Changes in Esterification of the Uronic Acid Groups of Cell Wall Polysaccharides during Elongation of Maize Coleoptiles[1]

Jong-Bum Kim and Nicholas C. Carpita*

Department of Botany and Plant Pathology, Purdue University, West Lafayette, Indiana 47907

ABSTRACT

Cell walls of grasses have two major polysaccharides that contain uronic acids, the hemicellulosic glucuronoarabinoxylans and the galactosyluronic acid-rich pectins. A technique whereby esterified uronic acid carboxyl groups are reduced selectively to yield their respective 6,6-dideuterio neutral sugars was used to determine the extent of esterification and changes in esterification of these two uronic acids during elongation of maize (Zea mays L.) coleoptiles. The glucosyluronic acids of glucuronoarabinoxylans did not appear to be esterified at any time during coleoptile elongation. The galactosyluronic acids of embryonal coleoptiles were about 65% esterified, but this proportion increased to nearly 80% during the rapid elongation phase before returning to about 60% at the end of elongation. Methyl esters accounted for about two-thirds of the total esterified galacturonic acid in cell walls of unexpanded coleoptiles. The proportion of methyl esters decreased throughout elongation and did not account for the increase in the proportion of esterified galactosyluronic acid units during growth. The results indicate that the galactosyluronic acid units of grass pectic polysaccharides may be converted to other kinds of esters or form ester-like chemical interactions during expansion of the cell wall. Accumulation of novel esters or ester-like interactions is coincident with covalent attachment of polymers containing galactosyluronic acid units to the cell wall.

Growth and differentiation in plants is manifest in the coordinated expansion of the existing cell wall matrix of each cell and the assembly of new polymers onto cellulose microfibrils. Newly synthesized and secreted precursors are soluble until they reach extracellular sites of assembly, and several kinds of chemical modifications of polymers have been identified that may participate in maintenance of solubility (7). For example, the uronic acid units of PGAs[2] are methyl esterified during synthesis (17), and one purpose may be to prevent premature cross-linking with Ca^{2+} during secretion. Esterification eliminates the electrical charge and, hence, the

binding site for Ca^{2+}. Cleavage of the methyl esters by pectin methylesterase not only permits cross-linking of neighboring carboxyl groups with Ca^{2+} to form gels (24) but also generates a negatively charged matrix that may induce a localized decrease in pH involved in the growth process (21, 22).

In grasses, pectins constitute a very small portion of the cell wall (6), and their role in providing a localized charged environment is perhaps replaced functionally by the glucosyluronic acids of the GAXs. The contiguous $(1\rightarrow4)\alpha$-D-linked galactosyluronic acid units form a contorted orientation during binding to Ca^{2+} to form tight junction zones, whereas the glucosyluronic acids of GAX are single units widely spaced along the xylan backbone. Although the GlcA units are unable to complex with Ca^{2+} in the same manner as the GalA units of PGA, it is still not known whether the GlcA units are also esterified as part of the synthesis and secretion pathway and later deesterified by an enzyme with an action similar to that of pectin methylesterase.

Maness et al. (20) developed an elegant procedure for indirect determination of the total uronic acid units that are esterified. The methoxy group, or any other substitution, is a good leaving group for reduction by sodium borohydride of the esters to aldehydes and, subsequently, to their respective neutral sugars. We modified this technique slightly to reduce the carboxylic esters in imidazole buffer with sodium borodeuteride to generate 6,6-dideuteriosugars, which we could distinguish from nascent neutral sugars by GC-EIMS of their alditol acetates (Fig. 1). The free uronic acids are not reduced, but after preliminary reduction of the esters, the sample is divided into two fractions. In the first fraction, the remaining uronic acids are activated with a carbodiimide and reduced with sodium borodeuteride to give total uronic acid in the presence of nascent sugar. The second fraction is reduced with sodium borohydride to yield the proportion of esterified uronic acids when compared to the total sugar acids. This technique revealed that the GlcA units of the grass arabinoxylans were not capable of being reduced by $NaBD_4$ alone at any stage of coleoptile elongation and, hence, did not appear to be esterified. The GalA units were esterified, and the apparent degree of esterification increased during the rapid phase of elongation and decreased slightly when growth ceased. To our surprise, however, the proportion of methyl esters decreased slightly during elongation and was not associated with the increase and decrease during elongation in the apparent esterified GalA revealed by reduction with $NaBD_4$. In this report, we document the alterations in apparent ester-

