Isolation and Characterization of Lignin from Hard Red Spring Wheat Bran[1]

P. B. SCHWARZ,[2] V. L. YOUNGS,[3] and D. R. SHELTON[2]

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ABSTRACT

Dioxane-extractable lignin (DEL) preparations were isolated from hard red spring (HRS) wheat bran (bran DEL) and HRS wheat outer pericarp tissue (pericarp DEL). Alkali lignin was prepared from the bran (bran AL). Prior to lignin extraction, the bran and outer pericarp tissues were treated to remove nonlignin materials. Treatments included pin milling, air classification, and enzymatic digestion. Bran DEL contained 0.86% nitrogen and 18% carbohydrate, whereas the bran AL and pericarp DEL contained, respectively, 1.95 and 1.06% associated nitrogen and 0.9 and 2.2% associated carbohydrate. Esterified hydroxycinnamic acids were detected in the bran DEL preparation. These consisted largely of *trans*-ferulic acid with lesser amounts of *trans*-*p*-coumaric acid. Ultraviolet spectroscopy indicated the absence of these compounds in the bran AL and pericarp DEL isolates. Bran DEL was characterized by nitrobenzene oxidation. The molecular weight distributions of the bran DEL and AL isolates were determined by gel permeation chromatography. The bran AL isolate had a high average molecular weight, whereas that of the bran DEL isolate was quite low. All lignin preparations were obtained only in low yields, perhaps representing less than 5% of the total tissue lignin.

Lignin may play an important role in several of the physiological effects believed to be associated with dietary fiber. These effects include the binding of bile acids and a hypocholesteremic effect (Story et al 1982), and the binding of carcinogens (Paden et al 1983, Reddy 1985, Rubio et al 1979). However, much of this work has been based upon in vitro studies, and the exact role of lignin in the diet remains unclear (Gyory and Chang 1983; Heaton et al 1971; Hillman et al 1983, 1985; Linder and Moller 1973; Thiffault et al 1970). In addition, many of these studies employed Klason lignins or lignins of wood origin. Klason lignins are extensively modified during the hydrolysis of polysaccharides in concentrated acid and can no longer be considered representative of the native lignin (Lai and Sarkanen 1971). Wood lignins may also be equally nonrepresentative of those from herbaceous food species, as it is well known that the composition of lignins varies between species (Sarkanen and Hergert 1971). Variation in lignin composition was shown to affect in vitro assays (Kay et al 1979, Rubio et al 1979), and has been suggested to influence the results of in vivo studies (Vahouny et al 1980). The study of lignins from herbaceous food sources is, therefore, of interest.

Although lignins have been isolated from a small number of herbaceous species (Gordon 1978), most research has been in wood chemistry. At present two lignin preparations, milled wood lignin and cellulytic enzyme lignin, are considered especially suited for the investigation of wood lignins (Fengel and Wegener 1983). Milled wood lignin is prepared from ball-milled wood meal following extraction with a suitable organic solvent, typically aqueous dioxane. Cellulytic enzyme lignin is isolated in much the same manner, except that the wood meal is treated with cellulase enzymes prior to extraction.

The application of these methods to herbaceous species, however, has not been as successful. Lignins isolated from herbaceous plants generally contain large portions of associated carbohydrate and protein (Harkin 1973). The tissues of herbaceous species are not as extensively lignified and also often contain a number of nonlignin phenolic compounds.

Milled wood lignin has been isolated from wheat straw (Erickson et al 1973, and Scalbert et al 1985) and other grasses (Higuchi et al 1967, and Himmelsbach and Barton 1980). Scalbert and co-workers (1985) reported a wheat straw milled wood lignin preparation that contained 7% associated carbohydrate and a portion of alkali-labile ferulic and *p*-coumaric esters. Both Scalbert et al (1985, 1986) and Himmelsbach and Barton (1980) suggested covalent cross-links between grass lignins and cell wall hemicelluloses. Hydroxycinnamic acids may be involved in these cross-links.

Scalbert and co-workers (1985) also reported the isolation of cellulytic enzyme lignin from wheat straw. Although yield was again low, cellulase treatment increased the yield of extractable lignin more than five times over that of straw milled wood lignin.

Isolation of lignins through alkaline extraction is a method that has been recommended as particularly suited for grass samples. Grass lignins can be solubilized with dilute aqueous alkali under mild conditions that should not cause chemical modifications beyond the saponification of esterified hydroxycinnamic acids (Lai and Sarkanen 1971). However, the number of reports involving the isolation of alkali lignin from grass samples is limited (Kuc and Nelson 1964, and Scalbert et al 1985).

In this study, several methods of lignin isolation were evaluated for the preparation of wheat bran lignin. Wheat bran was chosen because it represents one of the richest sources of dietary lignin (Gordon 1978) and is a common fiber source. The suitability of each bran lignin isolate was evaluated by preliminary characterization involving factors of yield, molecular weight distribution, and associated carbohydrate and nitrogen.

MATERIALS AND METHODS

Sample and Sample Preparation

A composite sample of the hard red spring (HRS) wheat variety Len, grown in 1985 at university agricultural experiment stations in Casselton and Minot, ND, Crookston, MN, and Redfield, SD, was used throughout this study.

Bran was obtained from the above sample by milling on a Miag 55 cwt pilot-scale mill. A portion of the adhering endosperm was removed by passing the bran through a Buhler bran duster.

