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PECTIC POLYSACCHARIDES OF MESOPHYLL CELL WALLS OF PERENNIAL RYEGRASS LEAVES

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Key Word Index—*Lolium perenne*; Gramineae; ryegrass; mesophyll; cell wall; pectic polysaccharides; homogalacturonan; rhamnogalacturonan.

Abstract—Extraction of mesophyll cell walls from leaves of perennial ryegrass with CDTA, a chelating agent, removed 25 mg uronic acid g^{-1}, largely in the form of a polymer which spontaneously precipitated on removal of the CDTA or during subsequent purification. Methylation analysis, before and after reduction, showed that the precipitated polymer was a 1,4-linked homogalacturonan essentially free from neutral sugar residues, with a low degree of acetylation (3.6%) and methyl esterification (3.3%). Hot water (HW) extracted further acidic material (5 mg uronide g^{-1} cell wall) which could be resolved by ion-exchange chromatography into neutral mixed-linked glucan and bound rhamnogalacturonan fractions. The latter co-chromatographed with sugar residues typical of 3-, 4- and 6-linked galactan and arabinoxylan. Pectin esterase promoted the release from cell walls of HW-soluble rhamnogalacturonan by polygalacturonase, but had no effect on the release of CDTA-soluble uronide. The presence of both homogalacturonan and rhamnogalcturonan, typical of dicotyledons, suggested that the pectic polysaccharides of the Gramineae differed from those of other plants in amount only, rather than nature.

INTRODUCTION

Chemical studies made predominantly with pectic material extracted from the walls of cells growing in culture have demonstrated a commonality of structure amongst what was once considered a particularly heterogeneous group of polysaccharides. Fractions of pectic polysaccharides from the primary walls of dicotyledonous plants now can be categorized as homogalacturonans essentially free of neutral sugars or as one of two rhamnogalacturonans, RG-I [1] and RG-II [2, 3], of more complex structure in which neutral sugars and other compounds make a substantial contribution The presence and relative proportions of these pectic fractions varies depending on the phylogenic origin of the plant and the cell type from which they were extracted.

There is also good evidence that the pectic fraction of dicotyledonous walls forms a discrete network independent of, but linked to a xyloglucan-cellulose network. Tomato cells adapted in culture to an inhibitor of cellulose and xyloglucan synthesis still form a functioning wall consisting only of a cross-linked network of pectic polysaccharide [4]. Further evidence for a dual polysaccharide network was provided by the microscopic examination of replicas of freeze-fractured onion cell walls. Chemical extraction of pectic material caused the disappearance of one network leaving a second intact network of cellulose and xyloglucan [5]. Although the latter was an observation made of a monocotyledonous plant, *Allium* spp. are known to possess higher levels of pectic substances than most monocotyledonous species [6]. It remains to be demonstrated how far these observations extend to other monocotyledons and, in particular, to the Gramineae which contain the majority of monocotyledonous plants of commercial importance.

Pectic polysaccharides evidently play a different and possibly less significant role in the Gramineae. The amounts of pectic material found are substantially lower than those encountered in the majority of dicotyledonous plants and they are associated with different polymers [7, 8]. Their distribution within the wall also appears to differ since, unlike dicotyledons, tissues of members of the Gramineae are highly resistant to maceration by pectic enzymes [9]. In addition, the pectic network observed in tomato walls was not evident in barley endosperm walls adapted to the same inhibitor of cellulose synthesis [10]. However, walls of this type have a highly specialized function and cannot be taken as typical of the walls of vegetative cells.

There are few systematic studies of the pectic fraction of graminaceous cell walls and fewer still based on homogeneous starting material. Most of these have focused on cell wall development and therefore have made use of very young cells in culture [11] or those derived from developing coleoptiles [12]. The present work takes

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advantage of the ease of extraction and purification of grass mesophyll cells [13, 14] in order to determine the composition of the pectic fraction of the wall of a mature primary cell and to establish the degree of concordance with previously published data on pectic polysaccharides.

