Effect of Isolation Procedure on Molecular Weight Distribution of Wheat Straw Lignins

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Neutral detergent fiber (NDF) of wheat straw (WS) was ball-milled for 7, 14, 21, and 28 days in a porcelain rotary ball-mill and hydrolyzed by a cellulase for 4 days, and the residue was used for lignin extraction by either dioxane or 1 M NaOH. The effects of ball-milling duration (BMD) and extraction procedure on lignin yield and its high-performance size exclusion chromatography (HPSEC) features were examined in this study. Optimal BMD was 14 days, at which 80% and 100% of the permanganate lignin has been extracted by the dioxane and alkali systems, respectively. At a BMD of 14 days, carbohydrate content was 6% and 15% of the alkali lignin (AL) and dioxane lignin (DL), respectively. By increasing the BMD, there was an increase in the proportion of the high molecular weight fraction residing in the 23-130-kDa range of the ALs, reaching 26% of the total AL at a BMD of 28 days. At 100% yield of the permanganate lignin, 75% of the lignin molecules resided in the range 1.6–23 kDa, the entire lignin having a weight-average molecular weight ($\overline{MW}$) of 17.6 kDa and a dispersivity of 2.29. Ball-milling in a porcelain rotary BM for prolonged periods did not cause a subdivision of the lignin molecules.

INTRODUCTION

The lack of appropriate equipment for exploring the organization of cell wall (CW) matrix constituents in situ and the inability to completely isolate intact native lignin are the main reasons for the many unknown aspects related to CW ultrastructure. The forage-oriented, matrix CW giant macromolecule theory has been proposed by Morrison (1974). However, there is too little information for concluding whether this theory applies to the entire CW matrix mass. By using a 90% (v/v) dioxane–water mixture, Kondo et al. (1992) extracted from wheat straw a small fraction (3%) of the total Klason lignin. The isolate was polydisperse with an "weight average molecular weight" ($\overline{MW}$) of 2200. Since dioxane is not thought to cleave covalent bonds, it is assumed that at least a small portion of the lignin molecules were not a part of the proposed "giant matrix molecule". However, what about the remaining 97% of the lignin mass; how is it organized within the CW? Generally, there is very little information on the properties of lignin and its organization in situ. For exploring the features of lignin in a state closest to that in situ, maximum lignin should be extracted by using the mildest existing procedures (Obst and Kirk, 1988). The principles recommended to comply with the above mentioned are as follows: (i) a mild ball-milling (BM) of the CW for an extended period of time to difibrilize the CW, exerting minimal effect on lignin molecular sizes (Polcin and Bezuch, 1978); followed by (ii) an exhaustive cellulase hydrolysis (Obst and Kirk, 1988) for peeling off the lignin from most of its neighboring carbohydrate moiety; and (iii) to maximize the yield of lignin from wheat straw, residue extraction consecutively with 96% v/v and 50% v/v dioxane–water (Jung and Himmelsbach, 1989). Both fractions are associated with some carbohydrate.

The rotary porcelain BM is considered among the mildest BM methods, since during the milling process there is very little heating (Polcin and Bezuch, 1978). Increasing ball-milling duration (BMD) gives rise to higher yields of extracted lignin (Polcin and Bezuch, 1978; Jung and Himmelsbach, 1989). However, BMD might affect the molecular weight profile of the isolated lignins (Faix et al., 1981), and there is no information on the effect of rotary porcelain BMD on molecular weight distribution of wheat straw lignins.

The objective of this study was to explore the effect of BMD and extraction procedure on the molecular weight distribution pattern of wheat straw lignins.

MATERIALS AND METHODS

Lignin Isolation. Wheat straw (WS) ground to 1 mm was treated with neutral detergent according to the method of Goering and Van-Soest (1970). The neutral detergent fiber (NDF) was subjected to various periods of continuous ball-milling consisting of 7, 14, 21, and 28 days in a rotary porcelain ball-mill at 60 rpm, using 20-mm porcelain balls at a balls/NDF weight ratio of 50. The ball-milled NDF samples were subjected to an exhaustive cellase hydrolysis (Celluclast 200L Type N, Novo, DK) in citrate buffer pH 4.8 at 50 °C for 96 h. Lignin was isolated by using two extraction procedures:

(I) The dioxane–water system, consisted of two consecutive 24-h extractions with a 95:5 mixture, followed by two consecutive 24-h extractions with a 50:50 mixture. The extractions were performed using 1 g of residue to 15 mL of solvent, at room temperature, in darkness and under N_2. The four extractions were combined into one composited extraction per sample, and the dioxane was removed by a rotary vacuum evaporator. The dry extract was dissolved in 90% acetic acid in the proportion of 1 g of lignin extract to 20 mL of acid. The lignin solution in acetic acid was added dropwise with stirring to water at a ratio of 1 mL of extract to 10 mL of water. The lignin precipitated in water was centrifuged, washed with distilled water, recentrifuged, and freeze-dried. The dioxane lignin isolate will be denoted DL.

(II) NaOH (1 M) was used for the second procedure of lignin isolation. Cellulase-pretreated NDF residues ball-milled for 7–28 days were subjected to alkali extraction consisting of two consecutive 24-h periods at the residue:extractant ratio of 1 g to 15 mL. The alkali extraction was neutralized with HCl, and the precipitated lignin was centrifuged, washed with distilled water, recentrifuged, and freeze-dried. The alkali lignin will be denoted AL.

Since both the DLs and ALs contained some carbohydrate, the isolated lignins have been incubated again under similar conditions with NOVO cellulase to find out whether further

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Table 1. Effect of Ball-Milling Duration (BMD) and Method of Extraction on the Yield of Lignin (g/100 g of Permanganate Lignin) Extracted from Wheat Straw[a]

BMD (days)	extraction method	
	dioxane–water	1 M NaOH
7	50.8	86.3
14	79.2	100
21	82.4	95.0
28	86.8	87.3

[a] Containing neutral detergent fiber and permanganate lignin 80.1 and 9.66 g/100 g of dry matter, respectively.

purification of the lignin can be achieved. Lignin content of WS was determined according to the gravimetric method of Goering and Van Soest (1970).

High-Performance Size Exclusion Chromatography (HPSEC). Prior to the HPSEC analysis, both DLs and ALs underwent a complexation procedure with *N*-methyltridecylammonium chloride (N-MTAC) (Milstein et al., 1990). Lignin samples were dissolved in 3 mL of 1 M NaOH and 2 mL of a 50 mM N-MTAC solution in ethyl acetate. The tube was closed with a Teflon-sealed screw cap, shaken for 10 min, and centrifuged for 10 min at 2000*g*, to ensure completion of phases separation. One milliliter of the organic phase was transferred to a new tube and washed twice with an equal volume of 1% NaCl solution.

Aliquotes (0.4 mL) of the ethyl acetate phase containing the N-MTAC lignin complexes were transferred to sample vials and evaporated overnight under vacuum at room temperature. The samples were dissolved in 1.10 mL of tetrahydrofuran, and 40 μL of the solution was injected into the HPLC column for determination of the molecular weight distribution. The HPSEC system used for the determination of MW distribution of the alkali and dioxane lignins was developed by Majcheczyk et al. (manuscript in preparation) and was described by Milstein et al. (1990). The column system consisted of two Zorbax PSM 60S columns and one Zorbax PSM 1000 S column (DuPont, Wilmington, DE) packed with silica and coupled in order of the increasing pore size. The chromatography was run in an isocratic mode, the mobile phase being a 0.02 M solution of *N*-methyltridecylammonium chloride in freshly distilled tetrahydrofuran, at a flow rate of 1.2 mL/min. The HPLC system consisted of HP 1090 with diode array detector and Pascal Work Station (Hewlett-Packard, Palo Alto, CA).

The phenolic component of the ALs and DLs was used as an indicator for detecting the elution of the lignin at 280 nm. The number-average molecular weight ($\overline{MN}$), the weight-average molecular weight ($\overline{MW}$), and the dispersivity ($\overline{MW}/\overline{MN}$) were calculated by the data processing system from area/time and MW of each segment, as described by Faix et al. (1981), by using polystyrene standards in the range of 800–2 500 000 Da (Waters) for calibration.

Monosaccharide composition of the DL and AL isolates was determined after hydrolysis with 12 M H_2SO_4 for 1 h at 21 °C followed by 0.5 M H_2SO_4 for 5 h at 100 °C, as described by Miron and Ben-Ghedalia (1992). The free sugars were converted to alditol acetates and determined by gas–liquid chromatography (Blakeney et al., 1983). Uronic acids in the hydrolysates were determined colorimetrically (Blumenkrantz and Asboe-Hansen, 1973).

