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[1] Isolation of Lignin

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General considerations

Introduction

Significant gains have recently been made in understanding the biochemistry of the microbial degradation of lignin.[1] Further advances will be facilitated through studies using isolated lignins. This chapter presents some of the most useful methods for lignin isolation.

Critique of Lignin Preparations. Probably the best known isolated lignin is Klason lignin, which is obtained by treating wood with sulfuric acid. The polysaccharides are hydrolyzed to water-soluble sugars, and the lignin is recovered as an insoluble residue. Although this method for lignin isolation has great utility as an analytical means of determining lignin content (see chapter [12], this volume), the highly condensed and altered Klason lignin is generally unsuited for either chemical characterization or studies of biological modification and degradation. For such studies, what is needed is an isolated lignin that is representative of the lignin in the original lignocellulose (which is sometimes referred to as protolignin).

Because the methods for isolating lignin have been devised by wood scientists, the procedures discussed here are those used to isolate lignin from wood (see Table I). Often these methods will be suitable for other lignocellulosics, but sometimes modifications will be required. In particular, it may be desirable to treat certain plant materials, such as forages and immature woody tissues, to remove protein prior to lignin isolation. This can be accomplished by treatment with proteases or by extraction with hot, neutral detergent (see chapter [12], this volume).

The most useful lignin preparation is Björkman lignin, also known as Björkman milled wood lignin or simply milled wood lignin (MWL). Milled wood lignin is purified from the aqueous p-dioxane extract of finely milled wood, which has been first extracted with organic solvents to remove extraneous components. Although it has not been rigorously proved that MWL is representative of protolignin, it is considered to be appropriate for most chemical and biological studies. Milled wood lignin can be obtained

[1] T. Higuchi (ed.), "Biosynthesis and Biodegradation of Wood Components," Chap. 19–21. Academic Press, San Diego, California, 1985.

TABLE I
LIGNIN ISOLATION METHODS

Preparation	Methodology	Remarks
Milled wood lignin (MWL)	Aqueous dioxane extraction of finely milled wood	Obtained in about 20% yield considered to be representative of the original lignin
Milled wood enzyme lignin (MWEL)	Residue left after polysaccharidase hydrolysis of the carbohydrates in finely milled wood	Ninety five plus percentage yield, but contains 10–12% carbohydrate; not completely soluble in common lignin solvents
Cellulase enzyme lignin (CEL)	Solvent-soluble fraction of MWEL	Similar to MWL
Brauns' native lignin	Ethanol extract of ground wood (fine sawdust-size particles)	Lower yield and lower molecular weight than MWL
Brown rot lignin	Ethanol or aqueous dioxane extract of brown-rotted Wood	Probably not severely altered, but some demethylation of methoxyls and oxidation of side chains has occurred
Chemical lignins (kraft and sulfite)	Dissolution of lignin at high temperature and pressure with chemicals	Not representative of the original lignin; major by-products in pulp production to make paper
Klason lignin	Insoluble, condensed residue left after hydrolysis of polysaccharides with sulfuric acid	Not representative of the original lignin; often used as a measure of lignin content (see chapter [12], this volume)

in 20–30% yields, based on the total lignin. This method requires either a vibratory or rotary ball mill.

One lignin preparation which does not require any ball-milling equipment is Brauns' lignin (sometimes referred to as Brauns' native lignin or native lignin, which should not be confused with protolignin). The wood is first extracted with cold water and then with ether to remove extraneous components. Subsequent extraction of some of the lignin with ethanol followed by purification steps gives Brauns' native lignin. This lignin preparation has fallen into disfavor among wood chemists, who consider its low yield (about 8% based on the total lignin) and its low molecular weight to be disadvantageous for investigations of structure and reactivity. However, the low-molecular-weight distribution of Brauns' lignin may be beneficial

in some lignin biodegradation studies by providing greater accessibility of the substrate and increased degradation rates. Basically, the structure of Brauns' native lignin is similar to that of MWL except for its molecular weight and associated properties. Brauns' native lignin may be used as the first lignin substrate, and successful investigations can then move on to use other more representative isolated lignins.