RESULTS AND DISCUSSION

Although sequential extraction of mesophyll cell walls with CDTA and hot water solubilized 200 mg g^{-1} dry matter, only 30.6 mg g^{-1} mesophyll cell wall was recovered as uronic acid. This represented *ca* two-thirds of the total uronide content of the unextracted wall. The major part of the uronide was solubilized by CDTA (24.9 mg g^{-1} cell wall) and much of this (22.0 mg g^{-1} cell wall) often spontaneously precipitated on removal of the CDTA by dialysis. Once recovered and freeze-dried the spontaneously-precipitated material was insoluble in water even when converted to the sodium or potassium salt. A smaller fraction of the hot-water (HW)-soluble material (3.1 mg uronide g^{-1} cell wall) occasionally behaved in a similar manner, although this fraction was partially soluble in water after recovery. Analysis showed the material spontaneously precipitating from the CDTA extract was composed of uronic acid with only trace amounts of neutral sugars present (Table 1). Reduction with $NaBD_4$ of carbodiimide-activated carboxy groups and methylation analysis confirmed that the fraction was essentially a 1,4-linked homogalacturonan. The corresponding fraction from the HW extract closely resembled the CDTA homogalacturonan but was associated with a higher proportion of neutral sugars (Table 1). Both the CDTA and hot water precipitates showed a similar degree of acetylation (DA 3.6 and 4.7%, respectively), but differed in their degree of methyl esterification (DE 3.3 and 10.2%, respectively). Pectin esterase promoted the release of HW-soluble uronic acid from mesophyll walls by polygalacturonase, but had no detectable effect on the release of CDTA-soluble uronide. It is recognised that in grasses, highly esterified galacturonan appears to be localised in lignified cell-types, while developing and primary walls, like leaf mesophyll, accumulate pectic polysaccharides with a relatively low degree of methyl esterification [15]. Fractions precipitated by ethanol after removal of spontaneously precipitating material had a substantially lower molar percentage of uronide and far higher neutral sugar content (Table 1). These fractions were readily soluble in water and were subject to anion-exchange chromatography (Fig. 2). For reasons which remain unknown, spontaneous precipitation of homogalacturonan did not always occur on dialysis and concentration of the CDTA and HW extracts. In these cases, all of the polysaccharide in the extract was precipitated with ethanol and recovered as a single fraction with a composition that agreed with that predicted from the summation of the separated dialysis and ethanol precipitates (see

Table 1. Molar percentage composition of the precipitate formed spontaneously on dialysis and composition of CDTA and hot water extracts and fractions precipitated by ethanol after removal of the spontaneously formed precipitate

	CDTA extract		Hot water extract	
Component	Dialysis	Ethanol	Dialysis	Ethanol
Rhamnose	0.46	2.49	1.64	2.39
Fucose	nd	nd	0.32	0.11
Arabinose	0.40	7.55	1.0	8.85
Xylose	0.16	9.48	1.11	9.86
Mannose	0.09	0.94	0.15	0.44
Galactose	0.44	6.30	2.02	7.68
Glucose	1.03	14.74	2.85	19.92
Uronic acid	91.15	42.00	79.15	31.93
Methanol	2.97	4.15	8.04	8.31
Acetyl	3.31	12.35	3.71	10.51

nd: not detected.

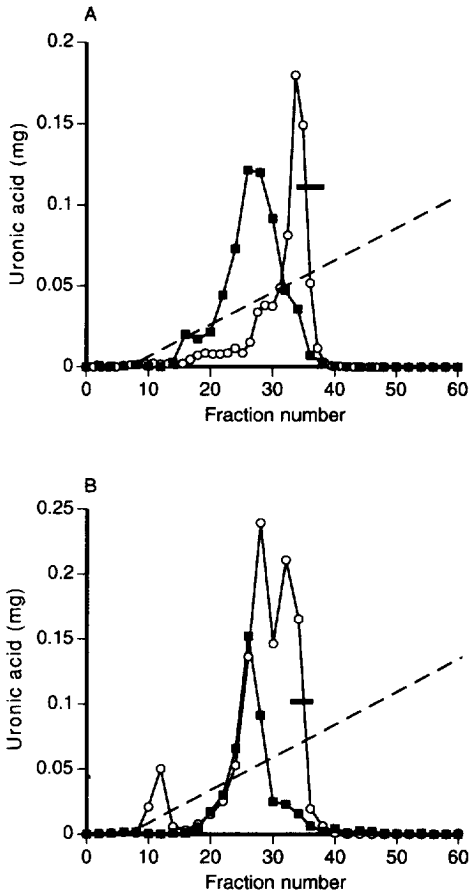

Fig. 1. Fractionation of CDTA-soluble (A) and hot water-soluble (B) extracts of leaf mesophyll on DEAE Sepharose. Samples were applied at pH 6.6 and eluted with a 0–1.0 M NaCl gradient (- - - -). Superimposed on each graph are separations of samples in which the homogalacturonan precipitated and was removed before application to the column (■) and where the homogalacturonan failed to precipitate and was included in the separation (○). The bar indicates the presence of a precipitate in the eluted sample from separations which included homogalacturonan.

