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FRACTIONATION OF HEMICELLULOSES FROM MAIZE CELL WALLS WITH INCREASING CONCENTRATIONS OF ALKALI

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Key Word Index—*Zea mays*; Gramineae; maize; biochemistry; cell walls; hemicellulose; xylans; delignification.

Abstract—Hemicelluloses were solubilized from depectinated walls of maize coleoptiles and leaves with increasing concentrations of alkali to yield three major fractions of polymers. A highly-substituted glucuronoarabinoxylan released by dilute alkali from walls of coleoptiles was present only in very small amounts in the walls of the leaves. The stepwise extractions with increasing concentrations of alkali resolved a relatively unbranched xylan from a mixture of mixed-linked glucan, xyloglucan and additional xylan from walls of young leaves. Delignification in acidic sodium chlorite solubilized a small amount of substituted xylan from walls of both coleoptiles and leaves, and rendered about one-half of the unextracted hemicellulose soluble in only 0.02 M potassium hydroxide solution. Delignification prevented the detection of highly-substituted xylans released by dilute alkali.

INTRODUCTION

Hemicelluloses are generally defined as polymers that are solubilized by concentrated alkali [1]. Although alkali disrupts the hydrogen-bonding between hemicellulose and cellulose microfibrils, alkali also hydrolyses the ester linkages between sugars and hydroxycinnamic acids to release polymers that alone may display little capacity for hydrogen-bonding [2]. Because lignification during secondary wall formation in differentiating cells could influence the solubility of hemicellulosic substances, wall material is often 'delignified' prior to extraction with alkali; the most common delignification procedure is extraction with acidic sodium chlorite, which increases the amount of material removed from walls upon subsequent alkali extraction [3]. Buchala *et al.* [4] demonstrated that delignification of grass cell walls does not remove substantial amounts of hemicellulose.

Dilute alkali solubilizes highly-substituted glucuronoarabinoxylans (GAX I) from walls of maize coleoptiles [5]. Extraction with alkali of increasing concentrations removed additional glucuronoarabinoxylans, mixed-linked glucans and xyloglucans which are bound more tenaciously to cellulose [5]. The highly-substituted GAX I was not found in dilute alkali extracts of walls from mature leaves, so the possibility that lignification might hamper the extraction of such polymers was examined. Consequently, the influence of delignification by acetic acid–sodium chlorite [3] on the composition of hemicellulosic material extracted from purified walls of coleoptiles and young leaves was determined.

RESULTS AND DISCUSSION

The highly-substituted xylans found in coleoptile walls [5] were present only in very small amounts in walls of either immature or mature leaves (Fig. 1a). The small amount of polymeric material extracted by dilute alkali comprised mostly arabinose and xylose (Table 1) and nearly equal amounts of branched and unbranched xylosyl residues (Table 2). In contrast to the walls of coleoptile cells, the walls of both immature and mature leaves were resolved primarily into two hemicellulosic fractions. GAX II was extracted with 0.2–1 M potassium hydroxide (Fig. 1a) and consisted primarily of xylose (Table 1), whereas MG-GAX required at least 2 M potassium hydroxide for extraction (Fig. 1a) and contained substantial amounts of glucose in addition to xylose.

GAX II from both immature and mature leaves contained a much higher proportion of 4-xylosyl; only one of every four xylosyl residues in this polymeric mixture was branched (Table 2). Terminal arabinosyl linkages constituted a major side-group (Table 2). In contrast, GAX II from coleoptile walls contained nearly equal amounts of branched and unbranched xylosyl residues (Table 2), although an unbranched xylan similar to that in the leaf wall was resolved after Sepharose 4B chromatography [5]. This fraction from leaves was probably heterogeneous also, and unbranched xylans possibly could be separated from substituted xylans by similar chromatography.

