Lignin Biochemistry of Normal and Brown Midrib Mutant Sorghum

Dennis L. Bucholtz, Ronald P. Cantrell, John D. Axtell, and Victor L. Lechtenberg*

A phloroglucinol–HCl assay for lignin, which detects aldehyde groups, was performed on tissue of normal and brown midrib 6 (bmr-6) sorghum (*Sorghum bicolor* L. Moench). The color formed in this lignin assay was greater in the brown midrib mutant 6 than in the normal tissue. The mutant lignin contained ~3 times more aldehyde groups than the normal lignin, but there was less lignin in the mutant than in the normal. Apparently, the mutation caused an accumulation of aldehyde lignin intermediates which were incorporated into the lignin polymer. The enzyme activity which catalyzed the reduction of aldehyde lignin intermediates, cinnamyl alcohol:NADPH oxidoreductase, was less in brown midrib mutant 6 than in normal tissue [i.e., 0.24 and 1.42 μmol min^{-1} (mg of protein)$^{-1}$, respectively].

Lignin is an amorphous biopolymer formed in vascular plants. Its function is not clearly understood, but it probably has a role in maintaining the structural integrity of plant tissues. The monomers of this complex plant constituent are members of the (4-hydroxyphenyl)propene series. Members of this series are synthesized in plants by deaminating tyrosine and/or phenylalanine. With the latter substrate, the phenylpropenoic acid intermediate is hydroxylated, producing (4-hydroxyphenyl)propenoic acid. This intermediate is further hydroxylated at the 3 position, which may then be methylated, forming (3-methoxy-4-hydroxyphenyl)propenoic acid. The hydroxylation and methylation may be repeated, but at the 5 position forming (3,5-dimethoxy-4-hydroxyphenyl)propenoic acid (Maule and Ride, 1976). The 4-hydroxy and its two methoxylated analogues are metabolized further. The carboxylic acid group of each of these analogues can be reduced through a coenzyme A intermediate to the corresponding aldehyde (Rhodes and Wooltorton, 1973; Wengenmayer et al., 1976). The aldehyde may be reduced to the corresponding alcohol (Mansell et al., 1974).

Theoretically, the acid, aldehyde, and alcohol of the 4-hydroxy, 3-methoxy-4-hydroxy, and 3,5-dimethoxy-4-hydroxy analogues may participate in the formation of lignin. Stafford (1960a,b) reported that the incubation of some of these lignin monomers individually with leaves of timothy (*Phleum pratense* L.) resulted in the formation of a lignin-like material. In many instances, the lignin monomer produced a colored lignin-like material. Abnormal amounts of the monomers would undoubtedly accumulate in this incubation system due to overloading of the enzyme systems. The color of the lignin-like material was probably due to the incorporation of abnormal amounts of some of the monomers.

Jorgenson (1931) reported a naturally occurring brown lignin in corn (*Zea mays* L.). It was subsequently found that four recessive genes would produce a peculiar corn lignin (Kuc' et al., 1968). Several brown lignin mutant genotypes have also been produced in sorghum (*Sorghum bicolor* L. Moench) as a result of chemical mutagen treatments (Porter et al., 1978). Some of these sorghum mutants were found to have not only a peculiarly colored lignin but also reduced lignin content. A reduced lignin content is a nutritionally desirable trait in forages. Lignin in forages is a major factor limiting the digestibility of polysaccharides by animals. Therefore, an understanding of the biosynthesis of lignin in forage is essential to the improvement of forage quality. The development of the lignin mutants affords an excellent opportunity to examine the complex process of lignin biosynthesis.

Agronomy Department, Purdue University, West Lafayette, Indiana 47907.

EXPERIMENTAL SECTION

Tissue. The seeds used in this experiment were obtained from the plants described by Porter et al. (1978). The mutant which was arbitrarily designated number six was used in the present experiment. Seeds of the normal and brown midrib 6 mutant were planted in separate 2-L plastic pots containing potting soil. The pots were placed in a greenhouse, and the plants were illuminated with sunlight and supplemented with fluorescent and incandescent lamps to provide a day length of 16 h. Water was supplied as needed. The distinguishing brown midribs of the mutant were first observed at the three-leaf stage. When the plants were in the five- to six-leaf stage, they were excised at the soil surface. The leaf blades were stripped from both sides of the midrib and discarded. The remaining midribs and stems were cut into pieces ~2 cm long. This tissue was used for subsequent analyses. It was either chilled in ice for immediate use in enzyme assays or dried and ground in a Wiley mill to pass through a 1-mm screen for chemical analyses.