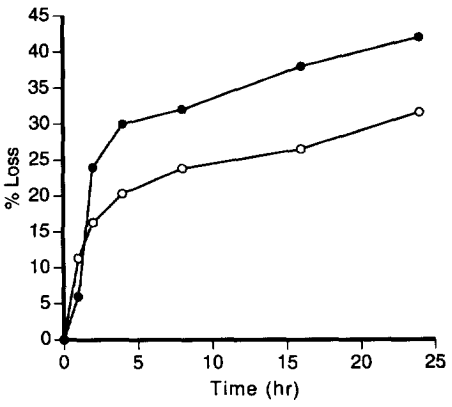

Fig. 2. Percentage loss of CDTA-soluble (○) and hot water-soluble (●) uronic acid from mesophyll cell walls incubated at 40° with polygalacturonase and pectin esterase.

Table 1). Homogalacturonan that failed to precipitate at this stage invariably precipitated on elution from ion-exchange columns (Figs 1a and 1b).

Much of the glucose found in both CDTA and HW extracts ran with the void volume when applied to anion-exchange columns (Table 2). Methylation analysis showed that this was in the form of 4-linked residues in the case of the CDTA extract (possibly leaf starch) and 3- and 4-linked glucan in the case of the HW extract. The ratio of 3-linked to 4-linked residues in HW extracts was 2.6:7.4, close to the 3:7 ratio typical of grass and cereal mixed-linked glucans [16]. The low amount detected and the ease of extraction of this polymer is consistent with the view of Carpita and Gibeaut [17] that mixed-linked glucan is a transient polymer of importance during growth, but of no structural significance in the mature wall.

Application of both extracts to ion-exchange columns allowed the separation of a uronide-rich peak (C2, H1) eluting immediately before the homogalacturonan peak (C3 and H2) when this was present (Fig. 1). The CDTA fraction contained an additional fraction (C1) which weakly bound to the anion-exchange column because of its low uronide content and it was readily eluted. The bound and eluted material from both extracts had a similar neutral sugar composition when allowance was made for the different uronide contents of the fractions (Table 2). Methylation analysis confirmed that the xylose was present in all of the fractions as terminal or 4-linked units and that two in every three residues carried a substituent at the 2-*O* (40%) or 3-*O* (60%) position. the extent of substitution was slightly lower than that reported for xylan extracted from maize coleoptiles or millet cells in culture [11]. Arabinose was found predominantly as terminal arabinofuranosyl units (85%), although small and variable amounts of 2-, 3- and 5-linked units were also detected. Although it is not possible to specifically assign the arabinose units to any one polymer, the higher proportion of arabinose and galactose present in fraction C2 compared to other fractions suggested that arabinose units were associated with both xylan and galactan polymers as would be expected from previous work [17]. Galactose was found as terminal, 4-,3- and 6-linked units; the presence of the 2,4-di-*O* methylgalactitol derivative was also detected indicating that the 3- and 6-linked units were associated in a manner typical of type II arabinogalatan [18, 19]. Similar partially methylated products were observed in maize coleoptiles and millet cells in culture [11], but it was thought that type II arabinogalactans were not structural components of the wall but components of transient peptidoglycans. Most rhamnose units were extensively substituted (80%) and recovered as the 3-*O*-methyl derivative. Terminal and 2-linked units were rarely detected as appears typical in rhamnogalacturonan of grasses [11, 20].

Reduction of fractions with borodeuteride before methylation gave the expected increase in recovery of terminal and 4-linked galactose units and, in addition, an increased recovery of the corresponding glucose units. The major fragments produced by EI-mass spectrometry containing the C-6 of the terminal (*m/z* 233/235) or in-chain (*m/z* 205/207) hexitols were monitored. The *m/z* 235 ion predominated in the spectrum of terminal glucose units indicating that most derived from glucuronic acid. Fragments from 4-linked glucose units, however, did not show a 2 mu increase and *m/z* 207 was not detected. The reason for the increased recovery of 4-linked glucose after reduction is not known.