MG-GAX of coleoptiles and immature and mature leaves were similar, except that the walls of leaves had lower amounts of 3-glucosyl and 4-glucosyl from mixed-linked glucan and *t*-xylosyl, 4-linked glucosyl and 4,6-glucosyl from xyloglucan. The ratio of substituted to unbranched xylan was only 1 : 4 (Table 2).

Data here are consistent with other analyses of the hemicellulosic constituents in 4 M potassium hydroxide extracts of grass coleoptiles and leaves [6–8], although the alkali gradient resolved a large xylan fraction essentially free of mixed-linked glucan and xyloglucan. At least 2 M potassium hydroxide was required to release substantial amounts of mixed-linked glucan from the wall matrix (Tables 1 and 2) [5]; it was not clear if the additional xylan extracted by 2 M potassium hydroxide was a result of entrapment by glucan or covalent linkages between xylan and glucan.

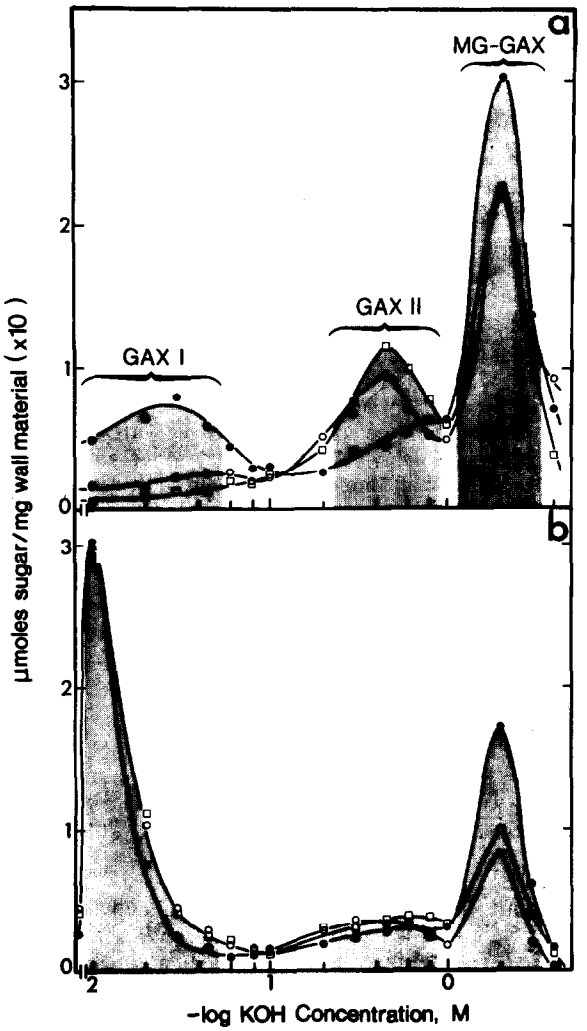

Fig. 1. Fractionation of hemicellulosic polysaccharide from walls of maize coleoptiles and leaves in an alkali-gradient. Fifty mg portions of depectinated walls of coleoptiles (●), immature leaves (○) and mature leaves (□) were extracted sequentially in a step-gradient of 0.01–4.0 M containing 3 mg/ml $NaBH_4$. Polymers solubilized by each solution were dialysed and lyophilized; each point represents the quantitation of alditol acetate derivatives after TFA hydrolysis of the lyophilized polymers. (a) Untreated walls; (b) walls delignified in acidic sodium chlorite.

Because other investigators had not reported the highly-substituted GAX I in coleoptiles or leaves, some alternative procedures for the extraction of pectic substances and for the delignification of wall material were evaluated.