Permanganate Lignin Determination. The lignin content of tissue used in this study was determined by the gravimetric procedure described by Goering and Van Soest (1970). A known weight of the dried and ground tissue (generally 1.000 g) was refluxed with 100 mL of 0.05 M cetyltrimethylammonium bromide in 1 N sulfuric acid. The resultant residue was collected on a tared fritted glass crucible by filtering with suction. The residue and crucible were dried and weighed. The crucible was then partially submerged in a buffered solution of potassium permanganate. The depth of the permanganate solution was sufficient to completely cover the residue in the crucible. This residue was filtered and washed with an acidic ethanolic solution of oxalic acid and then with 80% ethanol. The residue and crucible were dried and weighed. The weight loss of the residue caused by the permanganate treatment was attributed to the loss of lignin. This procedure was performed on tissue from both the normal and the mutant plants.

Phloroglucinol–HCl Reaction. Two volumes of a freshly prepared ethanolic (95%) 0.1 M solution of phloroglucinol was mixed with one volume of concentrated hydrochloric acid just prior to use. Two-tenths gram of dried and ground tissue was weighed into a test tube, and 2 mL of the phloroglucinol–HCl reagent was added. A dark pink color formed almost immediately. The intensity of the color of the normal and mutant tissue was compared visually.

Lignin Preparation. Dried and ground tissue (150 g) was extracted with 400 mL of ethanol (95%). The ethanol was removed by filtration with suction. The residue was

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suspended in 750 mL of a benzene–ethanol (2:1 v/v) mixture with continuous stirring for 3 h. This solvent was removed by filtration with suction, and the residue was resuspended in 750 mL of a benzene–toluene–ethanol (1:1:1 v/v/v) mixture with continuous stirring overnight. The solvent was removed by filtration with suction. This residue was then boiled for 1 h with 750 mL of 4% (v/v) aqueous HCl. This hydrolysis was repeated a second time. The residue was recovered by filtration with suction. This hydrolyzed residue was incubated in 750 mL of 1% HCl (v/v) in 95% ethanol overnight at 60 °C. The filtrate was collected with suction. The filtrate was evaporated under reduced pressure to a volume of ~50 mL. Distilled water was added to the concentrated ethanol solution, and a brown flocculent precipitate formed. The precipitate was collected on a fine fritted glass crucible and washed with water and then with diethyl ether. This precipitate, the lignin preparation, was dried to constant weight. This extraction procedure was conducted on both the normal and the mutant tissues.

Two milliliters of both of the lignin solutions, 10 mg dissolved in 250 mL of 95% ethanol, and of ethanol (the blank) was added to 1 mL of concentrated HCl. Two milliliters of a freshly prepared 0.1 M ethanolic solution of phloroglucinol was added to each of the lignin–HCl solutions and to the blank. The absorbance of the solutions was measured at 550 nm 1 min after the addition of the phloroglucinol solution. (The absorbance was dependent on time.) A series of (3-methoxy-4-hydroxyphenyl)propenal ethanolic solutions differing in concentration were prepared, and 2-mL aliquots were assayed with HCl and phloroglucinol as described above. A graph of absorbance vs. (3-methoxy-4-hydroxyphenyl)propenal concentration was prepared which was used to calculate the amount of aldehyde equivalents in the lignin preparations.

Enzyme Preparation. The freshly cut stalks and midribs were weighed and homogenized with 0.05 M phosphate buffer (pH 7.3) containing 5 mM EDTA and 20 mM 2-mercaptoethanol. Generally, 3 mL of buffer was used for each g of fresh tissue. Immediately after homgenization, polyvinylpolypyrrolidone (generally 1 g/3 g of tissue) was added, and the mixture was stirred occasionally for the next hour. The homogenate was squeezed through four layers of cheesecloth. This extract was centrifuged at 23000*g* for 10 min, and the supernatant was collected. Ammonium sulfate was added to the supernatant to make it 40% saturated. This was centrifuged at 23000*g* for 10 min, and the supernatant was collected. Ammonium sulfate was added to this supernatant to make it 70% saturated. This was centrifuged at 23000*g* for 10 min, and the supernatant was discarded. The pellet was resuspended in a 0.05 M phosphate buffer (generally 5 mL) containing 20 mM EDTA and whose pH was 7.3. No reducing sulfhydryl reagents were added to this buffer because they were found to reduce the aldehyde substrate, spontaneously. All procedures were performed at 4 °C. Protein concentration was measured by using the Bio-Rad protein assay, using bovine serum albumin as a standard.