RESULTS AND DISCUSSION

For lignin characterization purposes, the ideal procedure would be the one which is able to isolate the entire, pure intact lignin. Such a method is not available yet, and the existing ones strive for the best compromise. The effect of ball-milling duration (BMD) on yield of lignin extracted by dioxane and alkali is shown in Table 1. Lignin has been entirely isolated (100% of the permanganate lignin) by 1 M NaOH from NDF of WS after 14 days of BM. Its yield decreased following an extension of that period. The precise reason for this phenomenon is not clear; it is known, however, that extended periods of BM might cause a loss in the organic matter of the lignocellulose (Jung and Himmelsbach, 1989). Table 1 shows that ~80% of the WS permanganate lignin can be isolated by the dioxane system after 14 d of BM. Increasing BM duration over 14 days yielded very little additional DL. For comparison,

Table 2. Effect of Ball-Milling Duration (BMD) and Additional Cellulase Application on Carbohydrate Content (g/100 g of Lignin) and Profiles (g/100 g of Monosaccharides) of Dioxane Lignins Isolated from Wheat Straw

carbohydrate	BMD (days)							
	7		14		21		28	
	NAC[a]	AC[a]	NAC	AC	NAC	AC	NAC	AC
total monosaccharide content	16.4	14.8	15.2	15.5	15.3	15.8	14.2	14.3
profile								
xylose	63.5	67.2	64.8	67.1	65.1	67.0	62.6	62.4
arabinose	11.6	11.1	11.3	11.7	11.4	10.7	11.2	11.4
galactose	2.57	2.18	1.99	2.13	2.24	2.34	1.48	1.91
mannose	1.10	1.02	1.19	0.77	1.25	0.76	0.92	0.82
uronic acids	7.53	8.53	8.92	9.82	9.71	10.0	10.1	9.47
glucose	13.7	9.97	11.8	8.38	10.4	9.20	13.7	14.0

[a] NAC, no additional cellulase; AC, additional cellulase applied.

Table 3. Effect of Ball-Milling Duration (BMD) and Additional Cellulase Application on Carbohydrate Content (g/100 g of Lignin) and Profiles (g/100 g of Monosaccharides) of NaOH Lignins Isolated from Wheat Straw

carbohydrate	BMD (days)							
	7		14		21		28	
	NAC[a]	AC[a]	NAC	AC	NAC	AC	NAC	AC
total monosaccharide content	13.1	6.53	6.27	5.15	5.03	4.82	3.85	3.78
profile								
xylose	69.4	62.4	38.2	32.9	39.7	37.0	32.4	35.1
arabinose	8.18	7.50	9.20	9.30	10.3	8.51	9.61	7.40
galactose	3.38	6.13	6.20	7.90	6.00	5.19	6.49	7.10
mannose	0.11	0.09	0.10	0.09	0.10	0.09	0.10	0.08
uronic acids	10.5	9.67	15.5	15.0	14.1	12.9	17.4	14.6
glucose	8.44	14.2	30.8	34.8	29.8	36.3	34.0	35.7

[a] NAC, no additional cellulase; AC, additional cellulase applied.

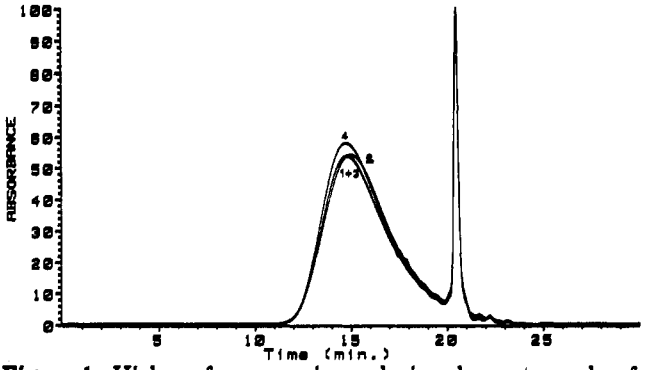

Figure 1. High-performance size exclusion chromatographs of dioxane lignins isolated from wheat straw NDF ball-milled for 7, 14, 21, and 28 days (1, 2, 3, and 4, respectively) and treated with cellulase.

