Doctoral dissertation

Development of Lignocellulosic Solution and Its Application to Chemical Analysis of Plant Cell Wall Components

(リグノセルロース溶液の開発とそれを用いた植物細胞壁構成成分の化学分析)

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Chapter 1

Introduction

1.1 Constitution of plant cell wall

The plant cell walls are important raw materials in the chemical industries to produce, for example, pulp, bio-ethanol and so on. In higher plants, the cell walls, which positioned in the outermost compartment of the plant cells, are constituted of three main polymeric components, i.e. cellulose, hemicelluloses, and lignins.[1] Polymeric structures of lignins are constituted by radical-coupling reactions of primarily p-hydroxycinnamyl alcohols after the polysaccharides have been laid down in the wall. Lignin provides a matrix in which the polysaccharides become embedded and possibly cross-linked. It is generally assumed that physical and/or chemical interactions between cellulose, hemicelluloses, and lignin form an extensive three-dimensional polymeric network as a whole.[1] Other components, such as proteins, together with pectin are considered in conjunction with the main polymers in the cell walls.[1,2] The cell wall is built up by several layers, namely middle lamella (ML), primary wall (P), outer layer of the secondary wall (S1), middle layer of the secondary wall (S2), and inner layer of the

secondary wall (S3). These layers except the warty layer consist of cellulose, hemicellulose, lignin, and other minor components. Proportion of these components differs depending on the layer. The constitution of wood cell wall and the distributions of wood cell wall components are illustrated in **Fig. 1.1**.

The effective utilization of abundant plant materials is inseparable from the characterization of each plant cell wall component and their interactions. Therefore, evaluation of the structure of each polymer and the interactions between these polymers have been key subjects in the field of wood chemistry. From this point of view, non- or low-degradable methods for the separation of each cell wall component are quite important.

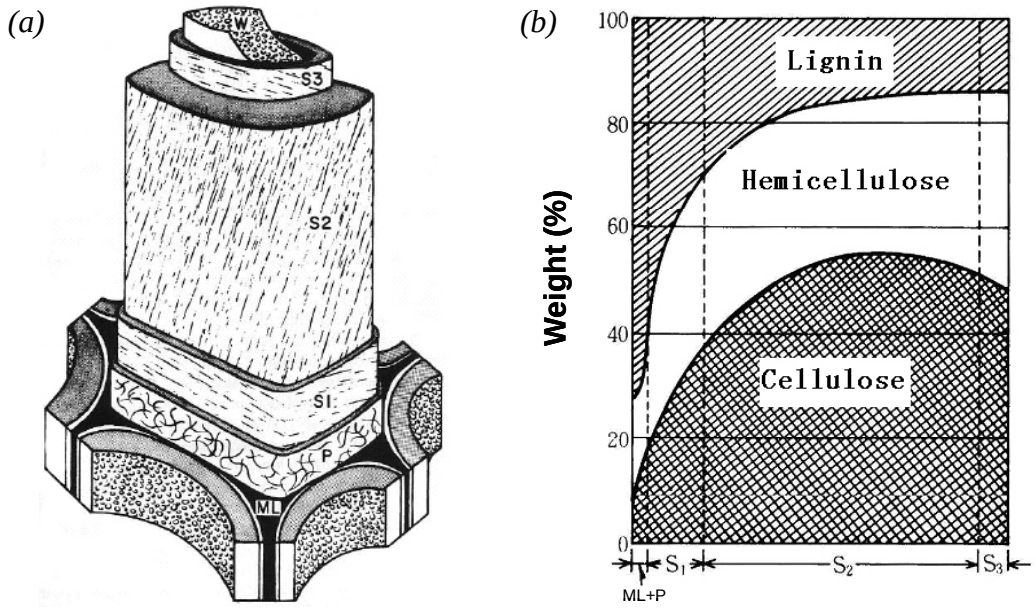

Figure 1.1 *(a)*. Simplified structure of a woody cell, showing the middle lamella (ML), the primary wall (P), the outer (S1), middle (S2), and inner (S3) layers of the secondary wall.[3]

(b). The distributions of wood cell wall components in hardwood.[4]

1.1.1 Polysaccharides in plant cell wall

Cellulose and various hemicelluloses are main polysaccharides found in the plant cell walls, and occupied around 50% and 20-30% in the wood materials, respectively.[3] Cellulose is predominantly found in the secondary cell wall along with the bulk of the lignin. Among the three main polymer components in plant cell wall, cellulose is well-characterized and is generally described as a long chain biopolymer of (1→4)- β-D-glucopyranose units, as shown in **Fig. 1.2**.[3]

The term "hemicellulose" is used for polysaccharides that normally occur in plant tissues together with cellulose and can be isolated by extraction either with water or, more frequently, aqueous alkali.[5-7] Galactoglucomannan is the principal hemicellulose in softwoods (about 20%). Their backbone is a linear chain built up of (1,4)-linked β-D-anhydroglucopyranose and β-D-anhydromannopyranose units.[3] Even if hemicelluloses in various hardwood species differ from each other both quantitatively and qualitatively, the major component is an *O*-acetyl-4-*O*-methyl glucurono-β-D-xylan, sometimes called glucuronoxylan. Often the xylose-based hemicelluloses in both softwoods and hardwoods are termed simply xylans.[3] The hemicelluloses together with cellulose and lignin, build up the cell walls in a fashion that gives combination of mechanical support and transport properties.[3,8,9]

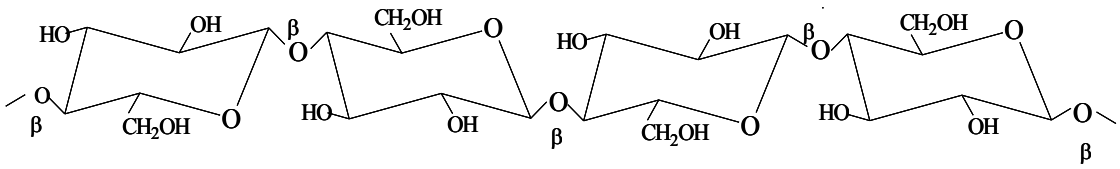

Figure 1.2 Structure of cellulose.

1.1.2 Lignin in plant cell wall

Lignin is generally distributed in the spaces of primary and secondary walls.[3] The functions of lignin are considered to connect cells to one another and to make the cell walls of xylem tissues hard and repellent to water. Lignins also gives rigidity to the cell walls and, in woody parts, act as permanent bounding agents between cells generating a composite structure outstandingly resistant not only physically towards impact, compression and bending, but also chemically towards many types of aging reactions including those brought about biologically. Thus, lignins play many parts in the function of the whole plant, such as protection against predators, storage of nutrients, skeletal structure within the plant.[3,10] The multiple functions of lignins are essential to the life of the plant.

Lignin is a heterogeneous macromolecule that represents one of abundant natural polymeric material on earth.[11] Lignin is an aromatic biopolymer composed by phenylpropane (or C9) units of the p-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) types **(Fig. 1.3)**. These monomeric units are linked in lignin macromolecule by various ether and C-C bonds.[11,12] It is known that the bulk of lignin in wood consists of aryl-glycerol-β-O-aryl ether (β-O-4) units as a major linkage unit[13] together with less abundant other units, such as phenylcoumaran (β-5), resinol (β-β), biphenyl (5-5), diaryl ether (4-O-5), and dibenzodioxocins (5-5/β-O-4, α-O-4).[14] Furthermore, lignin is most presumably covalently linked to carbohydrates[15,16] forming a lignin-carbohydrate network made up of benzyl-ether,[15,17] benzyl-ester,[15,18] and phenyl-glycoside[19,20] bonds although direct evidence for these bonding has not been obtained except for benzyl ether type. [21]

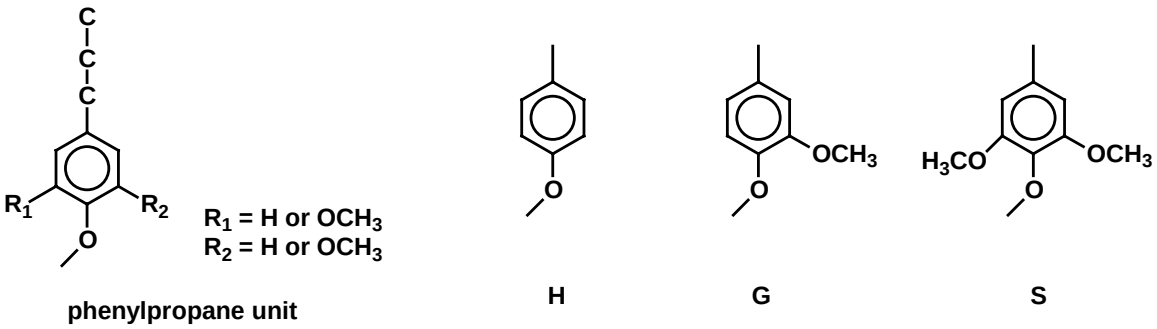

Figure 1.3 Phenylpropane (C9) unit, p-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) types of aromatic nuclei.

1.1.3 Lignin-carbohydrate network in plant cell wall

Many reports have proposed linkages between lignin and carbohydrates (LC bonds) to exist in wood and chemical pulp.[22-26] The occurrence of stable lignin-carbohydrate (LC) bonds is one of the main reasons preventing selective separation of the wood components in biorefining processes. For example, in chemical pulping, cellulose dominates the surface chemistry, however, precipitation of lignin onto the fibers during the latter stages of pulping may alter this fact.[27] Bleaching is often used to remove the surface lignin, which causes discoloration or darkening of the pulp, making it unsuitable for use in the subsequent manufacture of fine paper grades.[28] Thus, understanding interactions between lignin and carbohydrates is of great interest in the functionalization of wood components and their applications. Linkages between lignin and carbohydrates also create significant problems in the selective isolation of lignin fraction from lignocellulosics. In spite of extensive studies on lignin and lignin-carbohydrate complex (LCC) chemical structures our knowledge in this field is still insufficient. Further progress requires development of new analytical methodologies.

1.2 Analyses of lignin in plant cell wall based on the isolation procedures

Most methods for lignin analysis require the isolation of lignin fractions from lignocellulosic materials. Important structural features of lignin have been obtained by the isolation of lignin and subsequent analysis by the sophisticated methods, for example, NMR. The most of degradation methods, such as alkaline nitrobenzene oxidation, permanganate oxidation and ozonation analysis, can usually be conducted without isolation of lignin. But in order to take advantages of sophisticated analytical methods, isolation of lignin without undesirable structural changes becomes more important.

Among many isolated lignins, MWL (Milled Wood Lignin), CEL (Cellulolytic Enzyme Lignin) and Björkman LCC (Lignin-Carbohydrate Complex) seem to be the most desirable because no chemical treatments are involved for the preparation of these lignins except enzymatic hydrolysis employed for CEL preparation, and therefore less structural modification during its isolation procedures could be expected. However, main problems in lignin isolation are associated with the complex structure of the cell wall and the interaction of its components. As almost all lignin is linked to polysaccharides, it is impossible to isolate pure lignin without any chemical degradation. An appropriate isolation procedure should produce a representative lignin preparation and minimize structural changes during isolation.[29-32]

1.2.1 Isolation of Milled Wood Lignin (MWL)

Isolation method of milled wood lignin (MWL) was originally proposed by Björkman, who extracted lignin from finely ball-milled wood with aqueous dioxane.[29] (**Fig. 1.4**)

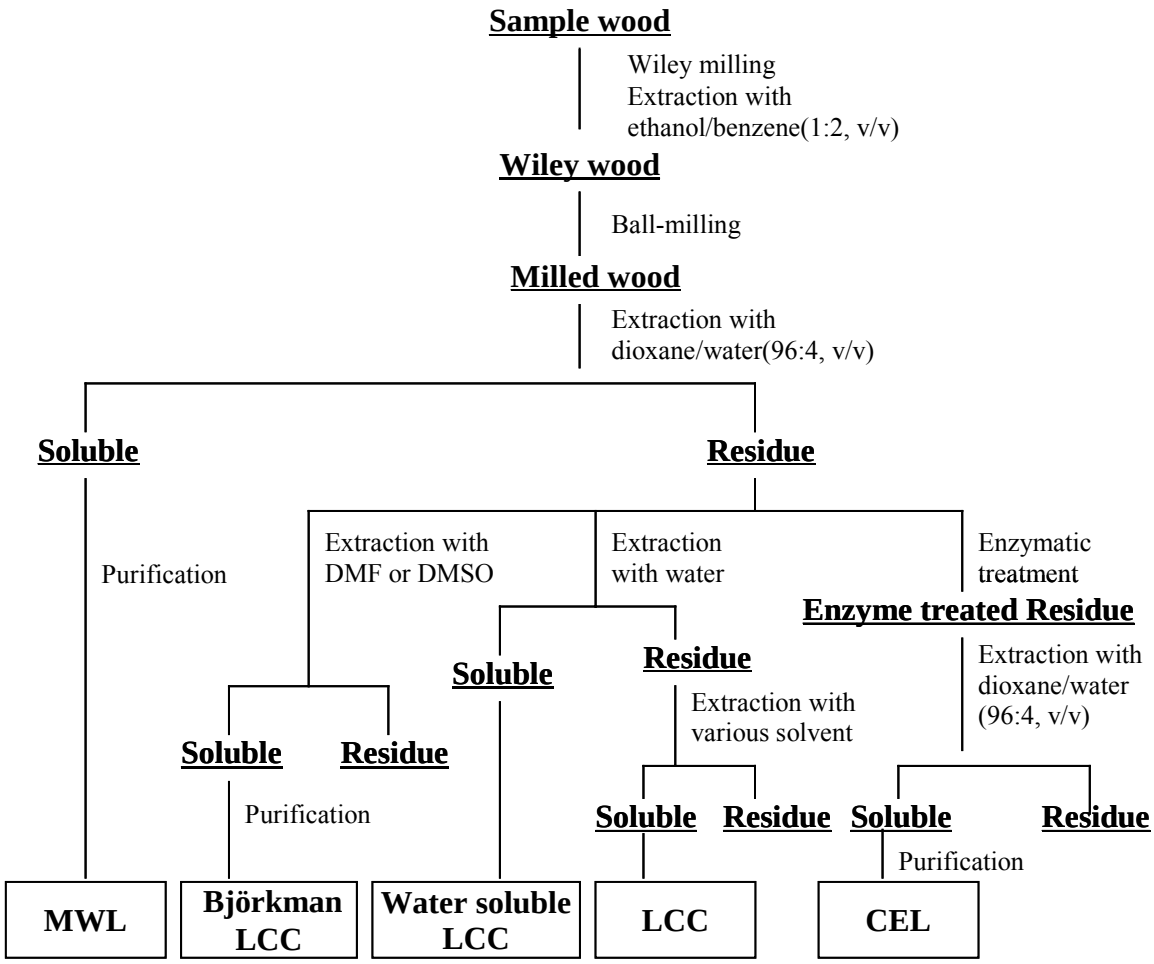

Figure 1.4 Isolation procedures of MWL, CEL, and LCC.

As the morphological origin of MWL, there are opposite opinions based on different fractionation and analysis methodologies. Lee *et al.*[33] revealed that a MWL of earlier milling time was rich in the guaiacyl unit and longer milling time resulted in a gradual increase of syringyl unit by determination of the five MWL fractions prepared from birch wood at different milling times with the sequential extractions. The results further indicated that the MWL fraction initially extracted from wood meal primarily originated from the compound middle lamella region, which was suggested to be rich in guaiacyl

unit,[34-36] and secondary wall lignin became extractable gradually with longer milling time. Later Matsumoto *et al*.[37] observed by ozonation analysis that lignin with lower *erythro/threo* ratio of the β-O-4 structure was first extracted as MWL, and with the progress of milling time, lignin with higher *erythro*/*threo* ratio gradually became extractable. According to the results obtained by Akiyama *et al*.,[38,39] the high *erythro*/*threo* ratio is associated with high S/G ratio, thus, high *erythro*/*threo* ratio could also be a characteristic of secondary wall lignin. Therefore, the ozonation results also suggest that MWL is rich in a fraction derived from compound middle lamella at least at the initial stage of milling.

On the other hand, Whiting *et al*.[40,41] concluded that MWL is not representative of the whole lignin in wood but originates primarily from the secondary wall of the cell by fractionation of finely ground spruce wood into high and low lignin content fractions with differential sedimentation methods and the successive milling (1.5 h) and MWL extraction of each fraction. They explained that the fractions of high lignin content (60%) were rich in compound middle lamella tissue and from which only 10.4% MWL could be obtained. The fractions of low lignin content (22%) were rich in tissue from the secondary wall, and from which the yield of MWL is 39.2%. However, it should be considered that milling effect on each morphological region could be different depending on whether each region is present in undestroyed cell or present as an isolated form as Whiting's sample. From this point of view, Whiting's sample could not be representative of compound middle lamella lignin fraction *in situ*,[42] and their conclusion does not seem to be fully justified.

The 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. This is currently the most common procedure for the isolation of lignin from wood. However, usually the yield of MWL is relatively low in spite of rather high yields reported by Björkman.[29] Furthermore, lignin yield is dependent on milling time.[43] Therefore,

concerns exist over the similarity between MWL and native lignin because of the low yields and structural alterations due to ball-milling. It has been debated long time whether MWL represents the whole lignin in wood or not. There are two main issues when transferring knowledge on the structure of MWL to lignin *in situ*: how representative of the whole wood lignin MWL preparations with typical yields of 20-30% are, and how significant lignin degradation is during the milling.

1.2.2 Isolation of Cellulolytic Enzyme Lignin (CEL)

Further improvements in the yield of lignin isolation from ball-milled wood have arisen through the use of cellulolytic enzymes[29,44,45] to remove carbohydrates prior to aqueous dioxane extraction. Cellulolytic enzyme lignin (CEL) was found to be structurally similar to MWL,[29] but was obtained in higher yield with less degradation, and hence is more representative of the total lignin in wood.[29] More recently Chang *et al*.[44,45] isolated lignin by extracting MWL first, and the residue was treated with cellulolytic enzymes followed by 96% aqueous dioxane extraction to isolate CEL.[44] The isolation procedure to obtain CEL are shown in **Fig. 1.4**. Comparison of the chemical structure of MWL and CEL using chemistry and modern NMR spectroscopy revealed that MWL is slightly more condensed than CEL, suggesting that MWL may contain a higher proportion of lignin from the middle lamella.[44-46]

Argyropoulos *et al*.[13,47] suggested a more complex 3-stage isolation procedure involving ball-milling, enzymatic hydrolysis followed by acidolysis of the residue to obtain enzymatic mild acidolysis lignin (EMAL). This method provides higher yields of lignin preparations at the same milling time than the CEL protocol. However, the maximal yield obtained (below 75%)[13,48] was lower than the that of CEL obtained without acidolysis (up to 86%).[49] The lignin released after the enzymatic hydrolysis was extracted first (equivalent to CEL) and then only the insoluble residue was used for

acidolysis to avoid the acidic degradation of the CEL fraction.[50]

No important differences have been detected in the chemical structure of lignin in MWL (with yields above ca 20%) and CEL[13,47,49,51,52] except the degradation of some acid-labile lignin units in EMAL.[50] Some structural differences observed[48] were very likely due to very low yields of the MWL used for comparison. As purified MWL contains much less carbohydrates than the corresponding CEL, it is more suitable for precise analysis of the lignin structure. While, CEL can be very useful for the analysis of lignin-carbohydrate linkages. Complete enzymatic hydrolysis of milled wood results in a preparation called milled wood enzymatic lignin (MWEL).[53] MWEL is very representative as it contains most of the lignin-carbohydrate linkages of the original wood. However, in contrast to CEL it is not completely soluble. This limits its analysis with high-resolution spectroscopic methods. Moreover, as LC bonds are partially degraded during acidolysis, the use of EMAL preparation for the analysis of LCC is less suitable than that of CEL. At the same time, much higher carbohydrate content in the EMAL than that in purified MWL results in less accurate quantitative lignin analysis. Therefore, optimal application of EMAL preparation is still to be found.

Furthermore, the effect of milling on the structure of MWL and CEL lignin is independent of the milling intensity and apparatus used (but might be dependent of milling time or milling degree).[49,54] In contrast, the composition of EMAL is significantly affected by milling intensity.[13] This implies that intensive milling affects the subsequent acidolysis stage of the EMAL protocol.[13] Therefore, the optimized EMAL method requires very mild milling resulting in very long experimental time. For example, intensive ball-milling can produce ca 30% yield of MWL (or 60% yield of CEL) within 1-2 hours. However, to obtain ca 45% yield of EMAL, a mild milling of almost one month is required.[13]

1.2.3 Isolation of Lignin-Carbohydrate Complex (LCC)

Originally, LCC (Lignin-Carbohydrate Complex) is a conceptional term that expresses the existing manner of lignin and carbohydrates that are present together in the cell wall not only as a simple mixture but also as a complex with strong chemical or physical interaction. Most researchers assume that there is a chemical linkage between lignin and carbohydrates. Considering that any kind of lignin preparation is not free from the co-existing carbohydrates, or contrary, any kind of carbohydrates preparation from wood is not free from co-existing lignin, even isolated lignin or carbohydrates can be used to investigate the nature of LCC.

On the other hand, term of LCC is quite often used to indicate specific fraction obtained from cell wall. Various LCC samples can be obtained by methods similar to those used for lignin isolation. For example, partial enzymatic hydrolysis of the milled wood followed by separation allows the isolation of various LCC fractions from wood and pulps.[26] Extraction of the wood residue with DMSO after extraction of MWL produces Björkman LCC [22] (**Fig. 1.4**). Other solvents and water were also used to extract LCC from the residue obtained after MWL isolation (**Fig. 1.4**).[55,56] Most of lignin and LCC samples obtained give information on lignin, carbohydrates and LCC structure and from this point of view can be normally defined as carbohydrate-rich LCC (Björkman LCC and similar ones) and lignin-rich LCC (MWEL, CEL).

Aimi *et al.*[55,56] extracted LCC with low lignin content (5.3%) with water from the residue of milled wood lignin (MWLR). The gel filtration chromatography analyses of the obtained LCC, LCC treated with carbohydrate-degrading enzymes, and that treated under mild alkaline condition indicated that lignin in this LCC is present as small fragments attached to high molecular weight polysaccharide at least partly by alkali-unstable linkages. They[55,56] also revealed from ozonation analysis that the lignin in this LCC was richer in the threo type structure carrying C-aryl linkages at β-position

(β-5 and/or β-1) than other lignin fractions present in MWL, LCCs extracted by other solvents (dimethylformamide, dimethylsulfoxide, and others), and their extraction residues. This result suggested that the lignin in this fraction is rich in detached side-chain structure such as β-1 structure. This suggestion was later confirmed by the finding that the content of glyceraldehydes-2-aryl ether structure was significantly higher than other fractions.[57] In addition, from nitrobenzene oxidation analysis they indicated that the syringyl/guaiacyl (S/V) ratio of this LCC was higher than other lignin fractions. These results suggested that the chemical structure of lignin closely associated to carbohydrates was different from that of the main part of lignin. It is likely that lignin existed near lignin-carbohydrate linkages has more endwise-type features than other lignin fractions.

Although it is very difficult to estimate the frequency of LC bonds, several trials have been made. For example, according to a trial performed by Obst, the frequency of LC bonds in wood is estimated to be only about 0.03 per C9-unit.[53] The low frequency of LC bonds is the major problem in detailed analysis of most LCC samples. From this point of view LCC-AcOH is very interesting as this LCC is proposed to contain large amounts of LC linkages.[58] LCC-AcOH is obtained during the purification of the crude MWL with 90% AcOH.[58] Similar preparation (LCC-W) was obtained earlier[59] using a more complex protocol. In spite of a lower yield, as compared to Björkman LCC and others,[22,55,56] it is not proper to regard LCC-AcOH less representative as the frequencies of LC bonds is proposed to be much higher. It has been reported[58] that LCC-AcOH qualitatively represents both the middle lamellae and secondary wall regions of the cell wall. However, whether the amounts of various LC linkages in the LCC-AcOH are proportional to their amounts in the whole cell wall is still under investigation.

1.2.4 Effect of ball-milling on the structure of plant cell wall components

As all the methods discussed require ball-milling pretreatment, for example, the yield of MWL varies depending on the extent of milling, ranging from 25 to 50%.[43] It is believed that by increasing the extent of milling, and thus MWL yield, a lignin sample more representative of the total lignin in wood is produced.[43,44] However, it is proposed that severe chemical modification of the lignin occurs during ball-milling. Therefore, it is important to understand how significant lignin and/or other wood cell wall components are degraded during the milling.

It is known that some changes in the lignin structure occur during ball-milling, such as increases in carbonyl content[22,30] and phenolic hydroxyl content, as well as decreases in molecular weight[30] and cleavage of aryl ether linkages.[60] Recent studies showed that ball-milling did not cause changes in aromatic ring of lignin units, but resulted in some cleavage of β-O-4 structures in the whole wood lignin.[44,49,54] Namely, ball-milling primarily affects the side-chain structure of the C9 phenyl-propane units in lignin.[44,54] Ikeda *et al*.[44] 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. It was found that phenolic β-O-4 structures increased continuously at the expense of etherified β-O-4 structures during ball-milling. It indicated that ball-milling reduces the degree of polymerization, creating new free phenolic hydroxyl groups through cleavage of β-aryl ether linkages and α-carbonyl groups increases via side chain oxidation.[30,44,54]

Recently, Fujimoto *et al*.[54] used ozonation to quantitatively analyze the change in β-O-4 linkages in sweet-gum lignin from wood meal under different ball-milling conditions. They observed a steady decrease in β-O-4 linkages with increasing ball-milling intensity even at the same milling time. On the other hand, they revealed the dependence of changes in lignin structures on the extractable lignin yield. The facts

that the *erythro* ratio (E/T ratio) and the total yield E+T decreased linearly with the increase in the extractable lignin yield suggested that extractable lignin yield is a better indication of the extent of ball-milling than milling time.[54] When milling conditions, apparatus, intensities, and so on are different, milling time cannot be employed as a criterion of milling degree to compare milling effect on lignin structure. Instead, the yield of extractable lignin from milled wood can be used as a general criterion of milling degree.

Besides lignin, some reports also concerned the effect of ball-milling on the cellulose and/or hemicellulose fractions as they also act as main components in plant cell wall.[61-63] The milling was considered to have influence on the degree of polymerisation (DP) and the crystallinity of ground cellulose and on the carboxyl content. As the effect of ball-milling on the ultrastructure of plant cell walls, the studies made by Maurer and Fengel[64] showed that the compound middle lamellae and the cell corners are most resistant to milling. The secondary wall 1 (S1) is very sensitive to mechanical stress as it is loosened at an early stage of milling and is separated from the compound middle lamella as well as from the S2 layer. Fengel observed a total breakdown of the cell-wall structure during intensive ball-milling.

1.3 Promising methodologies for plant cell wall analyses based on the solubilization procedures

The plant cell walls are structurally complex composite materials consisting of cellulose, hemicelluloses and lignin, which interact with each other physically and chemically, forming a network structure. The diversity of polymers in the wall and their chemical and physical associations make it difficult to isolate the component polymers in pure form. Because of its highly regular and crystalline form, cellulose is most readily separated, although it is an energy-demanding process to free cellulose from hemicelluloses and lignin. Wood cellulose is purified industrially in the kraft-pulping processes by typically treating the cell walls in approximately 1-2 M caustic soda and sodium sulfide at 170℃ for 2 h. Hemicelluloses comprise a class of polysaccharides with considerable structural complexity. Fractions can be isolated by various extraction procedures, but these are usually accompanied by degradation.[65] The difficulty and problems regarding the extraction and isolation of lignin were already mentioned in the previous sections.

Analysis of the lignin component has been achieved by destructive methods, or by spectroscopic methods such as nuclear magnetic resonance (NMR) measurement of isolated lignins.[14,66] Solid-state NMR does not have the resolution required to provide sufficient structural detail. As the yield of MWL has been considered insufficient to represent the whole lignin in wood, significant efforts were made recently to increase the yield of the isolated lignins. The increase in the extent of milling is considered to be one of such methods. However, it is proposed that unavoidable chemical modification of the lignin occurs during ball-milling as mentioned before.

