Characterization of Some Cell Wall Components of Untreated and Ozone-Treated Cotton Stalks

Edith Yosef,† Daniel Ben-Ghedalia,*,† Joshua Miron,† Aloys Huttermann,‡ Andrzej Majcherczyk,‡ Oleg Milstein,‡ Hans Dietrich Ludemann,§ and Rudiger Frund§

Metabolic Unit, Volcani Center, ARO, Bet-Dagan 50250, Israel, Forstbotanisches Institut, Universität Göttingen, D-3400 Busgenweg 2, Germany, and Institut für Biophysik und Physikalische Biochemie, Universität Regensburg, 8400 Regensburg, Germany

Untreated (CS) and ozone-treated (OCS) cotton stalks were studied to determine the effects of ozonation on the content of water-soluble phenolics, on the chemical features of cell wall (CW) matrix complexes (MC), and on in situ structural and chemical properties as reflected in solid-state cross-polarization/magic angle spinning (CP/MAS) ^{13}C NMR spectroscopy. The concentrations of water-soluble total monomeric phenolics were 20.1 and 48.2 mg/100 g of dry matter in the CS and OCS materials, respectively. The major increase was in protocatechuic acid (PA), whose concentration increased nearly 10-fold. Matrix complexes extracted by N NaOH from neutral detergent fiber (NDF) of CS and OCS pretreated by ball-milling plus *Trichoderma reesei* cellulase were analyzed by high-performance size exclusion chromatography (HPSEC) to assess the molecular weight distribution. Two-thirds of the MC of CS were in the molecular weight range 800–10 000 with a weight-average molecular weight (M_w) of 8500. The MC isolated from OCS showed a more dispersed pattern, with a higher proportion of molecules in the range higher than 10 000 and a (M_w) of 15 000. The MC isolates from CS and OCS contained 54% and 39% carbohydrates, respectively, with xylose as the major component and uronic acid as the major potentially branching unit. Solid-state CP/MAS ^{13}C NMR spectroscopy of NDF preparations of CS and OCS showed that the 89 and 65 ppm peaks (the "crystallinity peaks") were affected by ozonation. The intensity of the signals found in the 160–110 ppm chemical region, assigned to aromatic carbons, declined. The quantitative data on carbon distribution in the various chemical entities of the NDF samples analyzed by solid-state CP/MAS ^{13}C NMR showed a decline in the proportion of aromatic carbons from 13% in CS to 7.4% in OCS. This is the first evidence to show that CS lignin degradation by ozone is mediated via ring cleavage.

Ozone is an oxidizing agent that can upgrade the nutritional value of dicotyledonous lignocelluloses (Ben-Ghedalia et al., 1980; Solomon et al., 1992). Therefore, interest has focused mainly on the structural carbohydrates which serve as energy-yielding substrates for both the rumen microflora and the host animal. The effects of the ozone treatment on the structural carbohydrates of cotton stalk cell walls have been studied at the monomer level using solubilization and in vitro studies (Miron and Ben-Ghedalia, 1981; Shefet and Ben-Ghedalia, 1982) and in vivo experiments with sheep (Ben-Ghedalia and Shefet, 1983; Solomon et al., 1992). However, very little attention has been given to the phenolic components of the cell walls.

Treating cotton stalks with ozone oxidizes up to 50% of the lignin, mainly to formic and acetic acids and some CO_2 (Ben-Ghedalia et al., 1982). However, the E_{280} of the water extract of ozonated cotton stalks also is increased, indicating that phenolic materials were released (Ben-Ghedalia et al., 1980). The nature and concentrations of these compounds should be investigated, because free phenolics may interfere with the attachment of rumen fibrolytic bacteria (Varel and Jung, 1986).

Matrix components frequently are related to biodegradation barriers (Shefet and Ben-Ghedalia, 1982; Jung and Ralph, 1990). Therefore, this study was aimed at increasing our knowledge regarding their characteristics.

The objectives of this study were to (i) assess the effect of the ozone treatment on the release of monomeric phenolic compounds from cell walls (CW) of cotton stalks, (ii) determine the molecular weight distribution and sugar composition of matrix macromolecules in untreated (CS) and ozone-treated cotton stalks (OCS), and (iii) study the effect of ozonation on NMR spectroscopy features of the cell walls of CS.