[2] Abbreviations: PGA, polygalacturonan; CMC, 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-p-toluenesulfonate; GAX, glucuronoarabinoxylan; RG, rhamnogalacturonan; EIMS, electron impact MS.
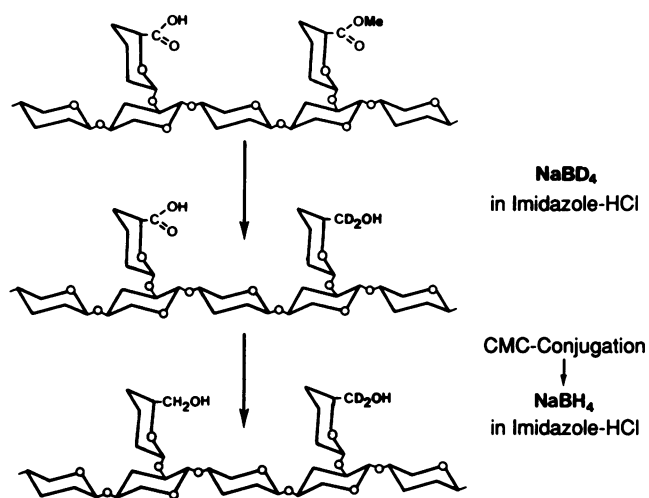
Figure 1. Reaction scheme for the selective reduction of esterified uronic acids. Uronic acids esterified with methyl or other leaving groups are reduced selectively by NaBD₄ to yield their respective 6,6-dideuteriosugars. The free acids remain unreduced but are activated by a water-soluble carbodiimide and are reduced subsequently with NaBH₄ to their respective neutral sugars. In practice, carbodiimide-activated uronic acids in an identical sample are reduced with NaBD₄ to yield total uronic acid in the presence of its respective neutral sugar.

ification of GalA during cell elongation and show that the putative nonmethyl esters are primarily in galactosyluronic acid-rich material retained by the cell wall matrix after extraction of the chelator-soluble pectins.

MATERIALS AND METHODS

Plant Material

Seeds of maize (*Zea mays* L. cv FR1141 × FR33) were sown in water-saturated beds of medium vermiculite and incubated in darkness at 28°C for as many as 8 d. Samples (10–50 coleoptiles minus leaves) were harvested intermittently throughout the period of elongation, and the lengths were recorded. The coleoptiles were frozen in liquid N_2 and kept at −20°C until all samples could be processed. Results represent data from two independent experiments with two samples taken at each time.

To prevent enzymic cleavage of esters during preparation of the cell walls, the frozen coleoptiles were suspended in 70% ethanol at 70°C for 30 min to inactivate pectin methylesterase (18). The coleoptiles were then washed with water and homogenized in a glass-glass Duall grinder in ice-cold 100 mM NaCl. The wall material was pelleted at 1200g for 5 min, resuspended and washed sequentially three times with water, twice in chloroform:ethanol (1:1, v/v; 45°C, 30 min), twice in ethanol, and four times in water, and stirred vigorously overnight in 90% DMSO to remove starch (8). Samples of walls were saved for uronic acid and methanol determination, and the remainder was used for chemical reductions. In some experiments, chelator-soluble pectins were extracted from the purified cell wall (20–50 mg) two times with 20 mL 0.5% ammonium oxalate, pH 7.0, at 80°C. Extracted polymers

were filtered through a GF/F glass fiber filter mat to remove wall fragments, dialyzed against running deionized water, and lyophilized.

The ammonium oxalate fraction was dissolved in 10 mM Na citrate, pH 5.5, by brief sonication in a water bath, and undissolved material was removed by centrifugation 2500g for 10 min. The solution was applied to a 1- × 6-cm column of DEAE-Sephadex A-50 (Pharmacia, repackaged by Sigma) equilibrated in the same buffer. The elution was carried out first with a 40-mL linear gradient to 50 mM NaCl in 10 mM Na citrate, pH 5.5, and then with a 100-mL linear gradient to 600 mM NaCl in 10 mM Na citrate. Flow rate was 8 mL/h, and 4-mL fractions were collected. Each fraction was analyzed for uronic acid, and two major fractions were pooled. A small portion of soluble material remained bound to the column in 600 mM NaCl and was extracted from the gel with 3 M LiCl in 10 mM Na citrate. The three fractions were dialyzed against running distilled water and lyophilized. The materials were portioned for analysis of uronic acid and methanol content and subjected to the double reduction.

