Chapter 26
Methods for Extraction and Purification of Lignin and Cellulose from Plant Tissues

Ksenija Radotić and Miodrag Mićić

Abstract Isolation of the two most abundant plant macromolecules, lignin and cellulose, from the plant tissues is reviewed. Isolation methods depend on the plant tissue that is used as the source of the polymers. Assessment of purity has an important place in the whole isolation process. Extent of needed purity depends on the further application of the obtained polymers. Different lignin extraction procedures are presented. Lignin isolation via preceding extraction of pulp is usually applied on wood as a starting material. The procedure preceded by cell wall extraction and combined with lignin complexation with thioglicolic acid is used for plant tissues containing various kinds of cells or low lignin content comparing with protein content. Lignin purification with ionic liquids, as well as those obtaining Brauns' native lignin, kraft lignin, or lignosulfonate are also described. The review of cellulose isolation procedures is presented, such as the alkaline procedure, as well as the method using ultrasound treatment, or the method applying enzyme technology. Dilute acid pretreatment in cellulose isolation is also depicted.

Keywords Cell walls • Wood • Pulp • Ionic liquids • Sulfate lignin • Sulfite lignin • Extractive free cell walls • Thioglicolic acid • Plant waists • Cryocrushing

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1 Introduction

This chapter deals with isolation of the two most abundant plant macromolecules, lignin and cellulose from the plant tissues. These polymers have a key role as the main building compounds in the plant cell walls, mainly providing mechanical support and protection from various kinds of stress [1, 2], but also being involved in the regulation of the plant growth [3]. On the other side, since cell wall makes the main part of plant biomass on Earth, these polymers have been extensively studied in the context of the bio-based sources for energy production. Cellulose in plant biomass is the principal component used for biofuel production. It is a polymer made of sugars (glucose). Lignin is another major component of the plant cell wall that contributes significantly to the resistance of the biomass to degradation. This polymer, consisting of phenylpropanoid monomers, is difficult to break down, thus various treatments are applied to remove it from cellulose [4–7] (Fig. 26.1).

As it is illustrated in Fig. 26.1, lignocellulosic matrix is a complex structure in which the cellulose is surrounded by a monolayer of hemicellulose and embedded in a matrix of hemicellulose and lignin [8, 9]. This explains why it is difficult to separate lignin from cell wall and from cellulose in the isolation process.

Isolation methods depend on the plant tissue that is used as the source of the mentioned polymers. Isolation procedure from wood may differ in certain steps from the isolation taking green parts of plant (leaves, stems), as well as from those which contain different kinds of cell/tissue types.

Purification is the next crucial step in obtaining satisfactory material from the starting plant material. Thus assessment of purity has an important place in the whole isolation process. Extent of needed purity depends on the further application of the obtained polymers. For example, if the aim of isolation is obtaining fine cellulose fibers for further application in certain branches such as textile industry, one should pay attention to the preservation of the structural integrity of the fibers [10].

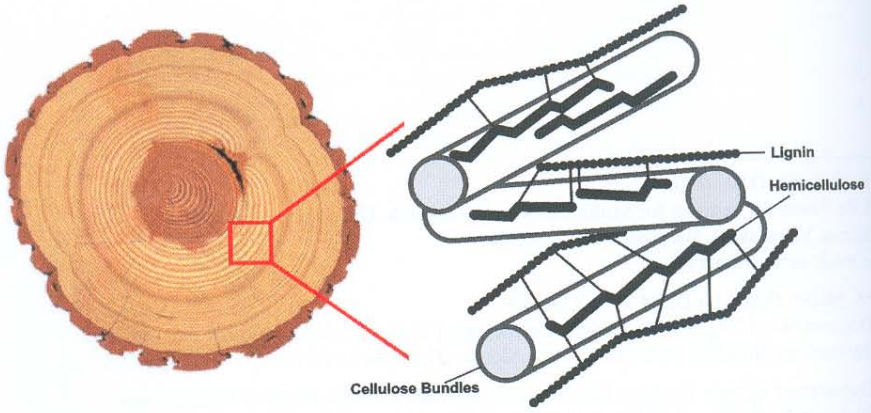

Fig. 26.1 Schematic presentation of the connections between main polymers in the cell walls of higher plants (scheme of a detail of cell walls adapted from [8])

2 Lignin Isolation Methods

It is almost impossible to isolate lignin in unaltered, native form, since it is necessary to apply drastic methods for lignin separation from the cell walls and cellulose, which partly changes its native conformation [11]. However, efforts have been made to simplify methods for lignin isolation and made them more ecologically friendly, as well as to obtain polymer with more preserved native structure. This polymer has shown potential in various medical applications, as bio-based support for drugs and food supplements, or as curing agent for certain diseases [12–14]. Also, use of lignin as a base for production of materials designed for various applications has been reported. These include materials for automotive brakes, wood panel products, biodispersants, polyurethane foams, and epoxy resins for printed circuit boards. It is also used as a binding and dispersing agent in different industries [15, 16].