Thus, the method to dissolve whole plant cell walls without severe degradation would provide promising and significantly improved methods for cell wall structural analysis

and allow standard solution-state derivatization and other chemical reactions to be more effectively applied. In addition, because isolation methods currently employed for the study of each components are based on the degradation of other components, it is principally impossible to observe the interaction between each cell wall components. Therefore, it is reasonably expected that such a solubilization method will not only provide more detailed information of each cell wall component bust also give a new insight into the interaction of cell wall components which can never be obtained by hitherto existing methods.

1.3.1 Solubilization of cellulose, holocellulose, and chemical pulp

In recent decades, various solvent systems have been found to dissolve cellulose.[68-72] The system N,N-dimethylacetamide/LiCl (DMAc/LiCl) shows an enormous potential for the analysis of cellulose and for the preparation of a wide variety of derivatives. However, several problems were reported when this solvent system is applied to pulp dissolution. When softwood bleached kraft pulp with kappa number 18 is applied to this system, the dissolution is incomplete.[73,74] Berthold *et al*. reported that unbleached softwood kraft pulp by a derivatization with ethyl isocyanate could be dissolved in 8% DMAc/LiCl.[75] The mixture 1,3-dimethyl-2-imidazolidinone (DMI) and LiCl was found to be suitable to dissolve cellulose.[76,77] The advantages of the nowadays commercially available DMI lies in its thermal stability and low toxicity. DMI/LiCl is able to dissolve cellulose samples with DP values as high as 1200 and concentrations of 2-10% (w/w) when applied in the same procedure as used for DMAc/LiCl, where an activation of the polymer by a heat treatment or a step-wise solvent exchange is absolutely necessary. Experimental details for a simple dissolution procedure are given in the literature.[78,79] However, more than 2 weeks were required to dissolve softwood kraft pulp although the lignin content was relatively low, about 2%.[80] A novel solvent

for cellulose consists in the mixture DMSO/tetrabutylammonium fluoride trihydrate (TBAF). The advantage of DMSO/TBAF is that cellulose with a degree of polymerization as high as 650 dissolves without any pretreatment within 15 min. An interesting finding was that no dissolution occurs if the fluoride is changed to other halides.[81] It should be mentioned that the solutions contain a certain amount of water because TBAF is used as commercially available trihydrate and the cellulose is air-dried only. Thus, there is no good solvent system for dissolving pulp with high lignin content without derivatization. The development of the solvent system for pulps with high lignin content is required not only to examine the nature of residual lignin and interactions between pulp components but also to utilize such pulps as a new source of fibrous materials.

1.3.2 Solubilization of wood meal

One promising method to analyze entire lignin fraction in cell wall is an application of complete wood solution. Spectroscopic analyses, for example, solution state NMR analysis, of such a solution may bring about new information on the entire lignin fraction, which can never be obtained by analyses of any isolated lignin. Kilpeläinen *et al*.[82] demonstrated that both hardwoods and softwoods are dissolved in various imidazolium-based ionic liquids under gentle conditions. However, the complete wood dissolution can be achieved at high temperature (80-120℃). Becausse ionic liquids are rather complicated, it must be difficult to apply wood solution in these solvents for the direct spectroscopic analyses, such as NMR and UV absorbance. In addition to this, Kubo *et al*.[83] reported that a non-phenolic β-O-4 type lignin model compound is almost completely converted into the corresponding enol ether compound during the dissolution process in imidazolium-based ionic liquids, which indicates that ionic liquids are not inert to lignin. Lu and Ralph[67] described that finely ground plant cell

wall including wood can be dissolved in dimethyl sulfoxide and tetrabutylammonium fluoride (DMSO/TBAF) or dimethyl sulfoxide and N-methylimidazole (DMSO/NMI) solvent systems. An ability to dissolve wood meal without serious degradation of lignin would provide significantly improved methods for the analysis of the entire lignin fraction. By the use of DMSO/NMI solvent systems, they suggested a method to prepare “acetylated cell walls”, which allowed the characterization of wood components by solution-state NMR that provides much higher resolution than solid-state NMR. However, the DMSO/NMI solvent system requires a rather long milling time to dissolve wood meal. It should be emphasized that milling of wood meal always causes structural change of lignin depending on the degree of milling as described in previous sections.[49,54] Therefore, efforts are still needed to develop a novel solvent system that can completely dissolve wood meal with as short a milling time, i.e. as less structural change of lignin, as possible.

1.4 Objective of this work

The main components of lignocellulosic material are polysaccharides (cellulose and hemicelluloses) and lignin. Lignin has complicated relationship with polysaccharides, which will affect the efficiency of polysaccharides utilization such as bio-ethanol production. Therefore, it is significant for us to clearly understand the chemical characteristics of "*in-situ* lignin" and its relation to other components in the plant cell wall for the efficient utilization of lignocellulosic material in more economically and environmentally-friendly way. One promising method to analyze entire lignin fraction in cell wall is an application of complete wood solution. Spectroscopic analyses, for example, solution state NMR analysis, of such a solution may bring about new information on the entire lignin fraction, which can never be obtained by analyses of any isolated lignin.

Wherein, the objective of this work is focused on dissolution of lignocellulosic materials in common solvents and chemical analysis of plant cell wall components and elucidation of the structures using the complete lignocellulosic solutions. The author tried to develop new solvent system for solubilization of lignocellulosic materials and to evaluate the achievement of dissolution of lignocellulosic materials by using different pretreatment methods, which cause less or no degradation of lignocellulosics compared to conventional methods. The second object of this work is to establish new fractionation method of cell wall components by the use of developed solvent system.

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Chapter 2

Dissolution of milled wood and characterization of wood solution

2.1 Introduction

The main components of wood cell wall are cellulose, hemicellulose, and lignin. Because monolignols polymerize in the cell wall and are embedded in already accumulated polysaccharide gels, molecular associations and covalent bonds are possibly produced between lignin and carbohydrates.[1] Phenylpropane units are linked by various types of ether and C-C bonds in a lignin macromolecule, and the frequency of each bonding type varies depending on the portion in the cell wall and on the kind of cell. Such diversity of polymers in cell walls and the chemical and physical associations with carbohydrates make it impossible to isolate the entire lignin fraction without serious degradation.[2-4] Consequently, analysis of complete lignin polymer has not been achieved by the use of isolated lignin.[3,4] Milled wood lignin (MWL) has commonly been used for the analysis of cell wall lignin. However, preparation of

MWL requires rather extensive milling, causing noticeable changes in lignin structure depending on the degree of milling.[5-8] Recently, Fujimoto *et al*. succeeded in quantitatively expressing the relationships between the degree of milling and structural change of lignin caused by the milling of wood meal.[7,9] In addition, the chemical structure of MWL is strongly dependent on which portion of cell wall is its origin. It is known that the occupation of compound middle lamella lignin is greater in the extracted MWL at the early stage of milling than that at the later stage.[4,10] Therefore, it should be emphasized that there are some limitations in discussing the structure of cell wall lignin on the basis of the structure of MWL. The effect of milling and the effect of the portion in the cell wall on the lignin structure were termed as "artificial change" and "native difference", respectively.

One promising method to analyze entire lignin fraction in cell wall is an application of complete wood solution. Spectroscopic analyses, for example, solution state NMR analysis, of such a solution may bring about new information on the entire lignin fraction, which can never be obtained by analyses of any isolated lignin. As such a solution system, ionic liquids have attracted wide attention recently. Kilpeläinen *et al*. demonstrated that both hardwoods and softwoods are dissolved in various imidazolium-based ionic liquids. However, the complete wood dissolution can be achieved at high temperature (80-120℃).[11] As ionic liquids are rather complicated, it seems to be difficult to apply these solvents for the spectroscopic analyses, such as NMR and UV absorbance. In addition, change of lignin structure during the dissolution process can't be overlooked. Kubo *et al*. reported that a nonphenolic β-O-4 type lignin model compound is almost completely converted into the corresponding enol ether compound during the dissolution process in imidazolium-based ionic liquids, which indicates that ionic liquids are not inert to lignin.[12] Conversion of β-O-4 linkage into enol ether linkage is fatal disadvantage of this solution system for analytical purpose because the stereo chemical characteristics of β-O-4 linkage, which is one of the most

important structural feature of lignin, is lost by this conversion. Furthermore, because the enol ether structure is very unstable under oxidative or acidic condition, lignin is converted into unstable form by the dissolution into ionic liquids, which is a serious disadvantage for the analytical purpose.

On the other hand, Lu and Ralph described that finely ground plant cell wall including that of wood can be dissolved in dimethyl sulfoxide and tetrabutylammonium fluoride (DMSO/TBAF) or dimethyl sulfoxide and N-methylimidazole (DMSO/NMI) solvent systems. If a serious change of lignin doesn't take place during the whole process, an ability to dissolve wood meal would provide significantly improved methods for the analysis of the entire lignin fraction.[13] However, the DMSO/NMI solvent system requires a rather long milling time to dissolve wood meal. It should be emphasized that milling of wood meal always causes structural change of lignin depending on the degree of milling.[7,9]

Therefore, in this chapter, the effort was made to develop a novel solvent system which can completely dissolve wood meal with as short a milling time as possible. It was reported recently that lithium chloride/dimethyl sulfoxide (LiCl/DMSO) can be used as a solvent system for the dissolution of regenerated cellulose.[14] In this chapter, the results when a LiCl/DMSO solvent system was applied to the dissolution of milled wood will be described. Because DMSO-d6 is commercially available, wood solution can be readily subjected to the measurement of NMR, which can be a significant advantage of this solution system.

2.2 Experimental

2.2.1 Materials

Beech (*Fagus crenata* Blume) and spruce (*Picea abies*) woods were ground in a Wiley mill and extracted with ethanol/benzene (1:2, v/v) for 8 h. The extracted Wiley wood was dried under air and subsequently under vacuum. The dried Wiley wood (2 g) was milled in a planetary ball-mill (Fritsch GMBH, Idar-Oberstein, Germany) for 1-10 h to give milled woods with different milling degrees. A zirconium dioxide bowl (45 ml) with 18 zirconium dioxide balls (1 cm diameter) was used in the milling. The milling frequency was 600 rpm. The milling was conducted in a cold room (-20℃), and 5 min intervals were provided between every 15 min of milling to prevent overheating. The process to obtain milled wood is illustrated in **Fig. 2.1**.

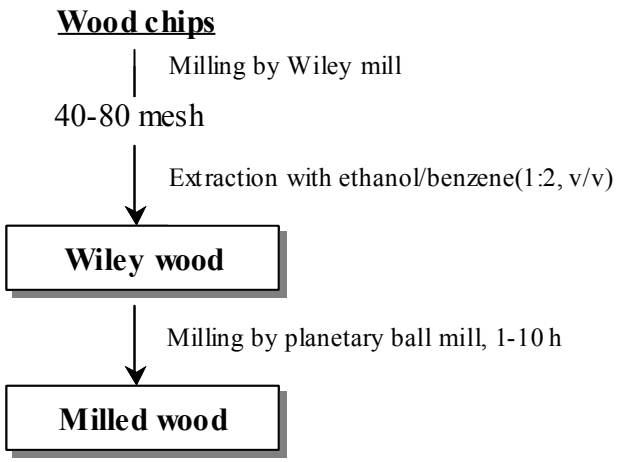


Figure 2.1 Preparation of beech and spruce milled woods.

2.2.2 Determination of lignin content and extractable lignin yield

The Klason method[15] was used for the determination of lignin content in the beech Wiley and milled woods.

The yield of extractable lignin from the milled wood was determined according to the method described by Fujimoto *et al*.,[7] and calculated as the weight percentage based on the total lignin content (Klason residue + acid soluble lignins) of the corresponding milled wood. The milled wood (20 mg) was extracted with dioxane/water (10 ml, 96%, v/v) for 2 days. After centrifugation, $NaBH_4$ (1 mg in 1 ml of 0.05 M NaOH) was added to the supernatant (5 ml) and kept for 1 day. The solution was filled up by acetic acid to 10 ml. The amount of extracted lignin from the milled wood was estimated based on the UV absorbance at 280 nm of the solution by the use of 13 as gram absorptivity.[7]

2.2.3 Alkaline nitrobenzene oxidation

Alkaline nitrobenzene oxidation analyses were applied to the Wiley and milled woods according basically to the common procedure.[16] Samples (10 mg) were added to 4 ml of 2 M NaOH and 0.25 ml nitrobenzene, and the mixture was kept at 170 ℃ for 2 h. As the internal standard solution, 1 ml of 0.1 M NaOH solution containing 3-ethoxy-4-hydroxybenzaldehyde (0.4-0.2 g/l) was added. The reaction mixture was extracted three times with dichloromethane (15 ml). The aqueous phase was acidified with 4 M HCl to ~pH 1 and extracted twice with dichloromethane (20 ml) and once with ethyl ether (15 ml). The combined organic solvent phase was washed once with water (20 ml) and then dried over Na_2SO_4. After the removal of the insoluble inorganic materials by filtration, the solution was evaporated to dryness and silylated with N,O-bis(trimethylsilyl)acetamide (BSA) at 100℃ for 10 min and, then, analyzed by gas chromatography under the following conditions.

Gas chromatography: GC-17A with FID (Shimadzu Co., Kyoto, Japan)

Column: NB-1 (fused-silica capillary column, 30 m, 0.25 mm i.d) (GL Science Inc., Tokyo, Japan)

Column program: kept for 15 min at 150℃, raised by 3℃/min to 180℃, and 10℃ /min to 280℃

Injection temperature: 250℃; Detector temperature: 280℃

Syringyl ratio, S/(S+V), was calculated based on the following formula.

S/(S+V) = (syringaldehyde + syringic acid)/(vanillin + vanillic acid + syringaldehyde + syringic acid)

Where syringaldehyde is 4-hydroxy-3,5-dimethoxybenzaldehyde, syringic acid is 4-hydroxy-3,5-dimethoxybenzoic acid, vanillin is 4-hydroxy-3-methoxybenzaldehyde, vanillic acid is 4-hydroxy-3-methoxybenzoic acid.

2.2.4 Ozonation

Ozonation was performed according to the method of Akiyama *et al*.[17] Samples (50 mg) were added to 30 ml of acetic acid/water/methanol (16:3:1, v/v/v). Oxygen containing 3% of ozone was bubbled into the solution at the rate of 0.5 L/min for 2 h at 0℃ with stirring (Nippon Ozone type ON-3-2 was used as an ozone generator). Main ozonation products were erythronic and threonic acids arisen from the *erythro* and *threo* type β-O-4 structures, respectively. Ozonation products were silylated in dimethyl sulfoxide (DMSO) with hexamethyldisilazane (HMDS) and trimethylchlorosilane (TMCS) at 60℃ for 30 min and, then, analyzed by the GC-17A under the following conditions.

Gas chromatography: GC-17A with FID (Shimadzu Co., Kyoto, Japan)

Column: NB-1 (fused-silica capillary column, 30 m, 0.25 mm i.d) (GL Science Inc., Tokyo, Japan)

Column program: kept for 5 min at 120℃, raised by 4℃/min to 170℃, and 10℃ /min to 280℃

Injection temperature: 250℃; Detector temperature: 280℃

Two ozonation products, erythronic acid (E) and threonic acid (T), obtained from *erythro* and *threo* forms of β-O-4 structure respectively, were quantified. The proportion of E to the total amount of E and T gives the *erythro* ratio in β-O-4 structure, E/(E+T), and the total yield of (E+T) gives information on the content of β-O-4 structure.

2.2.5 X-ray diffraction analysis

The Wiley wood and milled woods from beech were converted into pellets using a disk apparatus for IR measurement and subjected to X-ray diffraction analysis from 5° to 35° diffraction angle 2θ using the reflection method by means of a Rigaku RINT 2000 with Ni-filtered Cu Kα radiation (λ= 0.15418 nm) at 40 kV and 40 mA.

2.2.6 Dissolution of milled wood in various solvent systems

Solvent systems examined in this chapter were dimethyl sulfoxide (DMSO) containing 1-6% LiCl (LiCl/DMSO), dimethylacetoamide containing 1-6% of LiCl (LiCl/DMAc), mixtures of DMSO and N-methylimidazole (DMSO/NMI) (2:1, v/v), and a mixture of DMSO and ethylenediamine (DMSO/EDA) (1:1, v/v). The structural formulas of various reagents were shown in **Fig. 2.2**. The milled wood was suspended into the above solvent systems with different concentrations (1-10%, w/w), and stirred at room temperature for 2-24 h. Detailed information is shown in **Table 2.1**.

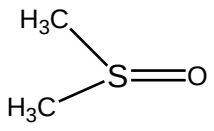
2.2.7 Spectroscopic analysis of complete wood solution

2.2.7.1 NMR analysis

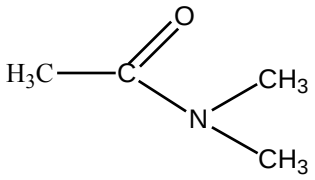
The milled wood (2 h) were dissolved in 6% LiCl/DMSO-d6. The concentrations of wood solutions were 0.5% and 5%, respectively. The analysis of NMR was performed on a JEOL Alpha 500 spectrometer (JEOL, Japan).

2.2.7.2 UV absorbance

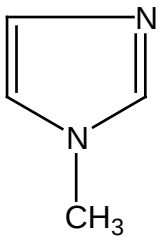
The UV absorbance at 280 nm of the milled wood solutions was determined using a Shimadzu UV-VIS spectrophotometer (UV-240).



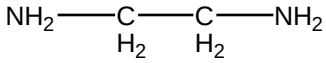
Dimethyl sulfoxide (DMSO)



Dimethylacetoamide (DMAc)



N-methylimidazole (NMI)



Ethylenediamine (EDA)

Figure 2.2 Structural formulas of various reagents.

2.3 Results and Discussion

2.3.1 Dissolution of milled wood in LiCl/DMSO solvent system

Beech and spruce woods were finely milled by planetary ball-milling. The particular milling conditions (milling intensity and temperature) with various milling times were applied to the preparation of milled woods with different degrees of milling. These milled woods were dissolved in many kinds of solvent system to evaluate the ability of each system as a solvent of milled wood. The results obtained for the beech wood are shown in **Table 2.1** and **Fig. 2.3**. Among the systems examined, the 6% LiCl/DMSO system completely dissolved both beech and spruce milled woods prepared by 2 h of milling.

Other various milled woods, prepared from paulownia, eucalyptus, and even nonwood samples such as rice grass, were also completely dissolved in the 6% LiCl/DMSO system by using the 2 h milling. The DMSO/NMI system[13] also dissolved the milled woods completely, but 5 and 6 h milling were required for the beech and spruce woods, respectively.

The concentration of the milled woods in the 6% LiCl/DMSO system was as high as 10%, which was higher than the value (8%) reported for solutions of some ionic liquids.[11] As a role of LiCl in the LiCl/DMSO system in the dissolution of regenerated cellulose, Petrus *et al*. suggested that undissociated ion pairs of LiCl molecules in a polar aprotic solvent (DMSO) might interact with the oxygen atoms of hydroxyl groups and, thus, disrupt and prevent re-formation of hydrogen bonds between cellulose molecules, whereby the dissolution is facilitated. LiCl/DMSO, as a cellulose solvent system, can dissolve only regenerated cellulose and,[14] thus, is not regarded as a general cellulose solvent. LiCl/DMAc is a better solvent system than LiCl/DMSO to dissolve cellulose. On the contrary, LiCl/DMSO is much better solvent system for

milled wood than LiCl/DMAc, as shown in **Table 2.1**. In addition, the degree of lignin degradation during the milling of the milled woods was very small when the milling time was 2 h, as will be seen in the following sections.

Table 2.1 Solvent systems examined for the dissolution of milled woods from beech with different milling time.

Milling time (h)	Property	LiCl/DMSO (wt 6%)	DMSO/NMI (2:1, v/v)	LiCl/DMAc (wt 6%)	DMSO/EDA (1:1, v/v)
1	Solubility[a]	-	-	-	-
	Concentration(%)	1	1	1	1
	Stirring time(h)	24	24	24	24
2	Solubility	+	-	-	-
	Concentration(%)	1-10	1	1	1
	Stirring time(h)	24	24	24	24
4	Solubility	+	-	+	-
	Concentration(%)	1-10	1	1	1
	Stirring time(h)	2	24	24	24
5	Solubility	+	+	+	-
	Concentration(%)	1-10	1-10	1	1
	Stirring time(h)	2	24	24	24
6	Solubility	+	+	+	-
	Concentration(%)	1-10	1-10	1	1
	Stirring time(hr)	2	3	24	24
8	Solubility	+	+	+	-
	Concentration(%)	1-10	1-10	1	1
	Stirring time(h)	2	3	24	24
10	Solubility	+	+	+	+
	Concentration(%)	1-10	1-10	1	1
	Stirring time(h)	2	3	24	24

[a] +, complete soluble. - incomplete soluble.

Because the milled woods containing lignin with relatively small structural change are completely dissolved in the LiCl/DMSO system as shown here, there are many expected potential applications of this system, for example, to the structural analysis of the entire lignin fraction in wood cell wall and isolation of cell wall components.

Other ligniocellulosic materials, such as chemical pulp, cellulose and holocellulose could also be completely dissolved in 6% LiCl/DMSO after 2 h of milling by planetary ball-milling as expected.

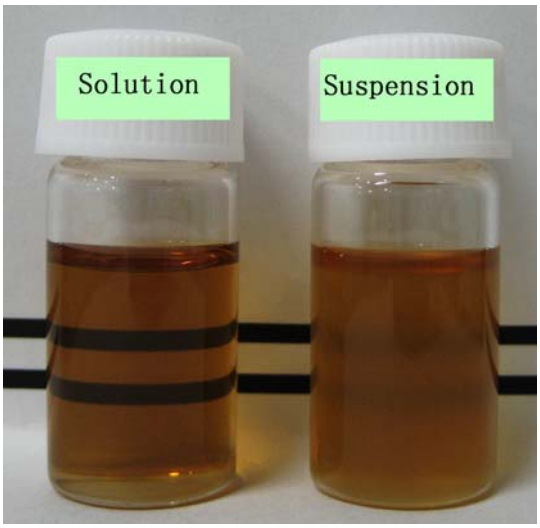


Figure 2.3 Photos of beech milled wood solution and suspension in 6% LiCl/DMSO with 1% concentration after 24 h stirring. (Photos were taken by using white paper marked with two black lines as background. Left: milled wood with 2 h of milling could be completely dissolved in 6% LiCl/DMSO. Right: milled wood with 1 h of milling could not be completely dissolved in 6% LiCl/DMSO.)

2.3.2 Effect of milling on wood features

The maintenance of native structure of entire wood cell wall is an important prerequisite for wood solution when it is applied to various analyses. To dissolve entire wood cell wall components in 6% LiCl/DMSO, 2 h of milling is required. Therefore, effect of milling on wood features by different milling time is necessary to be evaluated.

2.3.2.1 Effect of milling on the structure of aromatic part of lignin

The nitrobenzene oxidation was performed to examine the milling effect on the structure of aromatic part of lignin (**Fig. 2.4**). The syringyl ratio, S/(S+V), was not affected by the milling, but the yield of the nitrobenzene oxidation products, S+V, decreased with the progress of the milling. For examples, the yield decreases were 2.6% and 6.5% after the 4 and 10 h millings, respectively. However, both the yield of S+V and the ratio of S/(S+V) were maintained when 2 h of milling was conducted. These results indicated that the 2 h of milling had almost no or quite low effect on the structure of aromatic part of lignin.

When milling conditions, apparatus, intensities, and so on are different, milling time cannot be utilized as a criterion of milling degree to compare milling effect on lignin structure. Instead, the yield of extractable lignin from milled wood can be used as a general criterion of milling degree. Fujimoto *et al*.[7] and Hu *et al*.[9] showed that when the yields of extractable lignin from milled woods are the same, the structural changes of lignin caused by the milling are similar regardless of the difference in milling conditions and apparatus. **Fig. 2.5** shows the S/(S+V) and S+V values as functions of the yield of extractable lignin. The S+V value decreased with the increase in the extractable lignin yield from the milled wood. However, the changes of both values of

S/(S+V) and S+V were not significant when the milling time was 2 h, in other words, when the yield of extractable lignin from the milled wood reached 23%. On the basis of these results, it can be stated that although the structure of the aromatic part of lignin changes depending on the milling time, the degree of the change is negligible after 2 h of milling.

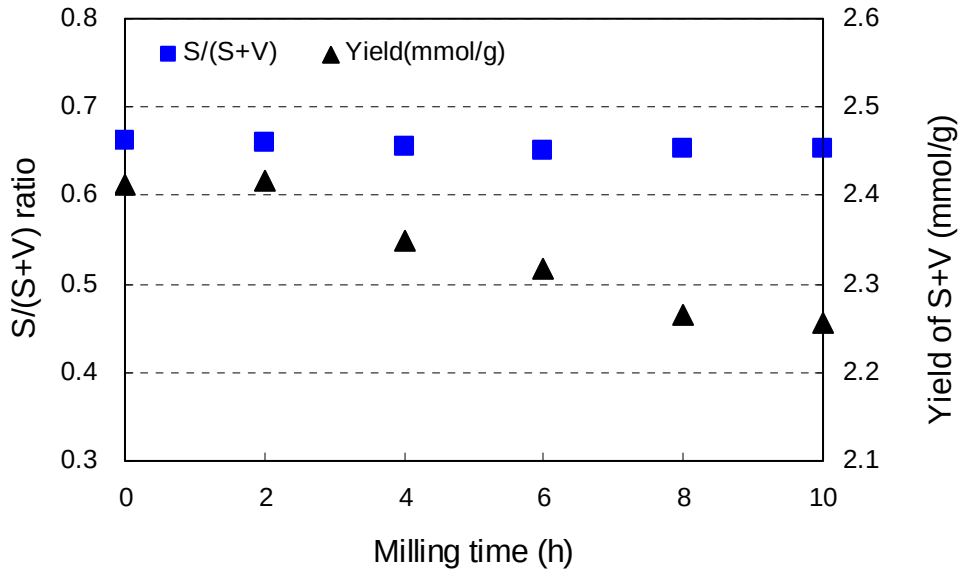


Figure 2.4 Changes of the syringyl ratio (S/(S+V)) and yield of alkaline nitrobenzene oxidation products (S+V) of the beech milled wood prepared by different milling time. (S/(S+V) = (syringaldehyde + syringic acid)/(vanillin + vanillic acid + syringaldehyde + syringic acid))

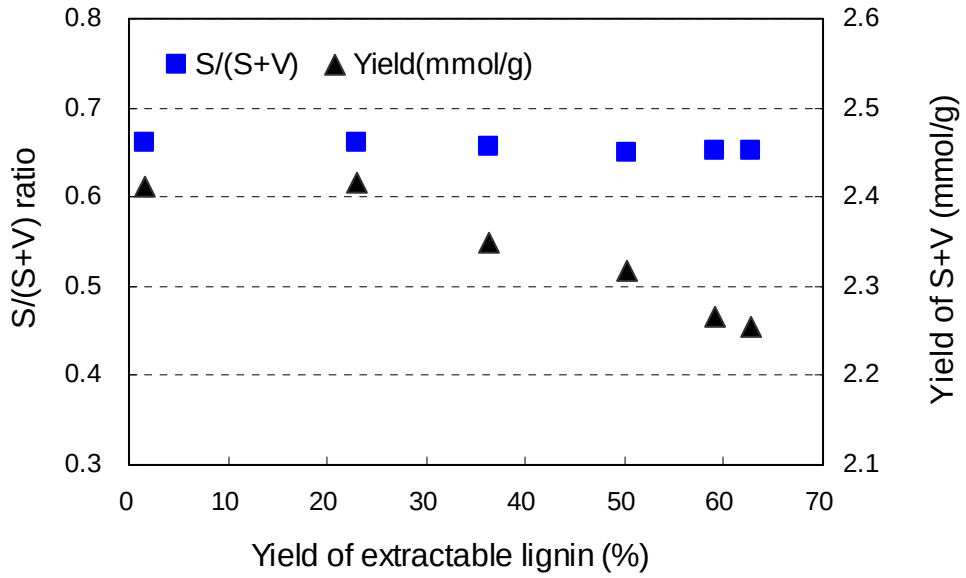


Figure 2.5 Dependence of the syringyl ratio (S/(S+V)) and yield of alkaline nitrobenzene oxidation products (S+V) on the yield of extractable lignin in beech milled wood prepared by different milling time.