Reduction of the Uronic Acid Groups

Cell wall materials (16–20 mg) were suspended in small beakers containing 10 mL ice-cold 1 M imidazole-HCl, pH 7.0, and stirred vigorously for 30 min in an ice bath. NaBD₄ powder (200 mg) was added, and the mixtures were stirred for 5 min. Second and third batches of 200 mg NaBD₄ were then added 5 min apart, and the mixtures were stirred for an additional 1 h. Glacial acetic acid was added dropwise to destroy excess borodeuteride, and the suspensions were dialyzed against running deionized water for at least 40 h. The dialyzed suspensions were divided into two equal portions, frozen, and lyophilized.

Samples of the material from the primary reduction were saved for analysis of uronic acids. The remaining duplicate samples were suspended and gently stirred in 10 mL water, and pH was adjusted to 4.75 with dilute HCl. CMC powder (250 mg) was added, and the pH was kept at 4.75 by dropwise addition of dilute HCl (25). The pH stabilized after about 2 h, the solutions were chilled to ice temperature, and 2.5 mL 4 M imidazole(HCl), pH 7.0, was added. Two batches of 200 mg each of NaBH₄ or NaBD₄ were added to the paired samples, and the suspensions were stirred in an ice bath at least 1 h. The excess borohydride(borodeuteride) was destroyed by dropwise addition of glacial acetic acid, and the mixtures were dialyzed against running deionized water at least 40 h. Samples were frozen and lyophilized. Samples of polygalacturonic acid with varying degrees of methyl esterification for use as standards to test the method were graciously donated by Dr. James BeMiller, Whistler Center for Complex Carbohydrates, Purdue University, and Dr. Kevin Hicks, U.S. Department of Agriculture-Agricultural Research Center, Eastern Regional Research Center.

Chemical Analyses

Uronic acid in 0.5 mg/mL aqueous suspensions was determined by a carbazole assay in which sulfamate was added to reduce neutral sugar interference (14) or a modification in

which sulfamate and *m*-hydroxydiphenyl were used to completely eliminate interference by neutral sugars (12). Methanol from 5- to 10-mg samples of cell wall (or 1–2 mg of material from ammonium oxalate extracts) was released by saponification essentially as described by Wood and Siddiqui (26) but with some slight modifications that increase sensitivity and reproducibility. The samples were suspended in 0.75 mL water in Eppendorf centrifuge tubes, and 0.25 mL 1.5 M NaOH was added. The samples were mixed and let stand at ambient temperature for 30 min. The samples were chilled on ice, and 0.25 mL 4.5 M H_2SO_4 was added. The mixtures were centrifuged to sediment the wall material, and 1.0 mL of the solution was withdrawn for analysis. The assay was carried out as described by Wood and Siddiqui (26) except that dilution of the arsenite with water was omitted and fresh pentane-2,4-dione reagent was always used.

Cell wall material (approximately 1-mg samples) after the secondary reduction was hydrolyzed in 1 mL 2 M TFA containing 1 μmol *myo*-inositol (internal standard) at 120°C for 90 min. *tert*-Butyl alcohol (1 mL) was added, and the mixture was evaporated in a stream of nitrogen. The sugars were reduced with $NaBH_4$, and alditol acetates were prepared as described previously (10). Derivatives were separated in a 0.25-mm × 30-m column of SP-2330 (Supelco) temperature programmed from 160 to 240°C at 5°C/min with a 5- to 10-min hold at the upper temperature. Helium flow was 1 mL/min with a splitless injection. EIMS was with a Hewlett-Packard MSD at 70 eV and a source temperature of 250°C. The system was autotune programmed on the day of the sampling, and all samples were run in duplicate under the same tune. The proportion of 6,6-dideuterioalditol acetate was calculated as the mean of ratios of *m/z* 187/189, 217/219, 259/261, and 289/291. Because the uronic acids sometimes constituted a small fraction of the corresponding undeuterated neutral sugar and the fragments used to assay the amount of deuterium contained as many as 12 carbons, the ^{13}C spillover of M^+ + 2 was sometimes significant and was subtracted. Galactose and glucose standards were used to determine the M^+ + 1 and M^+ + 2 spillover. Because only half of the fragments of the borodeuteride-reduced uronic acid contain the two deuterium atoms, 100% GalA will yield 6,6-dideuteriogalactitol hexacetate, giving nearly equal amounts of *m/z* 187 and 189, and so forth. Hence, the proportion of uronic acid was determined by the equation:

$$\frac{M^+ + 2(-^{13}C)}{[M^+ - M^+ + 2]/2 + M^+ + 2(-^{13}C)}$$

Two samples from each of two experiments were derivatized, and each sample was run in duplicate; all values reported are the means of two experiments with each time point representing eight MS determinations, and each MS determination is the mean of four diagnostic mass ratios.