2.1 Lignin Isolation via Preceding Extraction of Pulp

This process is usually applied on wood as a starting material, to obtain pulp that is further delignified. Lignin composition and yield is affected by the extraction method, solvent type, time, and temperature being all important variables. The choice of extraction method also depends on the type of starting material.

2.1.1 Obtaining Pulp

Method 1 comprises alkaline extraction of material. Wood chips or powder obtained by wood milling are pre-extracted by using cyclohexane/ethanol (1:1) (v:v) [17], or toluene/ethanol (2:1) [18] in a Soxhlet apparatus. Further extraction is performed by using dioxane/water mixture (9:1) (v:v) using HCl as catalyst at 90 °C. After the extraction the pulp is washed extensively with dioxane/water.

Method 2 includespressurized low-polarity water (PLPW) processing [19, 20]. Dry wood pieces are milled to a particle size range between 0.25 and 1 mm, and placed in the extraction cell, filled with water or 0.47 M NaOH. The extraction temperature is 100, 140, or 180 °C and pressure is 5.2 MPa. Total processing time is 82 min. The pH of the PLPW extract is reduced to 5.5 by addition of 6 N HCl.

2.1.2 Lignin Isolation from Pulp

Several different enzymatic, chemical, and mechanical methods have been developed for the isolation of lignin from pulp. However, due to the heterogeneous nature of wood and pulp fibers and the heterogeneity that exists between individual fibers, no method is currently available for the quantitative isolation of native or residual

lignin without the risk of structural changes during the isolation. Even if the perfect isolation technique could be found, the product would at best represent the average structure of native or residual lignin components.

Lignin Isolation by Enzymatic Hydrolysis of Pulp

This procedure is based on selective hydrolysis and dissolution of carbohydrates in pulp by commercial cellulolytic enzymes, leaving lignin behind as an insoluble residue ([21, 22]). Pulps are typically subjected to several successive enzyme treatments to ensure complete dissolution of the carbohydrates and to increase the amount of recovered insoluble lignin residue. The residual lignin from this isolation method is typically obtained in good yield, especially from unbleached kraft pulps. The enzymes employed in this enzymatic hydrolysis technique are effective enough to hydrolyze practically all pulp polysaccharides. The insoluble lignin residue remaining after enzymatic hydrolysis is subsequently extracted with 0.5 M sodium hydroxide and the soluble fraction is acid-precipitated, yielding the residual lignin sample. The residual lignin samples contain 65–80 % lignin, 7–8 % carbohydrates, and the remaining impurities from proteins acquired during the enzymatic treatment.

There are several drawbacks of utilizing the enzymatic hydrolysis method for isolating residual lignin. All residual lignins isolated with this procedure contain some carbohydrates that cannot be removed by prolonged and repetitive enzymatic treatments or by purification methods that are commonly employed for milled wood lignins. In addition, residual lignin sample obtained by this method contain protein impurities originating from the enzymes used in the hydrolysis stage. The carbohydrate and protein impurities from the enzymatic hydrolysis method complicate subsequent analyses of the lignin structure. However, the residual lignin obtained from this isolation technique is considered to be chemically unchanged and the yield is quite good.

Lignin Isolation by Acid Hydrolysis of Pulp

The extracted pulp is refluxed under an inert atmosphere (i.e., nitrogen or argon) with 0.1 M HCl in 9:1 dioxane:water (azeotrope boiling point of 88 °C). The solubilized lignin is then recovered from solution [23–25]. This technique offers a residual lignin that is free from carbohydrates and other impurities. The acid hydrolysis isolation method is a comparatively rapid way of obtaining pure residual lignin for further analysis when compared to enzymatic hydrolysis. The mechanism believed to be responsible for the liberation of lignin is the hydrolysis of covalent linkages between lignin and carbohydrates. One drawback of this method is the rather strong acidic conditions that are necessary to liberate the lignin from pulp fibers. The acid hydrolysis conditions can be expected to cause some structural modifications to the lignins including the cleavage of some aryl and alkyl ethers in benzyl alcohol units.