Enzyme Assay. One milliliter of the enzyme preparation (containing 1–2 mg of protein) was added to a cuvette. In a test tube, NADPH was generated from NADP, glucose 6-phosphate, and baker's yeast glucose-6-phosphate dehydrogenase in a 0.05 M phosphate buffer with 20 mM EDTA and a pH of 7.3. The reduction of the nucleotide reached equilibrium in less than 10 min, as evidenced by the absorbance at 340 nm. After the absorbance at 340 nm had stabilized, an aliquot of the NADPH-generating system containing initially 1 μmol of NADP, 10 μmol of glucose 6-phosphate, and 1 enzyme unit of glucose-6-phosphate dehydrogenase was added to the cuvette containing the enzyme preparation. The volume of the NADPH–enzyme preparatin solution was increased to 4.1 mL with phosphate buffer. The reaction was started with the addition of 0.2 μmol of (3-methoxy-4-hydroxyphenyl)propenal in 0.25 mL of phosphate buffer. The rate of the reduction of the aldehyde was monitored at 415 nm.

The molar extinction coefficient of the phenolic aldehyde at 415 nm was dependent on pH. The absorption spectra of related phenols was shown by Lemon (1947) to be dependent on the pH of the solution. A series of aldehyde solutions with pH values ranging from 7.1 to 8.9 were prepared, and the molar extinction coefficient (ϵ_{415nm}) of each was calculated. Over this narrow range of pH values, the relationship between ϵ_{415nm} and pH was found to be linear ($r = 0.992$; $\epsilon_{415nm} = 13750 \times \text{pH} - 94880$). The pH of each enzyme assay solution was measured at the conclusion of the assay, and the observed pH was used to calculate the corresponding molar extinction coefficient. The contribution of the other components used in the assay to the total absorbance at 415 nm was less than 1% of that of the aldehyde substrate.

Analysis of the Enzyme Product and Synthesis of (3-Methoxy-4-hydroxyphenyl)propenol. An enzyme assay was conducted as described above except that the amounts of the components were doubled. The reaction was allowed to proceed until the change of absorbance at 415 nm ceased. The incubation was then terminated by extracting the assay solution twice with diethyl ether. The ether was evaporated, and the residue was redissolved in 0.5 mL of 95% ethanol. A 0.1-mL aliquot of the ethanol solution was applied to Whatman No. 1 chromatography paper.

A (3-methoxy-4-hydroxyphenyl)propenol standard was synthesized chemically by adding 2 mg of potassium borohydride to an aqueous solution of (3-methoxy-4-hydroxyphenyl)propenal (0.5 mg in 5 mL). Potassium borohydride selectively reduces the aldehyde group of α,β-unsaturated carbonyl compounds. The yellow solution of the aldehyde turned colorless almost immediately after the addition of the potassium borohydride. The reaction products were extracted with diethyl ether. The ether was evaporated, and the residue was redissolved in ethanol. An aliquot was applied to the chromatography paper alongside the enzyme assay extracts. The chromatograms were developed with 1-butanol saturated with 2% ammonium hydroxide as described by Pearl (1959). A spot from each extract was detected on the developed chromatogram with UV light. The compound from each extract was eluted with ethanol, and an absorption spectra of each solution was determined with a Beckman Acta II recording spectrophotometer.

RESULTS AND DISCUSSION

The reaction of the phloroglucinol–HCl reagent with plant tissue has been used as an assay for lignin in histochemical studies. Adler et al. (1948) found that this reagent reacts with the aldehyde group in the lignin polymer. The phloroglucinol–HCl reagent was used in this study to measure the aldehyde content of the lignin of the normal and mutant tissue. When the phloroglucinol–HCl reagent was reacted with dried and ground tissue from the normal and mutant plants, the intensity of the pink color that developed was greater in the mutant than in the normal tissue. The greater color intensity developed in the mutant tissue suggests that more aldehyde-containing lignin was present in this tissue than in the normal tissue.