Ball-milled wood, prepared in the same manner as that used for MWL extractions, may be treated with polysaccharidase enzymes to solubilize the carbohydrate components. In this way, a lignin residue is produced which contains nearly all of the lignin in the wood. This lignin, termed milled wood enzyme lignin (MWEL), has not been severely modified by any chemical treatment. Milled wood enzyme lignin is the most representative of all the isolated lignins. Unfortunately, it contains a relatively high residual carbohydrate content of 10–12%, a result of covalent linkages between lignin and polysaccharide fragments. Also, due to its high molecular weight, it is not completely soluble in common lignin solvents, such as aqueous dioxane, acetic acid, dimethylformamide, and dimethyl sulfoxide. This insolubility presents experimental difficulties in handling, purifying, and analyzing MWELs.

Fractionation of MWEL, based on solubility in dioxane–water, is a means of preparing a soluble lignin which can then be purified in the same manner as MWL. This lignin was originally termed cellulase enzyme lignin (CEL). It is thought to be more representative of protolignin than MWL but has a lower yield than MWEL. Milled wood lignin is probably adequate for most studies, and the additional steps in preparing CEL are usually not justifiable.

There are numerous other lignin preparations, including hydrochloric acid lignin, periodate lignin, cuoxam lignin, enzymatically liberated lignin, alcohol-HCl lignin, thioglycolic acid lignin, acetic acid lignin, dioxane-HCl lignin, phenol lignin, and hydrogenolysis lignin.[2] These preparations usually are not adequate as substrates for biochemical studies of protolignin. However, "enzymatically liberated lignin," or brown rot lignin, may be useful in certain circumstances. When wood is rotted by brown rot fungi, the lignin is not substantially degraded while the carbohydrates are removed. The rotted wood, largely lignin, is extracted with lignin solvents and the lignin is purified; yields of over 20% of the original lignin are obtained. Although brown rot fungi demethylate aromatic methoxyl groups and cause a limited amount of oxidation, the lignin is not otherwise severely damaged. The demethylation may even be considered advanta-

[2] D. Fengel and G. Wegener, "Wood, Chemistry, Ultrastructure, Reactions," p. 50. de Gruyter, Berlin, Federal Republic of Germany, 1983.

geous: subsequent methylation of phenolic hydroxyls using a ^{14}C label would provide a substrate for monitoring lignin degradation by measuring the evolution of labeled carbon dioxide or for studying demeth(ox)ylation.

Another class of lignins is produced by chemical pulping processes. Most of the chemical pulp produced in the United States is by the kraft process. Kraft lignin is highly modified; it is lower in molecular weight, has a higher phenolic content and a lower methoxyl content, and has undergone extensive side-chain reactions.

Lignosulfonate, as implied by its name, is the sulfonated lignin removed from wood by sulfite pulping. Lignosulfonates have higher molecular weight than kraft lignin but are not representative of protolignin. Hydrolysis reactions have occurred, and sulfonation (to give a water-soluble product) can be extensive.

Whereas kraft lignin and lignosulfonates are not suitable for studies modeling the behavior of protolignin, they are important in their own right as industrial by-products, and research is warranted on their biodegradation and bioconversion.

Finally, after a lignin has been isolated and purified, it is essential to analyze it to be certain that it is not grossly contaminated. Often, determinations, such as carbohydrate content, methoxyl content, and ultraviolet absorption, are sufficient, but sometimes more detailed analyses are required. An overview of quantitative lignin determinations is given in chapter [12] in this volume.

Isolation of Lignin

Milled Wood Lignin (Björkman Milled Wood Lignin, Björkman Lignin)

The wood used for lignin isolation should be sapwood; heartwood often contains polyphenolics which are difficult to remove and may even have condensed with the lignin. Air-dried wood is milled in a Wiley mill to pass 40 mesh and extracted first with acetone : water (9 : 1, v : v) by percolation at room temperature and then with ethanol : benzene (2 : 1, v: v). Lignin is heat-sensitive, so extractions should not be at the boiling point of the solvents. For this reason also, the extracted wood should never be oven-dried, but rather dried in a vacuum desiccator over phosphorus pentoxide or other efficient desiccant.