RESULTS

Efficacy of the Technique

PGA and methyl-esterified PGAs (37–100%) were used to determine the specificity and efficacy of the $NaBD_4$ reduction. Total uronic acids were measured colorimetrically before and

after reduction with $NaBH_4$ alone, and the percentage esterification was calculated from the difference in these measurements. Reduction of methyl-esterified PGA gave values about 90% of theoretical based on uronic acid and methanol content of the standards (Fig. 2). The remaining uronic acids were reduced with $NaBH_4$ after activation of the carboxyl groups with a water-soluble carbodiimide (25). After carbodiimide activation of the carboxyl groups, the solutions were chilled, and ice-cold imidazole buffer was added before addition of the $NaBH_4$. This modification buffered the reduction near neutral pH, but, more important, the imidazole seemed to slow the decomposition of the borohydride. The pH slowly increased to between 8 and 8.5 during the procedure. Reduction of the carbodiimide-activated carboxyls was generally >90%, even for PGA, before or after primary reduction with $NaBH_4$ alone (Fig. 2). We did not have methyl esters of glucosyluronic acids for standards, but there is little reason to suspect that the chemistry would be different. To determine whether Ca^{2+} cross-linked carboxyl units tightly enough to mimic esterification, $CaCl_2$ was added to solutions of PGA before addition of the imidazole buffer and $NaBH_4$. Gelling was noticeable with 1 mg/ml PGA in 10 mM $CaCl_2$, but addition of imidazole buffer partially dissolved the gels, and no PGA was reduced even in 1 M $CaCl_2$ (Fig. 2, inset). These experiments gave us confidence that $NaBD_4$ specifically reduced ester linkages or linkages that behaved chemically like esters, that the reductions were stoichiometric, and that Ca^{2+} cross-linked carboxyl groups could not be reduced.
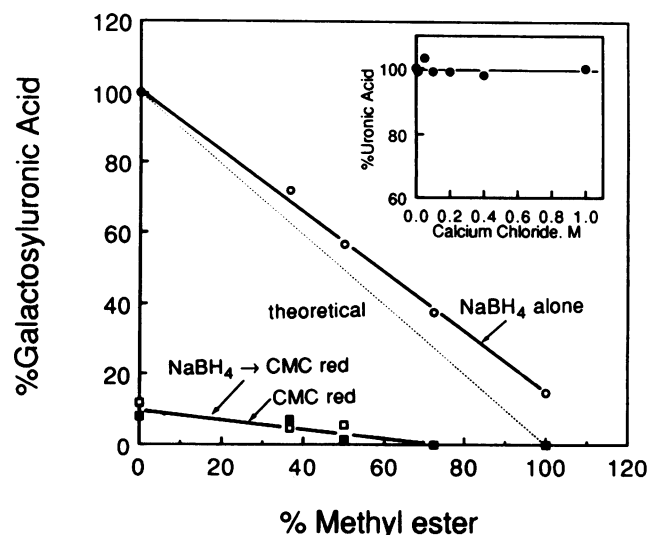

Figure 2. Stoichiometry of selective reduction of esterified uronic acids. Pectins with varying degrees of methyl esterification (37–100%) were reduced with $NaBH_4$ alone, and uronic acid content was determined before and after reduction. Other samples, with ($NaBH_4$ → CMC red) or without (CMC red) prior reduction with $NaBH_4$ alone, were then carbodiimide activated, and all uronic acids were reduced with $NaBH_4$. Theoretical line, Percentage of galactosyluronic acid expected to remain depending on the degree of methyl esterification. Inset, Inability of Ca^{2+} to catalyze the reduction of uronic acids with $NaBH_4$. Samples of unesterified PGA were preincubated with varying concentrations of $CaCl_2$ up to 1 M to form gel complexes, and $NaBH_4$ was added alone as described for the esterified uronic acids.