Jarvis *et al.* [9, 10] reported that chilled sodium carbonate and Ca^{2+}-chelating agents removed pectic substances without the extensive degradation of uronic acid-rich polymers commonly expected with hot ammonium oxalate/citrate mixtures. Graminaceous species are low in pectin, and hot ammonium oxalate removed much of this material [5]. Alkaline sodium carbonate and sodium carbonate + EGTA were expected to remove some hemicellulosic material [9] and, indeed, removed small amounts of polymer from depectinated coleoptile walls (Table 3). The addition of EGTA increased the amount extracted by over 80%. The material was rich in both arabinose and xylose (Table 3) and comprised primarily of terminal-arabinosyl, 4-xylosyl and 2 + 3,4-xylosyl residues (Table 4). The ratio of branched xylose (2 + 3,4-xylosyl) to unbranched xylose (4-xylosyl) was *ca* 6:1 (Table 4) and identical to GAX I described previously [5]. Urea extracted less than 5% of the depectinated coleoptile wall, but, unlike any potassium hydroxide fraction, the material extracted was enriched in 4-mannosyl units (Tables 3 and 4). Other derivatives found in the extract indicated that small amounts of arabinoxylan and glucan material co-eluted with the mannan (Table 4).

Delignification of the coleoptile wall solubilized a small amount of material enriched in arabinose and xylose, but the ratio of substituted to unbranched xylan was less than 4:1, perhaps as a result of the loss of some arabinofuranosyl linkages in the acidic sodium chlorite solutions or from the extraction of additional unbranched xylan (Table 3). Similar material was solubilized from the leaf wall (not shown).

Although delignification removed little hemicellulosic material from either the coleoptile or the leaf wall, the treatment substantially altered the distribution of hemicellulosic materials in subsequent alkali extractions. Most of the hemicellulosic material was extracted with 0.01–0.02 M potassium hydroxide and comprised a mixture of arabinoxylan, xyloglucan and mixed-linked glucan (Tables 1 and 2) and was enriched in uronic acid-rich material (Table 1). These data indicated that lignification or covalent interactions between hemicellulosic substances and phenolic residues were responsible for the high concentrations of alkali required for the removal of hemicelluloses from untreated wall material. The acetic acid–chlorite causes oxidative degradation of these phenolic cross-linkages and thus solubilizes polymers held by such linkages [3]. Alternatively, the acidic sodium chlorite may have altered hemicellulosic structure to the extent that it is no longer hydrogen bonded to cellulose or other polymers. The GAX II and MG-GAX fractions remaining after delignification were similar to those extracted from the original material except that they were enriched in the amounts of glucan, perhaps as a result of preferential loss of xylans to the fraction extracted by dilute alkali (Tables 1 and 2).

Delignification is necessary to increase the amount of hemicellulosic material extracted in subsequent alkali treatment from only heavily-lignified walls [3]; it is unnecessary for walls of Gramineae or unlignified tissues. GAX I was identified in growing coleoptile walls only after extraction in dilute alkali [5], so delignification in acidic sodium chlorite should be avoided as a general procedure for unlignified walls because (1) the arabinofuranosyl residues are acid-labile, and warm acetic acid treatment could result in loss of some of these residues, and (2) subsequent extraction of delignified walls with dilute alkali will yield substantially more hemicellulosic materials which prevent the detection of these highly-substituted xylans.

The results here do indicate that substantial amounts of hemicellulosic material are held by phenolic cross-linkages from either protein or hydroxycinnamic acid residues. The walls of cereals generally do not stain with phloroglucinol/hydrochloric acid, although fluorescence microscopy revealed that phenolic acids are components

Table 1. Influence of delignification on the sugar composition of fractions resolved by alkali-gradient extraction of depectinated walls of maize coleoptiles and leaves*