Table I. Permanganate Lignin Content in bmr-6 Mutant and Normal Sorghum Genotypes

genotype	permanganate lignin, % of dry wt
mutant	4.02
normal	5.06
$LSD_{0.05}$	0.38

Table II. Aldehyde Equivalents in Lignin Preparations from bmr-6 Mutants and Normal Sorghum Genotypes

genotype	aldehyde equiv,[a] μg/mg of lignin
mutant	7.68
normal	2.50
$LSD_{0.05}$	1.12

[a] See the text for a description.

However, the permanganate lignin analysis indicated that the lignin content in the mutant was less than in the normal tissue (Table I). This apparent anomaly is explained if the mutation caused an accumulation of an aldehyde lignin monomer, which was then incorporated into the growing lignin polymer.

It was difficult to estimate the aldehyde content of the lignin from the normal and mutant tissues by visually observing the resulting color when the phloroglucinol–HCl reagent was added. Therefore, it was necessary to solubilize the lignin from both mutant and normal tissues for spectrophotometric measurements of the phloroglucinol–HCl color reaction with lignin. Many lignin isolation procedures are destructive. However, one of the mildest isolation procedures is alcoholysis. This procedure was selected to isolate lignin from mutant and normal sorghum tissue. Interfering substances in the tissues were removed by extraction with organic solvents. The hemicellulose was hydrolzyed with hot 4% aqueous hydrochloric acid. Lignin is insoluble in water; therefore, only small amounts of lignin should have been lost by this procedure. Some lignin was solubilized by alcoholysis. These lignin preparations were liberally washed with water and then with diethyl ether in an attempt to eliminate compounds which might interfere with the quantification of the aldehyde groups in the lignin preparation. After these precautions were taken, it was found that the mutant lignin preparation contained ~3 times the aldehyde content of comparably prepared normal lignin (Table II).

The accumulation of an intermediate in a biosynthetic pathway is caused by increased anabolism and/or decreased catabolism of that intermediate. In a simple enzyme system, a mutation would likely cause a reduced activity of an enzyme. Therefore, an assay was developed to measure the rate of catabolism of the lignin aldehyde monomer, (3-methoxy-4-hydroxyphenyl)propenal, to the corresponding alcohol. The enzyme activity of comparably prepared extracts from mutant and normal plants was measured. The specific activity of cinnamyl alcohol:NADP oxidoreductase was 0.24 and 1.42 μmol min^{-1} (mg of protein)$^{-1}$ for extracts from mutant and normal plants, respectively (Table III). The amount of protein extracted from equivalent weights of mutant and normal tissue was comparable.

The chromatographic and spectrophotometric characteristics of the enzyme reaction product and synthetic (3-methoxy-4-hydroxyphenyl)propenol were compared. The R_f of both on the paper chromatogram was 0.80, and the absorption maximum in 100% ethanol for both was 277 nm.

Table III. Specific Activity of Cinnamyl Alcohol:NADP Oxidoreductase in Extracts from bmr-6 Mutant and Normal Sorghum Genotypes

genotype	sp act., μmol min^{-1} (mg of protein)$^{-1}$
mutant	0.24
normal	1.42
$LSD_{0.05}$	0.20

The ability of sorghum to reduce the (3-methoxy-4-hydroxyphenyl)propenal to the alcohol appears to be severely diminished by the bmr-6 gene. This diminished ability in the bmr-6 apparently caused an accumulation of the aldehyde intermediate which was incorporated into lignin at a higher concentration than in the lignin of the normal genotype. The incorporation of abnormal concentrations of precursors into the polymer is a unique possibility for the formation of lignin. Unlike other biopolymers which are formed by highly structured mechanisms, lignin is formed by a random process. The lignin precursors, the (4-hydroxyphenyl)propenes, are acted on by peroxidase and hydrogen peroxide to form free-radical species (Freudenberg, 1965). These free-radical species react with one another and with other cell constituents to form lignin and the lignin–carbohydrate complex. Therefore, the chemical constitution of lignin is dependent on the concentration and reactivity of each of the free-radical species.

The bmr-6 mutant in sorghum has been shown to increase polysaccharide digestion by ruminant animals (Porter et al., 1978). Since this mutant was induced and inasmuch as there are numerous lignin precursors, there is a good possibility that an abnormal lignin can be induced in other plant species either genetically or by chemical inhibitors.

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