MATERIALS AND METHODS

Cotton Stalks and the Ozone Treatment. Cotton stalks chopped to pass through a 6-mm screen were moistened to 50%, placed in a 80 × 8 cm perspex column, and flushed with a stream of ozone gas generated from air by a Fischer ozonator (Bon-Bad Godesberg, Germany) until completely bleached, as described by Ben-Ghedalia et al. (1982). Untreated and ozonated cotton stalks were freeze-dried, ground to 1 mm, and used for water extraction of phenolic compounds and for the preparation of neutral detergent fiber (NDF) fractions for further analysis. The preparation of NDF and the determination of lignin permanganate followed procedures of Goering and Van Soest (1970).

Determination of Water-Soluble Free Phenolic Compounds. Ground (1 mm) CS and OCS were added to distilled water (5 g/100 mL), boiled for 10 min, and filtered through Whatman No. 41 filter paper. The filtrate was acidified with 1 N H_3PO_4 to pH 2 to allow the precipitation of polymeric aromatic material by centrifugation. Ethyl acetate was added to the supernatant fluid to extract the free aromatic compounds. A portion of the ethyl acetate phase was transferred to a silylation tube, evaporated to dryness, and silylated with a 1:1 mixture of pyridine/*N*-methyl-*N*-(trimethyl-silyl)trifluoroacetamide (MSTFA). Samples were analyzed by a HP GC–MS system with peaks identified by matching the spectra with those of a mass spectral data bank, including a library of standard mass spectra of over 40 000 compounds stored in the computer system. A capillary

* Author to whom correspondence should be addressed.
† Volcani Center.
‡ Universität Göttingen.
§ Universität Regensburg.

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Table 1. Concentration of Water-Soluble Phenolic Compounds in Untreated (CS) and Ozone-Treated (OCS) Cotton Stalks (Milligrams per 100 g of Stalks DM)

lignocellulose sample	phenolic compound							
	PHBAL[a]	VAN[a]	PHBA[a]	VA[a]	PA[a]	PCA[a]	FA[a]	total
CS	0.08	4.36 ± 0.03	2.31 ± 0.11	3.35 ± 0.13	3.96 ± 0.15	0.39	5.60 ± 0.99	20.1
OCS	traces	4.11 ± 0.59	traces	3.75 ± 0.45	35.2 ± 0.41	0.21	4.97 ± 0.48	48.2

[a] PHBAL, *p*-hydroxybenzaldehyde; VAN, vanillin; PHBA, *p*-hydroxybenzoic acid; VA, vanillic acid; PA, protocatechuic acid; PCA, *p*-coumaric acid; FA, ferulic acid.

column HP-Ultra-2, with a 0.33-μm film thickness, was used for the separation of phenolic compounds.

Isolation of Matrix Complexes (MC). The 1 mm NDF preparation was mildly ball-milled for 72 h in a rotary porcelain ball mill with 20-mm porcelain balls at a weight ratio of balls/NDF of 50. The objective of this step was to defibrilize the cellulose component of NDF while minimally affecting the matrix components. The ball-milled NDF samples underwent a *Trichoderma reesei* cellulase (Novo Alle, Bagsvaerd) hydrolysis in citrate buffer, pH 4.8, at 50 °C for 96 h. This cellulase source was chosen for having a strong cellulolytic but weak xylanolytic activity (Poutanen et al., 1987), with the objective of obtaining a high-matrix NDF residue. This residue was washed, freeze-dried, and used for the extraction of matrix complexes by shaking 1 g of residue with 10 mL of 1 N NaOH for 48 h at room temperature. About 50% of the permanganate lignin present in the NDF was extracted.