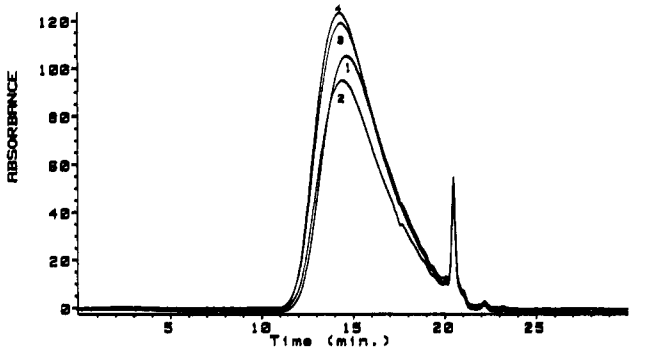

Figure 2. High-performance size exclusion chromatographs of alkali lignins isolated from wheat straw NDF ball-milled for 7, 14, 21, and 28 days (1, 2, 3, and 4, respectively) and treated with cellulase.

by using a different BM system for 8 + 4 days of cellulase hydrolysis, Jung and Himmelsbach (1989) isolated ~70% of the WS lignin with the same dioxane system. However, the carbohydrate content of their lignin isolate was not presented in that paper.

The carbohydrate contents and profiles of the DLs and ALs isolated after 7, 14, 21, and 28 days of BM are shown in Tables 2 and 3, respectively. Since in the CW of monocots, lignin is thought to be extensively bound to carbohydrates through ester links (Scalbert and Monties, 1986; Wallace et al., 1991), ALs are expected to be the lowest in carbohydrate. Indeed, this was the case as shown by comparing the two tables. Application of additional cellulase (AC) for further purification of the lignin isolates, proved successful only for the 7 days BM of the AL. Irrespective of length of BM or AC application, DLs were higher in carbohydrate content (14.2–16.4 g/100 g of DL) than the ALs (4–6 g/100 g of AL). DLs and ALs differed also in their monosaccharide profiles. Xylose was the major component (62.4–67.2%) in DLs with arabinose, uronic acids, and glucose, each in the 10% range, as the secondary monosaccharides. Both xylose and glucose, in the range of 30–40%, were the major sugars in ALs. The high proportion of uronic acid and glucose found in ALs hint to the possible role of these sugars in interlinking the heteroxylan to lignin and cellulose.

The effect of BMD on the distribution of molecular weights in DLs and ALs is shown in Figures 1 and 2 and in Tables 4 and 5. BM is an important factor introduced in the procedure for maximizing lignin yield (Obst and Kirk, 1988). However, BMD has an impact not only on the yield of lignin but also might affect the molecular weight profile of the isolated lignin (Faix et al., 1981). Tables 4 and 5 show for both DLs and ALs that increasing BMD did not cause lignin depolymerization. The DL isolated from WS after 28 days of BM, accounting for 87% of the permanganate lignin, had the same molecular weight profile and $\overline{MW}$ as the DL obtained after 7 days of BM, accounting for only half the permanganate lignin. Thus, the present data support the view of Polcin and Bezuch (1978) with regard to the suitability of the porcelain rotary BM as both a mild and effective CW pretreatment prior to lignin isolation. Alkali proved more effective than dioxane for lignin extraction, yielding 86 and 100% of the permanganate lignin after 7 and 14 days, respectively (Table 1).

The pattern of molecular weight distribution of AL at BMD of 7 days was very similar to those of the DLs. By increasing BMD, there was an increase in the proportion of the high molecular weight fraction residing in the 23–130-kDa range of the ALs. This fraction reached 26% of total AL at BMD of 28 days as compared to 16% in the DLs. It is clear from these data and from the dispersivity figures presented in Tables 4 and 5 that the increase in yield (Table 1) was associated with a more extensive extraction of high molecular weight lignins (Table 5). This particular finding is in accord with the results of Bland and Menshun (1967) who showed an increase in the $\overline{MW}$ of the isolated lignins with increasing BMD, but does not coincide with the data of Faix et al. (1981) showing an opposite trend.