The bran was pin milled and air classified according to a modification of the procedure of Dick (1976). Bran was milled on an Alpine-Augsburg pin mill (Alpine-American Corp., Sommerset, MA) at 14,000 rpm. Pin-milled bran was then passed through an Alpine-Microplex air classifier with the feed gate set at 5 and the air-flow at 25°. The coarse fraction obtained following air classification was retained, whereas the fine fraction was discarded. This coarse fraction was passed through the air classifier an additional two times with the respective feed gate/air flow

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[2]Postdoctoral research associate and assistant professor, respectively; Department of Cereal Science and Food Technology, North Dakota State University, Fargo, 58105.

[3]Current address: 3401 N. 1st St., Fargo, ND 58102.

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settings of 7.5/30° and 7.5/35°. The resultant fine fractions were discarded. Following the third air classification step, the coarse fraction was pin milled a second time, again at 14,000 rpm. This material was passed through the air classifier three additional times with the respective feed gate/air flow settings of 5/25°, 6.5/30°, and 7.5/35°. The fine fractions were again discarded whereas the final coarse fraction was retained.

Prior to the extraction of lignin, the coarse bran fraction was treated as outlined in Figure 1. Bran (300 g) was extracted with 3.0 L of benzene/ethanol (2:1, v/v) for 24 hr in a Soxhlet apparatus. The bran was next similarly extracted with 95% ethanol. The extract-free bran, when dry, was milled with a rotary ball mill (Norton Chemical Process Division, Akron, OH). Extracted bran (250 g) was combined with 4.5 kg of burundum grinding balls (38-mm diam.) in 11.3-L burundum milling jars. The milling jars were operated at 42 rpm for 24 hr at −20.0°C.

The ball-milled bran (200 g) was combined with 4.5 L of 0.05*M* potassium phosphate buffer, pH 7.5 (50 ppm Ca^{++}), in a 6.0-L Erlenmeyer flask. When the sample had become fully hydrated, 2.0 g of Termamyl 120 L α-amylase (Novo Laboratories, Wilton, CT) was added, and the temperature was slowly increased to 94 ± 3°C with continuous stirring. At this point an additional 1.0 L of buffer was added, and the solution was stirred for 1 hr at 85 ± 5°C. The flask was then transferred to a 45°C water bath where constant stirring was maintained. When the solution reached a constant temperature, 400 mg of protease (*Streptomyces griseus* no. P5147, Sigma Chemical Co., St. Louis, MO) was added, and digestion was continued for 18 hr. At the completion of the protease digestion, the sample was filtered with aid of an IEC 8-in. perforated basket rotor on an IEC model K centrifuge (International Equipment Co., Needham Heights, MA) operated at 3,000 rpm at ambient temperature. Once filtered, the sample was washed on the centrifuge with an additional 2–4 L of water.

The filtered bran was resuspended in 5.5 L of 0.05*M* potassium acetate buffer pH 4.5. Amyloglucosidase (700 mg *Rhizopus* sp. no. A7255, Sigma) was added, and digestion was continued 18–24 hr at 45°C. The sample was again recovered and washed with the aid of the basket rotor. This material was freeze-dried.

In addition to bran, lignin was also extracted from wheat outer pericarp tissue. Outer pericarp tissue was isolated by blending whole wheat in water at reduced speeds for time periods up to 1.5 hr as described by Schwarz et al (1988). The isolated outer pericarp was ground on a Wiley mill to pass a 0.5-mm screen, after which it was extracted, ball milled, and enzyme treated as described for bran.

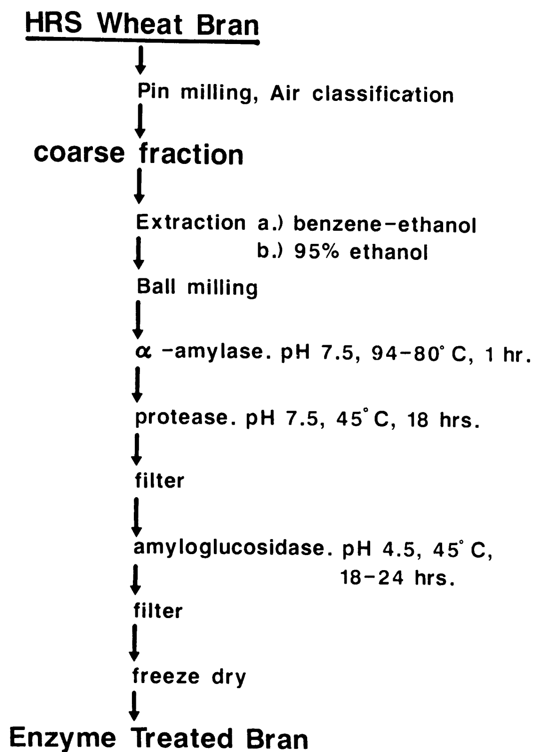

Fig. 1. Mechanical and enzymatic treatments of bran used in the isolation of lignin.

Dioxane-Extractable Lignin (DEL and HDEL)

Lignin isolates were prepared from the ball-milled, enzyme-treated bran (or outer pericarp) according to the milled wood lignin procedure of Harkin as described by Freudenberg and Neish (1968). In order to avoid confusion, the wheat lignin isolates prepared in this study are referred to as dioxane-extractable lignins (DEL). Freeze-dried tissue (150 g) was extracted first by stirring with ethyl acetate (2.0 L) for 24 hr at 2°C. The residue was recovered by vacuum filtration and allowed to air dry.