The cleavage of the aryl ethers would result in the creation of phenolic hydroxyl groups, causing the content of this functional group to be higher than the actual amount present in the wood or pulp. Lignin condensation reactions under acidic conditions are also possible but believed not occur during the isolation. Another drawback to this isolation technique is that the yield is rather low compared to enzymatic hydrolysis (40–60 %) and is dependent on the severity of the isolation conditions. The yield may be increased by applying more severe conditions (i.e., higher acid concentration and longer reaction times).

2.2 *Lignin Purification with Ionic Liquids*

Currently, the use of volatile organic compounds—free solvents are increasing in importance to avoid the increase of atmosphere contamination; most of the investigations in this area are focused on minimization of the greenhouse effect. Ionic liquids are the most widely investigated green solvents, particularly in the area of biomass. The combination of reutilization with their low volatility is the reason why ionic liquids are considered as green solvents. Lignin obtained by alkaline (NaOH 7.5 %, 90 min 90 °C) or organosolv (60 % ethanol, 90 min 180 °C) processes is purified by combining treatment with ionic liquid [Bmim][$MeSO_4$] (1-butyl-3-methylimidazolium methylsulfate) as a new green solvent, and microwave radiation [26]. Dried lignin is introduced in a flask along with [Bmim][$MeSO_4$] in a solid:liquid ratio of 1:25 at 50 °C for 6 h in an inert atmosphere. Microwave radiation can also be added. The solution is then filtered, and the filter residue dried at 50 °C. Acidified water at pH 2 was added to the liquid fraction to recover the lignin from the ionic liquid. After centrifugation recovered lignin is separated, washed, and dried at 50 °C in an oven.

Extraction of lignin assisted by microwave irradiation at 60, 80, 100, and 120 °C, combined with using DMSO and $LiCl_2$, has shown to increase lignin yield by 2.4, 8.8, 13.5, and 24.6 % (% Klason lignin), respectively. The content of neutral sugars in these lignin fractions was relatively lower as compared with the milled wood lignin (MWL) obtained by the classical method [27].

2.3 *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 h 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 crunched

alternatively with water and ether until it becomes solid. The solid is filtered and dried. 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). The Brauns' native lignin separates as a fine tan-colored powder. The yield is about 8 % based on the lignin in the wood [28].

2.4 *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 NaOH. 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 °C, whereas 170–180 °C is required for softwoods (gymnosperm woods). The bomb is usually heated in an oil bath and rotated, end over end, to ensure mixing. Time at temperature depends on the pulp yield desired; 1–2 h is typical. The precipitated lignin is washed thoroughly with distilled water and freeze-dried [29].

2.5 *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 °C 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 h or more [30].

2.6 *Lignin Isolation via Preceding Cell Wall Extraction*

In cases when lignin is isolated from plant tissues containing various kinds of cells or low lignin content comparing with protein content, the recommended procedure is to isolate and purify cell walls first and then to isolate lignin from the obtained cell walls. It is recommendable to use techniques for cell wall isolation which involve cell breakage [31] and which enable obtaining perfectly pure extractive cell wall material as a transient step for lignin isolation.

2.6.1 Extractive Free Cell Wall Material

Dry plant material (72 h at 60 °C) is ground into a fine powder, mostly by milling. To obtain cell walls, the powder is homogenized in 80 % methanol. The homogenate is stirred for 1 h at room temperature and after low-speed centrifugation the pellet is re-extracted twice with 80 % methanol. The pellet is then subjected to the following washing steps [32, 33]: 1× (1 M NaCl, 0.5 % Triton X-100), 2× distilled water, 2× 100 % methanol, and 2× 100 % acetone (each step for 30 min). For more efficient extraction of the cell wall material a FastPrep-24 System (MP Biomedicals, Santa Ana, CA, USA) was recently used [34–36] in each isolation step. The FastPrep-24 has long been used for the lysis and homogenization of plant tissues, prior to molecular biology applications such as nucleic acid isolation [37]. The FastPrep-24 instruments and matrix tubes provide rapid and thorough, automated disruption of plant cell walls, which are difficult to homogenize/lyse. The FastPrep-24 has been fitted with a 50 ml tube adapter, BigPrep (MP Biomedicals, Santa Ana, CA, USA). Each sample is homogenized for 30 s at 4.5 m/s speed in 50 ml BigClean tubes filled with stainless-steel matrix (MP Biomedicals, Santa Ana, CA, USA). Thus usage of FastPrep system shortens and increases efficiency of the cell wall isolation process (Fig. 26.2).

After drying in vacuum, extractive free cell wall material is incubated in the Na-phosphate buffer pH 5.7 containing 0.5 % (w:v) cellulase and 2.5 % (w:v) pectinase, with gentle shaking for 48 h at 30 °C. After centrifugation, the pellet is washed with Na-phosphate buffer and distilled water subsequently, and dried in a vacuum. For more efficient washing FastPrep apparatus can be used (Fig. 26.2).