Table 2. Molar percentage composition of fractions of CDTA and hot water extracts separated by ion-exchange chromatography on DEAE Sepharose

	CDTA extract				Hot water extract		
Component	C_{void}	C1	C2	C3	H_{void}	H1	H2
Rhamnose	0.64	0.23	7.87	2.17	7.50	9.50	4.26
Fucose	3.11	1.98	2.40	1.77	0.51	0.87	0.42
Arabinose	18.17	30.12	17.84	3.76	17.65	12.99	5.72
Xylose	10.50	34.83	12.22	1.55	7.65	18.67	5.15
Mannose	11.23	2.10	0.60	2.35	0.80	0.41	0.23
Galactose	22.19	17.21	29.84	11.56	16.72	15.88	2.96
Glucose	32.51	8.83	3.97	8.81	33.49	1.88	0.68
Uronic acid	1.64	4.70	25.26	68.03	15.70	39.80	80.57

The ease of extraction of a homogalacturonan with the calcium sequestering agent strongly suggested that this polymer was not directly covalently linked to other wall components but existed in discrete form. It is possible that its retention within the wall and interaction with other polymers was simply physical, a product of interlacing with other polymers strengthened by the presence of intermolecular calcium-bridged junction zones. In which case, the structural role played by the rhamnogalacturonan fraction is obscure. However, treatment of mesophyll walls with pectic enzymes did not lead to fragmentation of the polymer and the rapid release of CDTA-soluble uronide (Fig. 2). In fact, the initial loss and the extent of hydrolysis was higher for HW-soluble uronide. The greater apparent availability of HW-soluble galacturonan may simply reflect a concentration of part of this fraction on the outside of the wall in the region of points of contact with other cells. However, as Ishii [9] found, treatment with pectic enzymes did not cause cell separation when applied to clumps of intact mesophyll cells. Extraction of rhamnogalacturonan required a modicum of hydrolysis (chemical or enzymatic) and was always accompanied by glucuronoarabinoxylan which co-eluted from columns. This argues that rhamnogalacuronan provides a covalent link to the glucuronoarabinoxylan network, presumably through the 4-linked galactan. The ratio of in-chain rhamnose to galacturonic acid found for C2 and H1 was lower than previously described for RG I-type structures [1]. The 'excess' galacturonic acid units, if found in contiguous regions essentially free of rhamnose, could provide regions of rhamnogalacturonan able to form junction zones with homogalacturonan providing an alternative or additional link between pectic and other wall polysaccharides.

Overall the results are consistent with the Type II cell wall model proposed by Carpita and Gibeaut [17] and indicate that the pectic polysaccharides found in Gramineae differ from those found in dicotyledonous plants in amount rather than nature. The minor differences between the present results and those obtained elsewhere, particularly in the extent to which polymer chains were substituted, probably reflect real differences in the data obtained from developing cells as opposed to the mature, differentiated primary wall found in leaf mesophyll.

EXPERIMENTAL

Plant material. Mesophyll cells were released from the leaves of freshly harvested perennial ryegrass (*Lolium perenne* L. var. Perma) grown as a pure sward by hand grinding in a mortar in ice-H_2O as previously described [13, 14]. Harvested mesophyll cells were then prepd as a thick aq. suspension and broken by passage through a French Pressure Cell (American Instrument Co., U.S.A) at 103.4 MPa. Cell contents were removed from broken cells by exhaustive washing with ice-H_2O and mesophyll cell walls (MCW) recovered by filtration and freeze-dried.

Extraction of pectic polysaccharides. MCW (*ca* 2 g) were first suspended in 0.05 M *trans*-1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetracetic acid (CDTA) in pH 6.5 acetate buffer for 24 hr at 20° with constant stirring [20]. The suspension was centrifuged at 1000 *g*, and the pellet resuspended in a fresh buffered CDTA soln and the extraction repeated. Filtrates were combined, dialysed against running H_2O at 4° and concd by rotary evapn. On most occasions, a gelatinous ppt. formed which was collected by centrifugation, washed with 80% EtOH and freeze-dried (yield *ca* 40 mg g^{-1} MCW). EtOH (× 4 vols) was added to the remaining concd supernatant and the ppt. formed also collected by centrifugation, washed with 80% EtOH, dissolved in H_2O and freeze-dried (yield *ca* 60 mg g^{-1} MCW).

