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PECTIC POLYSACCHARIDES OF MAIZE COLEOPTILES AND PROSO MILLET CELLS IN LIQUID CULTURE

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Key Word Index—*Zea mays*; *Panicum miliaceum*; Gramineae (Poaceae); coleoptile; cell suspension culture; cell wall; polysaccharides; pectins; arabinogalactans; rhamnogalacturonans; glucuronoarabinoxylans.

Abstract—The chemical composition of the pectin fraction of growing cells of two cereal grasses, maize and proso millet, was examined. Chelator-soluble pectin comprised less than 15 wt% of the cell wall. About one-third of this material was glucuronoarabinoxylan similar to that of the hemicellulose fraction, and about an equal amount was galacturonan and rhamnogalacturonan typical of dicot pectins. The rhamnosyl units were more highly branched than those typically found in dicots; side-chains were composed predominantly of (1→5)-linked arabinosyl and (1→4)-linked galactosyl units with numerous branches. A type II arabinogalactan containing (1→3)-, (1→6)-, and (1→3,1→6)-linked galactosyl units comprised the remainder of the pectic polysaccharides.

INTRODUCTION

The chemical composition of the primary cell walls of cereal grasses is remarkably different from that of dicots and even other monocots [1, 2]. Hemicellulose of dicots is mostly xyloglucan and constitutes only about 25% of the cell wall, whereas hemicellulose of cereal grasses is mostly glucuronoarabinoxylan (GAX) and is well over half of the total mass [1–4]. In dicots, hydroxyproline-rich glycoproteins also comprise a substantial amount of the wall and cross-link the carbohydrate polymers to form a rigid matrix [5]. In cereal grasses, these proteins have been replaced by esterified and etherified phenolic compounds [6, 7]. About one-third the primary wall of dicots is pectin, and it is composed of polygalacturonic acid and rhamnogalacturonans (RGs) substituted with arabinans, galactans, and type I and type II arabinogalactans (AGPs) [1, 8, 9]. The pectins are immobilized mostly by cross-linkages of the galactosyluronic acid units with Ca^{2+} or esterification of diphenyl hydroxycinnamic acids [1, 8]. Pectins constitute only a small fraction of the walls of cereal grasses [1–4], and there are few systematic studies of their chemical composition. Rhamnose and galacturonic acid residues constituted wall material from oat coleoptile [10], and type II arabinogalactans also comprised a portion of the pectin fractions of several cereal suspension cultures [11]. Shibuya and Nakane [12] reported the first thorough chemical analysis of the pectic polysaccharides of rice endosperm. Rhamnogalacturonans with expected arabinan and galactan side-chains similar to those of dicots were found, but a significant proportion was glucuronoarabinoxylan similar to that comprising most of the hemicellulose. Because of the role that pectins may play in control of growth-related activities of the cell wall [13, 14], the composition of this fraction in growing cells of two representative cereal grasses was investigated. Cells of proso millet in liquid culture contain mostly primary wall and exhibit little elongation or differentiation that accompanies stationary phase cultures of many dicots [15], whereas the cortical cells of the developing maize coleoptile grow mostly by expansion of cells formed during seed development [16]. In this report, the chemical composition of these comparable pectin fractions is documented.

RESULTS AND DISCUSSION

The polysaccharides extracted with hot ammonium oxalate constituted 11 and 13% of the total wall of maize and millet, respectively. Because of the enrichment in uronosyl residues in this material, samples were subjected to chemical reduction with $NaBD_4$ after carbodiimide activation of the carboxyl groups [17]. Comparison of sugar analyses before and after reduction reflected a substantial increase in galactose expected upon conversion of the galactosyluronic acid to its respective neutral sugar (Table 1). From chemical determination of uronic acid, the efficiency of reduction was about 85% for both maize and millet pectins; a second round of reduction did not improve the % reduction significantly (data not shown). The neutral sugar profiles of the two preparations were similar. Both contained about 5% rhamnose and a total of about 30% arabinose and xylose (Table 1). The maize pectin fraction contained about 36% uronic acid, which reduction converted mostly to galactose (Table 1). By comparison, only *ca* 25% of the millet pectin fraction was uronic acid, and the decreased proportion was compensated mostly by slight enrichment of arabinose and galactose (Table 1).