2.6.2 Lignin Isolation by Complexation with Thioglycolic Acid

This procedure extracts all detectable lignin from the cell walls and preserves lignin native structure. The preceding cell wall purification steps provide lignin free from proteins and other contaminations. Such lignin is especially suitable for accurate and sensitive assays of its structure [33, 38]. The extractive free cell wall is incubated for 4 h at 95 °C in solution containing thioglycolic acid (TGA) and 2 M HCl. After cooling at room temperature and centrifugation, the pellet is washed with deionized water. The lignin complex with thioglicolic acid (LTGA) is extracted by vigorous shaking at 30 °C for 18 h in 0.5 M NaOH. This step can be significantly shortened to 3 h by using FastPrep system (Fig. 26.2), with additional improvement of extraction efficiency. After centrifugation and repeated washings of the pellet with 0.5 M NaOH, the combined alkali extracts are acidified with concentrated hydrochloric acid. The LTGA precipitate that formed after 4 h at 4 °C is recovered by centrifugation and washed twice with distilled water.

Advantages of using FastPrep in the procedure for cell wall and lignin isolations are (1) better homogenization and consequently better extraction and purification (better contact with solvent); (2) shorter duration of the individual steps (incubations, stirring).

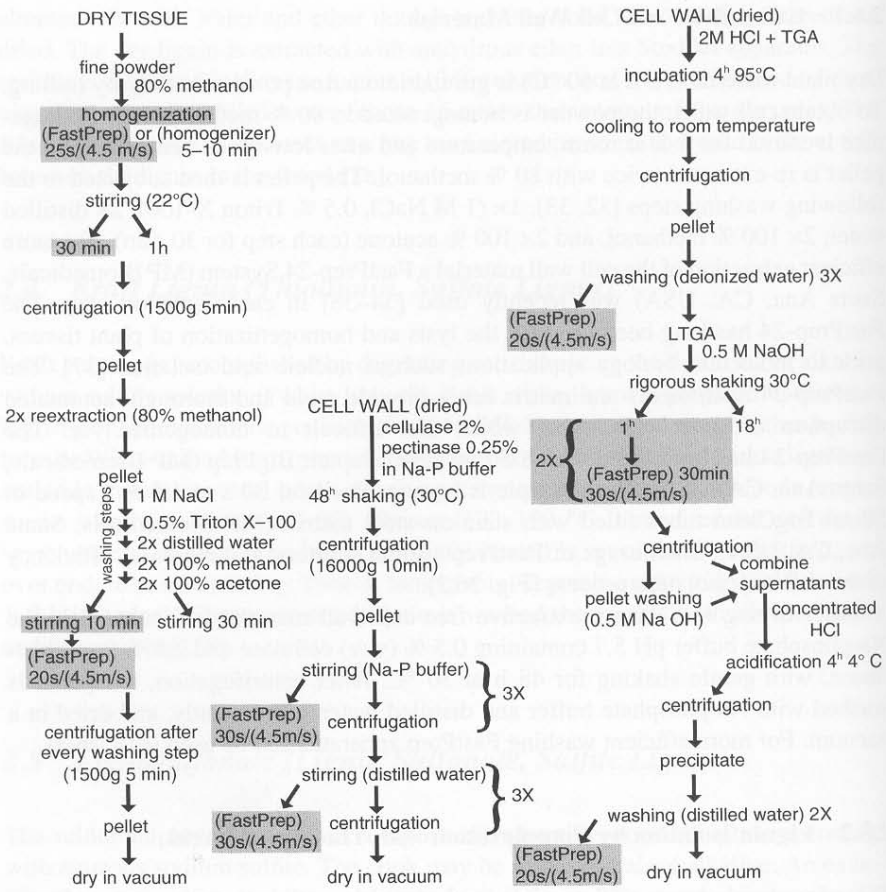

Fig. 26.2 Scheme comparing classical procedure and procedure with using FastPrep, for isolation and purification of extractive-free cell walls from the plant material (*left*), for subsequent cellulase/pectinase treatment of the isolated cell walls (*middle*) and for lignin isolation from the cell walls (*right*)

The FTIR spectroscopic analysis has shown that the structure of the cell walls and lignin extracted by using FastPrep system was not changed in comparison with the corresponding structures obtained without using this system.