2.3.2.2 Effect of milling on β-O-4 structure in lignin

The effect of milling on the β-O-4 structure in lignin was evaluated by applying the ozonation method. The yield ratio and total yield of two major ozonation products from the β-O-4 structure, erythronic acid (E) and threonic acid (T), provide important information on β-O-4 structure.[10,17] Furthermore, the *erythro* and *threo* forms of this structure are known to exhibit different chemical reactivities in some chemical reactions. If this is also the case for the any kind of reaction taking place during milling, even a slight change in the *erythro* ratio (E/(E+T)) should be a good indication for the occurrence of some chemical modifications of β-O-4 structure during milling. From the results shown in **Fig. 2.6**, it was found that both E/(E+T) and the total yield of the ozonation products (E+T) decrease depending on the milling time. The E+T value remarkably decreased from 0.96 to 0.79 mmol/g (17.7% decrease) with 4 h of milling.

The loss of β-O-4 linkage reached to about 26% after a milling time of 10 h. By the 2 h milling, the change of both E/(E+T) (0.35% decrease) and E+T (9.8% decrease) were much more moderate than the longer milling time.

The same results are expressed as a function of the extractable lignin yield (**Fig. 2.7**). This confirms that the β-O-4 structure is degraded during the milling process and that the *erythro* isomer is preferentially degraded, which is consistent well with the results of Fujimoto *et al*. [7] and Hu *et al*.. [9]

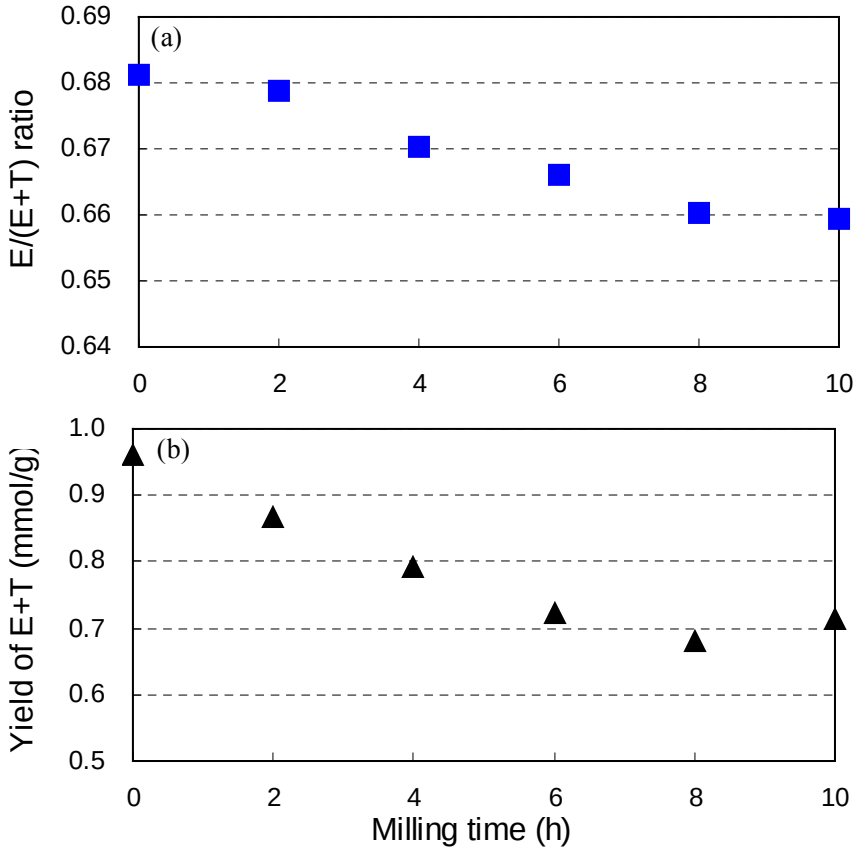


Figure 2.6 Changes of the *erythro* ratio (E/(E+T)) and yield of ozonation products (E+T) from the beech milled wood prepared at different milling times. (a) *erythro* ratio (E/(E+T)) versus milling time. (b) yield (E+T) versus milling time. (E/(E+T) = erythronic acid/(erythronic acid + threonic acid))

The results obtained by the nitrobenzene oxidation (**Fig. 2.4** and **2.5**) and ozonation method (**Fig 2.6** and **2.7**) indicate that the structural changes of lignin cannot be avoided during the milling. The degree of lignin degradation increases with the progress of milling. It is quite important for a solvent system to dissolve milled wood prepared by short milling time. As shown above, the structural change of lignin was not so significant when the milling time was 2 h, which is a significant advantage of the LiCl/DMSO system.

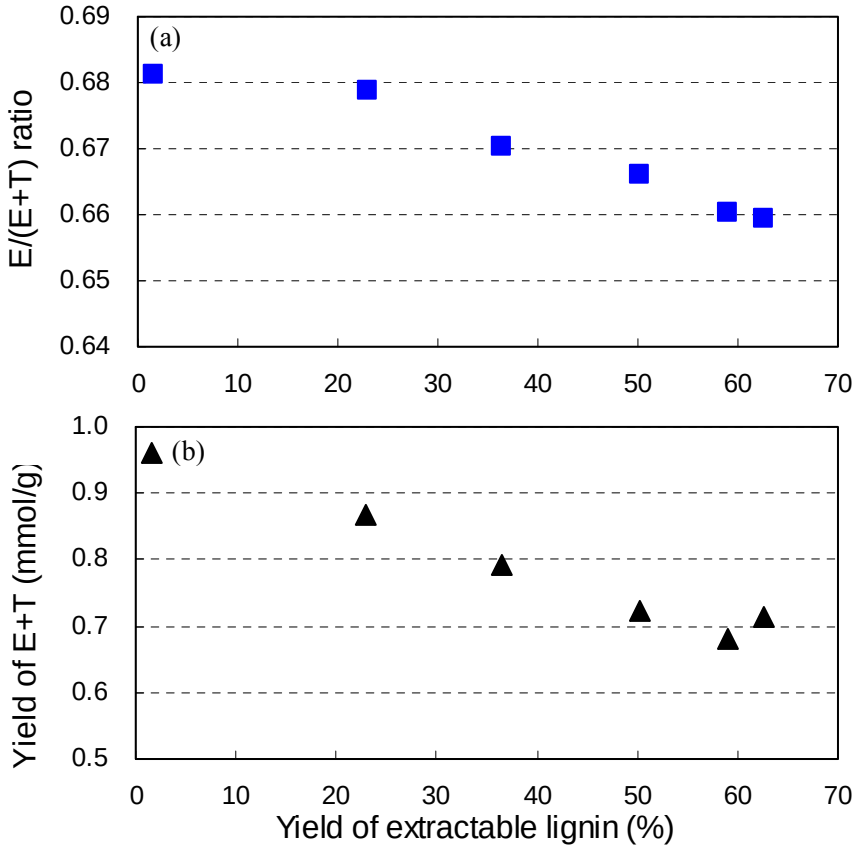


Figure 2.7 Dependence of the *erythro* ratio (E/(E+T)) and yield of ozonation products on the yield of extractable lignin in beech milled wood prepared at different milling times. ((a) *erythro* ratio (E/(E+T)) versus yield of extractable lignin. (b) yield of E+T versus yield of extractable lignin.)

2.3.2.3 Effect of milling on cellulose in milled wood

X-ray diffraction patterns of the beech Wiley and milled woods are shown in **Fig. 2.8**. The crystal region of cellulose disappeared when the milling time was as short as 1 h, indicating that the milling process affects the structure of not only lignin but also cellulose. The destruction of the crystal region should be one of the decisive factors for the dissolution process of the milled woods in the LiCl/DMSO solvent system.

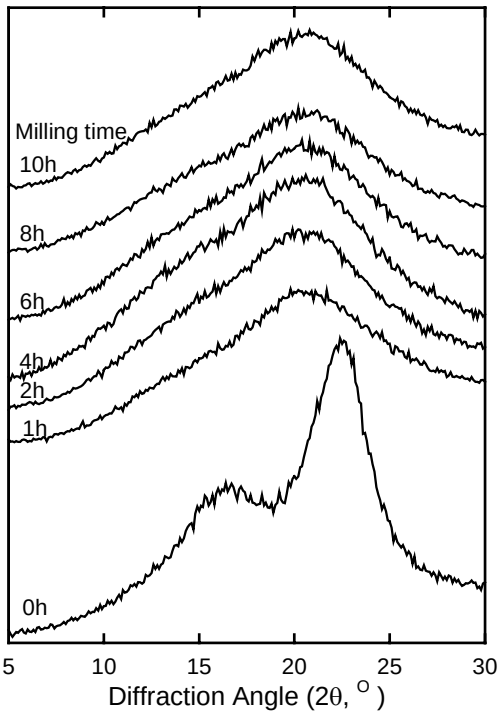


Figure 2.8 X-ray diffraction patterns of Wiley wood and milled wood prepared at different milling times from beech wood. (The individual diffractograms were shifted on y-scale for the total display. Y-axis represents intensity.)

2.3.2.4 Comparison with other wood solvent system

LiCl/DMSO can completely dissolve milled wood (both softwood and hardwood) prepared by planetary ball-mill with milling time, 2 h. But DMSO/NMI, which was reported by Lu and Ralph,[13] could not completely dissolve milled wood prepared by 2 h milling under the same condition. Only suspension was obtained as shown in **Fig. 2.9.**

Table 2.2 compares LiCl/DMSO system with DMSO/NMI system. From **Table 2.2**, DMSO/NMI can just dissolve hardwood prepared under 5 h of milling and softwood prepared under 6 h of milling. Syringyl ratio was not affected by the milling, but yields of the nitrobenzene oxidation products decreased with the progress of milling. On contrast to not detectable decrease by 2 h of milling, the yield of the nitrobenzene oxidation products obviously decreased 3.4% by 5 h milling. Both the *erythro* ratio and total yield of ozonation products decreased 1.9 and 21.9% respectively when milling time was 5 h. Compared with 5 h of milling, decrease of both *erythro* ratio and total yield of ozonation products by 2 h milling was moderate (0.3 and 9.8%). Moreover, the utilization of DMSO/NMI system on UV spectroscopic analysis encountered big difficulties due to the strong absorbance of NMI around 280 nm, which interfere with the absorbance of lignin. The direct NMR spectroscopic analysis of milled wood in DMSO/NMI also has not been reported yet. On the contrary, the new milled wood solution in LiCl/DMSO system can be directly used for both UV and NMR spectroscopic analyses without any derivatization.

LiCl/DMSO system can be widely used in the field of wood sciences, not only for NMR, UV analysis, but also will be widely used such as GPC separation and analysis, isolation of wood components, and as a reaction media for chemical modification. Therefore, LiCl/DMSO is an advanced solvent system for the analysis of plant cell wall components.

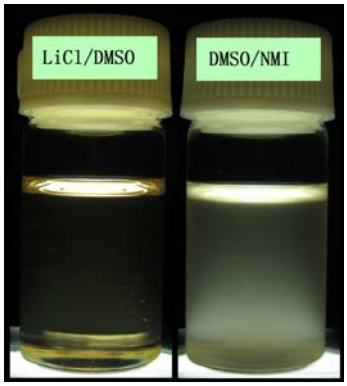


Figure 2.9 Photos of beech milled wood (2 h of milling) solution in 6% LiCl/DMSO and suspension in DMSO/NMI (2:1, v/v) with 1% concentration after 24 h stirring (Photos were taken in a dark room with a white light comes from bottom of the bottles).

Table 2.2 Comparison between LiCl/DMSO and DMSO/NMI system**.**

	LiCl/DMSO (wt 6%)	**DMSO/NMI (2:1, v/v)**
Dissolution ability (Required milling time for complete dissolution) (h)		
Hardwood (beech)	**2**	**5**
Softwood (spruce)	**2**	**6**
Degradation of lignin structure by the required milling time		
Decrease of aromatic part of lignin		
Ratio (%)	ND[a]	ND
Yield (%)	ND	3.4
Decrease of β-O-4 structure		
Ratio (%)	**0.3**	**9.8**
Yield (%)	**1.9**	**21.9**
Direct applicability to spectroscopic analysis[b]		
NMR	**+**	**-**
UV	**+**	**-**

[a] ND, not detectable. [b] +, can be directly used for spectroscopic analysis. -, cannot be directly used for spectroscopic analysis.

2.3.3 Characterization of the wood solution

LiCl/DMSO solvent system can completely dissolve milled wood prepared by 2 h of milling with planetary ball-mill. Nitrobenzene oxidation and ozonation analysis suggested that the structural changes of lignin caused by 2 h of milling were not very significant. Therefore, various spectroscopic analysis such as NMR, UV etc. can be applied to LiCl/DMSO system to give the basic information of original lignin structure, which could not be obtained by present isolation methods or other high degradative dissolution method. The high-resolution solution-state NMR analysis of complete wood solution will provide considerable insight into wood cell wall structure without the need for polymer fractionation. By subjecting the complete wood solution to UV measurement, the gram absorptivity of "in-situ" lignin will be obtained.

2.3.3.1 NMR analysis of the complete wood solution

Milled wood concentration in LiCl/DMSO system can be as high as 10%, which is much higher than some ionic liquid solutions.[11] Significant advantage of this solvent system is that d6-DMSO is commercially available and it doesn't interfere with the important peaks of lignin by NMR spectroscopy. Therefore, the obtained complete wood solution can be directly subjected to the analysis. **Fig. 2.10** shows an example of ^{1}H-NMR spectrum, which clearly shows the lignin aromatic part without any interfering overlapping peak at around 6.8 ppm. Analysis of originally present acetyl groups appearing at around 2.0 ppm is also quite interesting. ^{13}C-NMR spectrum of wood solution was obtained as shown in **Fig. 2.11**.

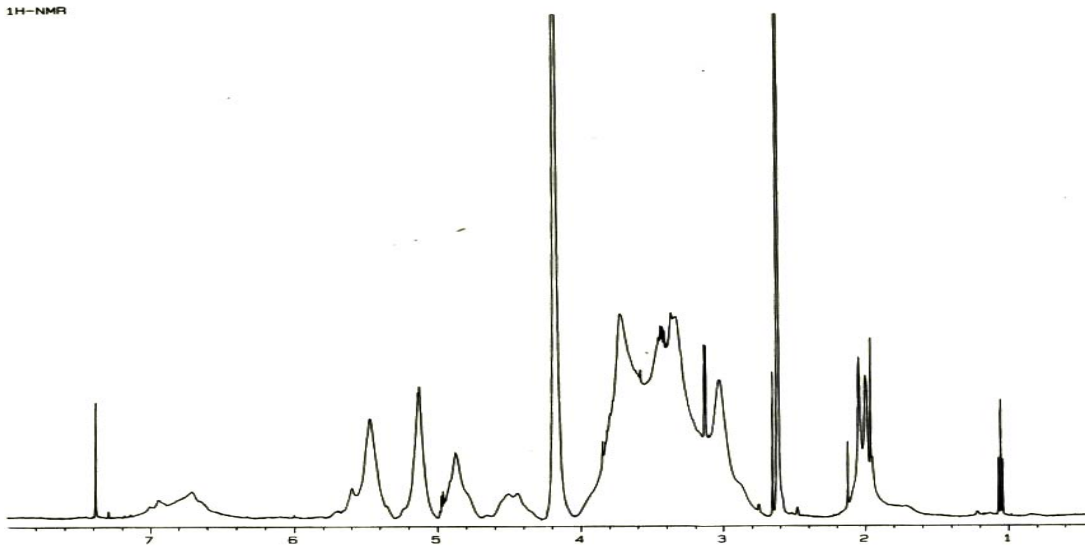


Figure 2.10 ^{1}H-NMR spectrum of complete wood solution of milled wood (2 h) in LiCl/DMSO-*d6*.

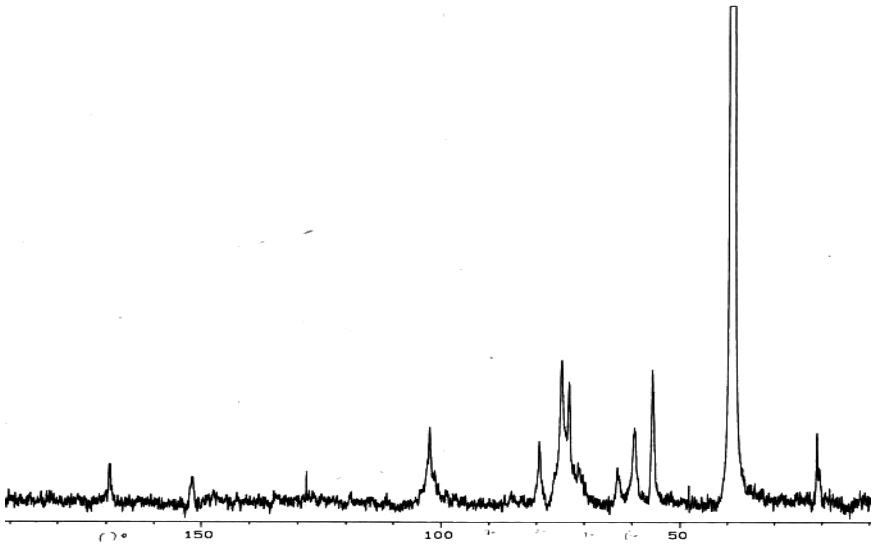


Figure 2.11 ^{13}C-NMR spectrum of complete wood solution of milled wood (2 h) in LiCl/DMSO-*d6*.

2.3.3.2 UV absorptivity of lignin in the complete wood solution

UV absorbance of lignin can be potentially applied to the determination of lignin in a sample, and in fact, it is widely used as a convenient method of lignin determination. However, in order to make it possible, we have to know the exact gram absorptivity of lignin. Usually, the gram absorptivity of lignin is obtained by the measurement of isolated lignin, such as MWL. However, considering the structure of MWL is affected by both "artificial change" and "native difference", it is impossible to ascertain whether or not the UV absorptivity of MWL is representative of the real value of native lignin in wood. Wood meal could be completely dissolved into LiCl/DMSO. Thus, we may easily think that we can straightly use complete wood solution to know gram absorptivity of original lignin in wood.

Fig. 2.12 shows the gram absorptivity at 280 nm of lignin in the completed wood solution in 6% LiCl/DMSO. However, from the results shown in **Fig. 2.12**, the gram absorptivity at 280 nm increased along with milling time. This result suggests that absorptivity of lignin in UV determination should be variable. The absorptivity should be different when condition of sample preparation is different such as milling time, even when the same sample or the same solvent is used. Therefore, we can suggest that gram absorptivity of lignin can be applied only to semi-quantitative determination.

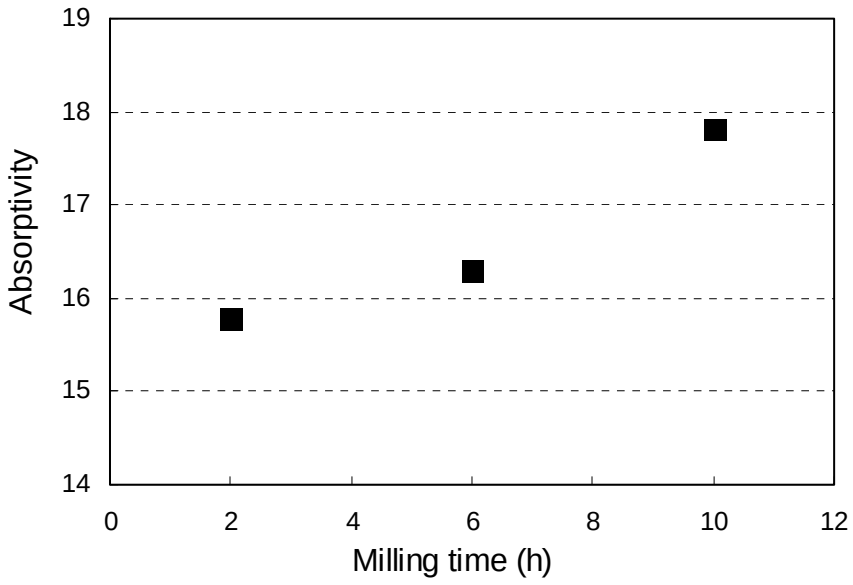


Figure 2.12 Gram absorptivity of lignin at 280 nm obtained for complete wood solution in LiCl/DMSO.

2.3.3.3 Gelation of the wood solution

Milled woods (milling time 2-10 h) were dissolved in LiCl/DMSO. At first, a viscous but clear solution was formed under room temperature by stirring for 1 day. If the wood solution was stood for 1 more day at relatively high concentration, it will form gel as shown in **Fig. 2.13.**

Furthermore, the concentration needed for gel formation was dependent on the milling time. From the results shown in **Fig. 2.14**, when we use longer milling time, for example 10 h, much higher concentration, 15.5%, is needed to form gel, compared to 2 h (only 7.6%). These results indicated that the wood solution is much easier to form gel when the milling time is less. It seems that less destruction of wood components is more suitable for gelation.

The reason for the gelation of wood solution is not clear at this moment, but if it is related to the ability of cell wall components to interact together, the gelation behavior will give quite important information. When wood components, cellulose,

hemicelluloses, and lignin, are present in the cell wall as rigid solid, probably the ability of these components to interact together doesn't appear fully. In other words, the ability is controlled and limited during the formation process of the cell wall. It seems that cell wall components are freed from such limitation and start to fully exhibit their ability to interact together when the cell wall becomes solution.

Figure 2.13 Wood solution (left,1% concentration) and gel (right, 10% concentration, bottle is up side down) prepared from milled wood (2 h) in 6% LiCl/DMSO.

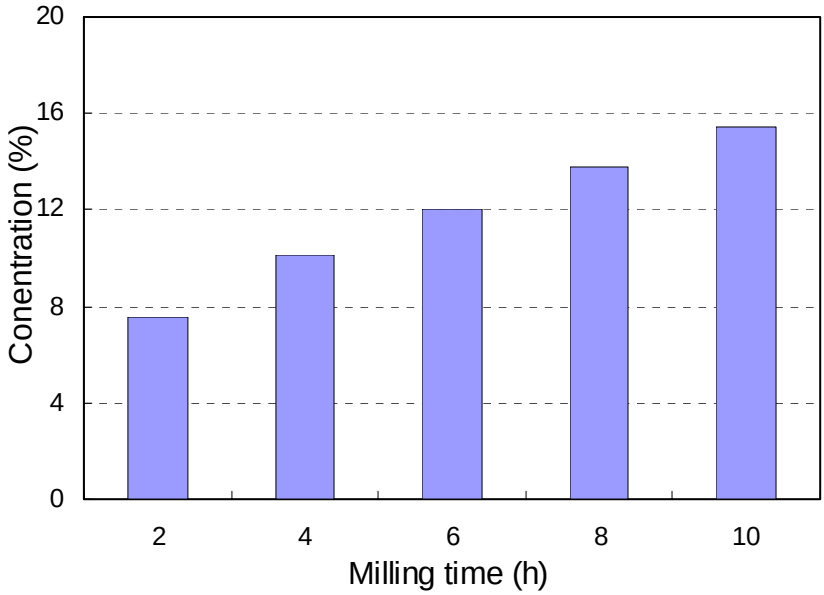


Figure 2.14 The relationship between the milling time and the concentration needed for gel formation in 6% LiCl/DMSO.

2.4 Conclusions

The novel solvent system developed in this chapter, LiCl/DMSO, completely dissolved milled wood prepared by as short as 2 h of milling using a planetary ball-mill. The nitrobenzene oxidation and ozonation analyses indicated that the structural change of lignin caused by the 2 h of milling is not significant. In contrast, the destruction of the cellulose crystalline region in the milled wood was serious even after 1 h of milling. Compared with already reported solvent systems, the LiCl/DMSO system, developed in this chapter, has advantages of high solubility and low degradation of lignin and is expected to be applied widely to the analysis of the entire lignin fraction in wood cell wall. The lignin in this wood solution can almost represent original entire lignin. Therefore, at the first time, the information of whole aromatic part of lignin structure was obtained by NMR of complete wood solution. The gram absorptivity of lignin at 280 nm was obtained by the use of complete wood solution. The absorptivity increased along with milling time, which suggested that gram absorptivity of lignin, which is widely applied for the determination of lignin, can be applied only to semi-quantitative level. The wood solution will form gel at relatively high concentration. The critical concentration for gelation increased along with milling time.

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Chapter 3

Dissolution of ethylenediamine pretreated lignocellulosic material with high lignin content

3.1 Introduction

Wood is an important reproducible natural composite consisting of different polymers, mainly cellulose, hemicellulose, and lignin. Evaluation of the interactions between these polymers has been one of the key subjects in the field of wood and pulping chemistry. It is important to clarify whether or not chemical bonds exist between these polymers in the native wood and corresponding pulp, because the difficulties encountered in the latter stages of delignification are partly attributed to such chemical bonds between lignin and carbohydrates. Although a lot of indirect observations have been reported examining these interactions, so far it has not been clarified whether these interactions are due to chemical linkages or physical adsorption. One promising method to examine these interactions is through pulp dissolution. However, there are no reported solvent systems for dissolving underivatized lignocellulosic material with relatively high lignin content.

In recent decades, many solvent systems have been reported to dissolve cellulose, which is the major component of chemical pulp.[1] However, direct application of cellulose solvent system to the dissolution of wood pulps has not been successful. For example, although lithium chloride/ N,N-dimethylacetamide (LiCl/DMAc) was presented as a good solvent system for dissolving cellulose,[2] several problems were reported when this solvent system is applied to pulp dissolution. When applied to softwood bleached kraft pulp, the dissolution is incomplete.[3,4] As well, the cellulose is degraded detrimentally by heating the system to facilitate dissolution.[5,6] LiCl/1,3-dimethyl-2-imidazolidinone (LiCl/DMI) solvent system[7] can give colorless solution when applied to unmodified cellulose,[8] but more than 2 weeks are required to dissolve softwood kraft pulp, even with relatively low, about 2% lignin content.[9] Cellulose solvent system has been used for the derivatization and dissolution of pulps. Berthold *et al.* reported that unbleached softwood kraft pulp derivatized with ethyl isocyanate could be dissolved in 8% LiCl/DMAc.[10] But, there is no good solvent system for dissolving pulp with high lignin content without derivatization. The development of a solvent system for pulps with high lignin content is required not only to examine the chemical nature of pulp components and interactions between them, but also to utilize such pulps as a new source of natural fibrous materials.

Petrus *et al.* reported that LiCl/dimethyl sulfoxide (LiCl/DMSO) can dissolve regenerated cellulose that is prepared from cellulose acetate.[11] In the Chapter 2, a novel solvent system, LiCl/DMSO was developed, which can dissolve wood meal finely ground by planetary ball-mill for 2 h. But the cellulose crystallinity of the ground wood meal is dramatically decreased even by 1 h of milling. Thus, a pretreatment method other than milling nor regeneration is a prerequisite to dissolve highly crystalline cellulose or pulp for analyzing entire plant cell wall components.

When cellulose is treated with ethylenediamine (EDA) it forms a “cellulose-EDA complex”. The structure and characteristics of cellulose-EDA complexes have been

investigated by Segal *et al.*,[12-15] Lee *et al.*,[16] and Wada *et al.*.[17] Gagnaire *et al.* reported that bacterial cellulose treated with 75% EDA can be dissolved in N-methyl morpholine-N-oxide/DMSO (NMMO/DMSO),[18] but they did not refer to the dissolution of lignocellulosic material containing lignin. It was found that EDA pretreated holocellulose, followed by a solvent exchange with DMI has improved solubility in LiCl/DMI, while maintaining the original crystaline structure albeit with a lower crystallinity.[19] In this chapter, LiCl/DMSO was applied to the dissolution of EDA pretreated lignocellulosic material with high lignin content, and the behavior during the pretreatment and dissolution processes was investigated.

3.2 Experimental

3.2.1 Materials

Microcrystalline cellulose (Whatman CF11), cotton, cellulose I, cellulose II and holocellulose were used as lignocellulosic samples as shown in **Table 3.1**. Beech and spruce wood meals were prepared as described in section 2.2.1. Holocelluloses (both of hardwood and softwood holocellulose) were prepared by the reaction of 2.5 g of wood meal with 1 g of $NaClO_2$ and 0.2 ml of acetic acid in 150 ml water. The mixture was kept in a water bath at 75℃ for 1 h. Then, the mixture was cooled to room temperature, and the reagents were added again. The addition of these reagents was repeated totally four times (A delignified wood meal, was used as holocellulose sample in this section). Cellulose I was prepared from holocellulose treated by 6% aqueous NaOH. Cellulose II was prepared form holocelluloe by soaking in 17.5% aqueous NaOH for 1 day at room temperature, followed by washing thoroughly with water and dry (**Fig. 3.1**).