The dry, extracted wood is then milled either in a vibratory ball mill[3] or

[3]Siebtechnik vibrating ball mills may be obtained from Tema, Inc., 11584 Goldcoast Drive, Cincinnati, Ohio 45249. We have successfully used mill type USM 12 to grind 200 g of wood at one time. For smaller amounts of wood (1 - 10 g), we have used the smaller

in a conventional rotating jar ball mill. The time of milling can vary from 1 hr in a very efficient vibratory ball mill, such as the NBS-type mill first used by Björkman: to 12 hr in a large Siebtechnik vibratory mill, to 3 weeks using a rotating jar mill. Thus, milling time and the amount of material milled must be optimized for each type of mill and grinding medium. Because the temperature of the milling jar will increase, it is desirable to cool the mill to avoid damaging the lignin.[3]

Often the ball milling is done in a nonswelling solvent such as toluene. The solvent excludes oxygen, and the milled wood may be recovered by centrifugation. However, the wood may be milled dry, preferably under carbon dioxide or nitrogen. In this case, the milled wood may conveniently be removed from the balls after conditioning in a high humidity environment by shaking the balls on screens in a Ro-Tap sieve shaker.[5]

The milled wood is dispersed in dioxane : water (96 : 4, v : v) and mechanically stirred; the ratio of wood to solvent is chosen to be convenient, for example, 10 g of milled wood and 250 ml of dioxane: water. After I day, the suspension is centrifuged, and the residue is redispersed in fresh dioxane :water and stirred for an additional day. Although lignin would continue to be extracted for many subsequent extractions, the bulk of the MWL is removed in the first two. The extracts are combined and then freeze-dried (or simply dried in a rotary vacuum evaporator) to give a crude MWL in about 20 - 30% yield; this lignin contains up to about 10% residual carbohydrate. (The milled wood may also be extracted with 9 : 1 dioxane : water, giving a higher yield of MWL but with more carbohydrate.) As is, this crude MWL is useful for many experiments.

In most cases, it is desirable to purify the crude MWL. This is accomplished by dissolving the lignin (the dioxane : water is removed by vacuum evaporation) in 90% acetic acid, using 20 ml of solvent for each gram of lignin. The acetic acid solution is then added dropwise, with stirring, to water (about 220 ml of water per gram of lignin). The precipitated lignin is centrifuged and then freeze-dried or air-dried, followed by drying in a vacuum oven. It is then dissolved with stirring in a mixture of 1,2-dichloro-

Siebtechnik mill or a custom-made mill patterned alter the NBS mill.[4] The temperature increase of the mill jars on the large mill is minimized by milling for no longer than 1 hr at a time, followed by 1 hr of cooling. A large fan is used to cool the jars during the entire process. The smaller mill may be placed in a cold room.

[4] A. Björkman, *Sven. Papperstidn.* 59,477 (1956).

[5] For the preparation of large amounts of milled wood, we have found that mechanical shaking on a sieve is a convenient way to remove the wood from the balls. We use a No. 7 (7-mesh) stainless-steel screen with a custom-made stainless inner collar to minimize ash contamination. Sieves and the Ro-tap may be obtained from W. S. Tyler, Inc., Mentor, Ohio.

ethane : ethanol (2 : 1, v : v) and centrifuged to remove solids. The lignin solution is added dropwise to *anhydrous* ethyl ether to precipitate the lignin. About 20 ml of solvent and 230 ml of ether are used for 0.5–1 g of lignin. After centrifugation, the insoluble MWL is washed three times With fresh ether. The yield of the purified MWL may be half that of the crude preparation, but its residual carbohydrate content is about 4%.[4] When prepared from light-colored woods, MWL is cream colored.

There are several ways significantly to reduce the carbohydrate content of MWL. However, yield losses may be substantial. Two such methods are those of Freudenberg and Neish[6] and Lundquist and Simonson.[7]

Milled Wood Enzyme Lignin

The wood is extracted and then ball milled as for milled wood lignin (see above).

For digestion of the carbohydrate in 100 g of milled wood, 3 g of Cellulysin (Calbiochem-Behring Corp., La Jolla, California 92307), which is a mixture of polysaccharidase enzymes, or a comparable preparation,[8] is dissolved in 40 ml of 0.5 *M* acetate buffer (pH 4.6) and 200 ml of distilled water. The enzyme solution is centrifuged to remove undissolved materials. Water is added to the suspension of milled wood in the enzyme solution to bring the total volume to about 1.5 liters. Several drops of toluene are added as a preservative. The digestion is carried out With stirring in a suitable glass container for a week to 10 days at 48°. The suspension is then centrifuged; the residue is washed with water and redigested in the same way two more times. The final residue is thoroughly washed and then freeze-dried. Yields based on the lignin in the wood are

[6] K. Freudenberg and A. C. Neish, "Constitution and Biosynthesis of Lignin," p. 52. Springer-Verlag, Berlin and New York, 1968.

[7] K. Lundquist and R. Simonson, *Sven. Pappersridn.* **78,** 390 (1975).