Tissue: Fraction†		Untreated					HOAc/chlorite treated				
		Ara	Xyl	Gal	Glc	Uronic acid	Ara	Xyl	Gal	Glc	Uronic acid
Coleoptile:											
GAX I	(4.9)	33.9	34.7	7.9	4.6	18.9	23.7	29.2	5.4	11.4	30.3
GAX II	(4.0)	20.9	27.0	8.1	32.2	11.7	4.2	17.0	2.2	51.1	25.6
MG-GAX	(10.4)	10.6	18.0	7.8	48.1	15.6	10.2	23.1	5.0	40.0	21.8
Immature leaf:											
GAX I	(2.0)	17.8	42.9	3.9	5.9	29.5	14.3	43.1	2.4	8.5	31.7
GAX II	(4.7)	9.7	63.3	1.4	2.4	23.2	9.6	56.5	2.0	10.8	21.2
MG-GAX	(5.6)	9.6	51.9	2.0	17.6	18.9	11.1	34.1	2.9	27.3	24.7
Mature leaf:											
GAX I	(1.4)	15.8	42.7	2.6	1.1	37.8	16.5	47.3	2.0	6.8	27.4
GAX II	(5.2)	8.3	68.3	1.3	2.4	19.6	11.3	64.4	0.8	12.8	10.7
MG-GAX	(5.0)	10.8	51.0	2.1	18.2	17.9	9.1	45.2	1.1	31.7	12.9

*Mol % of neutral sugar from GLC of alditol acetate derivatives and uronic acid analysis of fractions designated in Fig. 1a.

†mg of material per 50 mg of depectinated untreated wall material is given in parentheses.

Table 2. Linkage analysis of fractions resolved in alkali-gradient extraction of depectinated walls of maize coleoptiles and leaves*

Tissue:	Arabinosyl					Xylosyl			Galactosyl			Glucosyl			
Fraction	*t*-	2-	3-	5-	3,5-	*t*-	4-	2+3,4-†	*t*-	3-	4-	*t*-	3-	4-	4,6-
Coleoptile:															
GAX I	43.9	0.4	0.4	3.5	0.2	0.7	6.9	42.5	0.3	tr	0.7	tr	0.1	0.3	tr
GAX II	23.2	1.8	tr	3.1	0.9	1.8	31.2	26.9	0.7	tr	2.3	tr	1.0	7.2	tr
MG-GAX	11.9	1.8	tr	2.0	tr	3.2	6.9	16.5	1.4	0.3	3.4	0.3	9.5	37.8	5.0
Immature leaves:															
GAX I	26.8	tr	tr	1.7	tr	tr	20.3	33.8	9.1	tr	tr	tr	3.0	5.3	tr
GAX-II	11.7	0.5	tr	1.7	1.1	1.8	61.5	18.2	0.3	tr	0.2	tr	0.8	4.1	tr
MG-GAX	8.6	0.7	tr	1.9	0.3	4.3	31.9	14.9	1.1	0.5	0.9	0.3	5.4	21.8	7.2
Mature leaves:															
GAX I	32.2	1.0	tr	2.3	0.3	1.7	32.0	28.1	1.4	0.3	0.3	0.2	tr	0.3	tr
GAX II	14.3	0.6	tr	0.7	0.2	2.6	66.4	13.9	0.4	tr	0.4	tr	0.4	0.4	tr
MG-GAX	13.0	0.6	tr	1.6	0.4	2.6	45.9	11.8	0.4	0.2	0.4	tr	4.9	17.7	0.4

*Mol % of partially methylated alditol acetates separated by capillary GLC; tr = trace amounts.

†2,4-Xyl comprised 6–11% of this fraction based on selective ion monitoring of *m/z* 118 and 129 + 130.

Table 3. Sugar composition of sodium carbonate, urea and acidic sodium chlorite extracts from depectinated coleoptile walls

Fraction	Composition*				
	Ara	Xyl	Man	Gal	Glc
Na_2CO_3 (2.9)†	42.8	42.6	tr	8.1	6.5
Na_2CO_3/EGTA (5.3)	44.6	45.6	tr	5.5	4.3
Urea (2.3)	9.4	16.6	27.2	15.1	31.7
$NaClO_2$/HOAc (6.1)	45.7	49.2	tr	1.0	4.1

*Mol % of neutral sugar from GLC of alditol acetate derivatives; tr = trace amounts.