Unbleached and bleached kraft pulps with different kappa numbers from different origin were used as pulp samples. Pulp properties are shown in **Table 3.2**. Hardwood unbleached kraft pulps (HUKP) were produced from paulownia wood chips. Different cooking conditions were used to prepare the pulps with different lignin contents. Lignin contents of the prepared pulps were measured by Kappa number method (TAPPI test method, T 236 om-99). The obtained Kappa number of each pulp was converted to lignin content by multiplying by 0.15. A hardwood bleached (delignified) kraft pulp (HBKP) sample was prepared by the reaction of 2.5 g of $HUKP_{P3}$ with 1 g of $NaClO_2$ and 0.2 ml of acetic acid in 150 ml water. The mixture was kept in a water bath at 75 ℃ for 1 h. The mixture was then cooled to room temperature, and the reagents were added again. The addition of these reagents was repeated for a total of three times. Softwood unbleached and bleached kraft pulps (SUKP and SBKP, respectively) were kindly

provided by Oji Paper Industries Co., Ltd (Tokyo, Japan).

Table 3.1 Various celluloses or holocelluloses for dissolution.

Samples	Materials
CF11	Microcrystalline cellulose
Cotton	Cotton
Cellulose I	Prepared from beech wood meal
Cellulose II	Prepared from beech wood meal
Holocellulose*	Prepared from beech and spruce wood meals

*, A delignified wood meal, which contained amounts of lignin, was used as holocellulose sample in this section.

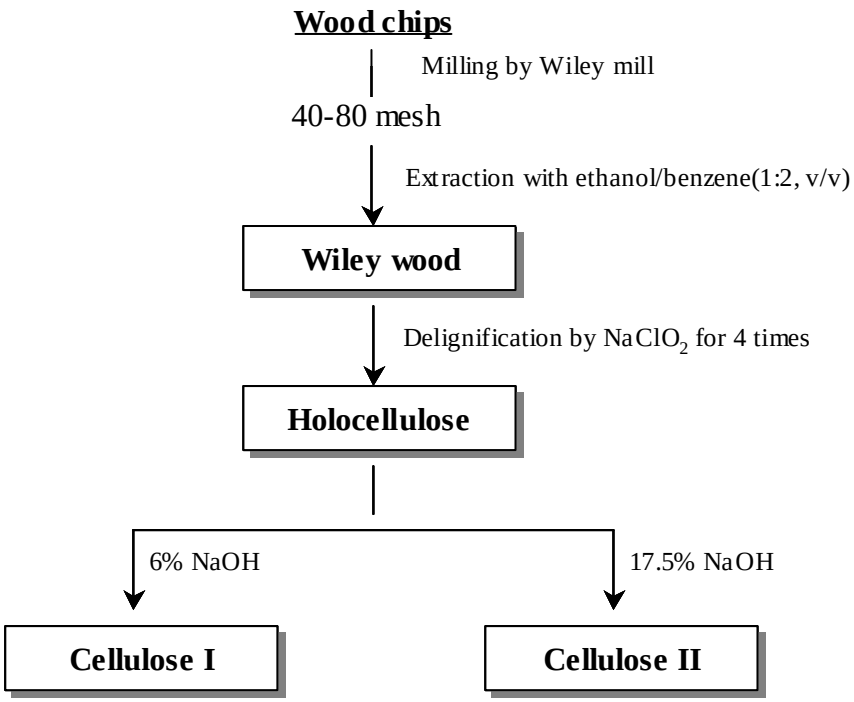

Figure 3.1 Preparation of holocellulose, cellulose I, and cellulose II from wood meal.

3.2.2 Pretreatment of lignocellulosic material with EDA

Various lignocellulosic materials, such as CF11, cellulose, holocellulose, hardwood, and softwood chemical pulps (0.5 g) were soaked in 30 ml of EDA, and stirred for 1 day at room temperature. The EDA treated lignocellulosic materials were then freeze-dried, and are referred to as "lignocellulose-EDA complex" as shown in **Table 3.3**. The EDA content of lignocellulose-EDA complex was determined gravimetrically by the gain in weight.

Table 3.2 Various pulps for dissolution.

Samples[a]	Wood species	Kappa number	Lignin content(%)[b]
$HBKP_{P0}$	Paulownia	Bleached	/
$HUKP_{P1}$	Paulownia	12.1	1.8
$HUKP_{P2}$	Paulownia	17.6	2.6
$HUKP_{P3}$	Paulownia	22.6	3.4
$HUKP_{P4}$	Paulownia	37.3	5.6
$HUKP_{P5}$	Paulownia	69.7	10.5
$HUKP_{P6}$	Paulownia	97	14.6
$SBKP_{M0}$	Mixture of softwood	Bleached	/
$SUKP_{M1}$	Mixture of softwood	30	4.5
$SUKP_{D1}$	Douglas fir	20	3.0
$SUKP_{D2}$	Douglas fir	30	4.5
$SUKP_{D3}$	Douglas fir	55	8.3

[a] HBKP, hardwood bleached kraft pulp. HUKP, hardwood unbleached kraft pulp. SBKP, softwood bleached kraft pulp. SUKP, softwood unbleached kraft pulp, subscript $_{P0 \ldots\ldots D3}$ represent pulps were prepared from different wood species (paulownia wood chips ..... douglas fir wood chips) with different kappa number (bleached ..... 55). [b] Lignin content was obtained from kappa number by multiplying by 0.15.

3.2.3 Dissolution of lignocellulose-EDA complex

CF11-EDA complex 50 mg was suspended in 2 ml of 8% LiCl/DMSO. By keeping the mixture stirring under room temperature for 30 minutes, a clear cellulose solution can be obtained. For other samples containing lignin, a lignocellulose-EDA complex (20 mg) was suspended in 2 ml of 8% LiCl/DMSO. The mixture was kept with stirring at room temperature for 24 h. The mixture was heated up to 75℃, and kept with stirring at this temperature for 1 h, and which point a clear solution can be obtained. Different concentrations of LiCl (2%, 4%, and 6%) in DMSO were applied to examine the solubility of the $HUKP_{P1}$-EDA complex by the above procedure under the same conditions.

3.2.4 X-ray diffraction analysis

Original lignocellulosic materials and lignocellulose-EDA complexes were subjected to X-ray diffraction analysis as described in section 2.2.5.

3.2.5 Spectroscopic analysis of lignocellulosic solution

3.2.5.1 NMR measurement

The CF11-EDA and $HUKP_{P1}$-EDA complex were dissolved in 8% LiCl/DMSO-*d6*. The concentration of CF11-EDA complex and $HUKP_{P1}$-EDA complex were 5% and 2% respectively. The analysis of ^{13}C-NMR was performed on a JEOL Alpha 500 spectrometer (JEOL, Japan).

3.2.5.2 Optical transmittance

Each of the LiCl/DMSO lignocellulose-EDA complexes (0.2% concentration based on the lignocellulosic material weight) solution was introduced into a cuvette (1 cm

width), and the transmittance was measured from 400 to 700 nm using a Shimadzu UV-VIS spectrophotometer (UV-240). The 8% LiCl/DMSO was used as a reference sample for the measurement of transmittance of the solutions.

3.2.5.3 UV absorbance

The UV absorbance at 280 nm of each of the LiCl/DMSO solutions of HUKP-EDA complexes was determined using the above described UV-240.

3.3 Results and Discussion

3.3.1 Effect of EDA treatment on the characteristics of the lignocellulosic materials

In this chapter, CF11, cotton, cellulose I, cellulose II, holocellulose, and various pulps with different kappa numbers (HBKP, HUKP, SBKP, and SUKP) were used as lignocellulosic samples as shown in **Table 3.1** and **3.2.** Various lignocellulosic samples were soaked in EDA with stirring for 1 day at room temperature followed by freeze drying. Because EDA is not removed completely by freeze drying, the EDA treated freeze dried lignocellulosic materials, referred to as "lignocellulose-EDA complex", contained about 18-20% EDA (**Table 3. 3**). This is in accordance with the result of Loeb *et al*.[12]

The crystal structure of the original cellulose and cellulose-EDA complex were investigated by X-ray diffraction patterns as shown in **Fig. 3.2**. The crystal structure of cellulose changed with EDA treatment, all of resulting cellulose-EDA complex samples showed similar diffraction patterns even the corresponding original cellulose had different crystal structure. Anyhow, the crystallinity of cellulose-EDA complex was maintained as high as corresponding original cellulose.

The corresponding results were also observed from pulp samples as shown in **Fig. 3.3**. By the EDA treatment, the crystal structure of cellulose in $HUKP_{P1}$ also changed to the similar patterns as all of above-mentioned cellulose-EDA complex samples but the crystallinity of the $HUKP_{P1}$-EDA complex appears to be as high as $HUKP_{P1}$.

Table 3.3 EDA content of lignocellulose-EDA complex.

Samples	Lignocellulose-EDA complex		
	Name	Content of EDA(%)	Solubility in 8% LiCl/DMSO [a]
CF11	CF11-EDA	22.5	+
Cotton	Cotton-EDA	18.5	+
Cellulose I	Cellulose I-EDA	22.8	+
Cellulose II	Cellulose II-EDA	21.7	+
Holocellulose	Holocellulose-EDA	22.9	+
$HBKP_{P0}$	$HBKP_{P0}$-EDA	20.1	+
$HUKP_{P1}$	$HUKP_{P1}$-EDA	20.8	+
$HUKP_{P2}$	$HUKP_{P2}$-EDA	20.8	+
$HUKP_{P3}$	$HUKP_{P3}$-EDA	20.6	+
$HUKP_{P4}$	$HUKP_{P4}$-EDA	20.5	+
$HUKP_{P5}$	$HUKP_{P5}$-EDA	19.6	+
$HUKP_{P6}$	$HUKP_{P6}$-EDA	18.3	-
$SBKP_{M0}$	$SBKP_{M0}$-EDA	18.9	+
$SUKP_{M1}$	$SUKP_{M1}$-EDA	19.5	+
$SUKP_{D1}$	$SUKP_{D1}$-EDA	18.4	+
$SUKP_{D2}$	$SUKP_{D2}$-EDA	19.4	+
$SUKP_{D3}$	$SUKP_{D3}$-EDA	19.8	+

[a] +, completely soluble. - incompletely soluble.

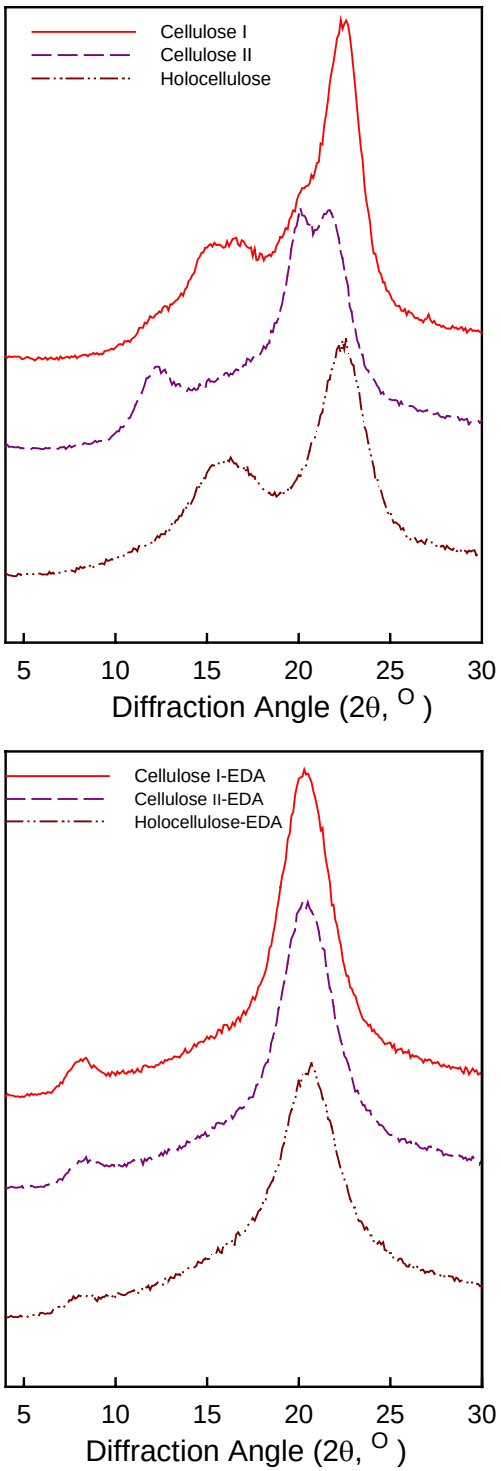

Figure 3.2 X-ray diffraction patterns of the original lignocellulose samples (above) and corresponding lignocellulose-EDA complex samples (below).

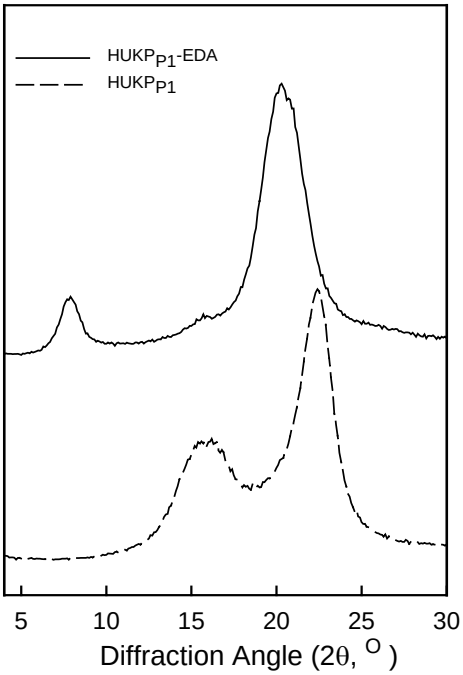

Figure 3.3 X-ray diffraction patterns of the original HUKP$_{P1}$ and corresponding HUKP$_{P1}$-EDA complex.

3.3.2 Dissolution of EDA pretreated lignocellulosic materials

Various lignocellulose-EDA complexes were subjected to LiCl/DMSO solvent system to examine whether or not they can be dissolved in this solvent system (Table 3.3, Fig.3.4-3.6). Among these samples examined, CF11-EDA complex is very easy to be dissolved completely within 30 minutes at room temperature. Cotton-EDA, cellulose I-EDA, cellulose II-EDA and holocellulose-EDA can be dissolved completely under stirring at room temperature for 24 h, followed by keeping at 75℃ for several minutes to 1 h, as shown in **Fig. 3.4.**

All the pulp-EDA complexes were dissolved completely under the conditions above described, except the pulp-EDA complex of HUKP$_{P6}$ which has rather high lignin content (**Fig. 3.5** and **3.6**). It is quite significant that transparent solutions were obtained. Even the pulp-EDA complex prepared from HUKP$_{P5}$ with high lignin content (10.5%) was also completely dissolved in this solvent system. Based on the data shown in **Fig.**

3.3, it is presumed that the crystallinities of the pulp-EDA complexes are maintained at the same levels as those of the corresponding original pulps. Consequently, the LiCl/DMSO solvent system is quite effective in dissolving pulps with high lignin content after EDA pretreatment. Similarly, pulps with fairly high lignin content, such as $HUKP_{P6}$ cannot be dissolved by a single EDA treatment. In these cases, only suspensions were obtained with low transparencies. Therefore, both the lignin content and the formation of the EDA complex seem to be important for the dissolution in LiCl/DMSO. Interestingly, about 70% of the coarse wood meal (40-80 mesh) sample could also be dissolved in LiCl/DMSO. In this case, however, EDA pretreatment and dissolution in LiCl/DMSO had to be repeated two times. Namely, the residue from the first dissolution (about 60%) was again treated with EDA and subjected to the dissolution in LiCl/DMSO. The second dissolution process left only about 30% of the original wood weight as a residue. The EDA untreated wood meals or pulps could not be dissolved in LiCl/DMSO unless they were finely ground as described in the Chapter 2.

Different concentrations of LiCl (2%, 4%, 6%, and 8%) in DMSO were also tried to dissolve the $HUKP_{P1}$-EDA complex using the above procedure under the same conditions. When the concentration of LiCl was less than 6%, the systems were unable to dissolve the $HUKP_{P1}$-EDA complex.

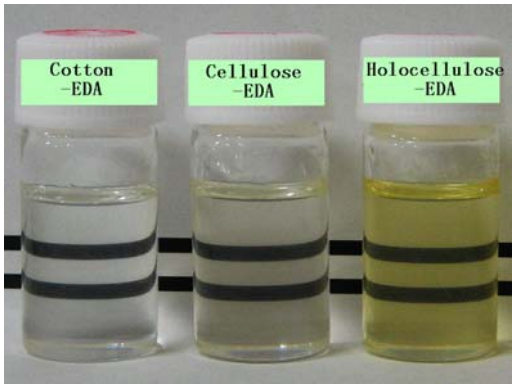

Figure 3.4 Photographs of solutions of lignocellulose-EDA complex in 8% LiCl/DMSO with 0.5% concentration (Photos were taken by using white paper marked with two black lines as background. Left: cotton-EDA, middle: cellulose I-EDA, right: holocellulose-EDA).

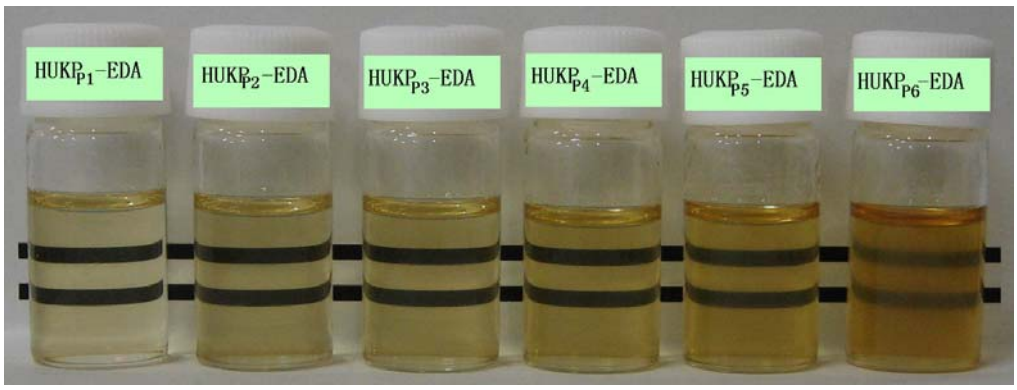

Figure 3.5 Photographs of solutions and suspension of the hardwood kraft pulp-EDA complex in 8% LiCl/DMSO with 0.5% concentration（From the left to right: HUKP$_{P1}$-EDA to HUKP$_{P6}$-EDA).

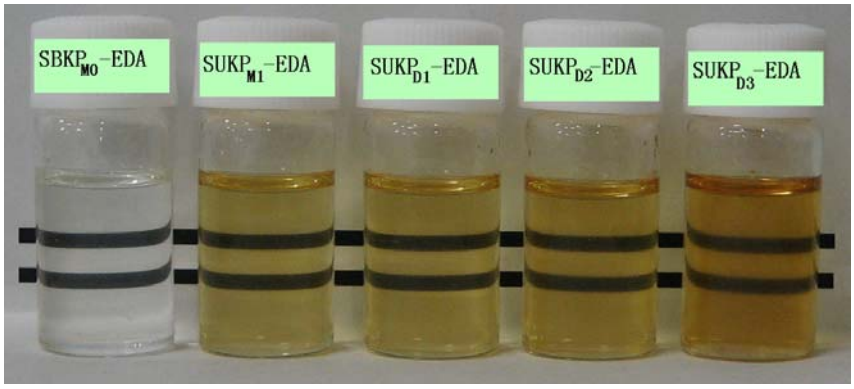

Figure 3.6 Photographs of solutions of the softwood kraft pulp-EDA complex in 8% LiCl/DMSO with 0.5% concentration.

If we compare LiCl/DMSO solvent system with the other systems, LiCl/DMAc and/or LiCl/DMI, significances of this system will be made clear. LiCl/DMAc was presented as a good solvent system for dissolving cellulose,[2] but it cannot dissolve holocellulose, chemical pulp even with high temperature or solvent exchange methods for long time.[3-6] LiCl/DMI solvent system[7] can give colorless solution when applied to unmodified cellulose,[8] but more than 2 weeks are required to dissolve softwood kraft pulp, even with relatively low, about 2% lignin content.[9] LiCl/DMSO solvent system can dissolve lignocellulosic materials even those with high lignin content 10.5% under mild conditions and relative short dissolving time after a EDA pretreatment.

3.3.3 NMR analysis of the lignocellulosic solutions

NMR spectra of cellulose dissolved in various solvent systems have been reported by many researchers. According to the results of Yanagisawa *et al*.,[20] no clear signal was observed in the ^{13}C-NMR spectrum of microcrystalline cellulose (CF11) with DP 200-300 when it was dissolved in 8% LiCl/DMAc or 8% LiCl/DMI. They consequently used cellulose with a DP 15 to observe a clear NMR spectrum.

In this chapter, the lignocellulose-EDA complex solutions were subjected to ^{13}C-NMR analysis. The ^{13}C-NMR spectra of the CF11-EDA complex and $HUKP_{P1}$-EDA complex dissolved in 8% LiCl/DMSO-d6 were shown in **Fig. 3.7** and **3.8**, respectively. The spectra are well resolved and show clear signals at around 101 ppm (C-1), 80 ppm (C-4), 74-75 ppm (C-5, C-3), 73 ppm (C-2), and 60 ppm (C-6). These ^{13}C-NMR analyses suggest that cellulose is dissolved in LiCl/DMSO without forming any derivatives, and LiCl/DMSO can be regarded as a true solvent. The peak of EDA appears at around 44 ppm. No clear peak of lignin is observed, because the amount of lignin in $HUKP_{P1}$ is only about 1.8%.

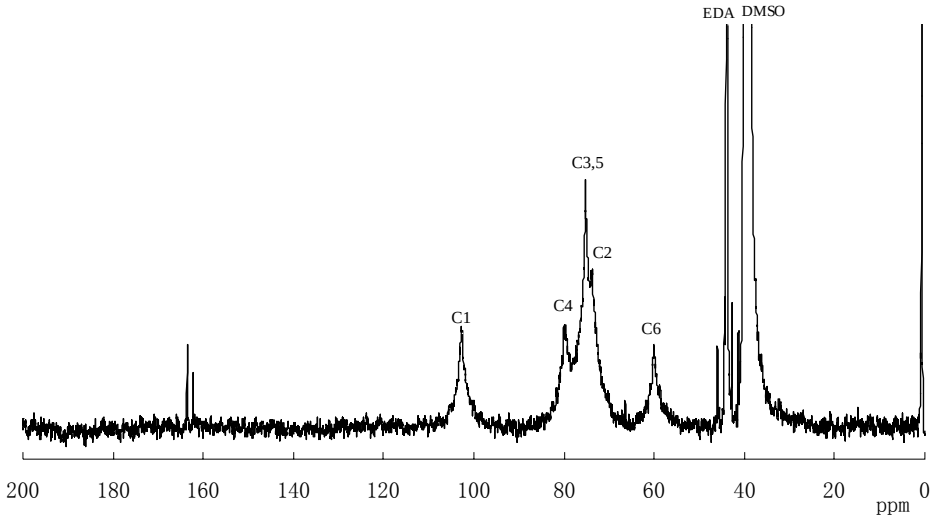

Figure 3.7 Solution-state ^{13}C-NMR spectrum of CF11-EDA solution in $LiCl/DMSO_{d6}$.

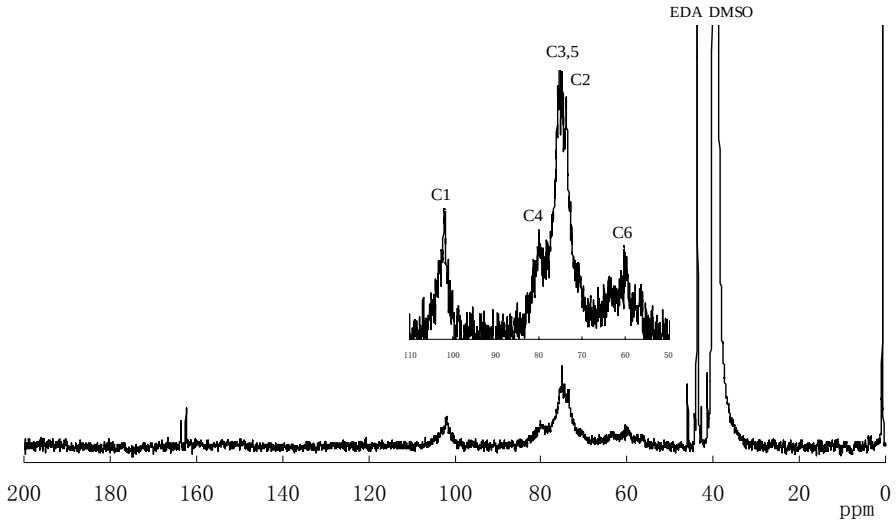

Figure 3.8 Solution-state ^{13}C-NMR spectrum of $HUKP_{P1}$-EDA solution in $LiCl/DMSO_{d6}$.

3.3.4 Light-transmittance of the lignocellulosic solutions

Light transmittances of different lignocellulosic solutions were measured at 0.2% concentration (**Fig. 3.9**). By dissolving lignocellulose-EDA in 8% LiCl/DMSO, transparent solutions with less color gave higher light transmittance. The distinct feature was observed for the light transmittance of holocellulose-EDA complex solution lies in the lower level in the short wavelength (400-500 nm) although this solution was transparent. In this chapter, the holocellulose was obtained by 4 times of $NaClO_2$ delignification of Wiley wood resulting in certain amount of lignin remaining in the so-called holocellulose sample. Therefore, color due to the derived structures from lignin present in holocellulose might affect the light transmittance.

The visible light transmittances of the pulp-EDA complex solutions were also measured at 0.2% concentrations. As shown in **Fig. 3.10** and **3.11**, if the pulp-EDA complex was soluble, the transmittance of the solution at the region of visible light was relatively high. The transmittance of $HBKP_{P0}$ and $SBKP_{P0}$ solutions were over 92.4% and 93% in the visible region, respectively. It can be seen that all the transmittances are relatively low in the short wavelength range, and the higher the lignin content of the original pulp, the lower the transmittance. It is known that new structures such as quinone structures are introduced in lignin during the pulping reactions. These structures, as well as the complexation of lignin with metals strongly affects the color of pulp, even though the native lignin itself doesn't absorb light in the visible region.[21,22] Therefore, the observed phenomena can be attributed to the presence of lignin in the pulp, as well as in the holocellulose mentioned before. The transmittance of the incompletely soluble $HUKP_{P6}$-EDA suspension was fairly low over the whole range, which must be the result of both color due to lignin and scattering effect due to the insoluble materials.

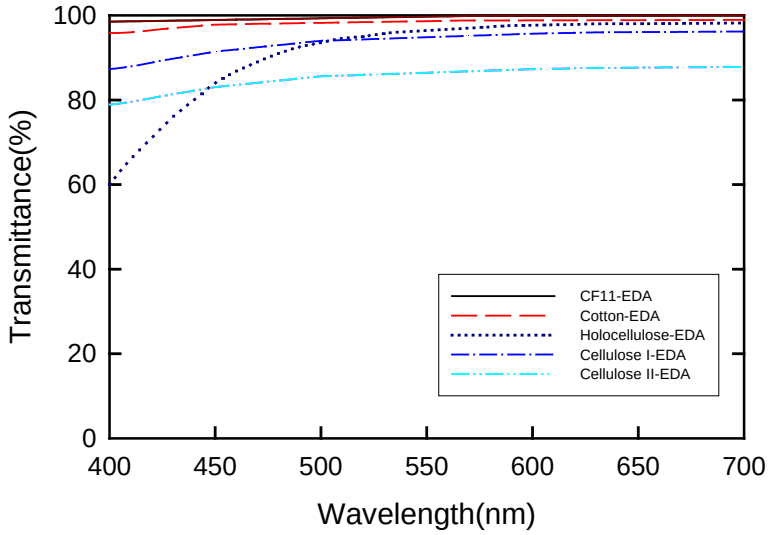

Figure 3.9 Visible light-transmittance of the cellulose-EDA complex solutions in 8% LiCl/DMSO with 0.2% concentration.