From reduction of the activated carboxyl group with $NaBD_4$, the former glycosyluronic acid units were identified as their 6,6-dideuterio derivatives. Thus, non-reducing terminal uronosyl units were identified from EIMS by a shift of the *m/z* 205 ion of the 2,3,4,6-Me_4-hexitol derivative to *m/z* 207, and likewise, the (1→4)-linked units were identified by a shift of the *m/z* 233 ion of the 2,3,6-Me_3-hexitol derivative to *m/z* 235 [18]. Methylation analysis revealed that most of the galactosyl units found were originally present as (1→4)-linked galactosyluronic acid units (Table 2). Together with (1→2)- and (1→2,1→4)-linked rhamnosyl units, the galactosyluronic

acid units comprise the backbone polygalacturonic acid and rhamnogalacturonan structures typically found in dicot walls [1, 2]. Identification of rhamnosyl-(1→4)-galactosyluronic acid in partial acid-hydrolysates of walls of oat coleoptiles and maize cobs gives supporting evidence that the rhamnosyl and galactosyluronic acid units are indeed in the same polymer [19]. Three of every four rhamnosyl residues were branched (Table 2). High proportions of branching were also observed in the RG of rice endosperm [12], but the dicot pectins generally have about equal amounts of (1→2)- and (1→2,1→4)-linked rhamnosyl units [1, 8]. From linkage analysis, it could be concluded that the side groups typically attached to the dicot rhamnogalacturonans are also found in these cereal pectic polysaccharides, with numerous linked and branched arabinofuranosyl units and (1→5)-linked arabinosyl units predominating (Table 2). The (1→4)- and (1→4,1→6)-linked galactosyl units found are also typical of the type I arabinogalactans of dicots [1, 8]. Considerable amounts of (1→3)-, (1→6)-, and (1→3,1→6)-linked galactosyl units were also found. However, these are probably components of type II arabinogalactans (AGPs). These AGPs are soluble peptidoglycans found in exudates and in the liquid incubation medium from both dicot and grass cell cultures [20, 21]. Through pulse-chase studies, Takeuchi and Komamine [22] found that these type II AGPs were transiently attached to the wall matrix of carrot cells before release into the medium; either covalent or strong ionic bonds must stabilize these otherwise soluble AGPs since they were not removed by extensive washings of the wall with high-salt buffers prior to ammonium oxalate extraction.

Linkages typical of other polysaccharides were also observed. The (1→4)- and (1→3,1→4)-linked xylosyl units and corresponding *t*-arabinofuranosyl and *t*-glucosyluronic acid units constituted about 30% of the total linkages and were from GAX, the hemicellulose typical of cereal grasses. These GAXs were of high degree of substitution; about 3 of every 4 xylosyl units of millet GAX and 4 of every 5 units of maize GAX were branch units (Table 2). Hemicellulosic GAXs with similar degrees of substitution were also found in dilute alkali extracts of both maize coleoptiles and millet cells [3, 23]. The walls of embryonal or dividing cells, such as those of the unexpanded coleoptile or cells in liquid culture, are enriched in (1→2)-, (1→3), (1→5)-, and (1→3,1→5)-linked arabinosyl units [4, 15]. The mole fractions of these units decrease markedly during cell expansion and *t*-arabinofuranosyl residues predominate [4]. This trend is apparent also in these pectin fractions. Non-reducing terminal

Table 1. Sugar analyses of the pectic polysaccharide fraction from maize coleoptiles and proso millet cell suspension cultures*

Sample		$NaBD_4$ reduction†	Rhm	Fuc	Ara	Xyl	Man	Gal	Glc	Uronic acids‡
Maize:										
	Total	–	4	1	15	18	2	10	6	43
		+	5	tr	14	17	1	52	11	—
Millet:										
	Total	—	8	2	26	10	2	11	10	31
		+	6	1	27	10	1	41	14	—
Fraction I		—	6	tr	39	18	tr	11	3	22
Fraction II		—	15	tr	25	5	tr	17	3	35

*Ammonium oxalate extracted material constituted 11% of the walls of maize and 13% of wall of proso millet; values are mol%.