†mg of material extracted per 50 mg of depectinated wall material is given in parentheses.

of the primary walls of coleoptiles [11]. The major phenolic acid is ferulic acid [2, 12, 13], and dimerization of ferulic acid to diferulic acid could result in polymer cross-linkages [14, 15].

Glycoproteins are also solubilized in acidic chlorite [16] indicating that these polymers could also be spliced by similar phenolic cross-linkages. The discovery of chlorite-labile isodityrosine in glycoprotein from primary walls of potato implies protein–protein interactions which could weave hemicellulosic polymers [17]. The possible organization of hemicellulosic constituents in the walls of Gramineae by these phenolic interactions bears close examination.

Table 4. Linkage analysis of sodium carbonate, urea and acidic sodium chlorite extracts from depectinated coleoptile walls*

	Arabinosyl					Xylosyl			Mannosyl		Galactosyl		Glucosyl			
Fraction	*t*-	2-	3-	5-	3,5-	*t*-	4-	2+4†	*t*-	4-	3-	4-	*t*-	3-	4-	4,6-
Na_2CO_3	24.0	2.1	1.3	1.7	tr	0.6	8.8	52.1	tr	tr	2.1	3.0	0.5	0.9	3.0	tr
Na_2CO_3/EGTA	18.4	3.4	1.3	1.7	tr	0.6	8.8	54.2	tr	tr	2.5	3.0	0.6	1.0	4.0	tr
Urea	3.5	1.6	0.4	0.5	tr	1.3	11.4	5.5	tr	21.6	8.5	3.0	0.7	4.9	30.9	6.1
$NaClO_2$/HOAc‡	40.7	0.8	0.9	3.2	0.1	0.5	11.3	37.4	tr	0.2	0.6	0.4	tr	0.4	3.6	tr

*Mol % of partially methylated alditol acetates separated by GLC; tr = trace amounts.

†2,4-Xyl was *ca* 8–10% of this fraction based on selective ion monitoring of *m/z* 118 and 129 + 130.

‡Partially methylated derivatives of this fraction were reduced and acetylated according to Blakeney *et al.* [25].

EXPERIMENTAL

Plant material. Seeds of corn (*Zea mays* L. cv WF9 × Bear 38) were from Customaize Research (Decatur, IL, U.S.A.) and were stored desiccated at 8° to maintain germinability. To obtain coleoptile tissue, seeds were soaked in running tap H_2O overnight and sown in trays of vermiculite saturated with deionized H_2O. Trays of seeds were covered with Al foil and incubated at 27° in darkness for 3 days. A 1 hr red light treatment was given on the second day to inhibit mesocotyl elongation. Coleoptiles 2–3 cm long were excised and floated on deionized H_2O before homogenization.

To obtain leaf tissues, seeds were sown in plastic cartons of vermiculite saturated with a nutrient soln [18] and incubated for 2 weeks at 25° in light at a fluence rate of 20 μE/m^2-sec delivered by two General Electric F40 PK (pink) fluorescent lamps. The third fully-expanded leaf (mature leaf) was excised *ca* 4 cm from the ligule, and the etiolated third leaf and meristem (immature leaf) were excised below the ligule. These tissues were floated on deionized H_2O before homogenization.

Purification of cell walls and fractionation. Tissues were homogenized in an equal vol. (w/v) of ice-cold 0.05 M Tes plus 0.01 M ascorbate (pH 7.2), and walls and cell debris were pelleted by centrifugation. Walls were washed as described previously [19] in 0.5 M KPi buffer (pH 7), H_2O, $CHCl_3$–MeOH (1:1, v/v), Me_2CO, MeOH and H_2O. Suspensions in H_2O were frozen and lyophilized. Each 60–75 mg sample of lyophilized walls was extracted in 20 ml DMSO for 24 hr with vigorous agitation with a magnetic stir-bar. This treatment removes starch [5, 20] and permits a more facile extraction of pectic substances [21].