The CDTA-insoluble residue was resuspended in 200 ml H_2O and heated under reflux for 90 min. The suspension was then filtered while hot through a sintered glass funnel. The residue was again extracted with hot H_2O, filtered and the two filtrates combined. The soln was dialysed against running H_2O at 4° for 24 hr, concd by rotary evapn and × 4 vols of EtOH added. The resulting ppt. was collected by centrifugation, washed with 80% EtOH, redissolved in H_2O and freeze-dried (yield 30–40 mg g^{-1} MCW). A small amount of gelatinous ppt. was occasionally produced during dialysis and concn. When this occurred, the ppt. was collected by centrifugation before addition of EtOH (Yield 0–10 mg g^{-1} MCW).

Purification of extracts. The EtOH-ppt. frs were dissolved in 20 mM, pH 6.6 Pi buffer and separately applied to a DEAE Sepharose CL-6B 30 × 1.6 cm i.d. column (Pharmacia). The column was washed with two bed vols of Pi buffer and bound material eluted by the application of a 0–1.0 M NaCl gradient in 20 mM, pH 6.6 Pi buffer. Column frs were assayed for total carbohydrate and uronic acid content.

Analytical methods. Total carbohydrate was measured by the PhOH–H_2SO_4 method [22] and uronide by the colorimetric method of ref. [23]. The degree of methyl esterification and acetylation was calculated from measurements of MeOH [24] and HOAc [25] released by treatment with M KOH. Neutral sugar residues in extracts were sepd and determined by GC as their alditol acetates [26] after acid hydrolysis. The pattern of glycosidic linkages amongst the neutral sugars was determined by methylation analysis as previously described [27]. Identities of parent uronic acids and their linkage pattern were also determined by methylation analysis following reduction with $NaBD_4$ of the carbodiimide-activated carboxyl groups [28]. Identities of partially methylated alditols were assigned on the basis of their retention coefficients relative to two internal standards added, methyl-β-D-allose and quebrachitol, and confirmed, when necessary, by MS.

Enzyme preparation and digest. Polygalacturonase (PG. EC 3.2.1.15) was prepd from a commercial pectic enzyme prepn, Rohament P. (Rohm GmbH, Germany). Crude enzyme (~ 4 g) was dissolved in 20 ml H_2O and dialysed against running H_2O at 4° overnight. The dialysed enzyme was adjusted to 20 mM pH 4.5 with acetate buffer and applied to a cation-excange column

(Pharmacia CM-Sepharose CL-6B). The column was washed with 30 ml 20 mM buffer and the bound protein eluted with a 0–1.0 M NaCl gradient in pH 4.5 buffer. Frs were assayed for PG activity (release of reducing groups from polygalacturonic acid, PGA) and those eluted between 0.5 and 0.6 M NaCl retained, pooled and dialysed to remove the salt. Pooled enzyme was applied to a PBE 94 chromatofocusing column (Pharmacia) previously equilibrated with 0.025 M, pH 7.4 imidazole–HCl buffer. After application, the column was washed with a further 30 ml of equilibration buffer, followed by 200 ml of pH 4 Polybuffer 74 (Pharmacia) to generate a pH 7.4 to 4.0 gradient. PG eluted as a single peak at its pI of 5.8. After dialysis to remove buffer, the PG prepn was tested and found to be free of detectable activities likely to be active against cell wall polysaccharides other than PGA. Pectin esterase (PE EC 3.1.1.11) *ex* orange peel (Sigma) was similarly partially purified by ion-exchange chromatography on CM-Sepharose equilibrated with 20 mM, pH 8.0 Pi buffer and eluted with a 0–1.0 M NaCl gradient. The second PE peak, detected by the release of MeOH from apple pectin [24], eluting from the column with 0.3–0.4 M NaCl was collected, dialysed and retained. This prepn was also free from demonstrable hydrolase activities likely to be active against cell wall polysaccharide.

Enzyme digests were made in duplicate with 20 mg samples of cell walls in 20 ml pH 5 acetate buffer at 40°. Sufficient enzyme was added to give a theoretical ten-fold excess when related to composition. After incubation, insoluble residues were recovered by filtration and freeze-dried. Residues were then sequentially extracted with CDTA and hot H_2O and the extracted uronide measured as previously described. Reference to control values allowed the amount of CDTA-soluble and HW-soluble uronide released by the enzyme to be calculated.

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