†Carboxyl groups were activated with CMC, and their mixed anhydrides were reduced with $NaBD_4$ [17].

‡Uronic acids were determined by a carbazole method [27] using sulphamate to minimize neutral sugar interference [28].

Table 2. Linkage analysis of mazie and millet pectin

	Rhm			Fuc	Ara							Xyl		(2+3),4-†
	t-	2-	2,4-	*t*-	*tf*-	*tp*-	2-	3-	5-	2,5	3,5-	*t*-	2-	4-	
Maize:															
total	tr	1	4	tr	9	tr	1	2	6	tr	1	1	nd	3	12
Millet:															
total	tr	1	4	1	4	tr	tr	2	14	1	2	1	1	1	5
Fraction I	tr	tr	3	tr	18	tr	tr	3	21	tr	2	1	tr	4	14
Fraction II	1	3	10	1	8	1	tr	3	17	1	3	tr	nd	1	4

*Mol % of partially methylated alditol acetates separated and identified by GLC-EIMS [18], quantified in paired runs by FID corrected by the effective carbon response method [32]. Tr, trace amounts less than 0.5%, nd, not detected.

†Based on selective ion monitoring of *m/z* 118 and *m/z* 129+130 [18], 2,4-xyl comprised less than 10% of the fraction in all samples.

arabinofuranosyl units comprised only 20% of the total arabinose of the millet preparation, whereas over 50% of the arabinosyl residues were non-reducing termini in the maize preparation (Table 2). This difference is also reflected in an increase in the proportion of GAX in the fraction.

Different polymers, particularly GAX and RG, constituted this pectin fraction. Shibuya and Nakane [12] obtained two major fractions from DEAE-cellulose by graded elution with 0.2 and 0.5 M NaCl, respectively. From methylation analysis, they deduced that the first fraction was enriched in GAX, whereas the second was mostly RG, although separation was far from complete [12]. To further explore possible separation, the maize and millet extracts were fractionated on DEAE-Sephadex but eluted in a bi-phasic linear gradient of Na citrate from 0.01 to 0.05 M and then to 0.6 M (Fig. 1). Two fractions of the millet pectins were resolved with relatively dilute citrate buffer, but some material remained on the column in the 0.6 M citrate. Wada and Ray [10] resolved several discrete fractions by paper electrophoresis, the fastest moving fractions containing mostly galactosyluronic acid. Polygalacturonic acid was not eluted from the ion-exchange gel (Fig. 1). The two fractions differed markedly in proportions of glycosyluronic acid, fraction I containing only 22% and fraction II containing 35% (Table 1). Both fractions contained RG, but fraction I was substantially enriched in the highly substituted GAX (Table 2). Linkages typical of type II AGP were also predominantly in fraction I, whereas (1→4)- and (1→4,1→6)-linked galactosyl units typical of type I AGP were principally with the fraction II RG (Table 2). Separation of GAX and RG was incomplete, even in these gradient elutions, and the ratio of uronic acid to total sugar and the neutral sugar profiles through each peak were consistent. These data indicate that subfractions of GAX interact with RG polymers and that the type II AGPs are associated primarily with the GAX fraction. Whether or not these associations are through direct covalent linkages or organized networks is unclear, but considering that the discrete fractions were obtained by gradient rather than step-wise elution, it is unlikely that co-elution of these different polymers is based purely on two populations with similar charge density. Such interactions of RG and GAX may reflect a ontogenic transition from the thin RG fundamental matrix to a RG-GAX transition matrix and finally to the hemicellulosic GAX matrix.