[8] Solubilization of the polysaccharides in milled wood and other lignocellulosic materials requires the concerted action of the cellulase system (endo- and exo-1,4glucanases) plus hemicellulose-depolymerizing enzymes. The latter include enzymes that hydrolyze substituted 1,4-xylans and substituted glucomannans, also 1,4-linked. Such mixtures of enzymes are produced commercially with the fungus *Trichoderma reesei (Trichoderma viride)* grown on delignified lignocellulosic substrates such as newsprint or on finely milled lignocellulosics, which have the necessary constituents to induce the enzymes. By suitable experimentation, the researcher should be able to produce the enzyme mixture without undue difficulty. General references to the production of cellulases and hemicellulases by *T. reesei* are as follows: K.-E. Eriksson and T. M. Wood, "Biosynthesis and Biodegradation of Wood Components" (T. Higuchi, ed.), pp. 469–503. Academic Press, San Diego, California, 1985; and R. F. H. Dekker, *in* "Biosynthesis and Biodegradation of Wood Components" (T. Higuchi, ed.), pp. 505–533. Academic Press, San Diego, California, 1985.

over 95%. The MWEL will have a carbohydrate content of approximately 10±296.9

The MWEL may be fractionated to remove some of the residual carbohydrate and to give a solvent-soluble lignin with lower molecular weight, termed CEL.[10] To prepare CEL, MWEL is twice extracted with 96% (or 90%) dioxane. This extract may be purified in the same manner as milled wood lignin as described above. The residual carbohydrate content of the CEL-96 is about 4%, whereas that of the CEL-90 is higher. The extracted residue may be further extracted with 50% dioxane. However, this extract is not completely soluble in dichloroethane : ethanol and cannot be purified by the MWL procedure.[10]

Brauns' Native Lignin (Brauns' Lignin, Native Lignin)

The wood is ground in a Wiley mill to pass a 100- to 150-mesh screen and extracted first with cold water and then with ethyl ether for 48 hr to remove extraneous components. The wood is then extracted by percolation with 95% ethanol at room temperature for 8–10 days or until the extract is colorless. A small amount of calcium carbonate is added to the extract to neutralize wood acids, and the alcohol is removed under reduced pressure. Water is added to the residue, and the evaporation continues to remove traces of the alcohol. The lignin residue is triturated alternatively with water and ether until it becomes solid. The solid is filtered and dried over an efficient desiccant. The dry lignin is extracted with anhydrous ether in a Soxhlet apparatus. The residue is dissolved in dioxane to give a 10% solution, and it is precipitated by dripping into stirred distilled water (about 15 times the volume of the dioxane). If a colloidal solution forms instead of a precipitate, a little sodium sulfate is added and the solution vigorously stirred to coagulate the lignin. The precipitate is filtered, washed with water, and dried in a desiccator. It is then dissolved in dioxane to give a 10% solution, centrifuged, and filtered. The solution is slowly dripped, with stirring, into anhydrous ethyl ether. The Brauns' native lignin separates as a fine tan-colored powder. It is washed sequentially with ether, high-boiling petroleum ether, and low-boiling petroleum ether and then dried in a desiccator over sulfuric acid and paraffin shavings. Precipitation into ether may be repeated until the methoxyl content of the Brauns' native lignin is constant. The yield is about 8% based on the lignin in the wood.[11]

Similar lignin preparations can be obtained from ground samples with

[9] J. R. Obst, *Tappi* **65,** 109 (1982).
[10] H.-M. Chang, E. B. Cowling, and W. Brown, *Holzforschung* **29,** 153 (1975).
[11] F. E. Brauns, "The Chemistry of Lignin," p. 5 1. Academic Press, New York, 1952.

other lignin solvents, including acetone: water, 9: 1 (v: v),[12] and aqueous dioxane.

Brown Rot Lignin [Enzymatically Liberated Lignin (ELL)]

The wood is decayed in soil block chambers (ASTM D 20 17-8 1) by a brown rot fungus, such as *Gleophyllum trabeum, Lentinus lepideus,* or *Poria vaillantii,* to weight losses of about 60–70%.[13] The dried, decayed wood is ground in a Wiley mill to pass a 60-mesh screen. Brown rot lignin is then obtained, employing the purification methods used for milled wood lignin or Brauns' native lignin. Alternatively, the Wiley-milled decayed wood may be extracted with 50% aqueous *p*-dioxane and the lignin purified by gel permeation chromatography on Sephadex G-25.[13]

Chemical Lignins (Kraft Lignin and Lignosulfonate)

Two types of commercially available lignin are kraft lignin and lignosulfonate.[14] Although the lignins from commercial sources may be adequate for many studies, it is recommended that this type of lignin be isolated from laboratory pulping experiments whenever possible. In this way, the entire history of the lignin is known and controlled.