A lignin-rich fraction was extracted by stirring the bran (or outer pericarp) with 1.9 L of dioxane/water (9:1, v/v) for six to seven days at ambient temperature. Both bran DEL and pericarp DEL were extracted in this manner. As an alternative to extraction at ambient temperature, a second bran sample (180 g) was extracted with 2.7 L of the dioxane/water mixture for four days in a Soxhlet apparatus. This preparation is referred to as bran HDEL.

After extraction, the bran residue was removed by vacuum filtration, and the filtrate was concentrated to 150–200 ml by evaporation under reduced pressure (50°C). Benzene (redistilled, 8 ml) was added dropwise with stirring to precipitate carbohydrate-rich material. Neutral alumina (2.0 g) was added to the solution, and the precipitate was removed by vacuum filtration. The dioxane extract was further concentrated (10–15 ml) and then freeze-dried.

Alkali Lignin

Alkali lignin (AL) was isolated from the ball-milled, enzyme-treated bran based upon the method of Scalbert et al (1985). Bran (150 g) was stirred with (4.5 L) 1.0*N* NaOH under nitrogen for 2 hr at 30°C. The bran was removed by centrifugation (10 min, 650 × *g*), followed by vacuum filtration of the supernatant through glass fiber filter paper. A lignin-rich fraction was precipitated by acidification to pH 1.0 through the dropwise addition of concentrated HCl. The precipitate was recovered by centrifugation (10 min, 10,000 × *g*), and the supernatant was discarded.

Lignin was extracted by stirring the residue with dioxane (700 ml) for 36–48 hr at ambient temperature. The extracted residue was centrifuged (10 min, 10,000 × *g*), and the dioxane extract was concentrated to 100 ml by vacuum evaporation at 50°C. This extract was added dropwise to 1.0*M* acetic acid (1.25 L) and the resultant precipitate recovered by centrifugation (15 min, 10,000 × *g*). This residue was dissolved in 25 ml of glacial acetic acid, which was then precipitated into diethyl ether (1.0 L). The ether was removed by centrifugation followed by aspiration.

Nitrogen, Starch, and Lignin

The nitrogen content of the lignin isolates (15–30 mg) was determined by AACC micro-Kjeldahl method 46-13 (AACC 1983) and bran starch content according to AACC glucoamylase-glucose oxidase method 76-11 (AACC 1983). Total bran lignin was determined by the AOAC indirect method 3.126 (AOAC 1980) as described in an earlier report (Schwarz et al 1988). All determinations were performed in duplicate.

Neutral Sugars

The hydrolysis of neutral sugars associated with the lignin isolates (7.0–15 mg) was performed in 4*N* trifluroacetic acid according to the method of Nesser and Schweizer (1984). Neutral sugars were determined as aldononitrile acetates by gas chromatography according to the procedure of McGinnis (1982).

Sample hydrolysis was performed in duplicate, and each derivative was chromatographed twice.

Uronic Acids

Galacturonic and glucuronic acids present in the lignin isolates were determined according to the procedure of Scott (1979), with the exception of sample hydrolysis. Sample hydrolysis (30–40 mg) was performed as described by Scalbert and co-workers (1985). Analysis was performed in duplicate.

Ferulic and *p*-Coumaric Acid

Hydroxycinnamic acids associated with the bran DEL isolate in the form of alkali-labile esters were determined by HPLC. Bran DEL (60–75 mg) was stirred with 1*N* NaOH (4.0 ml) under nitrogen for 1 hr at 20°C. Upon completion of the alkaline treatment, the suspension was adjusted to pH 1 with 6*N* HCl and then centrifuged (17,000 × *g*) for 15 min. The supernatant was transferred to a 10-ml volumetric flask, and the pellet was resuspended in 1.0 ml of water. After recentrifugation, the supernatant was transferred to the volumetric flask, and the volume was adjusted to 10 ml with water. The pellet received two additional alkaline treatments. The residue remaining after the third alkaline treatment was washed with 10 ml of water, freeze-dried, and retained for nitrobenzene oxidation and ultraviolet (UV) spectroscopic analysis.

Ferulic and *p*-coumaric acid in the alkaline hydrolysates (50 μl) were analyzed directly by reverse-phase HPLC following filtration through a 1.20-μm pore size filter disk. The column (3.9 mm × 15 cm Novopak, Waters Associates, Milford, MA) was protected with a Bio-Rad ODS-10 guard column (Bio-Rad, Richmond, CA). A solution of water, acetonitrile, and acetic acid (88:10:2, v/v) was used for isocratic elution at a flow rate of 0.8 ml/min. This solvent system was originally described by Charpentier and Cowles (1981) for the separation of phenolic compounds in pine extracts. The solvent system was prepared daily prior to use. Column temperature was maintained at 30°C. The effluent was monitored at 280 nm with a Waters model 440 absorbance detector.