High-Performance Size Exclusion Chromatography (HPSEC) of MC. The mixture was centrifuged, and the MC solubilized in the NaOH supernatant were extracted by using a 50 mM solution of *N*-methyltridecylammonium chloride (*N*-MTAC) in ethyl acetate. The tube was closed with a Teflon-sealed cap, shaken for 5 min, and centrifuged to ensure completion of phase separation. One milliliter of the organic phase was transferred to a new tube and washed with an equal volume of 1% NaCl solution. Aliquots (0.4 mL) of the ethyl acetate phase containing the isolated MC were transferred to sample vials and evaporated overnight in vacuum at room temperature. Samples were dissolved in 1.1 mL of tetrahydrofuran, and 40 μL of the solution was injected into the HPSEC system. The procedure for determination of the molecular weight distribution of the MC has been developed by Majcherczyk et al. (unpublished data) and was described preliminarily by Milstein et al. (1990). The column system consisted of two Zorbax PSM 60S columns and one Zorbax PSM 1000S column (DuPont, Wilmington, DE) packed with silanized silica and coupled in order of increasing pore size. The chromatography was run in an isocratic mode, the mobile phase being a 0.02 M solution of *N*-MTAC in freshly distilled tetrahydrofuran, at a flow rate of 1.2 mL/min. The HPLC Waters 840 system (Milford, MA) consisted of a single pump (Model 510), an automatic sample injector (WISP 710B), and a Digital 350 data processing computer. A UV detector (Model 450) was used for monitoring sample elution. The phenolic component of the MC was used as an indicator for detecting the elution of the MC at 280 nm. The number-average molecular weight (M_n), the Weight-average molecular weight (M_w), and the dispersity (M_w/M_n) were calculated by the data processing system from area/time and M_w of each segment, as described by Faix et al. (1981), by using polystyrene standards in the range 800–2 500 000 (Waters) for calibration.

Monosaccharide and NMR Analysis. The monosaccharide composition of the MC isolates was determined after hydrolysis with 24 N H_2SO_4 for 1 h at 21 °C followed by 1 N 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 quantified by gas–liquid chromatography (Blakeney et al., 1983). Uronic acids in the hydrolysates were determined colorimetrically (Blumenkrantz and Asboe-Hansen, 1973). Nuclear magnetic resonance (NMR) spectra and distribution of carbons in NDF chemical groups and components were determined by solid-state cross-polarization/magic angle spinning carbon-13 NMR analysis at 25.1 MHz on a Bruker MSL spectrometer at a spinning rate of 4 kHz. The contact time for the Hartmann–Hahn matching was set to 1 ms and the total recycle time was 5 s. The NMR spectra and assessment of carbon distribution followed procedures of Frund and Ludemann (1989). Figure 1 presents the layout of the analytical procedures.

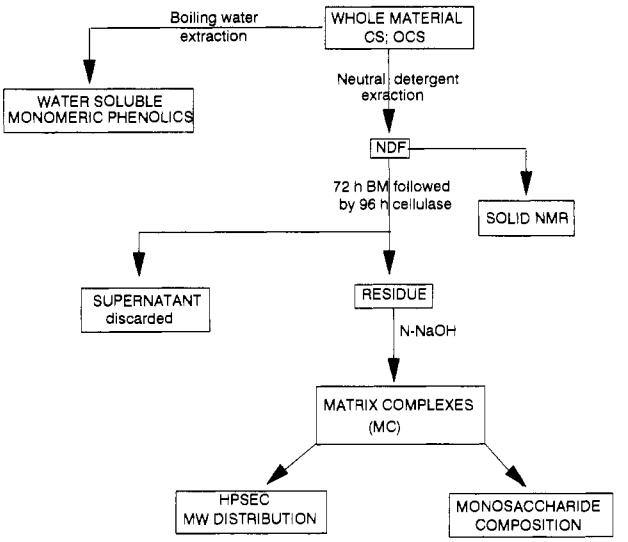

Figure 1. Layout of the analytical procedures.