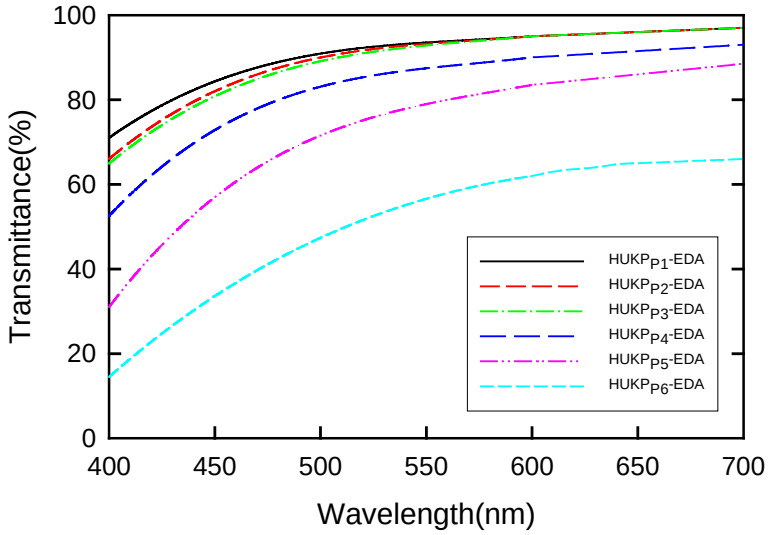

Figure 3.10 Visible light-transmittance of solutions and suspension of the hardwood kraft pulp-EDA complex in 8% LiCl/DMSO with 0.2% concentration.

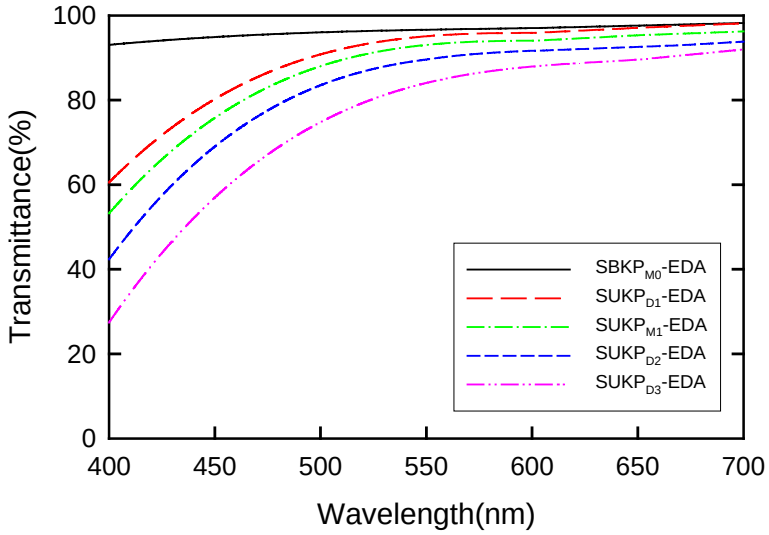

Figure 3.11 Visible light-transmittance of solutions of the softwood kraft pulp-EDA complex in 8% LiCl/DMSO with 0.2% concentration.

3.3.5 UV absorbance of the lignocellulosic solution

The UV absorbances of the HUKP-EDA complex solutions, which contain certain amounts of lignin, were analyzed at 280 nm (**Fig. 3.12**). A significantly high correlation was observed between the UV absorbance of the solutions and the lignin content expressed as kappa numbers of the corresponding HUKPs. This result was an unexpected result, as the structure of residual lignin in pulp differs depending on the stage of delignification. Thus, the absorption coefficient of lignin must be different in pulps with different amount of lignin. In addition, kappa number method detects not only lignin but also other conjugated structures including non-lignin compounds such as hexenuronic acid, which does not show as absorbance maximum at 280 nm. Nevertheless, the absorbance of the pulp solutions at 280 nm correlated very well to the kappa number of the corresponding pulp. At this moment, this result cannot be interpreted, but is important information to understand the structure of permanganate consuming substances which are expressed as kappa number.

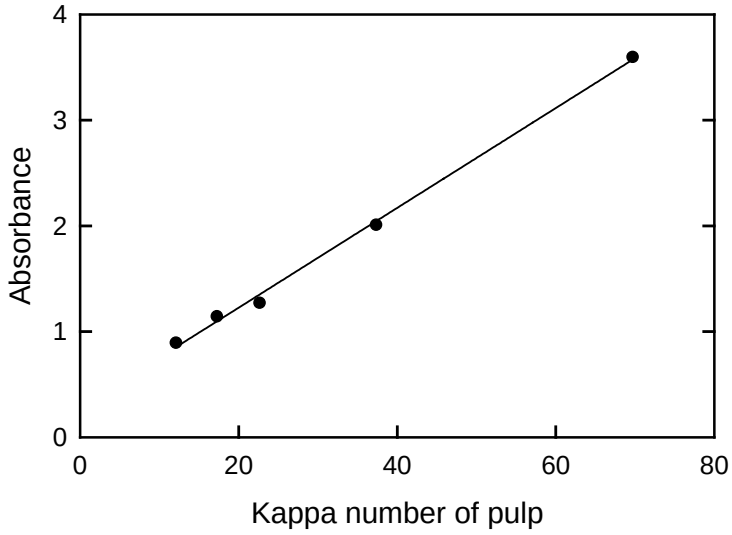

Figure 3.12 Relationships between UV absorbance of lignin at 280 nm in 0.2% HUKP solution in 8% LiCl/DMSO and kappa number of HUKP.

3.4 Conclusions

In the Chapter 2, finely milling was established as a pretreatment method to dissolve lignocellulosics with high lignin content in LiCl/DMSO solvent system. In this Chapter, in order to avoid the degradation of cell wall components during the pretreatment process, ethylenediamine (EDA) pretreatment was examined. The solvent system, lithium chloride/dimethyl sulfoxide (LiCl/DMSO) can dissolve various EDA pretreated lignocellulose samples, such as microcrystalline cellulose (Whatman CF11), cotton, cellulose I, cellulose II, holocellulose and kraft pulps (HKP, SKP) including those with relatively high lignin content. Although original Wiley wood can't be dissolved completely in LiCl/DMSO by this pretreatment, HUKP gave the transparent solution even the lignin content was as high as 10.5%. Interestingly, even in the case of coarse wood meal (40-80 mesh), about 70% could be dissolved after repeating the dissolving procedure two times. After the EDA pretreatment, the crystallinity of the lignocellulosic materials remained as high as the original samples, although the crystal structure changed. Because milling of the sample is not required, degradation of the cell wall components caused by milling does not take place. This is the first time that transparent solutions of underivatized pulps with high lignin content were obtained in a simple organic solvent system. The formation of a lignocellulose-EDA complex seems to be critical for the dissolution in LiCl/DMSO. The NMR spectrum of the EDA treated pulp solution had good resolution even though the DP of the cellulose in the pulp is very high. A very good relationship between UV absorbance of lignin at 280 nm in pulp solution and corresponding kappa number of pulp was obtained.

3.5 References

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Chapter 4

Fractionation and characterization of wood cell wall components by using LiCl/DMSO system with ball-milling pretreatment

4.1 Introduction

The occurrence of stable lignin-carbohydrate complex (LCC) is one of the main reasons preventing effective separation of the wood components in biorefining processes. The presence of LCC also causes significant problems in the research of lignin because selective and quantitative isolation of lignin fraction from lignocellulosics is almost impossible because of LCC.

As described in the Chapter 1, LCC is a conceptional term that expresses the existing manner of lignin and carbohydrates that are present together in the cell wall not only as a simple mixture but also as a complex with strong chemical or physical interaction. Most researchers assume that there is not only a physical interaction but also chemical linkage between lignin and carbohydrates. Such chemical linkage is termed LC bond in this theses. On the other hand, term of LCC is quite often used to indicate specific

fraction obtained from cell wall. The term of “Björkman LCC”, which is obtained as DMSO or DMF extract from the MWL extraction residue of milled wood, belongs to this category.

Many reports have proposed linkages between lignin and carbohydrates (LC bonds) to exist in wood or other lignocellulosic materials such as chemical pulp.[1-5] In spite of extensive works on lignin and LCC, our knowledge in this field is still insufficient. Evaluation of the interactions between lignin and carbohydrates has been one of the key subjects in the field of wood chemistry. Various LCC fractions can be obtained by methods similar to those used for lignin isolation. Extraction of the wood residue with DMSO after extraction of MWL produces Björkman LCC.[1] Recently, other solvents and water were also used to extract LCC from the residue of MWL isolation. Small lignin fragment retained in carbohydrate chain was successfully isolated and characterized.[6-9]

However, these isolation methods require rather extensive milling, causing noticeable changes in lignin structure depending on the degree of milling.[10-13] Furthermore, even when extensive milling is performed, there is still a limitation in the yield of MWL or LCC fraction. Recently, quantitative expression of the relationships between the degree of milling and structural change of lignin caused by the milling was established.[12,14] This finding suggests that as far as conventional solvents such as dioxane, DMSO or DMF are used to extract lignin and LCC fractions, it is almost impossible to increase the yield of these fractions without causing serious structural change of wood components.

In order to isolate each cell wall component in a yield close to quantitative level, the method employed is totally different from above mentioned sequential fractionation. For example, for the isolation of cellulose from wood cell, lignin has to be removed completely by oxidative degradation and alkaline extraction (preparation of holocellulose and α-cellulose). Holocelluloses can be obtained during its alkaline

extraction process. Contrary, in order to obtain lignin in a quantitative yield, carbohydrates have to be completely degraded by acid (preparation of Klason lignin) or periodate (preparation of periodate lignin). However, these fractionations are based on the destruction of cell wall components other than the targeted one. Therefore, such a fraction principally cannot give information on the interaction between cell wall components, even though it is obtained in high yield.

Consequently, problems of traditional methods for the isolation of lignin and LCC fractions can be summarized as following.

1. In order to gain the yield of soluble fraction, enhanced milling is required, which causes serious change in chemical structure of cell wall components.
2. Totally, only up to 20% of whole cell wall (about 50% of lignin) can be obtained as soluble fraction. Although the remaining 80% of whole cell wall (50% of lignin) embodies the “real LCC” that has stronger interaction between cell components than soluble fractions, it is usually left uninvestigated or cannot be analyzed by the comparable method with the soluble fraction.
3. Isolated each component based on the destruction of other components cannot give information on the interaction between cell wall components, even though it is obtained in high yield.

In order to overcome these problems, a quantitative fractionation method for wood cell wall components without seriously damaging the lignin moiety or LC bonds is required. From this point of view, the use of solvent system which can completely dissolve wood cell wall seems promising. The solvent system, lithium chloride/dimethyl sulfoxide (LiCl/DMSO) described in the chapter 2, can completely dissolve milled wood finely ground by planetary ball-mill for 2 h. The nitrobenzene oxidation and ozonation analyses indicated that the structural change of lignin caused by the 2 h of milling is not significant.

By controlling the solubilizing ability of the solvent system, different soluble and

insoluble fraction with different proportion of lignin, hemicelluloses, and cellulose will be obtained sequentially. Because even the hardest-to-dissolve fraction can be dissolved in the DMSO with 6% LiCl, every soluble and insoluble fraction can be finally obtained as soluble fraction, enabling the same solution state analyses applicable to all fractions. Based on this principle, following two basically different approaches for the study on LCC will be described in this Chapter.

1. Approaches starting from solid milled wood

   Scheme I: stepwise extraction by a series of solvents (aqueous dioxane with different water content and LiCl/DMSO with different LiCl concentration).

   Scheme II: individual extraction by a series of solvents (LiCl/DMSO with different LiCl concentration).

2. Approaches starting from whole wood solution

   Scheme III: individual precipitation in aqueous dioxane with different water content.

   Scheme IV: individual precipitation in DMSO with different LiCl concentration.

4.2 Experimental

4.2.1 Materials

Beech (*Fagus crenata* Blume) milled wood was prepared by 2 h milling in planetary ball-mill under the condition described in section 2.2.1.

4.2.2 Fractionation of different cell wall components from milled wood

The different soluble and insoluble fractions were separated by the stepwise extraction methods from milled wood according to Scheme I (**Fig. 4.1**) and by the individual extraction methods from milled wood according to Scheme II (**Fig. 4.2**).

In the Scheme I (**Fig. 4.1**), at first, the milled wood (10 g) was extracted by dioxane/water (96:4, v/v) to obtain milled wood lignin (MWL, *NO. 1.1*). Then, the residue was extracted by dioxane/water (50:50, v/v), DMSO, 1% LiCl/DMSO, 3% LiCl/DMSO sequentially. Finally, the residue, MR-DL3R (after 3% LiCl/DMSO extraction), was dissolved in 6% LiCl/DMSO. The volume of each solvent was 200 ml and the extraction time was 24 h under stirring at room temperature. The fractions named as MWL (*NO. 1.1*) and MR-DW50S (*NO. 1.2*), which represents the milled wood lignin and dioxane/water (50:50, v/v) soluble fraction of milled wood lignin residue, respectively, were freeze-dried. Other fractions were regenerated by dialysis with water, followed by freeze-drying.

In the Scheme II (**Fig. 4.2**), the milled woods were extracted individually with LiCl/DMSO containing different concentration of LiCl (0, 0.5, 1, 3, 6%). The amount of milled wood and the volume of the solvent used for each extraction were 1.5 g and 30 ml, respectively. Extraction time was 24 h under stirring at room temperature. Each soluble and insoluble fraction were freed from solvent by dialysis in water for 7 days, and then freeze-dried. Because 6% LiCl/DMSO can dissolve whole wood, this fraction

(MW-DL6S, *NO. 2.9*) gives “regenerated wood” by dialysis and freeze-drying. Yields of fraction were determined by the weight.

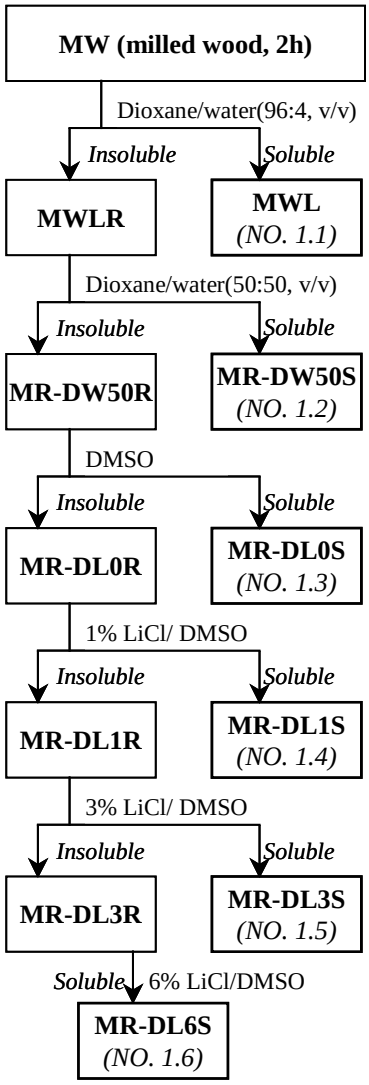

Figure 4.1 Scheme I: Isolation procedure of various fractions by the stepwise extraction from milled wood (2 h).

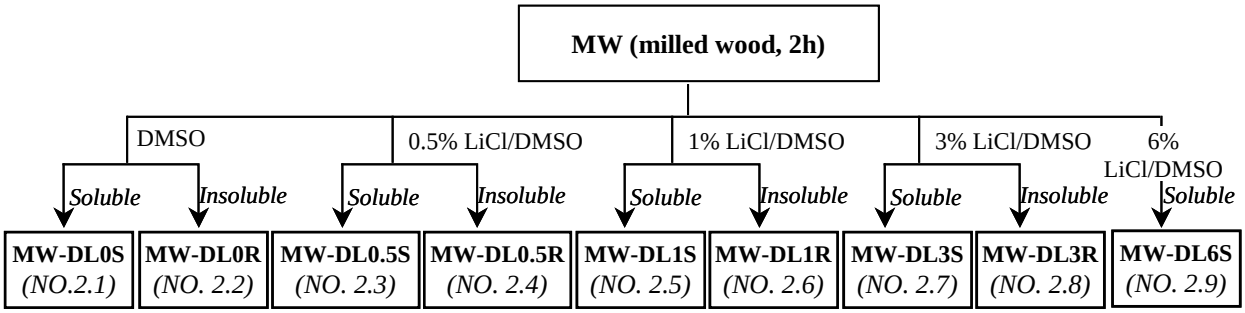

Figure 4.2 Scheme II: Isolation procedure of various fractions by the individual extraction from milled wood (2 h).

4.2.3 Fractionation of different cell wall components from wood solution

Wood solutions were prepared as described in section 2.2.6. The different soluble and insoluble fractions were separated from wood solution by precipitation with different solvents according to Scheme III and IV, as shown in **Fig. 4.3** and **4.4**, respectively.

In the Scheme III (**Fig. 4.3**), at first, the milled wood (1 g) was dissolved in 20 ml of 6% LiCl/DMSO completely to obtain wood solution. Then, the wood solutions were precipitated individually by adding into 200 ml of water, dioxane/water (50:50, v/v), dioxane/water (96:4, v/v), and dioxane to obtain different fractions, respectively. The precipitate was separated from the soluble part by centrifugation. In the Scheme IV (**Fig. 4.4**), the wood solutions were diluted individually with different amount of DMSO so that LiCl concentration can be adjusted to be 0.5, 1, and 3% respectively. The precipitate was separated from soluble part by centrifugation. Each obtained soluble and insoluble fraction of wood cell wall components was first dialyzed with water and then freeze-dried.

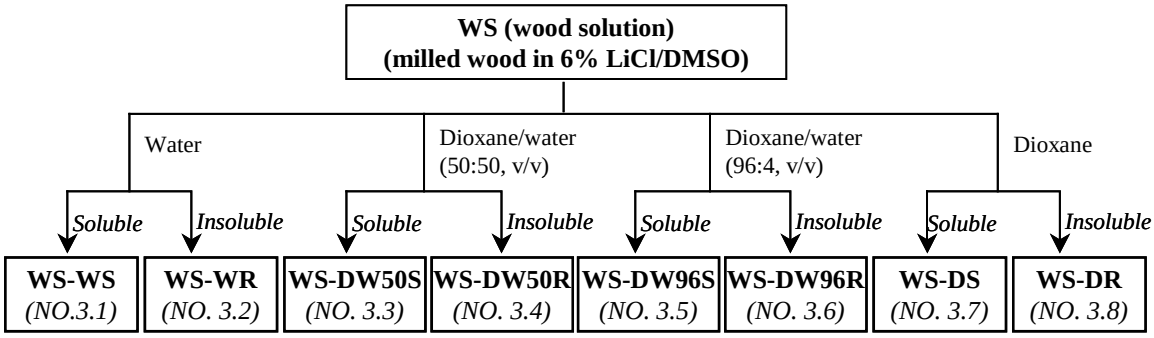

Figure 4.3 Scheme III: Isolation procedure of various fractions from wood solution.

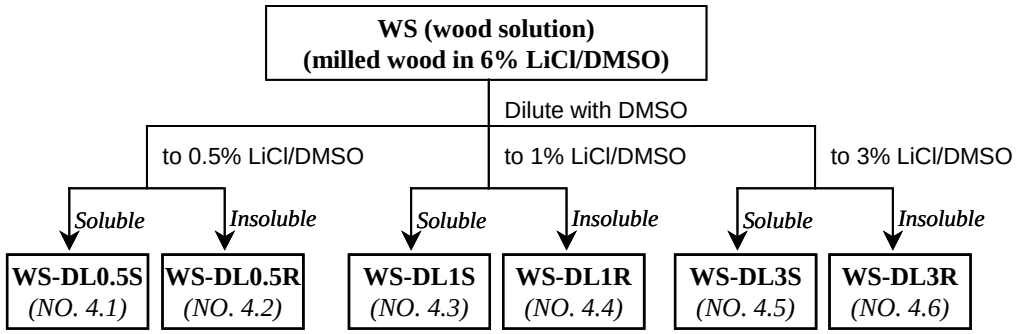

Figure 4.4 Scheme IV: Isolation procedure of various fractions from wood solution.

4.2.4 Lignin and neutral sugar analyses

The Klason method was used for the determination of lignin content in the samples.[15] Alkaline nitrobenzene oxidation analysis was applied to the samples according to the common procedure.[16] Detailed procedures are given in section 2.2.3.

Neutral sugars composing polysaccharides in the samples were determined by the alditol-acetate method.[17] One ml of 72% sulfuric acid was added to 100 mg of samples at room temperature for 4 h, and then, diluted with water to adjust the concentration of sulfuric acid at 4%. Hydrolysis in 4% sulfuric acid was performed in an autoclave at 120℃ for 1 h. After cool to room temperature, the hydrolyzed solution was filtrated by glass filter, and the filtrate was adjusted to 100 ml with water. As the internal standard solution, 1 ml of myo-inositol aqueous solution (~ 1 mg/ml) was added into 5 ml of cooled solution. The solution was neutralized by aqueous barium hydroxide solution to pH 5.5. About 20 mg $NaBH_4$ was added after centrifugation, and keep for 24 h. Excess $NaBH_4$ was removed by acetic acid. The mixture was evaporated to dryness, and heated in an oven at 105℃ for 15 min to ensure complete removal of the water. The dry residue was acetylated by 1 ml of acetic anhydride under 120℃ for 3 h, then, analyzed by gas chromatography under the following conditions.

Gas chromatography: GC-14b with FID (Shimadzu Co., Kyoto, Japan)

Column: TC-17 (fused-silica capillary column, 30 m, 0.25 mm i.d) (GL Science Inc., Tokyo, Japan)

Column program: kept for 20 min at 220℃

Injection temperature: 220℃

Detector temperature: 230℃

4.3 Results and Discussion

4.3.1 Fractionation of wood cell wall components from milled wood

Different soluble and insoluble fractions of wood cell wall components were obtained from milled wood by different scheme as shown in **Fig. 4.1** (Scheme I) and **Fig. 4.2** (Scheme II). In the Scheme I (the stepwise extraction method), aqueous dioxane was employed at the first two successive extraction stages. Increase of the water content in aqueous dioxane is known to be more favorable for the extraction of LCC rather than lignin. At the latter three extraction stages, LiCl/DMSO with increasing LiCl concentration was employed. By the increase in the LiCl concentration, LiCl/DMSO solvent system was expected to increase the solubility of cellulose-rich fraction because of the increased ability to cleave hydrogen bonding. Therefore, it is expected that by the stepwise extraction with these sequential solvent systems in the Scheme I, a part of lignin with less interaction with carbohydrate is first extracted, and then lignin with more interaction with carbohydrates gradually becomes soluble, and finally, fractions whose solubility were restricted by cellulose become soluble depending on the concentration of LiCl. In the Scheme II, the different LiCl/DMSO solvent system containing different concentration of LiCl was used to extract milled wood individually. The purpose to vary the LiCl concentration was the same as the Scheme I.

The yield of each soluble fraction and their lignin content obtained by the successive extraction according to Scheme I is shown in **Table 4.1**. The yield of milled wood lignin (MWL), obtained by dioxane/water (96:4, v/v) extraction, was only 7.72%. According to previous results in the chapter 2 and results reported by Fujimoto *et al.* [12,14] it was proposed that the degree of the structural changes of lignin is negligible when extractable lignin is obtained in such a low level. The sum of the yield of each fraction was 91.29%, due to the loss of wood components during dialysis process.

Table 4.1 Yield and lignin content of each fraction prepared by Scheme I.

NO.	Fraction	Yield (%)	Lignin content (%)
1.1	MWL	7.72	64.15
1.2	MR-DW50S	9.53	28.53
1.3	MR-DL0S	6.97	25.54
1.4	MR-DL1S	2.69	23.27
1.5	MR-DL3S	9.32	11.66
1.6	MR-DL6S	55.06	24.38

It is noticeable that there is more than 55% of the wood cell wall components remained as residue after 3% LiCl/DMSO extraction in Scheme I. This insoluble fraction is obtained as a solution by the next extraction with 6% LiCl/DMSO (MR-DL6S, *NO. 1.6*). This result is quite different from the fractions obtained by Scheme II. As shown in the **Fig. 4.5**, the yield of soluble fraction obtained by Scheme II increased with the increase in the concentration of LiCl in DMSO, leaving only 12.7% of the wood cell wall components as the residue of 3% LiCl/DMSO (MW-DL3R, *NO. 2.8*) extraction. The discrepancy of the solubility at the same LiCl concentration between Scheme I and Scheme II might be due to the different condition under which milled wood (or residue from the previous extraction) contact with LiCl in the increased concentration. In Scheme II, fresh dry milled wood contact with LiCl and DMSO simultaneously. When DMSO penetrates into the wood, LiCl is also carried, facilitating the enough contact between cell wall components and LiCl. On the other hand, in Scheme I, insoluble residue from the previous extraction is always wet with DMSO containing lower concentration of LiCl. When such sample contact with DMSO with LiCl in the increased concentration at the next extraction, the presence of DMSO inside of the sample may prevent the penetration of LiCl into the sample. Because of this, the effect of higher concentration of LiCl cannot appear well in the Scheme I.

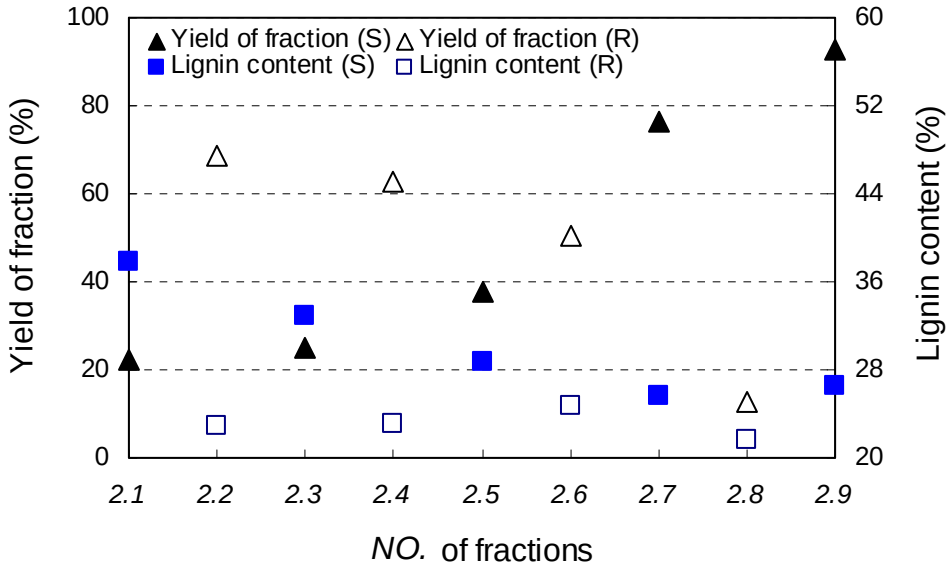

Figure 4.5 Yield and Lignin content of each fraction prepared by Scheme II.
(S: soluble fraction, R: insoluble fraction)

Either by Scheme I or Scheme II fractionation process, the lignin content in the soluble fraction decreased with an increase in LiCl concentration. The fractions rich in lignin were easily extracted by DMSO with relatively lower LiCl concentrations. On the other hand, the cellulose-rich fractions became extractable along with the increase of LiCl concentration. As Petrus *et al*. previously reported[18] that undissociated ion pairs of LiCl molecules in DMSO might interact with the oxygen atoms of hydroxyl groups and, thus, disrupt and prevent re-formation of hydrogen bonds between cellulose molecules, whereby the dissolution is facilitated.

By direct and complete dissolution of milled wood in 6% LiCl/DMSO (Scheme II), the regenerated wood could be obtained by dialysis with water. The yield of regenerated wood, MW-DL6S (*NO. 2.9*) reached 92.9%, and the total lignin content (26.6%) of MW-DL6S (*NO. 2.9*) was slightly lower than that of original wood meal (28.8%). These results were due to the loss of some low molecular weight fractions of wood

components during the dialysis process, which was also observed when Scheme I was conducted. In spite of the slight loss of low molecular weight fractions, the regenerated wood was considered to be able to represent almost the full information of entire wood cell wall components.