Standards were dissolved in the solvent and chromatographed for tentative identification by retention time. Both *trans*-ferulic and *trans-p*-coumaric acid exhibited a linear response ($r > 0.99$) in the range evaluated. The retention times, based upon a minimum of five injections for these two standards, respectively, were: ferulic acid 20.55 ± 0.09 min and coumaric acid 15.34 ± 0.34 min. *trans*-Ferulic and *trans-p*-coumaric acid in the bran DEL hydrolysates were quantitated from standard curves of concentration versus peak area.

The *cis*-isomers of these compounds were prepared by exposing solutions of the *trans*-isomers in a quartz cell to UV light (Kahnt 1967). The *cis*-isomers of ferulic and *p*-coumaric acid eluted at 22.69 and 16.16 min, respectively. The *cis*-isomers, however, were not detected in the bran DEL samples.

Two bran DEL hydrolysates were prepared, and chromatographic analysis was performed in duplicate.

Alkaline Nitrobenzene Oxidation

Alkaline nitrobenzene oxidation of the alkali-treated bran DEL (17 mg) was performed as described by Cymbaluk and Neudoerffer (1970) and Gee et al (1968) with two exceptions. First, a Teflon-lined Parr acid digestion bomb (model 4749, Parr Instrument Co., Moline, IL) was employed for oxidation rather than sealed glass tubes. Second, the phenolic acids and aldehydes resulting from oxidation were analyzed by reverse-phase HPLC, rather than by gas chromatography.

Following oxidation, the reaction mixture was purified by successive extractions with 1*N* NaOH, diethyl ether and methylene chloride as described by Gee and co-workers (1968). The final organic phase was concentrated to 10 ml under vacuum on a rotary evaporator. This material was transferred to a test tube and then brought to dryness under a stream of dry nitrogen at 30°C. The flask, which had been used for evaporation, was rinsed twice with (10 ml) diethyl ether, and the washes were transferred to the test tube and dried.

Prior to analysis, the dried reaction mixture was resuspended in 5.0–6.25 ml of the HPLC solvent (88:10:2, v/v, water/acetonitrile/acetic acid). This solution (50 μl) was chromatographed directly, following filtration through a 1.2-μm pore size filter disk. Chromatographic analysis was conducted as described in the preceding section.

Again, standards were employed for tentative identification of compounds present in the nitrobenzene oxidation mixture. These compounds and their respective retention times, based upon a minimum of five injections, were: *p*-hydroxybenzoic acid, 6.01 ± 0.03 min; vanillic acid, 7.56 ± 0.04 min; *p*-hydroxybenzaldehyde, 8.91 ± 0.08 min; vanillin, 12.04 ± 0.12 min; syringaldehyde, 14.89 ± 0.05 min; and *trans*-ferulic acid, 20.58 ± 0.09 min. Compounds present in the nitrobenzene oxidation mixture were quantitated from standard curves of concentration versus peak area. All standards exhibited a linear response ($r > 0.95$) in the range examined.

Nitrobenzene oxidation was performed on duplicate samples, and each oxidation mixture was chromatographed twice.

Gel Permeation Chromatography

Gel permeation chromatography of the bran DEL and bran AL isolates (3–5 mg) was performed as described by Connors et al (1980). A 1.6 × 90 cm Sephadex LH-60 column and a 1.6 × 93 cm Sepharose CL-6B column were eluted with *N,N*-dimethylformamide containing 0.1*M* LiCl. The Sephadex column was eluted at 0.5 ml/min, and the Sepharose column was eluted at 0.25 ml/min. Eight polystyrene standards (Pressure Chemical, Pittsburgh, PA) in the molecular weight range 575,000–2,000 were used for column calibration. Fractions of 3.0 ml were collected. Polystyrenes were monitored at 268 nm on a UV spectrophotometer, and lignin samples were monitored at 280 nm.

Statistical Analysis

Analysis of variance was performed with the ANOVA procedure of the Statistical Analysis System (SAS 1979).

RESULTS AND DISCUSSION

Isolation of Lignin

As the isolation of lignin from herbaceous species is frequently complicated by the presence of protein and a low tissue lignin content, the bran employed in this study was pretreated to reduce the protein content while concurrently increasing the concentration of lignin. A previous study in this laboratory had shown that selection of the coarse fraction, following pin milling and air classification of HRS wheat bran, provides material of elevated lignin content and reduced protein (W. H. Kunerth, A. E. Staley Manufacturing Co., Decatur, IL, *personal communication*, 1987). In this study, pin milling and air classification was combined with an enzymatic treatment as outlined in Figure 1. This treatment increased the lignin content to 9.8% from 4.4% in the original bran. Protein content was decreased from 18.7% in the original bran to 4.8%. Quantitative recoveries are not reported, because some bran was invariably lost during the centrifugation of the enzyme digests.

While it is unlikely that the presence of starch would interfere with the extraction of lignin, it did significantly hinder the filtration of the bran digests, and from this standpoint its removal was desirable. Treatment of the bran with α-amylase and amyloglucosidase as shown in Figure 1 greatly reduced the time required for filtration of the digests, and the presence of starch was not detected by the AACC (1983) glucoamylase-glucose oxidase procedure.

Bran DEL was prepared by extraction of the treated bran with 90% aqueous dioxane at room temperature. This material was obtained in yields of 4.7 mg/g of treated bran. Extraction of DEL at an elevated temperature (bran HDEL) resulted in similar yields. The yield of DEL from treated outer pericarp tissue (pericarp DEL) was quite low (0.5 mg/g treated pericarp). Pericarp DEL, bran DEL and bran HDEL were all obtained as dull orange powders. Bran AL was obtained as a dark brown powder at 0.40 mg/g treated bran.