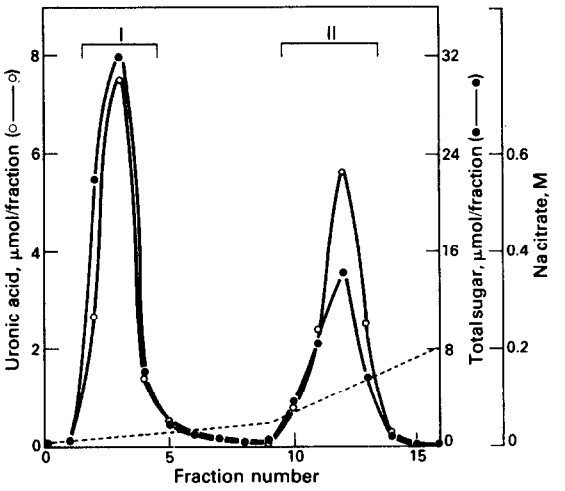

Fig. 1. Chromatography of pectic polysaccharides. Samples were dissolved in 10 mM Na citrate, pH 5.5, and eluted in a bi-phasic linear gradient to 50 mM and then 600 mM Na citrate, pH 5.5. Negligible material eluted at concentrations higher than 200 mM. Samples of each fraction were taken for determination of total sugar, uronic acid and neutral sugar composition. For fractions I and II, individual column fractions were pooled, and dialysed material was carboxyl reduced and prepared for the linkage analysis reported in Table 2.

The pectic polysaccharides of maize coleoptiles and proso millet cells were composed primarily of GAX, RG, and AGP and were similar in composition to the pectic fraction of rice endosperm. The GAX resembled that found predominantly in dilute alkali extracts of the cell wall. The composition of RG was similar to that of dicots, but comprised a much smaller proportion of the primary wall and was more highly branched. The chromatographic behaviour suggested that subfractions of the GAX and RG may be covalently linked but structural implications of such interactions were not resolved.

EXPERIMENTAL

Plant material. Seedlings of maize (*Zea mays* L. cv. LH74 × LH51) were grown in moist vermiculite at 28° for 3 days in

preparation and two fractions of the millet preparation*

Gal							Glc			GlcA‡		GalA‡	
t-	3-	4-	6-	3,4-	3,6-	4,6-	*t*-	4-	4,6-	*t*-	4-	*t*-	4-
2	1	4	1	tr	5	4	tr	6	2	1	tr	2	36
2	2	1	tr	tr	6	4	2	7	2	1	tr	3	32
3	1	1	tr	tr	4	1	tr	2	1	1	nd	2	18
2	tr	2	tr	tr	1	7	tr	1	tr	tr	tr	2	31

‡Deduced from 6,6-dideuterio derivatives in *t*- and 4- Glc and Gal, respectively.

darkness. Coleoptiles were elongating maximally at this time [4]. Cells of proso millet (*Panicum miliaceum* L. cv. Abarr) were grown in liquid cultures in MS medium [24; commercial preparation from GibCo] with hormone and vitamin supplements [15]. Cells were normally transferred biweekly and had been maintained continuously for over three years. Cells for this study were harvested during mid-logarithmic phase, 7 to 8 days after subculture.

Preparation of pectic substances. Coleoptiles were excised from the growing seedlings, frozen in liquid N_2, and homogenized with a motorized glass-glass grinder in 0.05 M K-Pi, pH 7.0, containing 0.03 M Na ascorbate and 0.05 M KCl. Cells of proso millet were collected by filtration through sintered glass funnels, washed with the K-Pi buffer, and 1 g aliquots were suspended in 30 ml of buffer. The cells were lysed by N_2 gas decompression [25], and the lysate was chilled quickly in an ice bath. The walls of each were washed sequentially with 0.5 M KCl, water, $CHCl_3$–MeOH (1:1) at 45°, MeOH, Me_2CO, and water as described [3, 4] and lyophilized. Starch from 50 mg samples of each material was extracted with 20 ml DMSO by vigorous stirring for 24 hr [26]. The unextracted material was washed several times with H_2O, and pectic polysaccharides were extracted twice with 20 ml 0.5% ammonium oxalate, pH 7, at 100° for 1 hr each with occasional stirring. The solns were cooled to ambient temp., and unextracted material was removed by centrifugation at 2500 g. The supernantant was cleared further by filtration through Whatman GF/F glass fibre mats; the two extracts were then pooled, dialysed against running deionized water overnight, and lyophilized.