4.3.2 Structural characterization of lignin in each fraction

In order to investigate the structural features of lignin aromatic part in each fraction, alkaline nitrobenzene oxidation was conducted. **Fig. 4.6** and **4.7** show the yield of nitrobenzene oxidation products, S+V, and the syringyl ratio, S/(S+V), of each fraction prepared by Scheme I and Scheme II.

It can be seen from **Fig. 4.6** that the S/(S+V) ratio of MWL is rather low compared with other fractions. As a common point, the MWL is the fraction that can be easily extracted from milled wood. Therefore, the lower S/(S+V) ratio in the MWL indicates that the fractions rich in guaiacyl nuclei are extracted more easily. This can be further proven by the results shown in **Fig. 4.7**, where both the yield of S+V and the S/(S+V) ratio of the soluble fractions were always lower than those of the corresponding insoluble fractions. Moreover, the S/(S+V) ratio of soluble fractions prepared by Scheme II increased with the increase in LiCl concentration in DMSO, but the S/(S+V) ratio of insoluble fractions decreased slightly. Combined with the formation features of lignin in the wood cell wall, it is proposed that the secondary cell wall lignin with higher S/(S+V) ratio is more difficult to be extracted than the primary cell wall lignin with lower S/(S+V) ratio. With the increase in the dissolving capacity of solvent by the increase in LiCl concentration, the fractions with higher S/(S+V) ratio became extractable, suggesting that contribution of secondary cell wall lignin to the soluble fraction became greater. It should be noted that these results support the previous findings in our laboratory. In the present work, the increase of the soluble fraction was achieved by the increase of LiCl concentration of the solvent. In the previous works, it

was achieved by prolonging the milling time to prepare milled wood. In both cases, the increase in the soluble fraction is accompanied by the dissolution of the lignin with higher S/(S+V) ratio, suggesting that the contribution of secondary wall lignin gradually increase with the increase in the soluble fraction. The present research proved that this tendency can be observed basically during the whole solubilization process of the cell wall. In the previous works, this tendency was suggested by the solubilization of only up to 30% of lignin.

Fig. 4.8 shows the relationship of lignin content with the yield of S+V or the S/(S+V) ratio in each fraction obtained from Scheme II. Both the yield of S+V and the S/(S+V) ratio of each fraction decreased with increase in the lignin content. The good correlations indicate that aromatic structures of lignin are related to the lignin content of the fraction regardless whether it is soluble or insoluble. It is proposed that the fractions with lower lignin content are richer in non-condensed type than those with higher lignin content.

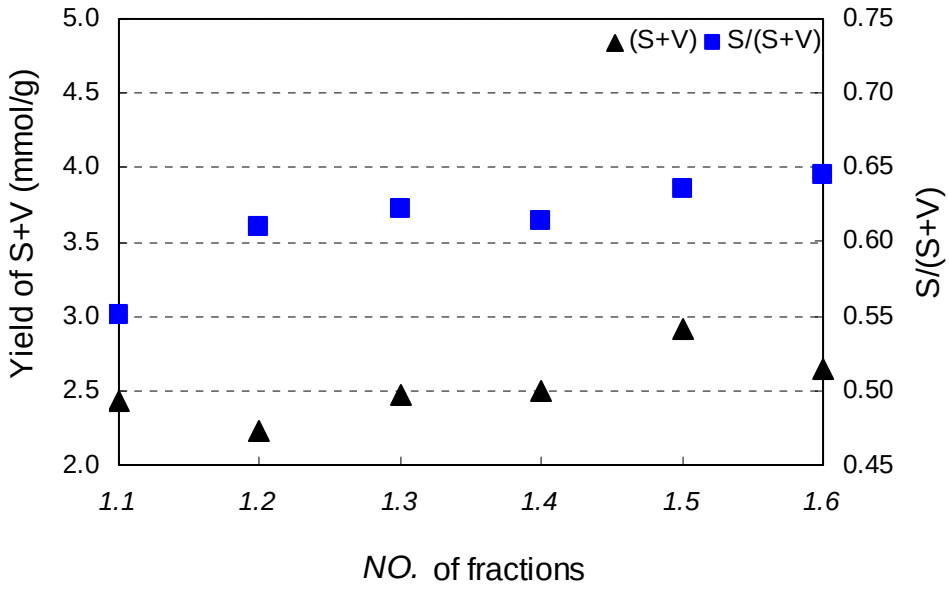

Figure 4.6 Yield of S+V and S/(S+V) ratio of each fraction prepared by Scheme I.

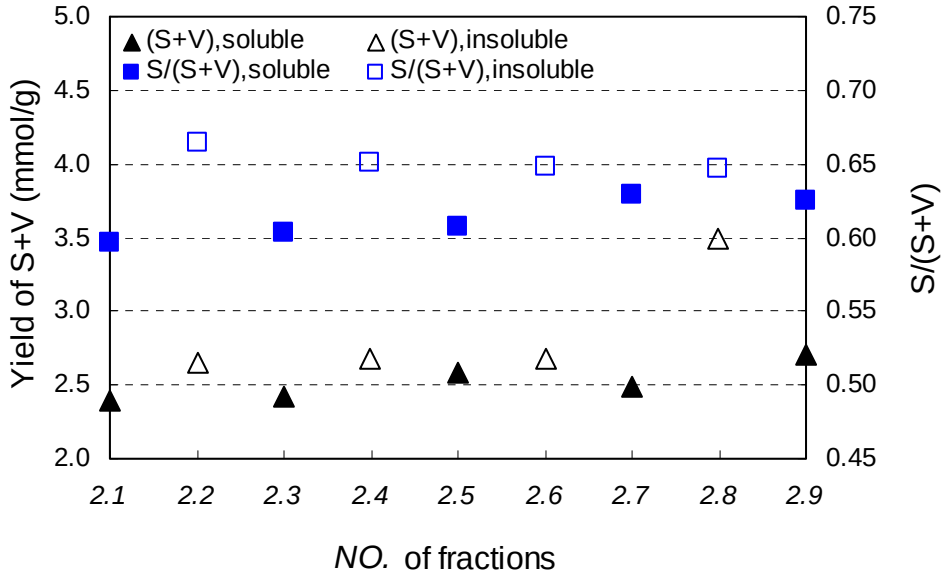

Figure 4.7 Yield of S+V and S/(S+V) ratio of each fraction prepared by Scheme II.

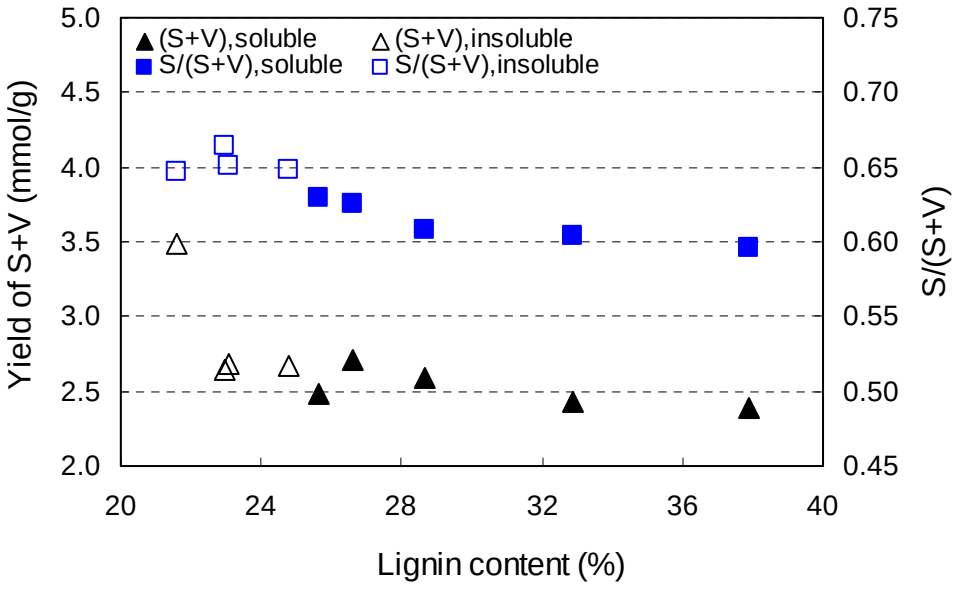

Figure 4.8 Dependence of the yield of S+V and S/(S+V) ratio on the lignin content of each fraction prepared by Scheme II.

4.3.3 Composition of neutral sugars in each fraction and the importance of 3% LiCl/DMSO insoluble fraction

The weight ratios of neutral sugars in each fraction prepared by Scheme I and Scheme II were shown in **Table 4.2** and **Fig. 4.9**, respectively. As shown in the **Table 4.2**, the weight ratios of glucose in MWL (*NO. 1.1*) and MR-DW50S (*NO. 1.2*) that represent the extractable fraction by dioxane/water were quite low, but, the weight ratios of xylose in them were very high. Thereafter, when LiCl/DMSO was used as extraction solvent, the weight ratio of glucose in the soluble fraction increased with increase in LiCl concentration except fraction MR-DL6S (*NO. 1.6*). The distinct feature of MR-DL6S (*NO. 1.6*) from other soluble fractions was due to its dual roles, i.e. it acts both the residue after 3% LiCl/DMSO extraction (equal to MR-DL3R somehow) and the final soluble fraction in 6% LiCl/DMSO.

Table 4.2 Weight ratio of neutral sugars of each fraction prepared by Scheme I.

NO.	Fraction	Weight ratio of neutral sugars (%)					
		Glu	Xyl	Man	Gal	Ara	Rha
1.1	MWL	15.69	77.94	ND*	ND	1.56	2.70
1.2	MR-DW50S	10.95	75.09	4.73	4.60	2.71	1.93
1.3	MR-DL0S	39.44	46.45	10.90	1.70	0.78	0.73
1.4	MR-DL1S	63.26	24.38	9.55	1.65	0.60	0.55
1.5	MR-DL3S	89.02	7.57	1.94	0.94	0.29	0.23
1.6	MR-DL6S	76.28	19.88	1.56	1.26	0.56	0.47

* ND, not detectable.

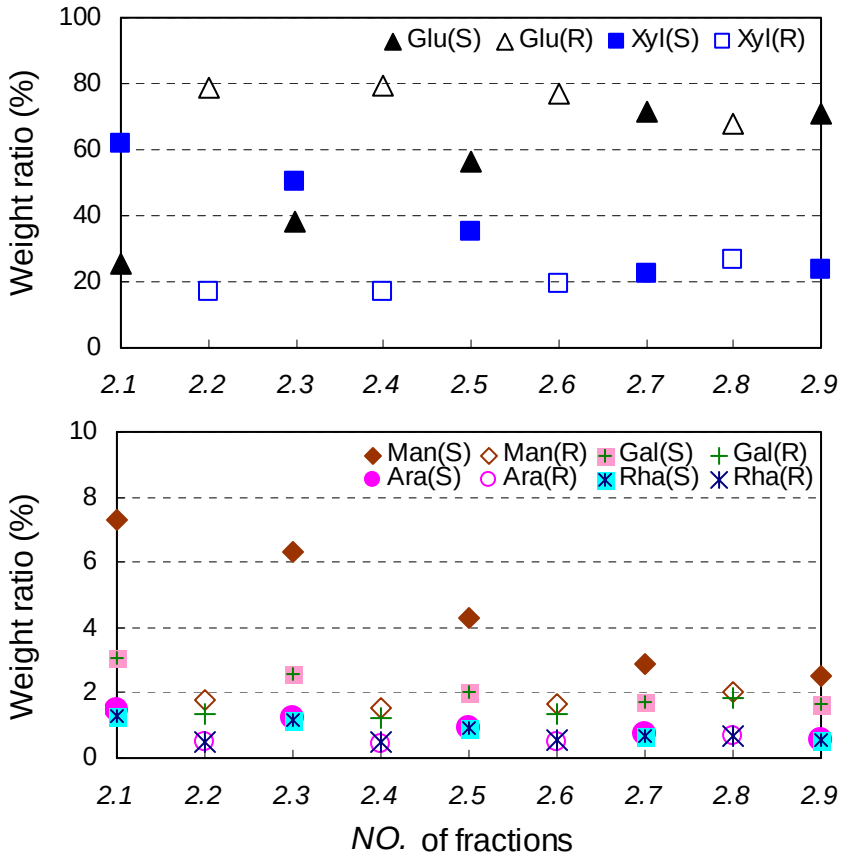

Figure 4.9 Weight ratio of neutral sugars of each fraction prepared by Scheme II. (S: soluble fraction, R: insoluble fraction)

As shown in **Fig. 4.9**, the weight ratio of glucose in soluble fraction were lower than those of corresponding insoluble fractions when concentration of LiCl were relatively low (0, 0.5, 1%). However, the weight ratio of glucose in soluble fractions increased with the increase in the concentration of LiCl in DMSO, indicating that more cellulose became soluble. Contrary, the weight ratio of glucose in insoluble fractions decreased. When concentration of LiCl reached 3%, the weight ratio of glucose in the soluble fraction MW-DL3S (*NO. 2.7*) became higher than that in the insoluble fraction MW-DL3R (*NO. 2.8*). This must be due to the increased ability of the solvent system to break and prevent hydrogen bonding working between cellulose molecules. These results suggest an idea that solubilization of milled wood by the LiCl/DMSO solvent

system is basically controlled by the solubilization of cellulose.

On the other hand, in the case of neutral sugars from hemicelluloses, basically the opposite trend was observed. The weight ratio of these sugars decreased in soluble fractions and increased in the insoluble fractions with the increase in LiCl concentration. The weight ratio of xylose in the MW-DL0S (*NO. 2.1*) was rather high (61.64%) when only DMSO (without LiCl) was used as solvent. However, along with increase in LiCl concentration, the weight ratio of sugars of hemicelluloses in soluble fractions decreased, i.e., the contribution of hemicellulosic component to soluble fraction became lower, and the contribution of cellulose to soluble fraction became higher. The solubilization behavior of carbohydrates and lignin were shown in **Fig. 4.10.** As to the solubilization behavior of carbohydrates, the most significant trend resulted from the increase in LiCl concentration of the solvent is the increase of the proportion of glucan (most presumably cellulose) in the fraction. It was safely stated that solubilization of cellulose controls the whole solubilization of milled wood. It was also clearly shown that about 40% of lignin and xylan dissolved independently from the cellulose solubilization but the solubilization of the rest was assisted by the cellulose solubilization.

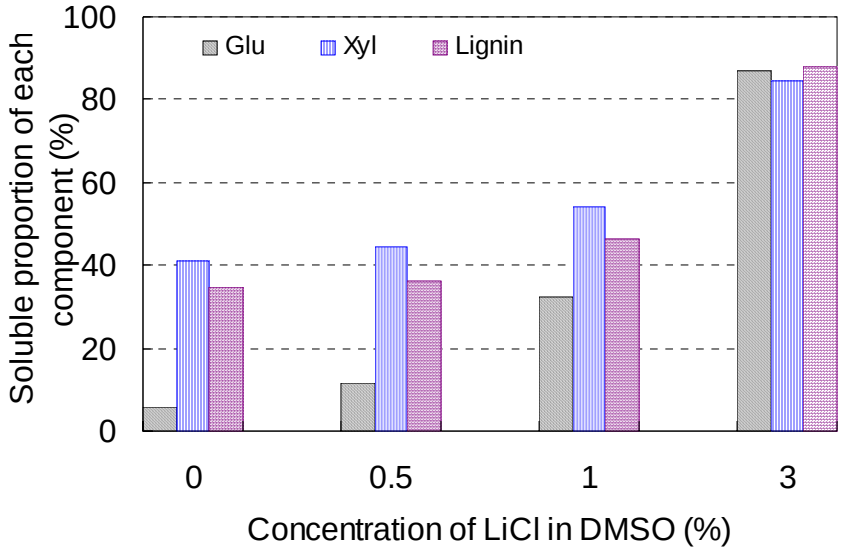

Figure 4.10 The solubilization behavior of carbohydrates and lignin.

The MW-DL3R (*NO. 2.8*) was the hardest-to-dissolve fraction to LiCl/DMSO system among the fractions prepared by Scheme II. In other words, the MW-DL3R (*NO. 2.8*) was the fraction that needs highest LiCl concentration (6%) in DMSO to be dissolved. It is very interesting that this insoluble MW-DL3R (*NO. 2.8*) fraction contained less glucose than other insoluble fractions, and more sugars from hemicellulose. The similar results were obtained also from Scheme I as shown in **Table. 4.2.** The harder-to-dissolve fraction MR-DL6S (*NO. 1.6*) contains less glucose and more xylose than the preceding soluble fraction MR-DL3S (*NO. 1.5*).

In order to explain these results, one hypothesis is necessary to be made. That is, the solubility of cellulose is simply affected by the LiCl concentration in LiCl/DMSO solvent system, but there is some special fraction, which cannot simply solubilized by the dissolution of cellulose. Based on this hypothesis, obtained results can be explained as following. As shown in **Table 4.3**, each fraction obtained by Scheme I and II can be basically classified into three groups. The first group is dissolved easily by the solvent containing no LiCl. The solubility of this group is basically controlled by the milling degree during the preparation of milled wood and by the property of the solvent itself. MWL and Björkman LCC belong to this group. The second group requires up to 3% concentration of LiCl to be dissolved, indicating that assist of cellulose solubilization is necessary to make this group soluble. As shown in **Table 4.4**, milled cellulose prepared in the same manner as the milled wood can be completely dissolved in LiCl/DMSO with 3% LiCl concentration. However, group 3 could not be dissolved by 3% concentration of LiCl. This suggests that not only the presence of cellulose but also another factor plays a role to prevent this group from being dissolved. The author believes this is the interaction between lignin, hemicelluloses, and cellulose, possibly. When fractions belonging to the group 2 become soluble by the increase of LiCl concentration, the contribution of group 3 becomes greater in the residual fraction. This should be the reason why the proportion of hemicelluloses in the insoluble fraction becomes greater with the progress of solubilization.

The group 3 can be regarded as the "real LCC" where the interaction between each cell wall component is the main force to prevent it from being dissolved. It should be emphasized that such fraction has never been obtained in previous studies. This is the advantage of the whole wood solution system in the LCC study.

Table 4.3 Classification of obtained fractions by Scheme I and II.

Group	Corresponding fraction in Scheme I	Corresponding fraction in Scheme II	Limiting factor for the solubilization	Overall solubility
1	MWL MR-DW50S MR-DL0S	MW-DL0S	milling degree property of solvent	high ↑
2	MR-DL0.5S MR-DL1S MR-DL3S	MW-DL0.5S MW-DL1S MW-DL3S	(*in addition to above*) cellulose solubilization	
3	MR-DL6S	MW-DL3R	(*in addition to above*) interaction between cell wall components	low

Table 4.4 Solubility of milled sample in LiCl/DMSO with different LiCl concentration and LiCl/DMAc.

Sample	LiCl/DMSO with					8.0% LiCl/DMAc
	0% LiCl	0.5% LiCl	1.0% LiCl	3.0% LiCl	6.0% LiCl	
Milled cellulose (2h)	-*	-	-	+	+	-
Cellulose	-	-	-	-	-	+**
Milled wood (2h)	-	-	-	-	+	-

* -, incomplete soluble under room temperature, +, complete soluble under room temperature.
** Cellulose was dissolved in 8% LiCl/DMAc under 130 ^{o}C.

4.3.4 Correlation between carbohydrate composition and lignin structure

A significantly high correlation was observed between the weight ratio of sugars and the lignin content of each fraction prepared by Scheme II as shown in **Fig. 4.11**. Except fraction MW-DL3R (*NO. 2.8*, lignin content is 21.6%), the weight ratio of glucose decreased with the increase in lignin content, at the same time, the weight ratio of sugars from hemicelluloses increased. Especially, when lignin content was more than 32.9%, the weight ratio of xylose was higher than that of glucose. The same trend could

also be observed from the fractions prepared by Scheme I except MR-DL6S (*NO. 1.6*). It is noticeable that fraction MW-DL3R (*NO. 2.8*) presented lowest lignin content and the lowest glucose to xylose ratio (glu/xyl) among all the insoluble fractions. As mentioned before, 3% LiCl concentration, which is enough to make milled cellulose soluble, could not assist this fraction to get solubilized. Relative enrichment of xylose in this hardest-to-dissolve fraction suggest that cleavage of the interaction of hemicellulose with other cell wall component requires higher concentration of LiCl.

The carbohydrate compositions were related not only with content of lignin but also with structure of lignin. As shown in **Fig. 4.12**, the weight ratio of glucose increased with increase in S/(S+V) ratio in all the soluble and insoluble fractions prepared by Scheme II. Meantime, the weight ratio of sugars from hemicelluloses decreased.

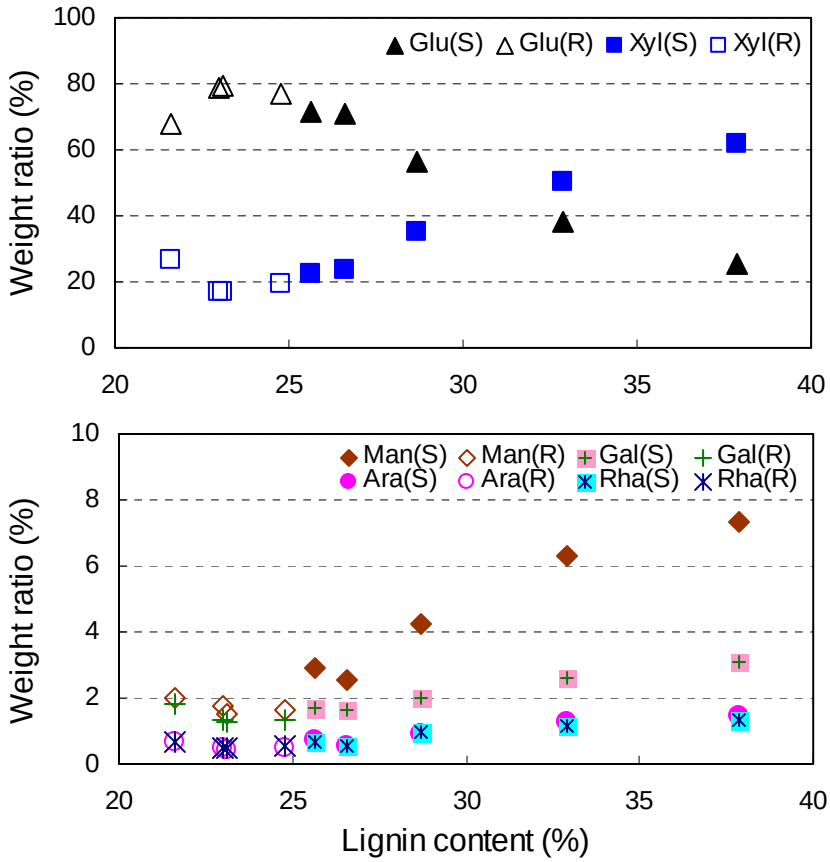

Figure 4.11 Dependence of the weight ratio of sugars on the lignin content of each fraction prepared by Scheme II (S: soluble fraction, R: insoluble fraction).

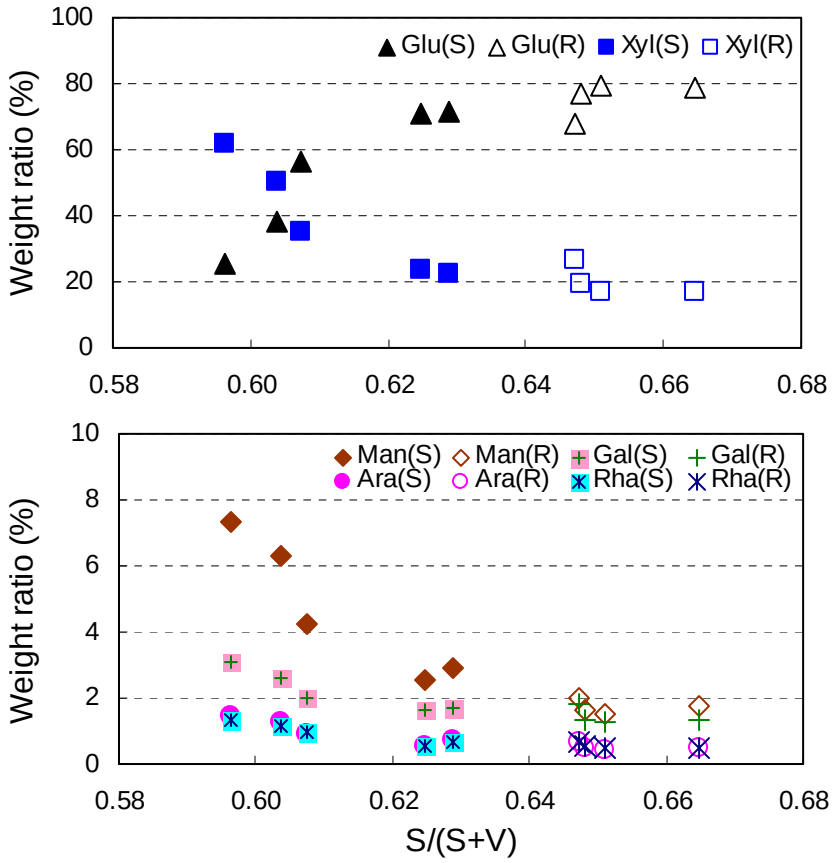

Figure 4.12 Dependence of the weight ratio of sugars on the ratio of S/(S+V) of each fraction prepared by Scheme II (S: soluble fraction, R: insoluble fraction).

4.3.5 Fractionation of wood cell wall components from wood solution

Most of lignin fractions, such as MWL, CEL, including the LCC fractions described in the previous sections, were obtained by a liquid-solid extraction process for dissolving some fraction in an appropriate solvent. Because all of them employ solid milled wood as starting material, involvement of a liquid-solid extraction is crucial. If isolation of wood cell wall components could be obtained not from milled wood (solid) but from whole wood solution (liquid), it will provide improved methods for the

analysis of cell wall components. In the chapter 2, the ball-milled wood (2 h) can be completely dissolved in 6% LiCl/DMSO. Therefore, the method different from liquid-solid extraction could be achieved by the use of this solution. In this section, different fractions were obtained by the precipitation of wood solution through adding the solution into different solvents individually.

The wood solutions were precipitated by water, dioxane/water (50:50, v/v), dioxane/water (96:4, v/v), and dioxane to obtain different fractions, as shown in Scheme III (**Fig. 4.3**). The yield, lignin content, yield of S+V, ratio of S/(S+V), and neutral sugar compositions of each fraction were shown in **Table 4.5**. As shown in **Table 4.5**, all of yield of soluble fractions were not very high. The yield of WS-DW50S (*NO. 3.3*) was 14.91%, which was higher than other soluble fractions. The yield of WS-DS (*NO. 3.7*) was quite low, only 1.45%. It is interesting that 6.63% of soluble fraction can be obtained (WS-WS, *NO. 3.1*) by precipitation with only water. It is noticeable that the soluble fraction WS-DW96S *(NO. 3.5*), which was obtained by dioxane/water (96:4, v/v), shows quite low yield compared with usual MWL (*NO. 1.1*). Meanwhile, the lignin content, the yield of S+V, and the ratio of S/(S+V) in the fraction WS-DW96S *(NO. 3.5*) are higher than that of MWL (*NO. 1.1*). It indicated that even both the fraction WS-DW96S (*NO. 3.5*) and the MWL (*NO. 1.1*) were obtained by using dioxane/water (96:4, v/v) system, they showed different characters according to different isolation methods (milled wood based liquid-solid extraction, or wood solution based liquid-liquid precipitation). And as shown in the **Table 4.5**, the weight ratio of xylose in every soluble fraction was higher than the weight ratio of glucose. It is interesting that the weight ratio of xylose in fraction WS-WS (*NO. 3.1*) was also very high even though the lignin content in this fraction was quite low. This fraction can be regarded as water soluble xylan fraction.

Table 4.5 Characterization of the fractions obtained by the Scheme III.