Nitrogen and Carbohydrate

All four lignin preparations contained associated nitrogen and carbohydrate. Nitrogen is not normally considered a component of lignins and is believed to reflect associated protein. The nitrogen content of the lignin preparations, when multiplied by 6.25, provide an estimate of associated protein. The respective protein estimates for the HDEL, AL, pericarp DEL, and bran DEL isolates are 21.0, 12.2, 6.6 and 5.4%, respectively. However, aminophenolics and amide conjugates of phenolic acids have been reported in cereal grains (Collins 1986), and as the absence of these compounds in the lignin isolates has not been firmly established, it is perhaps most prudent to consider only total nitrogen content. Bran HDEL contained the highest level of nitrogen (3.36% of dry matter), followed by bran AL (1.95%); pericarp DEL (1.05%) and bran DEL (0.86%) contained significantly less nitrogen.

While bran HDEL exhibited the highest level of nitrogen, it contained an intermediate amount of carbohydrate. The levels of associated carbohydrate expressed as total neutral sugars and glucuronic acid are shown in Table I. Bran DEL exhibited significantly the highest level of carbohydrate, and clearly the temperature of the DEL isolation influenced the levels of associated nitrogen and carbohydrate. Pericarp DEL and bran AL contained the lowest level of carbohydrate.

The low level of carbohydrate observed in the bran AL isolate is not surprising, as NaOH treatment would be expected to saponify the hydroxycinnamic esters that have been hypothesized to cross-link lignin to a cell wall hemicellulose (Scalbert et al 1985). Scalbert and co-workers (1985), however, reported a wheat straw AL isolate to contain 18% carbohydrate.

In wheat straw, these linkages are proposed to occur between lignin and an arabinoglucuronoxylan (Scalbert et al 1985). Large portions of both arabinose and xylose were detected in the bran DEL hydrolysates (Table I). However, only a small amount of glucuronic acid was detected in the bran DEL and bran AL preparations (Table I). No glucuronic acid was detected in the pericarp DEL sample. These results would seem to suggest that bran DEL occurs in association with an arabinoxylan. Arabinoxylans are the predominant cell wall polysaccharide of wheat bran (Selvendran et al 1980). In addition to arabinose and xylose, a large portion of glucose was detected in the hydrolysate of bran DEL. Glucose accounted for over one-half the neutral sugars in bran AL and pericarp DEL. Under the conditions of hydrolysis employed in this study, glucose most likely evolved from the small portion of glucan believed to be associated with bran cell walls (Selvendran et al 1980), or perhaps to some extent from cellulose. Some association of bran lignin and these cell wall polymers appears possible. The predominance of glucose

TABLE I
Neutral Sugars and Uronic Acids Associated with Bran Lignin Isolates[a] (percent of dry weight)

Sample[b]	n	Arabinose	Xylose	Mannose	Glucose	Galactose	Total Sugars	Glucuronic Acid
Bran DEL	2	2.0 a	8.3 a	t[c] b	7.6 a	t a	17.9 a	0.1
Bran HDEL	2	0.9 b	3.1 b	t b	4.8 b	t a	8.8 b	...[d]
Pericarp DEL	2	0.4 bc	0.1 c	nd[e] b	1.7 c	t a	2.2 c	nd
Alkali lignin	2	t c	t c	0.2 a	0.5 c	0.2 a	0.9 c	t
Bran	1	19.3	20.8	t	3.2	t	43.3	...

[a]Means within the same column exhibiting the identical letter are not significantly different ($P = 0.95$). Statistical differences were determined using Duncan's multiple range test. Trace quantities were considered as zero for statistical analysis.
[b]DEL = Dioxane-extractable lignin; HDEL = DEL extracted in a Soxhlet apparatus.
[c]Trace.
[d]Not determined.
[e]Not detected.