RESULTS AND DISCUSSION

Water-Soluble Monomeric Phenolics in CS and OCS. Compared to monocots, dicotyledonous forages are very low in alkali-labile phenolics (Jung, 1988). Moreover, the concentrations of phenolic acids in the rumen of sheep fed conventional forages are much lower than the levels expected to exert detrimental effects on rumen fibrolytic microflora (Akin, 1982; Jung et al., 1983). However, cotton stalks may contain up to 22% lignin which, when massively degraded by ozone, yields a mixture of water-soluble products including phenolic materials (Ben-Ghedalia et al., 1980). Therefore, we determined the concentrations of water-soluble monomeric phenolics in untreated and ozonated cotton stalks as shown in Table 1. Concentrations of water-soluble monomeric phenolics were very low, 20.1 and 48.2 mg/100 g of dry matter (DM) in CS and OCS, respectively. The major effect of ozonation was an increase in protocatechuic acid (PA) by 10-fold. The formation of PA (3,4-dihydroxybenzoic acid) as a product of lignin oxidation by ozone can be explained on the basis of studies of ozone oxidation of lignin model compounds (Tanahashi et al., 1975; Gierer, 1986). Cleavage of the aliphatic tail of terminal phenyl propanoid units, most likely via a 1,3-dipolar cycloaddition of ozone to the α and β double-bond carbons, resulted in its cleavage and the formation of the carboxyl group of PA. This mechanism also is operating in ring cleavage. While exposed to ozone, aromatic structures also may undergo an electrophilic substitution followed by loss of oxygen resulting in ring hydroxylation. The second possible hydroxylation mechanism operates via oxidative dealkylation, the final result being the substitution of the aromatic methoxyl by a hydroxyl group (Gierer, 1986).

HPSEC of Matrix Components. Treating CS with ozone results in a massive degradation of lignin and

Table 2. Solubilizing Effect of the Ball-Milling plus Cellulase Treatment on the Neutral Detergent Fiber (NDF) of Untreated (CS) and Ozone-Treated (OCS) Cotton Stalks

criteria	CS	OCS
NDF, g/100 g of CS dry matter	75.0	55.0
NDF solubilized (g/100 g of NDF)	49.2	79.1
permanganate lignin (PL) (g/100 go of NDF)	24.3	13.9
PL solubilization (g/100 g of PL)	0.91	2.66

solubilization of matrix components, phenomena thought to be associated with the increased biodegradability of the residual CW (Shefet and Ben-Ghedalia, 1982). These effects, however, raised the interest regarding the nature of the biodegradation-resistant matrix complexes. Therefore, properties such as molecular weight distribution and sugar composition of the undegradable matrix complexes of CS and OCS were investigated. To obtain a matrix-rich preparation, NDF of CS and OCS were mildly defibrilized by ball-milling and subsequently treated with *T. reesei* cellulase, known for its strong cellulolytic but limited xylanolytic activities (Poutanen, 1987). Table 2 shows the effect of this procedure on the NDF fraction and its lignin content. The combined ball milling plus cellulase action solubilized 50% and 80% of the CS and OCS NDF, respectively, but affected very little the permanganate lignin. The common feature shared, however, by both CS and OCS residues is their resistance to biodegradation.

The molecular weight data are presented in Table 3. Two-thirds of the MC of CS were in the molecular weight range 800–10 000 with a M_w of 8500. The OCS MC showed a more dispersed pattern, with a higher proportion of molecules in the range higher than 10 000 and a M_w of 15 000. Polydispersity is a property common to all isolated lignins and LCC fractions irrespective of the method of isolation. It is thought to be associated with the random degradation of the native CW by chemical attack during isolation, yielding soluble matrix complexes of different sizes (Faix et al., 1981). Table 2 shows that OCS NDF contains about 50% of the lignin originally found in NDF of CS. This fact and the above-mentioned dispersity feature raise the question as to whether the data shown in Table 3 represent the CW in situ or are merely a result of the random action of the isolation procedure.