After DMSO extraction, the wall material was washed once with deionized H_2O, and pectic substances were extracted in 20 ml 0.5% NH_4 oxalate at 100° 2 × for 1 hr each. The soln was stirred vigorously every 15 min to prevent the accumulation of material at the surface. The wall material was washed with deionized H_2O and lyophilized.

Fifty mg portions of coleoptile wall material were extracted further with 20 ml 0.05 M Na_2CO_3 (containing 3 mg/ml $NaBH_4$) or Na_2CO_3 + 2 mM EGTA (containing 3 mg/ml $NaBH_4$) at 4°, or 8 M urea at 23°. All extractions were for 18 hr under N_2 with vigorous agitation by a magnetic stir-bar. Unextracted wall material was pelleted by centrifugation, and the supernatants were filtered through Whatman GF/F glass-fibre filter paper, dialysed overnight against running deionized H_2O, and lyophilized.

Several procedures have been examined for the delignification of woody tissues [3]. Fifty mg portions of NH_4 oxalate-extracted wall material (depectinated wall) from coleoptile and leaf were delignified in 40 ml 0.34 M Na chlorite in 65 mM HOAc at 70° for 1 hr. An additional 1.25 g Na chlorite and 150 μl 18 M HOAc were added, and the mixture was incubated at 70° for an additional 1 hr. The wall material was collected by centrifugation, and the yellow supernatants were dialysed overnight against running deionized H_2O. Wall material was washed twice with deionized H_2O, and both the dialysed supernatants and wall materials were lyophilized.

Fractionation of hemicelluloses. Lyophilized lignified and delignified NH_4 oxalate-extracted wall material was extracted in a 17-step alkali gradient from 0.01 to 4 M KOH. Forty to 60 mg of material was suspended in 20 ml 0.01 M KOH containing 3 mg/ml $NaBH_4$ and was agitated vigorously with a magnetic stir-bar under N_2 for 1 hr. Unextracted material was pelleted by centrifugation and suspended in the next KOH soln. Supernatants were filtered through Whatman GF/F glass fibre filter paper, chilled in an ice bath, and neutralized with 18 M HOAc. Samples were taken for assay of uronic acid [22], and the remainder of the supernatants were dialysed overnight against deionized H_2O and lyophilized.

Sugar analyses. Lyophilized materials were hydrolysed in 2 M TFA, and alditol acetates prepared according to ref. [23]. Ac_2O was evapd in a stream of N_2 and replaced with CH_2Cl_2 prior to GLC. Derivatives were separated isothermally on a 0.25 mm × 30 m SP-2330 WCOT vitreous silica capillary column at 240°. N_2 carrier flow was 1.5 ml/min with a split ratio of 1:100. Injector and FID were at 250°, and 1–2 μl samples were injected.

Samples were partially methylated essentially according to Hakomori as described in ref. [24] except that K dimethyl sulfinyl anion was prepared and silylation-grade dry DMSO was from Pierce (Rockford, IL, U.S.A.). Partially-methylated polymers were extracted from DMSO with either $CHCl_3$, or by reverse-phase chromatography in MeCN on small C-18 columns (Sep-paks; Waters Inc., Milford, MA, U.S.A.). $CHCl_3$ or MeCN containing derivatized polymers was evapd in a stream of N_2, and partially-methylated alditol acetates were prepared after hydrolysis in 2 M TFA as described before [23]. Some materials were reduced in DMSO and acetylated with catalysis by 1-methylimidazole as described in ref. [25]. This technique avoids repeated evaporations with MeOH to remove trimethyl borate, and substantially increases the recovery of terminal arabinosyl derivatives.

Derivatives were separated on the same column and conditions were as for alditol acetates except the temp. was programmed from 170° to 240° at 2°/min. Derivatives were identified by GC/MS on the same column and chromatographic conditions at 70 eV and a source temp. of 160°. Spectra of $NaBD_4$-reduced derivatives were compared to those published in ref. [26]. Derivatives were quantified from FID detector response according to the effective carbon response calculated in ref. [27].

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