NO.	Fraction	Yield (%)	Lignin content (%)	S+V (mmol/g)	S/(S+V)	Weight ratio of neutral sugars (%)					
						Glu	Xyl	Man	Gal	Ara	Rha
3.1	WS-WS	6.63	15.32	1.62	0.69	11.87	73.39	5.83	4.63	2.11	2.17
3.2	WS-WR	84.95	29.23	2.57	0.63	74.67	20.32	2.57	1.42	0.52	0.50
3.3	WS-DW50S	14.91	49.37	2.24	0.58	9.64	76.54	3.10	5.45	2.82	2.45
3.4	WS-DW50R	75.92	23.98	2.58	0.65	75.19	20.31	2.58	1.00	0.44	0.48
3.5	WS-DW96S	2.99	76.29	2.64	0.60	22.51	63.07	5.87	5.78	0.00	2.78
3.6	WS-DW96R	88.49	25.74	2.59	0.63	69.59	25.10	2.64	1.46	0.66	0.55
3.7	WS-DS	1.45	74.46	ND[a]	ND	24.57	67.31	6.32	-	-	1.80
3.8	WS-DR	90.48	26.53	2.51	0.63	68.87	25.51	2.70	1.57	0.69	0.66

[a] ND, not determined.

One more fractionation method based on wood solution was carried out as shown in the Scheme IV (**Fig. 4.6**). The wood solutions were diluted with different amount of DMSO to adjust the LiCl concentration to 0.5, 1, and 3%. The yield, lignin content, yield of S+V, ratio of S/(S+V), and neutral sugar compositions in obtained soluble and insoluble fractions were shown in **Table 4.4**. The yield of soluble fraction increased with the increase in concentration of LiCl. The yield of WS-DL3S (*NO. 4.5*) reached 86.08%. Even when the concentration of LiCl was as low as 0.5%, the yield of WS-DL0.5S (*NO. 4.1*) was 52.15%. These yields of soluble fractions obtained by Scheme IV were significantly higher than the yields of the soluble fractions obtained by Scheme II, although the same concentration of LiCl was used. The characters of lignin and sugar in each fraction were also different from those in the fractions prepared from Scheme II under the same LiCl concentration. It is quite important to note that complete separation of cell wall components into “lignin”, “hemicellulose”, and “cellulose” was not possible.

The results obtained by Scheme III and IV were quite different from the results

obtained by Scheme I and II. This new fractionation method based on complete wood solution should be further examined.

Table 4.6 Characterization of the fractions prepared by Scheme IV.

NO.	Fraction	Yield (%)	Lignin content (%)	S+V (mmol/g)	S/(S+V)	Weight ratio of neutral sugars (%)					
						Glu	Xyl	Man	Gal	Ara	Rha
4.1	WS-DL0.5S	52.15	35.15	2.55	0.62	52.20	39.20	4.40	2.21	0.99	1.00
4.2	WS-DL0.5R	38.54	16.42	3.09	0.65	86.59	10.74	1.03	0.96	0.35	0.32
4.3	WS-DL1S	78.08	27.90	2.51	0.63	73.48	21.47	2.64	1.31	0.57	0.52
4.4	WS-DL1R	12.03	29.99	2.52	0.64	68.88	26.85	2.33	1.80	0.02	0.13
4.5	WS-DL3S	86.08	27.24	2.54	0.63	71.52	22.92	2.76	1.59	0.63	0.59
4.6	WS-DL3R	4.77	30.80	2.41	0.64	67.71	26.28	2.70	1.87	0.69	0.75

4.4 Conclusions

By the use of LiCl/DMSO solvent system which can dissolve whole milled wood, new methods for the separation of cell wall components were examined. Two basically different approaches were made to give different fractions.

Approach 1 starting from solid milled wood

Scheme I: stepwise extraction by aqueous dioxane with different water content and LiCl/DMSO with different LiCl concentration

Scheme II: individual extraction by LiCl/DMSO with different LiCl concentration

Approach 2 starting from whole wood solution

Scheme III: individual precipitation in aqueous dioxane with different water content

Scheme IV: individual precipitation in DMSO with different LiCl concentration

Approach 1

Based on the solubilization behavior and structural analyses, obtained fractions were classified into three groups. The solubilization of the first group is not restricted by the cellulose but basically by the milling degree during the preparation of milled wood and by the property of the solvent itself. Traditionally obtained MWL and Björkman LCC belong to this group. The second group is made soluble by the solubilization of cellulose. Because the main part of the cell wall belongs to this group, the solubilization of cellulose is a main cause of cell wall solubilization. However, the third group cannot be made soluble only by the solubilization of cellulose. Fractions in this group are the hardest-to-dissolve in the cell wall and seem to be prevented from getting solubilized by the strong interaction between lignin and carbohydrates. In this meaning, fractions in

the third group can be regarded as “real LCC” that could never be analyzed by the previous studies. The finding and structural elucidation of the fractions belonging to this group is the most significant achievement of this chapter. Structural features of lignin in this group are quite different from those of the group 1, so called MWL and Björkman LCC fraction. Namely, lignin in the group 3 has a nature of uncondensed type, which is a feature of secondary wall lignin. Glucose to xylose ratio (glu/xyl) of this group is much higher than group 1, but a little bit lower than the value of whole wood. Therefore, group 3 is featured by the non-condensed type lignin and hemicellulosic sugars. Whether or not fractions in this group originate from specific portion of cell is still unknown.

Approach 2

Fractionation of cell wall components starting from whole wood solution was examined. Interestingly, some part of xylan was obtained as water soluble xylan (fraction WS-WS from Scheme III). Other than this, it was impossible to separate cell wall components into “lignin”, “hemicellulose”, and “cellulose” fraction. Further examination of obtained each fraction and the establishment of more effective precipitation methods are required.

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Chapter 5

Fractionation and characterization of wood cell wall components by using LiCl/DMSO system with ethylenediamine pretreatment

5.1 Introduction

In the chapter 3, ethylenediamine (EDA) treatment was developed as a pretreatment method to dissolve pulp with high lignin content in LiCl/DMSO. Because this method doesn't require milling pretreatment, structural modification of lignin and the serious degradation of cellulose can be avoided. Various hardwood and softwood chemical pulps, including those with relatively high lignin content (up to ca.10.5%), were completely dissolved without milling in LiCl/DMSO after a pretreatment with EDA. In this chapter, new fractionation method was applied to wood for structural analyses of lignin, LCC and polysaccharide by the use of this dissolution method.

5.2 Experimental

5.2.1 Materials

Beech Wiley wood was prepared as described in section 2.2.1.

Delignification was conducted by treating 2.5 g of Wiley wood with 1 g of $NaClO_2$ and 0.2 ml of acetic acid in 150 ml water at 75 ℃ for 1 h. This treatment was conducted once to three times to give DW1 (delignified wood 1), DW2 (delignified wood 2), and DW3 (delignified wood 3), respectively, with different lignin content (**Fig. 5.2)**.

5.2.2 Pretreatment of lignocellulosic material with EDA

The wiley wood or partially delignified wood was treated with ethylenediamine (EDA) as a pretreatment of dissolution without milling. The pretreatment condition was the same as section 3.2.2. In this chapter, 10 g of samples were soaked in 100 ml EDA. The Wiley wood and delignifed woods treated with EDA are referred as “wood-EDA complex” and “DW-EDA complex” as shown in **Fig. 5.1** and **5.2**, respectively.

5.2.3 Fractionation of wood cell components

The fractions were separated by 8% LiCl/DMSO extraction from EDA pretreated Wiley wood and delignified wood. The wood-EDA complex and DW-EDA complexes were suspended in 8% LiCl/DMSO. The mixture was kept with stirring at room temperature for 24 h, and heated up to 75 ℃, kept with stirring at this temperature for several hours. The soluble and insoluble fractions were separated by centrifugation. As shown in **Fig. 5.1**, the wood-EDA complex was extracted by 8% LiCl/DMSO to obtain soluble fraction WE-DL8S (*NO. 5.1*) and insoluble fraction WE-DL8R (*NO. 5.2*). The residue WE-DL8R (*NO. 5.2*) was freeze-dried after regeneration by dialysis with water.

The dried WE-DL8R (*NO. 5.2*) fraction was treated with EDA again. The EDA treated WE-DL8R residue (WRE) was extracted by 8% LiCl/DMSO again. The soluble fraction WRE-DL8S (*NO. 5.3*) and insoluble fraction WRE-DL8R (*NO. 5.4*) were obtained.

The EDA pretreated delignified woods with different lignin content were extracted by 8% LiCl/DMSO to obtain soluble and insoluble fraction as shown in **Fig. 5.2**. All of the fractions were freeze-dried after regeneration by dialysis with water.

5.2.4 Lignin and neutral sugar analyses

The Klason method was used for the determination of lignin content in the samples.[1] Alkaline nitrobenzene oxidation analysis was applied to the samples according to the common procedure.[2] Detailed procedures are given in section 2.2.3. Neutral sugars in the samples were determined by the alditol-acetate method.[3] Detailed procedures are given in section 4.2.4.

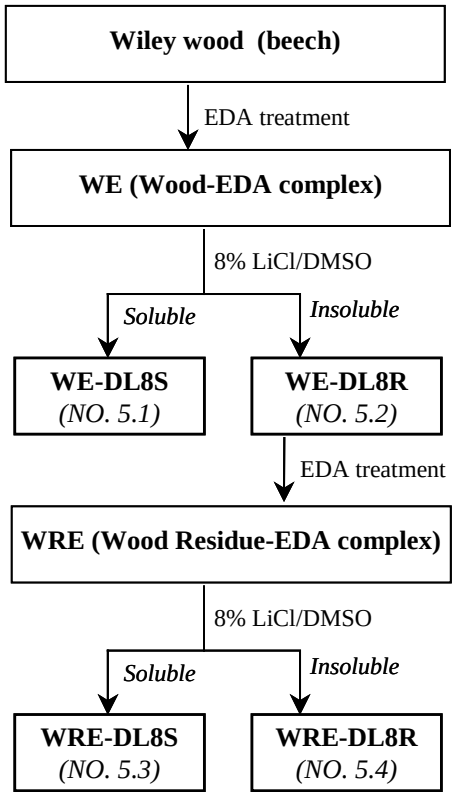

Figure 5.1 Scheme V: Isolation procedure of various fractions from EDA treated Wiley wood.

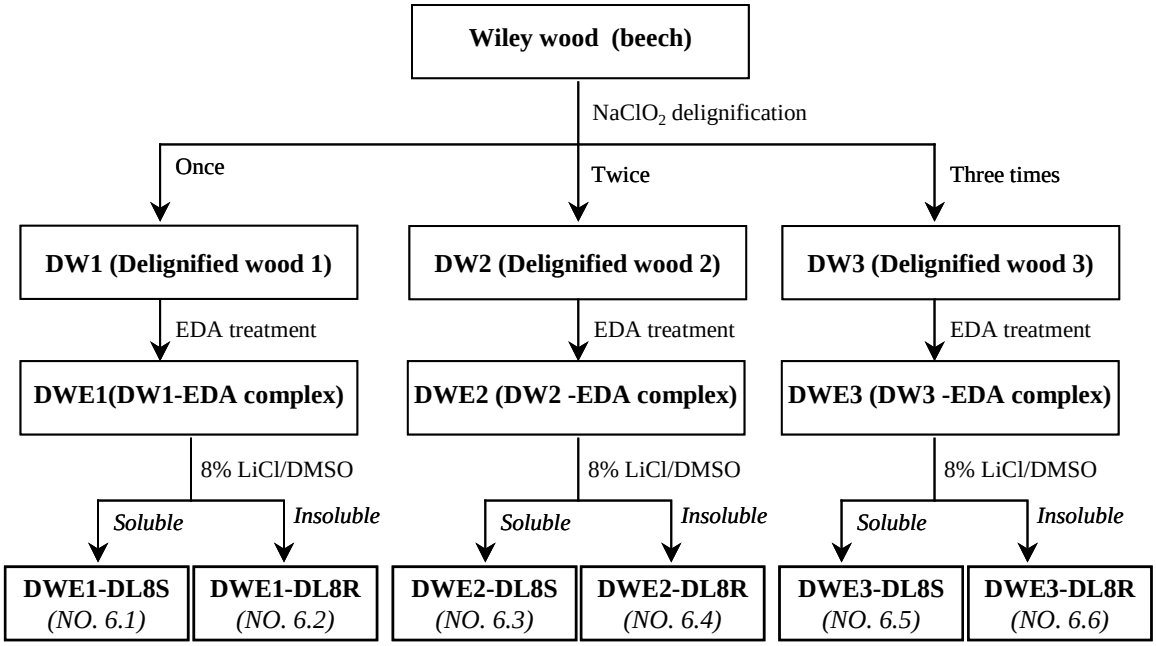

Figure 5.2 Scheme VI: Isolation procedure of various fractions from delignified wood.

5.3 Results and Discussion

5.3.1 EDA pretreatment of Wiley wood and delignified wood

As described in the chapter 3, various lignocellulosic materials such as CF11, cotton, cellulose I, cellulose II, holocellulose, hardwood and softwood chemical pulps, including those with relatively high lignin content, can be completely dissolved in LiCl/DMSO without milling after a pretreatment with EDA. When a chemical pulp contained about 14.6% lignin, it cannot be completely dissolved in LiCl/DMSO after EDA pretreatment. Therefore, it is quite important to understand the reason why insoluble fraction is left when lignin content is higher than certain level.

Wiley wood without milling was subjected to the dissolution process consisting of EDA pretreatment and dissolution in 8% LiCl/DMSO. Because the yield of the soluble fraction from this process was only about 20%, the residue was again subjected to the same dissolution process. The EDA treated freeze-dried wood is called as “wood-EDA complex”.

High lignin content was considered to relate to the incomplete solubility of EDA pretreated wood meal. Therefore, wood meals were delignified by $NaClO_2$ under acid condition. By repeating the delignification treatment, delignified woods with different lignin content were prepared. The yield of delignified wood and its lignin content were shown in **Fig. 5.3**. Both the yield and lignin content of the delignified wood decreased with the increase in repeating times. It should be mentioned that the lignin content of the DW3 (delignified wood 3) is still maintained to be 14.1% even though the delignification treatment was repeated 3 times. Delignified woods were soaked in EDA with stirring for 1 day at room temperature followed by freeze-drying.

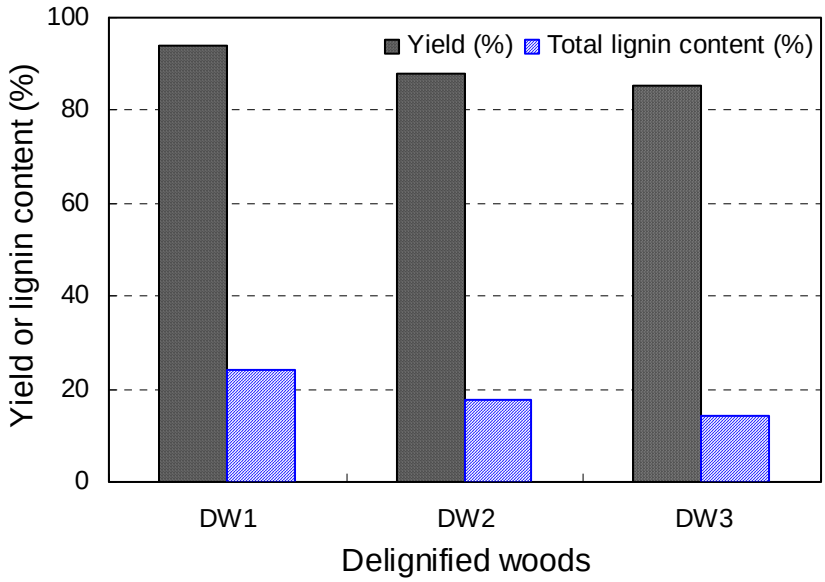

Figure 5.3 The yield and lignin content of the delignified woods. (Lignin content of original wood was 28.8%)

5.3.2 Fractionation of wood cell wall components

In the chapter 4, different cell wall fractions were obtained by varying dissolving capacity of the solvent system, which was controlled by LiCl concentration in LiCl/DMSO solvent system. On the contrary, in this chapter, the LiCl concentration was maintained at 8% in DMSO. Namely, the dissolving capacity of solvent system was fixed to the highest level. Instead, the different soluble and insoluble fractions of wood cell wall components were obtained by varying the solubility of each sample in 8% LiCl/DMSO system. One method to change the solubility of the sample was to conduct the second EDA pretreatment for the insoluble residue from the first dissolution (Scheme V, **Fig. 5.1**), and another was to partially delignify the wood before subjecting to EDA pretreatment (Scheme VI, **Fig. 5.2**). In the Scheme V (**Fig. 5.1**), at first, the EDA pretreated Wiley wood was extracted with 8% LiCl/DMSO, thereafter, the soluble fraction WE-DL8S (*NO. 5.1*) and insoluble fraction WE-DL8R (*NO. 5.2*) were

separated. Then, the insoluble fraction WE-DL8R (*NO. 5.2*) was treated with EDA and extracted by 8% LiCl/DMSO again. The second extraction process produced soluble fraction WRE-DL8S (*NO. 5.3*) and insoluble fraction WRE-DL8R (*NO. 5.4*). In the Scheme VI (**Fig. 5.2**), the delignified wood samples with different lignin content were treated by EDA and extracted by 8% LiCl/DMSO. Different soluble and insoluble fractions were obtained from delignified woods.

The yield of each soluble and insoluble fraction and their lignin content obtained by Scheme V and Scheme VI were shown in **Fig. 5.4** and **5.5** respectively. As shown in **Fig. 5.4**, the Wiley wood still could not be completely dissolved in 8% LiCl/DMSO by the second EDA pretreatment. In Scheme V, the yield of each soluble fraction was lower than that of corresponding insoluble fraction. As shown in **Fig. 5.4**, the first EDA treatment of Wiley wood yielded 24.8% of soluble fraction WE-DL8S (*NO. 5.1*) in 8% LiCl/DMSO. After the second EDA treatment of the remaining residue WE-DL8R (*NO. 5.2*), the yield of soluble fraction WRE-DL8S (*NO. 5.3*) could reach to 41.0% based on the weight of WE-DL8R (*NO. 5.2*) leaving 31.7% of the original Wiley wood as a residue.

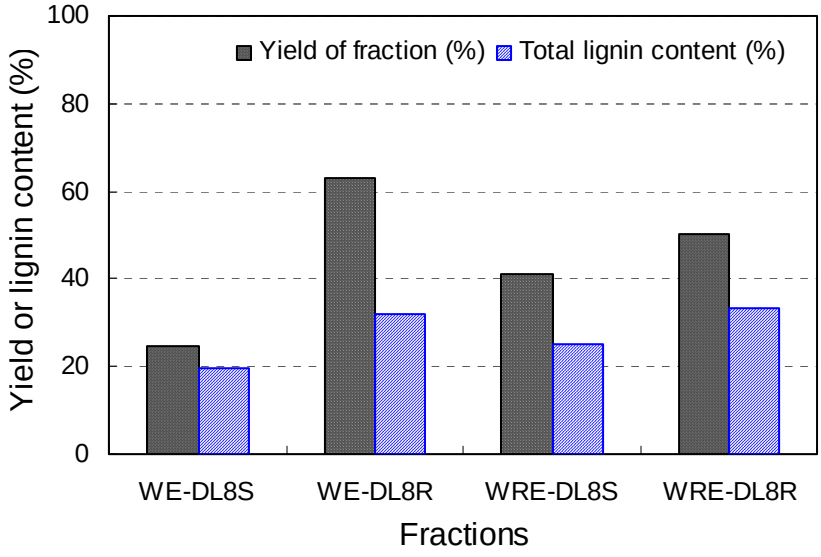

Figure 5.4 Yield and lignin content of each fraction prepared by Scheme V.

The yield of soluble and insoluble fraction separated from delignifed wood by Scheme VI was shown in **Fig. 5.5**. The yield of each soluble fraction obtained from Scheme VI was higher than that of corresponding insoluble fraction. Apparently, the results were due to the low lignin content. The yield of insoluble fraction decreased along with the increase in degree of delignification, i.e. the solubility of delignified wood in LiCl/DMSO increased along with the decrease in the lignin content of each delignified wood. From **Fig. 5.5**, the yield of insoluble fraction DWE3-DL8R (*NO. 6.6*) is only 9.2% when the lignin content of delignified wood (DW3) was relatively lower, 14.1% (**Fig. 5.3**). The results fully agreed with the results in the chapter 3, where the chemical pulp can be dissolved completely after EDA pretreatment when the lignin content of pulp was up to ca.10.5%, but, when the lignin content of pulp reached to 14.6%, only suspension was obtained. It is supposed if a lignocellulosic material contains relatively lower lignin content, the cellulosic components will be more accessible during EDA pretreatment and result in the better dissolution in 8% LiCl/DMSO.

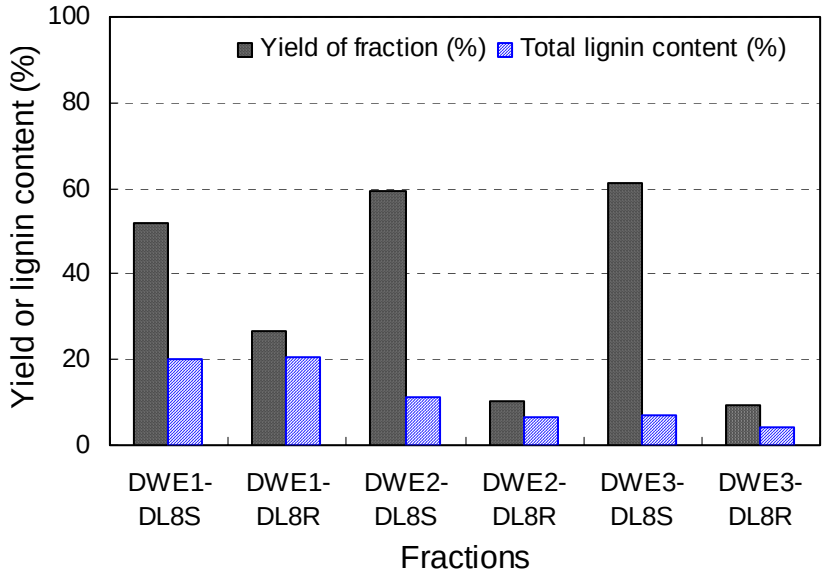

Figure 5.5 Yield and lignin content of each fraction prepared by Scheme VI.

The sum of the yield of each soluble and corresponding insoluble fraction separated by Scheme VI was 78.6%, 69.3%, and 70.2% respectively (**Fig. 5.5**). The weight loss during the dialysis is quite significant compared with the Scheme 1 to 4 (chapter 4) and the Scheme V. In the case of Scheme 1 to 4, which was conducted for milled wood, the sum of each fraction was around 92%. And the sum of the total fraction obtained by the Scheme V was around 90% (**Fig. 5.4**).

In the Scheme V, the lignin content in the soluble fraction was lower than that of corresponding insoluble fraction, as shown in **Fig. 5.4**. These results were quite different from that obtained from milled wood. The results suggest that EDA pretreatment of wood meal have more effect on polysaccharide fractions than lignin in wood cell wall. In Scheme VI, it is interesting that the lignin content in the soluble fraction of DWE2-DL8S (*NO. 6.3*) and DWE3-DL8S (*NO. 6.5*) was higher than that in the corresponding insoluble fraction DWE2-DL8R (*NO. 6.4*) and DWE3-DL8R (*NO. 6.6*) as shown in **Fig. 5.5**. It indicated that after a delignification process, the fraction rich in lignin were easily dissolved in 8% LiCl/DMSO after EDA pretreatment leading to the higher lignin contents in the soluble fraction. This is unexpected result because after $NaClO_2$ treatment (delignification), the remained residue-lignin should be more difficult to be degraded. However, after EDA treatment, the fractions rich in such hard-to-degrade lignin became soluble in LiCl/DMSO. This interesting and contradicted result needs the further study.

5.3.3 Structural characterization of lignin in each fraction

In order to investigate the structural features of aromatic part in lignin, alkaline nitrobenzene oxidation was conducted for the fractions separated from Scheme V and Scheme VI. **Fig. 5.6** and **5.7** showed the yield of nitrobenzene oxidation products, S+V, and the syringyl ratio, S/(S+V) of each fraction. From **Fig. 5.6**, the S/(S+V) ratio of the soluble fraction was slightly higher than those of the corresponding insoluble fractions.

And the yields of S+V of each fraction were almost the same. Thus, the lignins in soluble or insoluble fraction separated by Scheme V were characterized to have similar structural characters. The results were different from the results obtained from milled wood (Scheme I to IV, Chapter 4). In the milled wood, it was proposed that the secondary cell wall lignin with higher S/(S+V) ratio was more difficult to be extracted than the primary cell wall lignin with lower S/(S+V) ratio. With the increase in the dissolving capacity of solvent, more of the fractions with higher S/(S+V) ratio became extractable, suggesting that contribution of secondary cell wall lignin to the soluble fraction became greater with the progress of the solubility of the solvent. However, in the case of Scheme V (EDA pretreated wood), fractions corresponding to the group 1 (MWL or Björkman LCC fraction) found by Scheme I and II (**Table 4.3**) was not found. It means that milling was the decisive factor to obtain MWL and Björkman LCC.

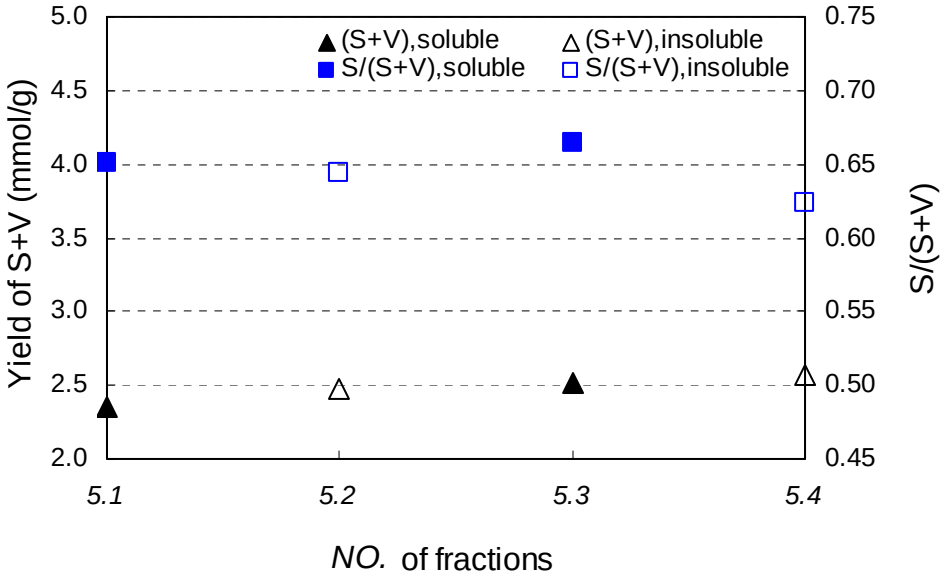

Figure 5.6 Yield of S+V and ratio of S/(S+V) of each fraction prepared by Scheme V.

On the other hand, from the results shown in **Fig. 5.7**, both the yield of S+V and the S/(S+V) ratio in the soluble fractions were always lower than those of the corresponding

insoluble fractions except the S/(S+V) ratio in DWE1-DL8S (*NO. 6.1*) and DWE1-DL8R (*NO. 6.2*). The high ratio of S/(S+V) in DWE1-DL8S (*NO. 6.1*) was due to the remained high lignin content in DW1 after only once delignification treatment, i.e. less degradation occurred in DW1, from which lignin with higher ratio of S/(S+V) was extracted by 8% LiCl/DMSO after EDA treatment. After the second and third delignification procedure followed by EDA treatment, the yield of S+V and ratio of S/(S+V) in both soluble and insoluble fractions decreased. Such decrease agreed well with the decrease in the yield of S+V and ratio of S/(S+V) in the delignified wood after repeating $NaClO_2$ treatment as shown in **Table 5.1**.