Table 4. Effect of Ball-Milling Duration (BMD) on Molecular Weight Distribution (% of Total Peak Area), Weight-Average Molecular Weight ($\overline{MW}$), Number-Average Molecular Weight ($\overline{MN}$), and Dispersivity ($\overline{MW}/\overline{MN}$) of Dioxane Lignins Isolated from Wheat Straw

BMD (days)	molecular weight ranges, Da × 10^3				$\overline{MW}$	$\overline{MN}$	dispersivity ($\overline{MW}/\overline{MN}$)
	0.8–1.6	1.6–10	10–23	23–130			
7	3.80 ± 0.41	36.0 ± 1.40	45.0 ± 0.70	15.2 ± 1.42	15072 ± 761	6961 ± 361	2.16 ± 0.05
14	3.60 ± 0.41	35.0 ± 1.74	45.2 ± 0.33	16.2 ± 1.89	15377 ± 847	7121 ± 384	2.16 ± 0.02
21	3.70 ± 0.39	35.0 ± 1.71	45.2 ± 0.45	16.1 ± 1.68	15251 ± 796	7085 ± 366	2.16 ± 0.01
28	3.80 ± 0.42	35.2 ± 2.21	45.1 ± 0.12	15.9 ± 2.30	15211 ± 1068	7038 ± 408	2.16 ± 0.04

Table 5. Effect of Ball-Milling Duration (BMD) on Molecular Weight Distribution (% of Total Peak Area), Weight-Average Molecular Weight ($\overline{MW}$), Number-Average Molecular Weight ($\overline{MN}$), and Dispersivity ($\overline{MW}/\overline{MN}$) of NaOH Lignins Isolated from Wheat Straw

BMD (days)	molecular weight ranges, Da × 10^3				$\overline{MW}$	$\overline{MN}$	dispersivity ($\overline{MW}/\overline{MN}$)
	0.8–1.6	1.6–10	10–23	23–130			
7	3.10 ± 0.25	34.5 ± 0.98	42.9 ± 0.62	19.5 ± 0.96	16459 ± 638	7381 ± 244	2.40 ± 0.03
14	3.00 ± 0.42	32.9 ± 3.16	42.6 ± 0.43	21.50 ± 3.62	17618 ± 1767	7671 ± 586	2.29 ± 0.06
21	2.80 ± 0.22	30.7 ± 1.50	41.7 ± 0.51	24.8 ± 1.77	19299 ± 1473	8039 ± 355	2.40 ± 0.08
28	2.70 ± 0.26	29.9 ± 1.64	41.5 ± 0.73	25.9 ± 2.16	19689 ± 513	8229 ± 428	2.39 ± 0.07

The origin of the residual 20% of lignin found in AL and absent from DL at BMD of 14 days is most interesting. Present study suggests this fraction is made mostly of large molecules. Lignin resides as large domains in the middle lamella and primary cell wall and in smaller domains embedded in and interlinking with the matrix carbohydrates as a part of the secondary layers of the CW. The middle lamella lignin is highly condensed and resistant to oxidation and to biodegradation (Mulder et al., 1992). The secondary layers of the CW are generally more extensively degraded in the rumen than the primary layer of lignified CW (Chesson et al., 1986; Engels, 1989). If an analogy is drawn between the CW biodegradational systems of the rumen and the AL isolation procedure which basically both apply a combination of mechanic comminution followed by hydrolysis, then it could be assumed that the residual extra 20% of the lignin extracted by alkali at BMD of 14 days originated in the middle lamella. Notwithstanding, Lai and Sarkanen (1971) and Faix et al. (1981) suggested that the lignin released at the early stages of milling originated from the large domains of lignin found in the middle lamella.

CONCLUSIONS

1. Ball-milling in a porcelain rotary BM for prolonged periods did not cause a subdivision of the lignin molecules. Optimal BMD for WS was 14 days, after which time the yield of lignin tended to decline.

2. NaOH (1 M) was found to be an effective extractant for WS lignins. At 100% yield of the permanganate lignin, 75% of the lignin molecules resided in the range of 1.6–23 kDa, the entire lignin having a $\overline{MW}$ of 17.6 kDa and a dispersivity of 2.29.

3. Since the isolation procedures applied in this study were based on peeling off the carbohydrates surrounding and linked to the lignin, with no apparent effect on its molecular sizes, it is speculated therefore that the data presented in Table 5 represents the molecular weight distribution of WS lignins in situ.

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