Chemical analyses. Small samples of the material were dissolved in water by mild sonication in a water bath for determination of uronic acid [27] by a procedure modified to include sulphamate to reduce neutral sugar interference [28] and for determination of total sugar [29]. The glycosyluronic acid residues in about 50 mg of material were activated with 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulphonate (CMC) and reduced with $NaBD_4$ according to Taylor and Conrad [17], except that solid $NaBD_4$ was added directly to the soln and the pH was permitted to rise to *ca* 8.5. Excess $NaBD_4$ was destroyed with glacial HOAc, and the material was dialysed against running deionized H_2O for 24 hr and lyophilized. Total sugar and uronic acid were determined to estimate reduction efficiency.

Neutral sugars before and after reduction of the glycosyluronic acids were determined after hydrolysis in 2 M TFA containing 1 μmol *myo*-inositol (internal standard) at 120° for 90 min with occasional vortex mixing. The TFA was evapd in a stream of N_2, and the sugars were reduced with $NaBD_4$ and acetylated using 1-methylimidazole as catalyst according to *ref.* [30], modified so that the reactions could be carried out in 1-dram vials [23]. Alditol acetate derivatives were separated in a 3-m × 0.2-cm packed column of 3% SP-2330 temp. programmed from 170 to 240° at 5°/min with a 10 min hold at the upper temp. Injector and FID were at 260°, and N_2 carrier flow was 30 ml/min.

Other samples of the material were per-*O*-methylated according to Hakomori [31] but with the K methylsulphinylmethanide anion and technical improvements designed to increase efficiency of permethylation and recovery [4, 23]. The permethylated polysaccharides were hydrolysed in 2 M TFA, and alditol acetate derivatives were prepared as described above. The derivatives were separated in a 30-m × 0.2-mm vitreous silica WCOT capillary column of SP-2330 temp. programmed from 160 to 210° at 2°/min and then to 240° at 5°/min. The split ratio was 50:1, the injector and MS interface oven were at 260°, and He carrier flow was 1.5 ml/min. EIMS were obtained at 70 eV and a source temp of 160° with a Finnigan/MAT 9610 GLC instrument coupled to a Finnigan/MAT 4021 quadrupole MS interfaced to a Finnigan/MAT 2100C INCOS data-system. Derivatives were identified by RR_t and fragmentation analysis after EIMS [18]; amounts of each derivative were quantified according to the effective carbon-response calculated by Sweet *et al.* [32] in paired runs with derivatives detected by FID.

Chromatography. Samples of the native material were dissolved by brief sonication in 10 mM Na citrate, pH 5.5, and a small amount of undissolved material was removed by centrifugation. The sample was loaded onto a 1 × 8-cm column of DEAE-Sephadex (citrate form) equilibrated in the same buffer and eluted first with a 40-ml linear gradient to 0.05 M Na citrate and then with a 100-ml linear gradient to 0.6 M Na citrate with a flow rate of 8 ml/hr. Four-ml fractions were collected, and samples were taken for estimation of total sugars and uronic acid. The two fractions resolved were dialysed against running deionized H_2O, the glycosyluronic acids were reduced with CMC and $NaBD_4$, and alditol acetates and per-*O*-methylated alditol acetates were prepared as described above.