Kraft Lignin (Thiolignin, Sulfate Lignin)

Kraft pulping is accomplished by degrading and dissolving the lignin in hot alkaline sodium sulfide solution ("white liquor"). Kraft white liquor is prepared by dissolving 16 g of sodium sulfide per liter of 1 *N* sodium hydroxide. The extracted wood, either in chip form or Wiley-milled form, plus white liquor at a 4:1 (w:w) liquor-to-wood ratio are sealed in a stainless-steel bomb. Cooking temperature for most hardwoods (angiosperm woods) is about 155°, whereas 170 - 180° is required for softwoods (gymnosperm woods). The bomb is usually heated in an oil bath and rotated, end over end, to ensure mixing. If industrial conditions are to be mimicked, the time to raise the bath from room temperature to the maximum cooking temperature should be about 90 min. Time at temperature will depend on the pulp yield desired; 1–2 hr is typical.

[12] T. K. Kirk and H.-M. Chang, *Holzforschung* **28,** 217 (1974).

[13] T. K. Kirk, *Holzjorschung* **29,** 99 (1975).

[14] Kraft lignin, and modified kraft lignins, may be obtained from Westvaco, Chemical Division, Box 70848, Charleston Heights, South Carolina 29415. Lignin sulfonates may be obtained from Reed Lignin, Inc., 100 Highway 51 South, Rothschild, Wisconsin 54474, and from Crown Zellerbach Corp., P.O. Box 4266, Vancouver, Washington 98662.

The bomb is then cooled with water, and the "black liquor" containing the lignin, some hemicellulose, carbohydrate degradation products, and inorganic chemicals is filtered from the pulp. The lignin may be precipitated by the addition of acid. Generally it is better to use acetic acid rather than mineral acids. Traces of sulfuric acid or hydrochloric acid are hard to remove, and they may cause the lignin to undergo condensation reactions when it is dried. Carbon dioxide may also be used to precipitate the lignin, but yields are usually lower than with acetic acid. The precipitated lignin should be washed thoroughly with distilled water and freeze-dried.

The kraft lignin may be purified through solvent (pyridine:acetic acid: water) fractionation.[15] Alternatively, kraft lignin may be purified by repetitive dissolution in 0.1 *N* sodium hydroxide and precipitated with acetic acid. Finally, it is washed with distilled water and freeze-dried.

Lignosulfonate (Lignin Sulfonate, Sulfite Lignin)

The sulfite pulping of wood is accomplished by treating wood at high temperatures with aqueous sodium sulfite. The cook may be acid, neutral, or alkaline. An example of neutral sulfite cooking conditions is as follows. The wood chips are heated from ambient temperature to 175° over 90 min in sulfite liquor at a 3 : 1 (w : w) liquor to wood ratio. The liquor contains 15% sodium sulfite and 1.5%. sodium carbonate, based on the dry wood. Time at temperature is 1 hr or more.

The sulfonated, water-soluble lignin (lignosulfonate) cannot be isolated by precipitation from the spent liquor with acid as are kraft lignins. However, this lignin may be purified by complexing with amines. For example, spent sulfite liquor, 408 ml containing 175 g of solids, is heated to 70–85° with mild stirring. *N,N*-Dimethylhexadecylamine (Armak, Chicago, Illinois) is added, and then the solution is adjusted to pH 3.5 with 10 N sulfuric acid. Four hundred grams of 1-octanol is added, and the solution is stirred for 5 min more, then left to stand. One hour is required for the layers to separate. The bottom, aqueous acidic layer is discarded.

To the alcohol layer is added 122 g of water, with stirring, and 28 g of 50% sodium hydroxide (the solution should be about pH 9.5). The solution is heated at 60–70° with stirring. The layers are allowed to separate over 30 min. The bottom, aqueous layer contains about 100 g of sodium lignin sulfonate and a trace of octanol. The alcohol is removed by vacuum evaporation.[16]

[15] K. Lundquist and T. K. Kirk, *Tappi* **63,** 80 (1980).

[16] S. Y. Lin (Reed Lignin, Inc., Rothschild, Wisconsin), personal communication.

Acknowledgment

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