McNaughton et al. (1967) have shown that the M_w of fractions obtained from different stages of kraft delignification increased from 1800 for the first fraction to 51 000 for the final fraction taken at the end of the cook; all fractions were polydisperse. With respect to our study, we assume that the residual lignin left in OCS following oxidation by ozone consisted mostly of highly condensed structures linked to matrix carbohydrates through non-esteric bonds. Such structures are thought to be found mostly in the middle lamella (Wallace et al., 1991). The focus of our study was on biodegradation-resistant MC. Sodium hydroxide, used in this study for the extraction of MC, acts through cleavage of the peripheral esteric bonds linking lignins to their matrix carbohydrate counterparts, most likely with little effect on the etheric or C–C intramolecular bonds of the lignin moiety or on the lignin–carbohydrate ether bonds. Sodium hydroxide is not an ideal agent but is effective for extracting biodegradation-resistant MC. Dioxane extraction requires a robust ball-milling which might cause lignin depolymerization (Faix et al., 1981); it is applied mostly for pure (low carbohydrate) lignin isolation (Jung and Himmelsbach, 1989). Unfortunately, in situ methods for the assessment of the molecular sizes of MC are not available.

Monosaccharide Composition of MC Isolates. The monosaccharide composition of the carbohydrate component of MC is given in Table 4. Although the molecular weight distribution was determined on the basis of the absorption of the phenolic component (at 280 nm), the NaOH MC isolates of CS and OCS contained 54% and 39% carbohydrates, respectively (Table 4), with xylose as the major component and uronic acid as the main potentially branching monosaccharide. The differences in molecular weight pattern are not reflected in the monosaccharide profiles. Moreover, considering the xylan-debranching effects incurred on the MC throughout the isolation procedure, it is obvious that the carbohydrates of the MC isolates reflect the basic compositional features found in the original matrix, as shown in Table 4. The composition and high content of the carbohydrate component in the NaOH MC isolates imply that more attention should be paid to this fraction and particularly to the interphase nonesteric bonds linking the matrix components.

Solid-State ^{13}C NMR Spectroscopy of NDF. The development of solid-state ^{13}C NMR spectroscopy (Schaefer and Stejskal, 1979) and its application to lignocelluloses (Himmelsbach et al., 1983; Frund and Ludemann, 1989) make it possible to investigate the chemical structures of the intact CW in situ and to address important issues such as cellulose crystallinity (Himmelsbach et al., 1986; Cyr et al., 1990) and in situ carbon distribution (Frund and Ludemann, 1989).

In our study, NDF preparations of CS and OCS were examined by solid-state ^{13}C NMR spectroscopy. The NMR spectra are shown in Figure 2 and carbon distribution data are presented in Table 5. Peak assignments were made according to the protocol of Frund and Ludemann (1989). The resonances up to 46 ppm were assigned to aliphatic carbons and from 46 to 110 ppm to cellulose plus hemicellulose carbons. Due to resolution limitations of the solid-state spectra, it was impossible to separate the syringyl C-2 and C-6 carbons from the anomeric C-1 carbon of cellulose, both classes having signals in the chemical range 100–110 ppm. The resonances between 110 and 160 ppm were assigned to aromatic carbons and those from 160 to 210 ppm to carboxylic carbons. The peak at 89 ppm is assigned to the C-4 of cellulose. Elofson et al. (1984) suggested that the sharpness of this peak can be used as an indicator of the degree of cellulose crystallinity. Himmelsbach et al. (1986) and Garleb et al. (1990) suggested both the 89 ppm peak and the cellulose's C-6 signal at 65 ppm as indices of cellulose crystallinity. These spectra, however, may contain also signals from amorphic

Table 3. Molecular Weight Distribution (Percent of Total Peak Area), Weight-Average Molecular Weight (M_w), Number-Average Molecular Weight (M_n), and Dispersity (M_w/M_n) of NaOH-Solubilized Matrix Complexes (MC) from Ball-Milled plus Cellulase Pretreated NDF[a] Preparations of Cotton Stalks (CS) and Ozone-Treated Cotton Stalks (OCS)

MC origin	molecular weight ranges				M_w	M_n	M_w/M_n
	800–1600	1600–10 000	10 000–23 000	23 000–125 000			
CS	7.56 ± 0.57	59.4 ± 0.90	30.2 ± 1.50	2.84 ± 0.02	8471 ± 179	4626 ± 160	1.84 ± 0.05
OCS		42.0 ± 0.80	48.6 ± 1.45	9.40 ± 0.15	15029 ± 308	4849 ● 179	3.51 ± 0.08

[a] NDF, neutral detergent fiber.