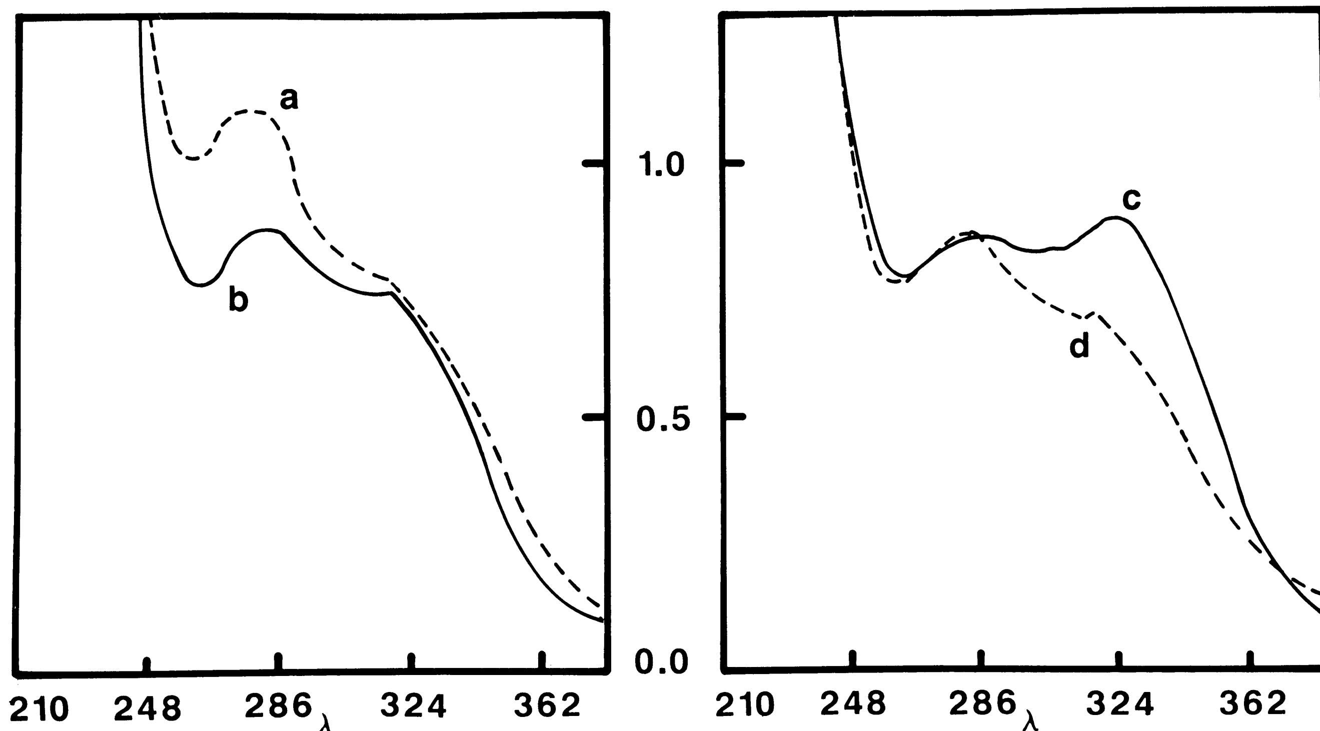

Fig. 2. Ultraviolet absorbance spectra of bran lignin preparations (in Methyl Cellosolve). Outer pericarp dioxane-extractable lignin (DEL) (**a**), bran alkali lignin (**b**), bran DEL (**c**), alkali-extracted bran DEL (**d**).

in the hydrolysates, however, does to an extent indicate that glucose was more stable than either arabinose or xylose under the conditions of hydrolysis in 4*N* trifluoroacetic acid (Nesser and Schweizer 1984).

At this point in the study, characterization of the bran HDEL isolate was discontinued, as it contained a higher level of associated nitrogen and carbohydrate than did bran DEL. Only limited characterization of the pericarp DEL and bran AL isolates was possible because of their low yields.

Associated Phenolic Acids and Nitrobenzene Oxidation

The UV spectra of the bran DEL, pericarp DEL, and bran AL isolates are shown in Figure 2. All preparations exhibit the basic UV spectrum typical of lignins, with a maximum near 205 nm, a shallow minimum near 260 nm, and a second maximum near 280 nm. The bran DEL also exhibits a third maximum at 330 nm. This maximum is undoubtedly associated with esterified ferulic or *p*-coumaric acid, because it disappears following alkaline (1*N* NaOH) treatment. Higuichi and co-workers (1967) clearly demonstrated this phenomenon in other samples of grass lignins. As expected, the bran AL isolate did not exhibit the maximum at 330 nm. Outer pericarp DEL exhibited only a small shoulder near 330 nm.

The alkali-labile hydroxycinnamic esters present in the bran DEL isolate were hydrolyzed by three successive NaOH treatments and then quantitated by HPLC (Fig. 3). Only *trans-p*-coumaric and *trans*-ferulic acid were detected, although the *cis*-isomers are reported in other grass cell wall samples (Hartley and Buchan 1979). Good separation of *trans-p*-coumaric and *trans*-ferulic acid was achieved. However, in the initial alkaline treatment, a portion of unidentified material was observed to elute from the system during the first 5 min (Fig. 3). Identification of this material was not attempted. However, amide conjugates with hydroxycinnamic acids are reported to be widespread in cereals (Collins 1986), and as such, represent an area for further investigation. After the initial NaOH treatment, the bran DEL lost its characteristic dull orange coloration and became light brown to tan in appearance. The results of the three treatments are given in Table II. *trans*-Ferulic acid was the predominant acid associated with the bran DEL isolate, constituting approximately 2.6% of the sample weight. *trans-p*-Coumaric acid accounted for less than 1% of the sample weight. In contrast to these results are those obtained for wheat straw milled wood lignin by Scalbert and co-workers (1985). They reported approximately 10 times more alkali-labile *p*-coumaric acid than ferulic acid.

In addition to the *p*-coumaric and ferulic esters, other material may have been solubilized and lost upon alkaline treatment. Approximately 50% of the sample weight was lost following the three successive NaOH treatments and as such the bran DEL isolate may represent more than a simple lignin-carbohydrate complex.

The bran DEL remaining after the three alkaline treatments should have been largely free of esterified hydroxycinnamic acids. The material remaining following the similar treatment of a corn preparation was referred to as the lignin core (Kuc and Nelson 1964). Alkaline nitrobenzene oxidation should thus provide information only on the composition of the bran DEL core.