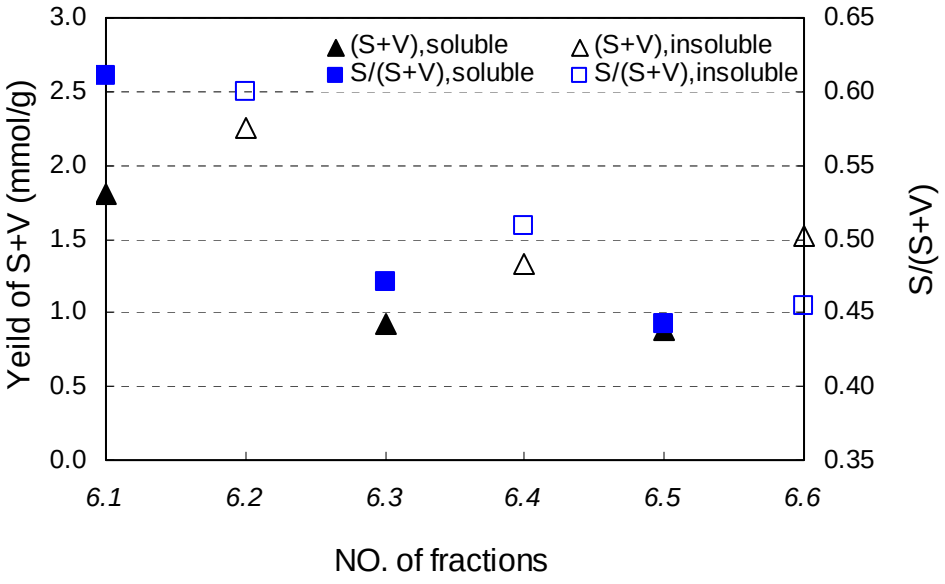

Figure 5.7 Yield of S+V and ratio of S/(S+V) of each fraction prepared by Scheme VI.

Table 5.1 Yield of S+V and ratio of S/(S+V) of the delignified woods.

Sample	S+V (mmol/g)	S/(S+V)
DW1(Delignified wood 1)	1.99	0.61
DW2(Delignified wood 2)	1.21	0.42
DW3(Delignified wood 3)	1.33	0.40

5.3.4 Composition of neutral sugars in each fraction

The weight ratios of neutral sugars in each fraction prepared by Scheme V and Scheme VI were shown in **Fig. 5.8** and **5.9**, respectively.

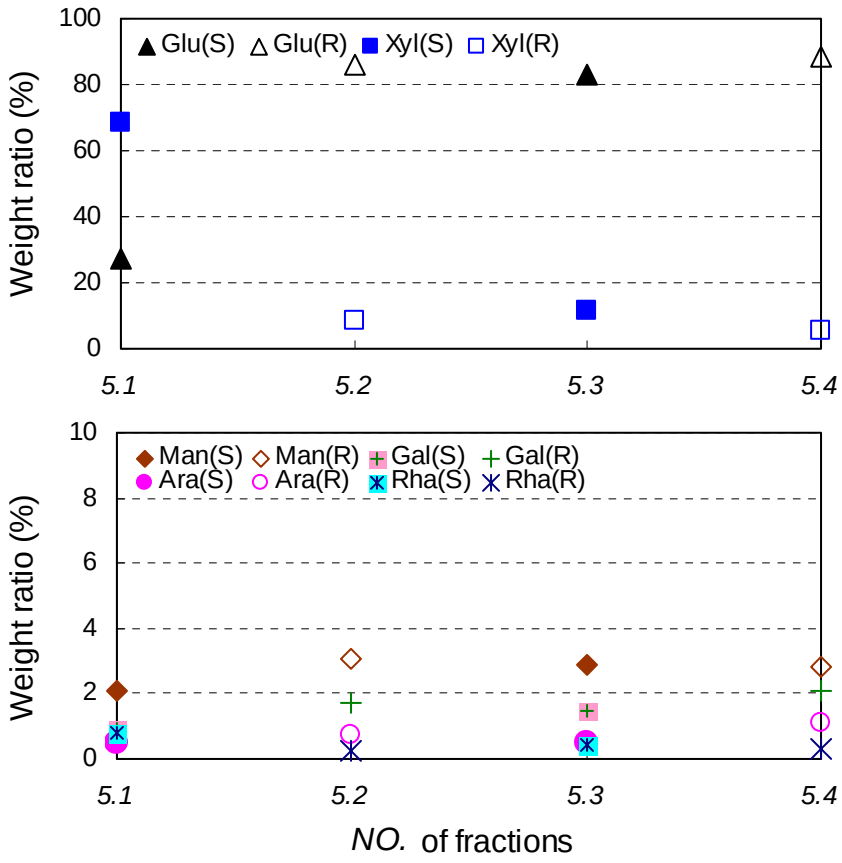

Figure 5.8 Weight ratio of neutral sugars of each fraction prepared by Scheme V. (S: soluble fraction, R: insoluble fraction)

As shown in **Fig. 5.8**, the weight ratio of glucose in WE-DL8S (*NO. 5.1*) was quite low and the weight ratio of xylose was very high. Because the lignin content was only 19.7%, which is extremely low compared with the first solubilized fraction by Scheme I and II (Chapter 4), main component of this fraction is xylan. Why only the solubilization of hemicellulose (xylan) was facilitated by the first EDA pretreatment? One possible explanation is that the high lignin content restricted the formation of cellulose-EDA complex in wood cell wall during the first EDA pretreatment. This idea can explain why cellulose was not solubilized. But the solubilization of xylan cannot be

fully understood. Another explanation is that the formation of cellulose-EDA complex is restricted by lignin-xylan complex, solubilization of which proceeds under high LiCl concentration. By the removal of this fraction during the first dissolution process, the formation of cellulose-EDA complex can be achieved during the second EDA treatment. By the second EDA treatment, the soluble fraction WRE-DL8S (*NO. 5.3*) obtained from WE-DL8R (*NO. 5.1*) showed just slightly higher or almost similar weight ratio of xylose compared with the corresponding insoluble fraction WRE-DL8R (*NO. 5.4*). It is reasonable because the amount of xylose in the fraction WE-DL8R (*NO. 5.1*) was already quite low after the first extraction. These results agreed well with the results obtained by Scheme VI. As shown in the **Fig. 5.9,** the weight ratios of glucose in soluble fraction were always lower and the weight ratios of xylose in soluble fraction were always higher than that in corresponding insoluble fraction.

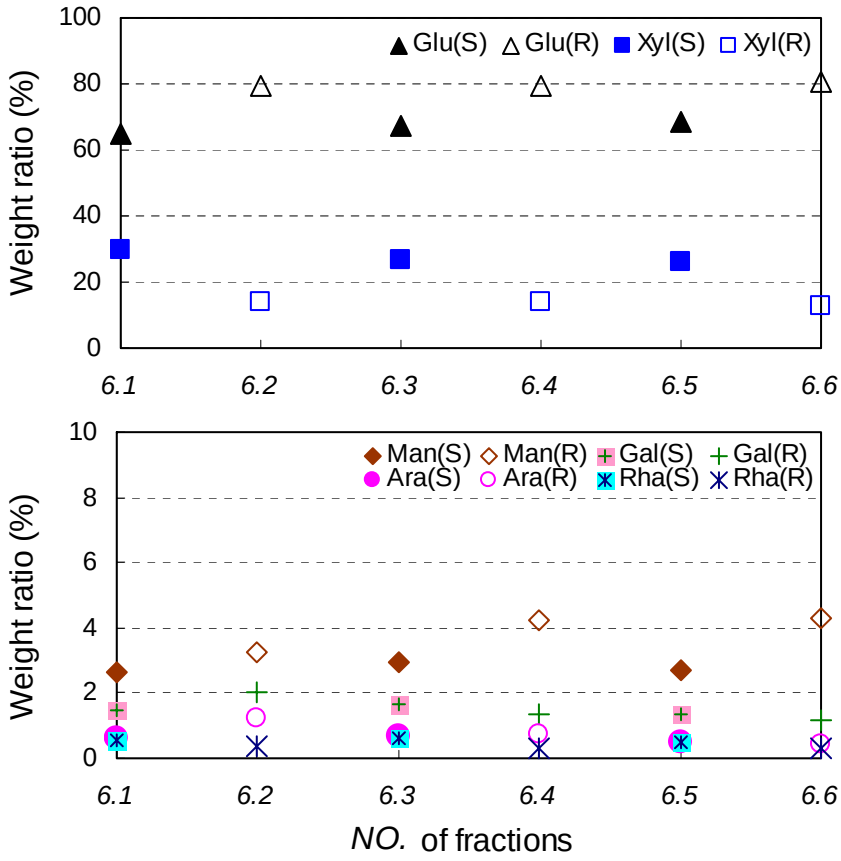

Figure 5.9 Weight ratio of neutral sugars of each fraction prepared by Scheme VI. (S: soluble fraction, R: insoluble fraction)

5.3.5 Correlation between carbohydrate composition and lignin content

The dependence of the weight ratio of sugars on the lignin content of each fraction prepared by Scheme V and VI was shown in **Fig. 5.10** and **5.11**. Except fraction WE-DL8S (*NO. 5.1*, lignin content is 19.7%), the weight ratio of glucose and the weight ratio of sugars from hemicelluloses were shown similar value although lignin content is different in each fraction. The results was quite different from the result obtained from Scheme I and II in chapter 4, where a significantly high correlation was observed between the weight ratio of sugars and the lignin content. Here, it appeared that the weight ratio of sugars of each fraction prepared by Scheme V and VI were not affected by lignin content.

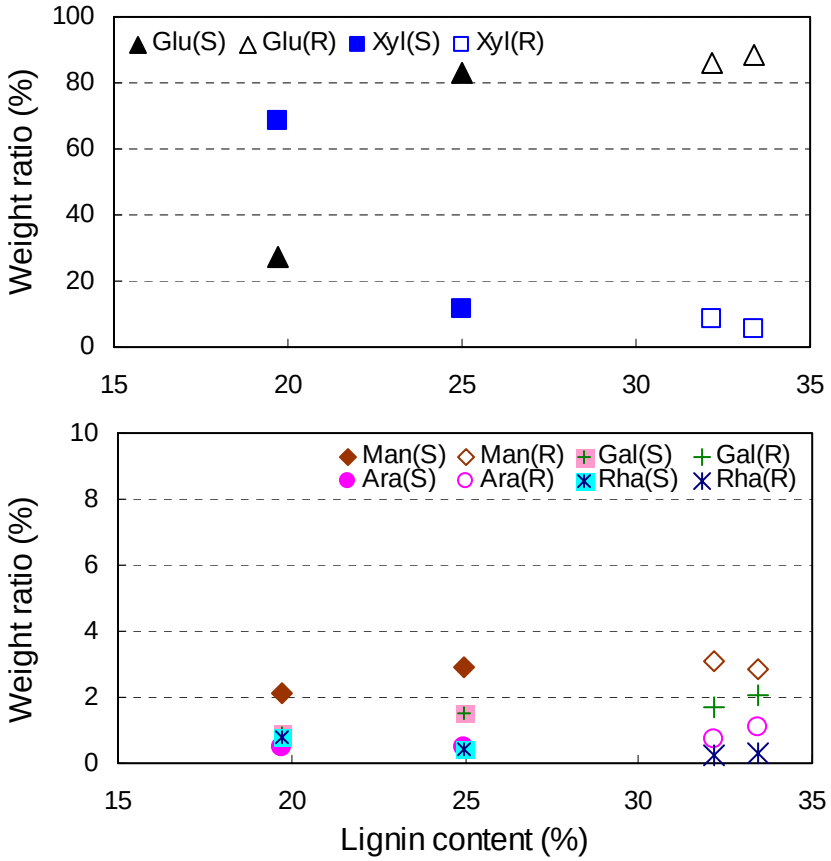

Figure 5.10 Dependence of the weight ratio of sugars on the lignin content of each fraction prepared by Scheme V (S: soluble fraction, R: insoluble fraction).

It is not unreasonable because in this chapter, it is the solubility of lignocellulosic materials that was varied by changing the EDA or delignification treatment times (Scheme V and Scheme VI). In the contrary, in the Chapter 4, it was the dissolving capacity of solvent system that was varied by controlling the LiCl concentration in DMSO. Here, because the dissolving capacity of solvent system was fixed by fixing the LiCl concentration to be 8% in DMSO, the characters of soluble or insoluble fraction was mainly decided by the features of sample after certain pretreatment. It is interesting that all of the weight ratio of glucose in the soluble fraction was lower than that in the insoluble fraction, meanwhile, the weight ratio of xylose in the soluble fraction was higher than that in the insoluble fraction, regardless of the lignin content. Obviously, the EDA pretreatment facilitated the dissolution of xylan, suggesting that the formation of EDA-complex is also necessary for the solubilization of xylan, or, lignin-xylan complex, as stated above. It should be pointed that, in Chapter 4, two specific fractions featured by xylan were obtained. One was a xylan-rich water soluble fraction obtained from the whole wood solution by the Scheme III, and, another was the hardest-to-dissolve fraction categorized as group 3 with relatively high xylose/glucose ratio in Scheme I and II. In all cases xylan was suggested to bind cell wall components, probably as lignin-xylan complex, in the native cell wall.

As was mentioned above, removal of such lignin-xylan complex during the first dissolution process by the Scheme V was necessary for the effective formation of cellulose-EDA complex, which result in the more effective solubilization during the second dissolution process. In case of Scheme VI, such lignin-xylan complex may be destroyed during the delignification process, which may be the reason why such a specific fraction is not obtained by Scheme VI.

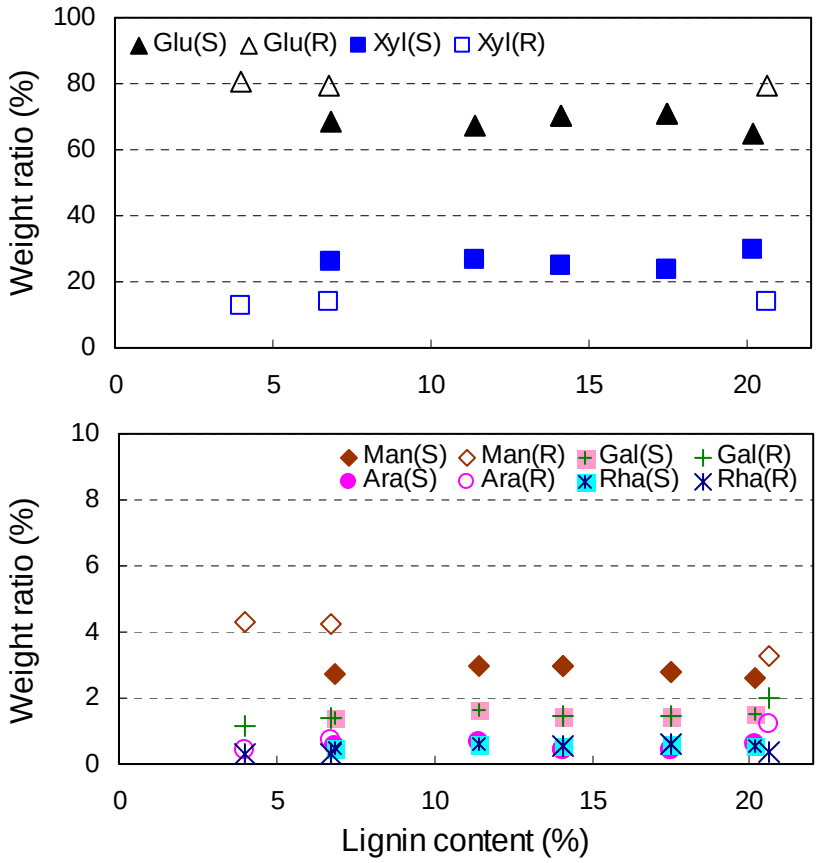

Figure 5.11 Dependence of the weight ratio of sugars on the lignin content of each fraction prepared by Scheme VI (S: soluble fraction, R: insoluble fraction).

5.4 Conclusions

By the dissolution process consisting of EDA pretreatment and dissolution in LiCl/DMSO, lignocellulosic materials including Wiley wood were found to be partially or completely dissolved depending on lignin content. Two methods to fractionate wood cell wall components were proposed on the basis of this finding. One method was to repeat this dissolution process (Scheme V). Namely, the insoluble residue from the first dissolution process was again subjected to the same process. Another was to conduct partial delignification on Wiley wood before subjecting to this dissolution process (Scheme VI). By varying the degree of delignification, different soluble and insoluble fractions were obtained.

When Wiley wood was subjected to the Scheme V, the yield of the final insoluble fraction WRE-DL8R (*NO. 5.4*) was 50.3% based on WE-DL8R (*NO. 5.2*, the residue from the first dissolution process) and was only about 32% based on the original Wiley wood. Even though this method was not a complete solubilization method, it is suggested that EDA pretreatment of wood cell wall is an efficient method for the solubilization of main part of wood cell wall while avoiding the destructive effect of milling on the structure of wood cell wall components. On the other hand, when the lignin content of lignocellulosic material is relatively low, it can be dissolved in 8% LiCl/DMSO after an EDA pretreatment.

Regarding the structure of lignin, either Scheme V or VI did not give specific fractions, indicating that the EDA pretreatment of wood meal have more effect on polysaccharides than on lignin in wood cell wall. Instead, a specific xylan-rich fraction, probably consisting of lignin-xylan complex, was obtained as the first soluble fraction from the Scheme V. It seemed that removal of this xylan-rich fraction was necessary to facilitate the formation of cellulose-EDA complex, which enables cellulosic fraction

soluble in LiCl/DMSO. Delignification pretreatment in the Scheme VI must destroy such lignin-xylan complex.

5.5 References

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Chapter 6

Summary

In this thesis, the researches were focused on the development of solution systems of lignocellulosic materials by the use of common solvents, and, on the chemical analysis of plant cell wall components on the basis of these solutions. Dimethyl sulfoxide containing lithium chloride (LiCl/DMSO) was found to be used as a new solvent system for lignocellulosic materials. The dissolution of lignocellulosic materials in LiCl/DMSO was achieved by using two different pretreatment methods, ball milling pretreatment and ethylenediamine pretreatment. Pretreatment conditions were designed so that structural change of cell wall components during the whole dissolution process could be minimized. The new fractionation methods of lignin, lignin-carbohydrate complex (LCC) and polysaccharides from the lignocellulosic solution were established. Main concern of the analyses of obtained fractions was to elucidate the relationships and possible interactions between cellulose, hemicelluloses, and lignin.

6.1 Dissolution of milled wood in LiCl/DMSO

The LiCl/DMSO solvent system completely dissolved milled wood prepared by as

short as 2 h of milling pretreatment using a planetary ball-mill. The nitrobenzene oxidation and ozonation analyses indicated that the structural change of lignin caused by the 2 h of milling is not significant. In contrast, the destruction of the cellulose crystalline region in the milled wood was serious even with 1 h of milling. Compared with already reported solvent systems, the advantages of the LiCl/DMSO system, developed in this thesis, are high solubility, low degradation of lignin and the simplicity of the solvent itself. Thus this solvent system can be applied widely to the spectral analyses of the entire lignin fraction in wood cell wall. For example, at the first time, the structural information of the whole aromatic part of lignin was obtained by measuring NMR of this complete wood solution. The gram absorptivity of lignin at 280 nm was obtained by measuring the UV absorbance of this solution. Interestingly, the absorptivity increased along with milling time, which suggested that gram absorptivity of lignin could be applied only to semi-quantitative determination. The wood solution forms gel at relatively high concentration by standing the solution at room temperature. The critical concentration for gelation increased along with milling time.

6.2 Dissolution of ethylenediamine pretreated lignocellulosic material in LiCl/DMSO

Milling pretreatment caused a minor but unavoidable structural change of lignin, cellulose and hemicellulose. Especially, in order to obtain the solution of lignocelluloses with high cellulose DP (degree of polymerization), other pretreatment method is required. From this point of view, the ethylenediamine pretreatment method was established for the dissolution of lignocellulosic material. Various lignocellulosic samples, such as microcrystalline cellulose (Whatman CF11), cotton, cellulose I, cellulose II, holocellulose or kraft pulps (softwood, hardwood) including those with relatively high lignin content, were soaked in ethylenediamine for a described period at

room temperature and then the bulk of ethylenediamine was removed by freeze drying. These ethylenediamine pretreated samples can be dissolved in LiCl/DMSO solvent system. Interestingly, even hardwood kraft pulp with as high as 10.5% lignin content gave the transparent solution. After the EDA pretreatment, the crystallinity of the lignocellulosic materials remained as high as the original samples, although the crystal structure changed. Because milling of the sample is not required, degradation of the cell wall components caused by milling does not take place. This is the first time that transparent solutions of underivatized pulps with high lignin content were obtained in a simple organic solvent system. The formation of a lignocellulose-EDA complex seems to be critical for the dissolution in LiCl/DMSO. The NMR spectrum of the EDA treated lignocellulosic solution had good resolution even though the DP of the cellulose in the pulp is very high. A very good relationship between UV absorbance of lignin at 280 nm in pulp solution and corresponding kappa number of pulp was obtained. Although the ethylenediamine pretreatment method is most effectively applied to partially delignified samples, about 70% of the original wood meal without milling or delignification was found to be dissolved by repeating the dissolution process consisting of ethylenediamine pretreatment and dissolution in LiCl/DMSO.

6.3 Fractionation and characterization of wood cell wall components based on LiCl/DMSO system with milling pretreatment

One new and simple fractionation method of wood cell wall components by the use of LiCl/DMSO solvent system was acheived by varying the dissolving capacity of LiCl/DMSO system, i.e. by changing the LiCl concentration in DMSO. When LiCl concentration was higher than 6%, milling-pretreated wood meal completely dissolved in LiCl/DMSO, but when it was lower than this value, some part of milled wood was

always left as an insoluble fraction depending on the LiCl concentration. Accordingly, different LiCl concentration gives different soluble and insoluble fractions, analyses of which must give new insight into the nature of cell wall components.

By the use of LiCl/DMSO solvent system which can dissolve whole milled wood, new methods for the separation of cell wall components were examined. Two basically different approaches were made to give different fractions.

Approach 1 starting from solid milled wood

Scheme I: stepwise extraction by aqueous dioxane with different water content and LiCl/DMSO with different LiCl concentration

Scheme II: individual extraction by LiCl/DMSO with different LiCl concentration

Approach 2 starting from whole wood solution

Scheme III: individual precipitation in aqueous dioxane with different water content

Scheme IV: individual precipitation in DMSO with different LiCl concentration

Approach 1

Based on the solubilization behavior and structural analyses, obtained fractions were classified into three groups. The solubilization of the first group (group 1) is not restricted by the cellulose but basically by the milling degree during the preparation of milled wood and by the property of the solvent itself. Traditionally obtained MWL and Björkman LCC belong to this group. The second group (group 2) is made soluble by the solubilization of cellulose. Because the main part of the cell wall belongs to this group, the solubilization of cellulose is a main cause of the whole cell wall solubilization. However, the third group (group 3) cannot be made soluble only by the solubilization of cellulose. Fractions in this group are the hardest-to-dissolve in the cell wall and seem to be prevented from getting solubilized by the strong interaction between lignin and

carbohydrates. In this meaning, fractions in the third group can be regarded as “real LCC” that could never be analyzed by the previous studies. The finding and structural elucidation of the fractions belonging to this group is the most significant achievement of this chapter. Structural features of lignin in this group are quite different from those of the group 1, so called MWL and Björkman LCC fraction. Namely, lignin in the group 3 has a nature of uncondensed type, which is a feature of secondary wall lignin. Glucose to xylose ratio (glu/xyl) of this group is much higher than group 1, but a little bit lower than the value of the whole wood. Therefore, group 3 is featured by the non-condensed type lignin and hemicellulosic sugars, especially xylan. Whether or not fractions in this group originate from specific portion of cell wall is still unknown.

Approach 2

Fractionation of cell wall components starting from whole wood solution was examined. Interestingly, some part of xylan was obtained as water soluble xylan (fraction WS-WS from Scheme III). Other than this, it was impossible to separate cell wall components into “lignin”, “hemicellulose”, and “cellulose” fraction. Further examination of obtained each fraction and the establishment of more effective precipitation method are required.

6.4 Fractionation and characterization of wood cell wall components based on LiCl/DMSO system with EDA pretreatment

By the dissolution process consisting of EDA pretreatment and dissolution into LiCl/DMSO, lignocellulocic materials including Wiley wood was found to be partially or completely dissolved depending on lignin content. Two methods to fractionate wood cell wall components were proposed on the basis of this finding. One method was to repeat this dissolution process (Scheme V). Namely, the insoluble residue from the first

dissolution process was again subjected to the same process. From the first and second dissolution process, different soluble and insoluble fractions were obtained. Another was to conduct partial delignification on Wiley wood before subjecting to this dissolution process (Scheme VI). By varying the degree of delignification, different soluble and insoluble fractions were obtained.

When Wiley wood was subjected to the Scheme V, the yield of the final insoluble fraction WRE-DL8R (*NO. 5.4*) was 50.32% based on the residue from the first dissolution process (WE-DL8R, *NO. 5.2*) and was only about 32% based on the original Wiley wood. Even though this method was not a complete solubilization method, it is suggested that EDA pretreatment of wood cell wall is an efficient method for the solubilization of the main part of wood cell wall while avoiding the destructive effect of milling on the structure of wood cell wall components. On the other hand, when the lignin content of lignocellulosic material is relatively low, it can be dissolved in 8% LiCl/DMSO after an EDA pretreatment.

Regarding the structure of lignin, either Scheme V or VI didn’t give specific fractions, indicating that the EDA pretreatment of wood meal have more effect on polysaccharides than on lignin in wood cell wall. Instead, a specific xylan-rich fraction, probably consisting of lignin-xylan complex, was obtained as the first soluble fraction from the Scheme V. It seemed that removal of this xylan-rich fraction was necessary to facilitate the formation of EDA-cellulose complex, which enables cellulosic fraction soluble in LiCl/DMSO during the second dissolution process. Such lignin-xylan complex must be degraded at least partly when delignification pretreatment was carried out in the Scheme VI, and this is the reason why such a specific fraction is not separated from other components and why solubilization proceeds effectively by the Scheme VI.

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Zhiguo Wang　(王　志国)

Publications

Journal Articles

1. Zhiguo Wang, Tomoya Yokoyama, Hou-min Chang, and Yuji Matsumoto. Dissolution of beech and spruce milled woods in LiCl/DMSO. *Journal of Agricultural and Food Chemistry* **2009**, 57, 6167-6170.

2. Zhiguo Wang, Tomoya Yokoyama, and Yuji Matsumoto. Dissolution of ethylenediamine pretreated pulp with high lignin content in LiCl/DMSO without milling. *Journal of wood chemistry and technology.* (In press)

3. Zhiguo Wang, Takuya Akiyama, Tomoya Yokoyama, and Yuji Matsumoto. Fractionation and characterization of wood cell wall components by using LiCl/DMSO system. Part I: pretreatment with ball-milling. *Journal of wood chemistry and technology.* (Submitted)

4. Zhiguo Wang, Takuya Akiyama, Tomoya Yokoyama, and Yuji Matsumoto. Fractionation and characterization of wood cell wall components by using LiCl/DMSO system. Part II: pretreatment with ethylenediamine. (Under preparation)

Patents

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2. Yuji Matsumoto, Zhiguo Wang, and Tomoya Yokoyama. “Dissolution method of lignocellulosic materials with high lignin contents and its application” Japan Patent Application, 2009-219964, **2009**.

Conference

International conference

[Oral]

1. Zhiguo Wang, Tomoya Yokoyama, Hou-min Chang, Yuji Matsumoto. “Dissolution of finely milled wood into organic solvent and its potential application for chemical analysis of cell wall components”. *10th European Workshop on Lignocellulosics and Pulp (EWLP 2008)*, Stockholm/Sweden, August **2008**.

2. Zhiguo Wang, Tomoya Yokoyama, Hou-min Chang, Yuji Matsumoto. “Studies on the effect of ball milling on lignin structure by the use of complete wood dissolution”. *International Conference on Pulping, Papermaking and Biotechnology (ICPPB 2008)*, Nanjing/China, November **2008**.

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Conference in Japan

[Oral]

1. Zhiguo Wang, Tomoya Yokoyama, Yuji Matsumoto. “Dissolution of finely milled wood into organic solvent”. *The 58th Annual Meeting of the Japan Wood Research Society*, Tsukuba/Japan, March **2008**.

2. Zhiguo Wang, Tomoya Yokoyama, Yuji Matsumoto. “Analysis of lignin by the use of whole wood solution consisting of DMSO/LiCl”, *The 53rd Lignin Symposium*, Tokyo/ Japan, October **2008**.

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[Poster]

1. Zhiguo Wang, Tomoya Yokoyama, Yuji Matsumoto. "Separation of wood cell components by the use of two dissolving procedures, 'ball-milling and dissolution in LiCl/DMSO' and 'EDA-treatment and dissolution in LiCl/DMSO'", *The 54th Lignin Symposium*, Shizuoka/Japan, October **2009**.