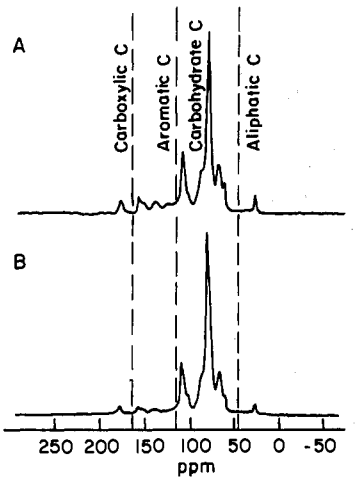

Figure 2. Solid-state 25.1-MHz cross-polarization/magic angle spinning carbon-13 nuclear magnetic resonance spectra of neutral detergent fiber preparation of (A) cotton stalks (CS) and (B) ozone-treated cotton stalks (OCS).

Table 4. Monosaccharide Profile of the Carbohydrate Component of the Matrix Complexes (MC) Isolated by N NaOH from Ball-Milled plus Cellulase-Pretreated NDF Preparations of Cotton Stalks (CS) and Ozone-Treated Cotton Stalks (OCS)

	CS		OCS	
monosaccharide	MC	orig matrix	MC	orig matrix
xylose	81.0	67.6	71.9	72.9
arabinose	0.94	5.72	1.28	2.71
galactose	0.61	4.72	0.92	2.23
mannose	0.37	2.96	0.79	2.76
rhamnose	0.80	0.20	0.61	0.20
glucuronic acid	13.2	18.8	13.7	19.2
glucose	3.08		10.8	
total	100	100	100	100
total monosaccharide content (g/100 g of MC)	53.9		39.1	

Table 5. Distribution of Carbon (Percent) in Chemical Groups and Components of Neutral Detergent Fiber (NDF) Preparations of Cotton Stalks (CS) and Ozone-Treated Cotton Stalks (OCS) Determined in Situ by Solid-State CP/MAS ^{13}C NMR Analysis

NDF sample	carboxylic C, 210–160 ppm	aromatic C, 160–110 ppm	carbohydrate C, 110–46 ppm	aliphatic C, 46–0 ppm
CS	2.60	13.0 (25.5)[a]	81.0 (68.5)[a]	3.40
OCS	2.20	7.40 (16.0)	86.5 (77.9)	3.90

[a] In parentheses, actual values of total lignin C and carbohydrate C corrected according to the method of Frund and Ludemann (1989).

carbohydrates (Cyr et al., 1990). Both crystallinity peaks found in cotton stalk NDF were affected by ozonation as demonstrated in Figure 2. The shoulder-like 89 ppm peak of CS (A) almost has been flattened, and the intensity of the signal at 65 ppm has been reduced (B) by ozonation. An additional effect of ozonation was a decline in the intensity of the signals found in the 160–110 ppm chemical region, assigned to aromatic carbons. Thus, the effects of ozone on dicot lignocelluloses are versatile, expressed not only in lignin degradation and matrix solubilization (Shefet and Ben-Ghedalia, 1982) but also in a decline in the degree of cellulose crystallinity. The term "lignin degradation" covers a broad array of processes, starting with lignin fragmentation and ending with carbon oxidation to CO_2. In the past, assessment of ozone treatment of CS has been based on the gravimetric permanganate procedure (Goering and Van Soest, 1970) which provides no information on the process itself. The advantage of the solid-state NMR analysis (Table 5) is associated with its ability to examine the process underlying lignin degradation. The quantitative data presented in Table 5 on carbon distribution in the various chemical entities of the NDF fractions analyzed by NMR were calculated according to the method of Frund and Ludemann (1989). The values of aromatic C were corrected for syringyl carbon overlap and multiplied by 1.7 to give the values of total lignin carbons and, consequently, the corrected carbohydrate C values; both groups of figures are given in parentheses (Table 5).

The most important point in Table 5 is the direct evidence that lignin degradation by ozone is the result of ring cleavage; this was shown by the decline in aromatic C from 13.0% in CS to 7.40% in OCS. The second point is the compatibility between lignin permanganate concentration (Table 2) and lignin derived from NMR data shown in Table 5.

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