3 Cellulose Isolation Methods

Cellulose is the most abundant naturally occurring polymer on Earth, being a candidate for providing such "nanoparticles" as a reinforcing agent [10, 39]. The inherent stiffness and high degree of crystallinity make it ideally suited for reinforcing and load bearing applications in composites. Apart from this, cellulose is a

sustainable resource, biodegradable in nature, and inexpensive and has a much lower density than most fillers that are in use today. For similar reasons cellulose can be used as a suitable material for bioethanol production [4, 40].

Cellulose isolation method from plant waists (usually of agricultural origin or wood) depends on the needed dimensions of the fibers [10, 41–43].

3.1 Cellulose Isolation by Using Alkaline Procedure

Initially, the dried plant tissue is digested at 80 °C in a 4 % sodium hydroxide solution for 4 h. This removes the greater part of lignin and a large part of hemicellulose. Because of persistent discoloration the product is subsequently bleached with a sodium chlorite/glacial acetic acid mixture to remove any residual lignin and hemicellulose that may have been present. The bleached cellulose fibers are washed repeatedly, initially with a 5 % aqueous NaOH and subsequently deionized water in order to attain a neutral pH [41]. In procedure of bleaching and washing of the material FastPrep system may be involved, to obtain material with higher purity.

3.2 Cellulose Isolation by Applying Ultrasound Treatment

The procedure for isolation of cellulose using alkaline peroxide with ultrasonic treatment comprises sequential treatment of the plant material with water at 55 °C for 2 h, then with ultrasonic irradiation for 40 min. In subsequent steps the material is treated with 0.5 M NaOH, 0.5 %, 1.0 %, 1.5 %, 2.0 %, and 3.0 % H_2O_2 in 0.5 M NaOH, and 2 M NaOH at 55 °C for 2 h. The insoluble residue is collected by filtration, washed with distilled water until the pH of the filtrate is neutral, and then dried at 60 °C [44].

By further processing of the isolated cellulose fibers, nanofibers can be obtained by using mechanical, chemical, physical, and biological methods. The choice of method mostly depends on the aimed final dimensions of the nanofibers [10].

3.3 Cellulose Isolation by Using Enzyme Technology

The application of enzymes in plant material processing has been based on the idea of selected hydrolysis of several components in the plant fiber (hemicelluloses, lignin) while retaining cellulosic portion [45].

Bio-treatment. Oven-dry bleached kraft pulp is used as a starting material. After soaking in water and autoclaving, the fungal culture is added to the fiber suspension with appropriate amount of sucrose and yeast extract to support the fungal growth. The fungus is left to act on the fibers at room temperature for different time duration

with slow agitation. The fibers are subsequently autoclaved, washed, and made into sheets of 10 % fiber consistency. Such fibers are sheared in a refiner for 125,000 revolutions.

Cryocrushing. The refined fibers are subjected to cryocrushing in which the fibers are frozen, using liquid nitrogen, and a high shear is applied, using a mortar and a pestle. This step is critical in liberating the microfibrils from the cell wall. The cryocrushed fibers are then dispersed into water suspension using a disintegrator and filtered through a 60-mesh filter. The filtrate, a dilute water suspension of microfibrils, is used for further investigation or applications.

3.4 *Dilute Acid Pretreatment in Cellulose Isolation*

This pretreatment is performed in order to more efficiently remove lignin and hemicelluloses from biomass during cellulose isolation [7, 46, 47].

Air-dried plant material is Wiley milled to pass through 0.05 mm pore size or 20-mesh screen. Wiley milled material is presoaked at room temperature (25 °C) while continuously stirring in an ~1 % dilute sulfuric acid solution at 5 % dry solids (w:w) for 4 h. The presoaked slurry is filtered and the solid material is washed with an excess of deionized water. This presoaked material is transferred to a 4560 mini-Parr 300 ml pressure reactor in an ~1 % dilute sulfuric acid solution at 5 % solids (w:w) and sealed. The vessel is heated to 160 °C over ~30 min (at ~6 °C/min). The reactor is held at 160 °C (6.4–6.8 atm) for the specified residence time: 2, 5, and 10 min. To halt the pretreatment process, the reactor is quenched in an ice bath (to cool to 70 °C). Then, the pretreated slurry is filtered to remove the solid residue and washed with an excess of deionized water and dried overnight at room temperature. All yields for biomass recovered after pretreatment range between 75 and 85 % by mass of the initial material.

Hot dilute sulfuric acid pretreatment of biomass increases digestibility through lignin redistribution and through hemicellulose dissolution [5, 7]. A side effect of hemicellulose removal and hydrothermal conditions is an annealing of cellulose that may increase crystallinity and may limit the efficiency of this pretreatment method. Changes in lignin structure during such pretreatment are evident [7], but the structural network of cellulose does not show signs of breakdown.

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