The phenolic acids and aldehydes resulting from oxidation of the bran DEL core are shown in Figure 4. The compounds resulting from nitrobenzene oxidation were identified by comparison with the retention times of standards. The retention times of compounds observed in the oxidation mixture, with the exception of the peak at 6.16 min, showed a close correspondence with those of the standards. The peak eluting at 6.16 min was tentatively identified as *p*-hydroxybenzoic acid.

The major products of alkaline nitrobenzene oxidation were vanillin, syringaldehyde, *p*-hydroxybenzaldehyde, and, tentatively, *p*-hydroxybenzoic acid (Table III). Yield of the first three products from nitrobenzene oxidation has been considered a diagnostic test for lignin (Sarkanen and Ludwig 1971), and

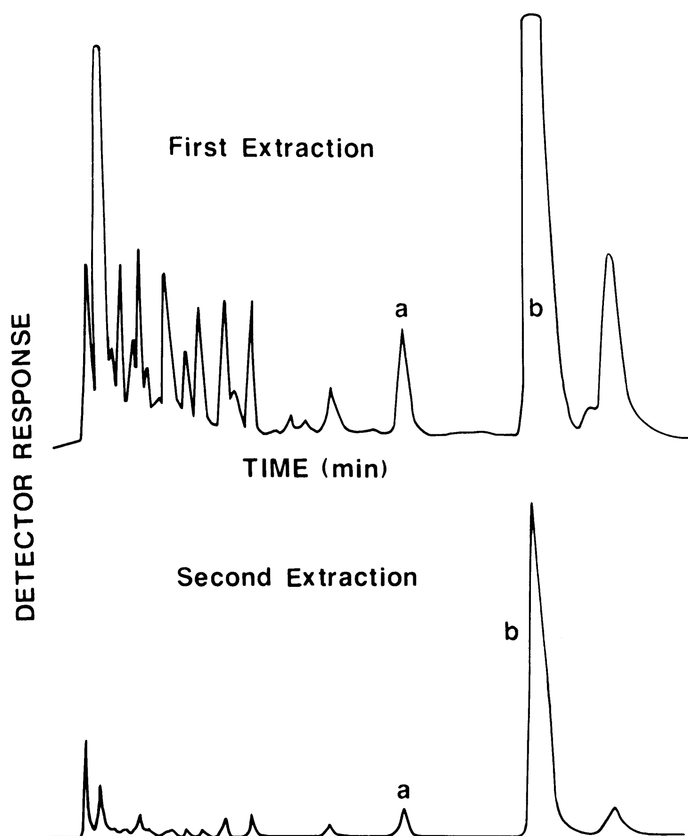

Fig. 3. Separation of *trans-p*-coumaric acid (**a**) and *trans*-ferulic acid (**b**) by high-performance liquid chromatography after successive extraction of bran dioxane-extractable lignin with 1.0*N* NaOH.

TABLE II
***trans*-Ferulic and *trans-p*-Coumaric Acid Content of Bran Dioxane-Extractable Lignin**

Treatment	Amount (μg/mg lignin)[a]	
	trans-Ferulic Acid	*trans-p*-Coumaric Acid
Alkaline hydrolysis		
1st	22.64 ± 0.04	0.71 ± 0.01
2nd	3.11 ± 0.58	0.11 ± 0.03
3rd	0.68 ± 0.02	t[b]
Total	26.44 ± 0.64	0.81 ± 0.04

[a]Mean and range of two determinations.
[b]Trace.

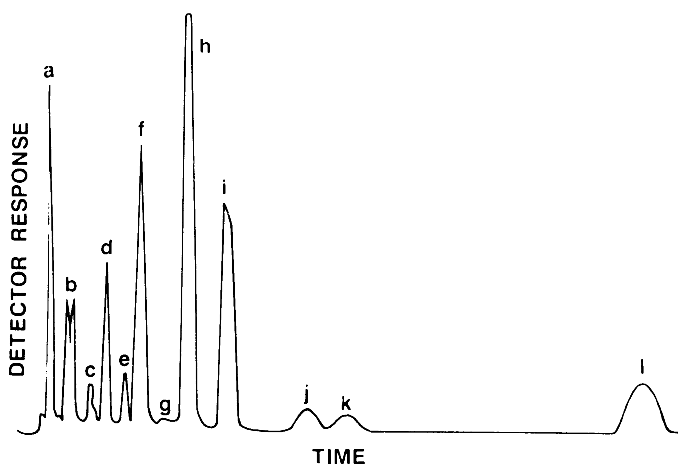

Fig. 4. Separation of phenolic acids and aldehydes by high-performance liquid chromatography after the oxidation of alkali-extracted bran dioxane-extractable lignin in alkaline nitrobenzene. Peaks **a, b, c, g, k,** and **l** are unidentified; (**d**) *p*-hydroxybenzoic acid (6.16 min); (**e**) vanillic acid (7.57 min); (**f**) *p*-hydroxybenzaldehyde (8.81 min); (**h**) vanillin (10.45 min); (**i**) syringaldehyde (12.01 min); and (**j**) *trans*-ferulic acid (20.57 min).

