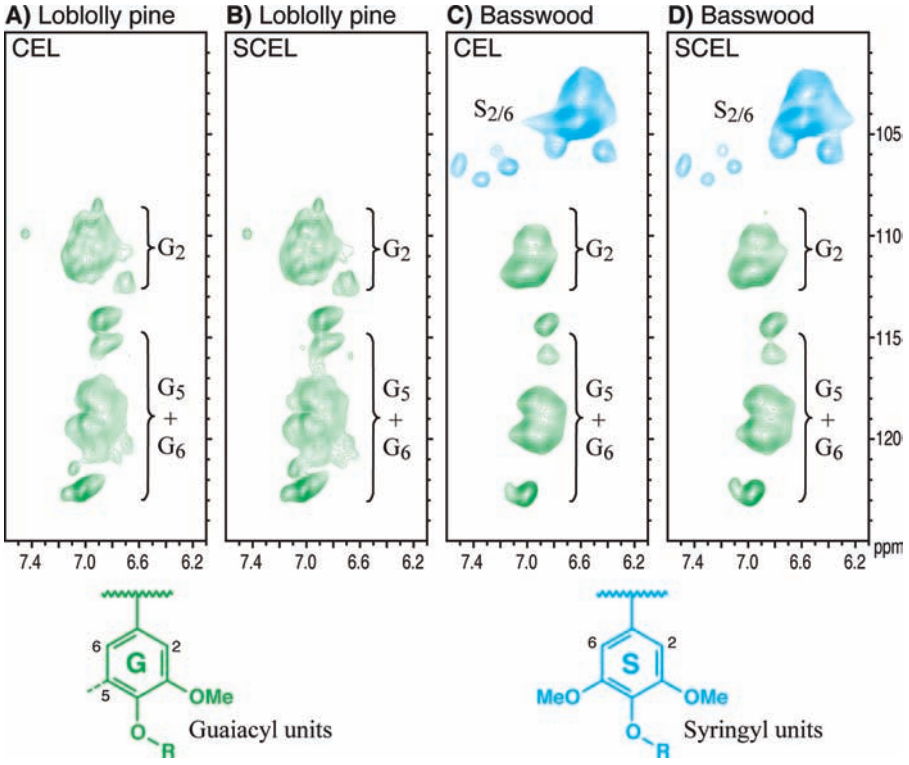

Figure 2. 2D HSQC NMR spectra of acetylated CEL and SCEL from loblolly pine and basswood, showing the aromatic C−H correlations from guaiacyl (in green) and syringyl (in blue) units in lignins.

Table 1. Yields and Compositional Estimation for CEL Preparations by HSQC NMR

sample	isolation yield[a] (%)	lignin content[b] (%)	lignin S:G	carbohydrates[b] (%)	"pure" lignin yields[c] (%)
pine CEL	30.5	85.8	0:1	14.2	26.2
pine SCEL	35.3	84.9	0:1	15.1	30.0
basswood CEL	36.5	88.7	1.9:1	11.3	32.4
basswood SCEL	45.8	84.6	2.2:1	15.4	38.7

[a] Yields are based on Klason lignin contents of the wood materials. [b] Molar percentages calculated from the following formulas: lignin (%) = integral of aromatic C_2-H_2 correlations/(integral of aromatic C_2-H_2 correlations + integral of anomeric C−H correlations); carbohydrates (%) = integral of anomeric C−H correlations/(integral of anomeric C−H correlations + integral of aromatic C_2-H_2 correlations). [c] Yields based on Klason lignin contents after correction for carbohydrates.

at δ_C/δ_H ~87−88/5.45−5.55, 49/3.85, and 65−66/4.2−4.4 are from the $C_\alpha-H_\alpha$, $C_\beta-H_\beta$, and $C_\gamma-H_\gamma$ correlations of the phenylcoumaran **B** (β-5) structures. The $C_\alpha-H_\alpha$, $C_\beta-H_\beta$, and $C_\gamma-H_\gamma$ correlations of the resinol **C** (β-β) structures, colored in purple, appear at δ_C/δ_H ~86/4.75, 54/3.05, 72/3.9, and 72/4.2. The $C_\alpha-H_\alpha$ and $C_\beta-H_\beta$ correlations from dibenzodioxocin **D** structures, colored in red, as labeled in the **Figure 1A**,**B**, are also distinguishable at δ_C/δ_H ~84/4.85 and 82.5/4.2, although the corresponding $C_\gamma-H_\gamma$ correlations are obscured by those from β-*O*-4-ether structures. As shown in these spectra, it is obvious that isolated CELs without purification contain significant amounts of carbohydrates as colored in gray in these spectra. However, for structural characterization, it is desired to have samples containing all structural features. Therefore, no purification was performed to avoid any possible loss of lignins. By comparison of the spectra of SCEL lignins isolated from DMSO/*N*-methylimidazole pretreatment with CEL lignins isolated directly (**Figure 1A** vs **B** and **Figure 1C** vs **D**), it is clear that spectra **A** and **B** are very similar, in terms of the structural features of both the lignins and their associated carbohydrates. The same conclusion can also be drawn when comparison is made between spectra **C** and **D**. The aromatic C−H correlations (**Figure 2**) from these lignin preparations also clearly suggested their similarity in basic structural units (S/G ratios) of lignin. The compositions of these lignin samples were estimated by integrating contour volumes corresponding to aromatic C_2-H_2 lignin correlations, including those in guaiacyl and syringyl units as well as those corresponding to carbohydrates' anomeric C_1-H_1 correlations (**Table 1**). Thus, the purity (estimated by NMR) of these lignin preparations ranges from 84 to 89% (molar percentages), although the SCEL samples have more carbohydrates in general. The overall isolated yields of the "pure" SCEL are higher than the corresponding CEL even after corrections are made on the basis of carbohydrate contents because the isolated yields of crude SCEL preparations were much higher than those of crude CEL preparations. From **Table 1** it is also noted that lignin compositions (S/G ratios) for SCEL and CEL isolated from basswood are similar, although the basswood SCEL preparation appears to contain slightly elevated syringyl levels.

It has been demonstrated that preswelling/dissolution pretreatment improves enzymatic hydrolysis of polysaccharides in ball-milled wood, thus increasing yields of lignins isolated by aqueous dioxane extraction. The improved (SCEL) method involves preswelling/dissolution of ball-milled wood in a DMSO/*N*-methylimidazole system followed by cellulolytic enzyme hydrolysis prior to lignin extraction. Using this method, two SCEL preparations from loblolly pine and basswood were produced in higher yields than those obtained by the traditional CEL method. Comparative studies, using 2D HSQC NMR, with the SCEL and traditional CEL preparations, revealed that both have similar

structural features and compositions except for slightly higher carbohydrate content in SCEL samples. The overall net yields of lignin in SCEL from loblolly pine and basswood are higher than those from CEL.

ABBREVIATIONS USED

G, guaiacyl; S, syringyl; HSQC, heteronuclear single-quantum coherence; MWL, milled wood lignin; CEL, cellulolytic enzyme lignin; SCEL, swollen cellulolytic enzyme lignin; TMP, thermomechanical pulp.

SAFETY

When distilling dioxane, one should always ensure that some dioxane remains in the flask before ending the distillation to avoid overconcentrating the peroxide, or distill dioxane over a reductant such as sodium borohydride (but still avoid distillation to dryness).

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