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REFERENCES

1. Bacic, A. J., Harris, P. J. and Stone, B. A. (1988) in *The Biochemistry of Plants* (Preiss, J., ed.). Academic Press, New York (in press).
2. Wilkie, K. C. B. (1979) *Adv. Carbohydr. Chem. Biochem.* **36**, 215.
3. Carpita, N. C. (1983) *Plant Physiol.* **72**, 515.
4. Carpita, N. C. (1984) *Plant Physiol.* **76**, 205.
5. Lamport, D. T. A. (1980) in *The Biochemistry of Plants* (Preiss, J., ed.) Vol. 3, p. 501. Academic Press, New York.
6. Carpita, N. C. (1986) *Plant Physiol.* **80**, 660.
7. Scalbert, A., Monties, B., Lallemand, J-Y., Guittet, E., and Rolando, C. (1985) *Phytochemistry* **24**, 1359.
8. Jarvis, M. C. (1984) *Plant Cell Environ.* **7**, 153.
9. Lau, J. M., McNeil, M., Darvill, A. G. and Albersheim, P. (1985) *Carbohydr. Res.* **137**, 111.
10. Wada, S. and Ray, P. M. (1978) *Phytochemistry* **17**, 923.
11. Burke, D., Kaufman, P., McNeil, M. and Albersheim, P. (1974) *Plant Physiol.* **54**, 109.
12. Shibuya, N. and Nakane, R. (1984) *Phytochemistry* **23**, 1425.
13. Cleland, R. E. (1981) in *Encyclopedia of Plant Physiology, New Series* (Tanner, W. and Loewus, F. A., eds.) Vol. 13B, p. 225. Springer, Berlin.
14. Taiz, L. (1984) *Annu. Rev. Plant. Physiol.* **35**, 585.
15. Carpita, N. C., Mulligan, J. A. and Heyser, J. W. (1985) *Plant Physiol.* **79**, 480.
16. Avery, G. S., Jr. and Burkholder, P. R. (1936) *Bull. Torrey Bot. Club* **63**, 1.
17. Taylor, R. L. and Conrad, H. E. (1972) *Biochemistry* **11**, 1383.
18. Carpita, N. C. and Shea, E. M. (1988) in *Analysis of Carbohydrates by Gas–Liquid Chromatography and Mass Spectrometry* (Biermann, C. J., MacGinnis, G., eds). CRC Press, Boca Raton, Florida, (in press).
19. Ray, P. M. and Rottenberg, D. A. (1964) *Biochem. J.* **90**, 646.
20. Anderson, R. L., Clarke, A. E., Jermyn, M. A., Knox, R. B. and Stone, B. A. (1977) *Aust. J. Plant Physiol.* **4**, 143.

21. Fincher, G. B., Stone, B. A. and Clarke, A. E. (1983) *Annu. Rev. Plant Physiol.* **34**, 47.
22. Takeuchi, Y. and Komamine, A. (1980) *Physiol. Plant.* **50**, 113.
23. Carpita, N. C. and Whittern, D., (1986) *Carbohydr. Res.* **146**, 129.
24. Murashige, T. and Skoog, F. (1962) *Physiol. Plant.* **15**, 473.
25. Carpita, N. C. (1985) *Plant Physiol.* **79**, 485.
26. Carpita, N. C. and Kanabus, J. (1987) *Anal. Biochem.* **161**, 132.
27. Knutsen, C. A., and Jeanes, A. (1968) *Anal. Biochem.* 470.
28. Galambos, J. T. (1967) *Anal. Biochem.* 119.
29. DuBois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A. and Smith, F. (1956) *Anal. Chem.* **28**, 350.
30. Blakeney, A. B., Harris, P. J., Henry, R. J. and Stone, B. A. (1983) *Carbohydr. Res.* **113**, 291.
31. Hakomori, S-I. (1964) *J. Biochem.* (*Tokyo*) **55**, 205.
32. Sweet, D. P., Shapiro, R. H. and Albersheim, P. (1975) *Carbohydr. Res.* **40**, 217.