TABLE III
Nitrobenzene Oxidation Products of Alkali-Treated Bran Dioxane-Extractable Lignin

Compound	Amount (μg/mg lignin)[a]
p-Hydroxybenzoic acid	21.45 ± 1.09
Vanillic acid	4.46 ± 0.85
p-Hydroxybenzaldehyde	7.29 ± 0.17
Vanillin	26.87 ± 1.28
Syringaldehyde[b]	30.93 ± 1.29
trans-Ferulic acid	2.77 ± 0.04

[a] Mean and range of two determinations.
[b] *trans-p*-Coumaric acid was observed to coelute with syringaldehyde in standard mixtures.

as such this bran isolate appeared to contain lignin. However, the spectrum of products provided only limited information on the nature of the lignin.

The presence of *p*-hydroxybenzaldehyde was at one time thought to be characteristic of grass lignins and indicative of *p*-hydroxyphenyl units within the lignin core. However, involvement of hydroxycinnamic acids in linkages other than the common carbon-carbon and alkyl-aryl ether linkages present in the core has been suggested (Erickson et al 1973, and Scalbert et al 1985). Scalbert and co-workers (1985) suggested the presence of hydroxycinnamic acids linked as ethers through their phenolic hydroxyl groups to lignin. The yield of *p*-hydroxybenzaldehyde then may also reflect the involvement of *p*-coumaric acid in linkages other than those commonly associated with the lignin core or alkali labile esters. Coumaric acid was not detected in the oxidation mixture, but at low concentrations it was observed to coelute with syringaldehyde in standard mixtures. In other grass samples, the yield of coumaric acid relative to syringaldehyde is small (Hartley 1971), and thus syringaldehyde probably reflects largely syringyl units (3,5-dimethyoxy-4-hydroxyphenyl) within the core. The presence of vanillin is generally considered indicative of guaicyl units (3-methoxy-4-hydroxyphenyl) within the core, although a portion of this could arise from ferulic acid bound to the core by linkages other than alkali labile esters.

Molecular Weight Distribution

In the final portion of this study, the approximate molecular weight distributions of bran DEL and AL were determined by gel permeation chromatography on Sephadex LH-60 and Sepharose CL-6B columns. These distributions should be considered as approximations, because the isolates did contain associated nitrogen and carbohydrate. The use of polystyrene molecular weight markers can be questioned due to potential differences in solution behavior exhibited by the lignin molecules and the synthetic polymer, but the presence of protein and polysaccharide would be expected to introduce greater uncertainties. The asymmetric nature of associated polysaccharide and potential denaturation of the protein in the organic solvent might inflate the results of molecular weight determinations.

The apparent exclusion limit for the Sephadex LH-60 gel was between 23,000 and 35,000. Bran AL eluted as a sharp peak within this range, whereas the bran DEL isolate exhibited a polydisperse distribution centered around 4,000. The AL preparation thus appears to be of a much higher average molecular weight.

In order to better define the distribution of the bran AL sample, chromatography was performed on a Sepharose CL-6B column. The bran AL sample again exhibited a sharp peak near the exclusion limit (175,000–575,000), and thus was not completely fractionated on this column. However, a second broad distribution was observed to range from 2,000 to near 175,000. The bran DEL isolate again appeared to be of a very low average molecular weight. This sample exhibited a broad distribution ranging from 23,000 to below 2,000. Several sharp peaks below 2,000 were observed in this sample.

The low apparent molecular weight of the bran DEL isolate was a serious concern. As previously stated, the molecular weight distributions determined in this study are perhaps inflated. However, if concerns regarding the presence of protein and carbohydrate were ignored, this material, on average, would represent the polymerization of less than 25 phenylpropane units. This assumes the distribution to be centered at 4,000.

CONCLUSIONS

DEL preparations were isolated from bran and outer pericarp tissue, and AL was isolated from bran. The bran DEL preparation contained a considerable portion of carbohydrate and is potentially associated with both a glucose-containing cell wall polymer and an arabinoxylan. Bran AL and pericarp DEL both contained low levels of carbohydrate. All bran and pericarp lignin preparations contained associated nitrogen, with bran AL exhibiting the highest level. The low level of carbohydrate and nitrogen associated with the pericarp DEL preparation may reflect the incomplete lignification of cells within the outer pericarp.

The UV spectrum of bran DEL indicated the presence of esterified hydroxycinnamic acids. These esters were released upon alkaline treatment and were found to consist primarily of *trans*-ferulic acid with lesser amounts of *trans-p*-coumaric acid. An additional component of the bran DEL sample was solubilized upon alkaline treatment. This material has not been identified, and as such bran DEL may represent more than a simple lignin-carbohydrate complex. However, the presence of lignin in the bran DEL sample appears to have been established through alkaline nitrobenzene oxidation. Yields of vanillin and syringaldehyde, as well as *p*-hydroxybenzaldehyde suggest that the lignin of bran is of the guaicyl-syringyl type, as are the lignins of other grasses (Sarkanen and Hergert 1971).

Of the four lignin isolates evaluated, bran AL is believed to offer the greatest potential for further investigation. This relates to the low level of associated carbohydrate and to its high apparent molecular weight. The bran DEL appeared to be on average of a very low molecular weight, which may represent less than 25 phenylpropane units. Bran AL should not have been extensively modified beyond the saponification of associated ferulic and *p*-coumaric esters.

However, a problem inherent to all of the lignin preparations was the low yield obtained. This is a serious concern because at most the preparations represented slightly less than 5% of the total bran lignin. Therefore, they may not be entirely representative of the total tissue lignin. In addition, the extent to which the isolates can be characterized is limited by the low yields.

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