Reduction of Coleoptile Cell Wall Esters

Coleoptiles are about 2 mm long in mature seeds and elongate to >5 cm in about 5 d, the most rapid growth occurring between 2 and 4 d after planting (Fig. 3). This elongation is also associated with synthesis of the wall whose mass increases about 14-fold between 1 and 5 d of growth (Fig. 3). Measurement of total uronic acid before and after reduction with NaBD₄ alone revealed that the percentage of esterification increased concomitantly with the rate of elongation and accumulation of wall mass (Fig. 3). The percentage of esterification decreased slightly as the rate of elongation waned. We then examined galactose and glucose after subsequent carbodiimide-activated reduction to determine which uronic acids were esterified and to what extent.

Analysis of the 6,6-Dideuteriosugars by GC-MS

Alditol acetates separated by GLC were subjected to EIMS, and the 6,6-dideuteriosugar alcohol derivatives were identified by a shift of 2 atomic mass units in one-half of the primary fragments (and their secondary fragments) of the symmetrical hexaacetate derivatives (Fig. 4). The diagnostic pairs were m/z 187/189, 215/217, 259/261, and 289/291, and the relative amounts of uronic acid were calculated after selective ion monitoring from the ratios of these diagnostic ion pairs. Selective ion monitoring of alditol acetates of sugars after primary reduction with NaBD₄ alone and paired secondary reduction with NaBH₄ and NaBD₄ of carbodiimide-activated units showed that GalA and GlcA units were present in a ratio of about 2:1 during rapid elongation, but only GalA units were esterified (Fig. 5). The percentage of esterification of the GalA specifically was calculated in two ways: (a) from the difference in the proportion of diagnostic ion pairs in cell wall samples reduced secondarily with NaBH₄ compared with samples reduced with NaBD₄, the latter giving total GalA in the presence of galactose, and (b) the relative reduction in total uronic acid (Fig. 3) and the contribution of GalA deduced from GLC-MS. The percentage of esterification of
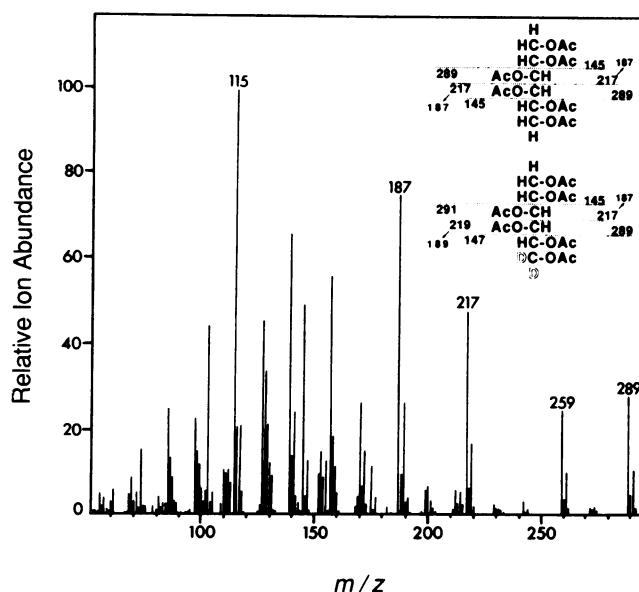

Figure 4. Ion spectrum from EIMS of a mixture of galactitol- and 6,6-dideuteriogalactitol hexaacetate derivatives separated from other alditol acetates by GLC. Esterified galactosyluronic acid units from walls of 72-h-old coleoptiles were reduced with NaBD₄ alone, and the remaining acids were carboxyl activated and reduced with NaBH₄. Diagnostic ions m/z 187, 217, 259, and 289 can arise from both derivatives, and, except for a small amount from undeuterated derivatives containing two ¹³C molecules, ions m/z 189, 219, 261, and 291 arise only from the 6,6-dideuterio derivatives. The amount of ¹³C spillover in each m/z fragment is calculated from galactose standards and subtracted from the total. For calculation of the ratio of uronic acid to sugar, dideuteriohexaacetates also contain an amount of m/z 187 equal to corrected m/z 189, for example, and this amount is subtracted from the total m/z 187, and so forth. Because the derivative lacking deuterium contains two m/z 187 and the dideutero derivative only one, one-half of the remaining m/z 187 is then compared with the corrected m/z 189 to give the ratio of each derivative.
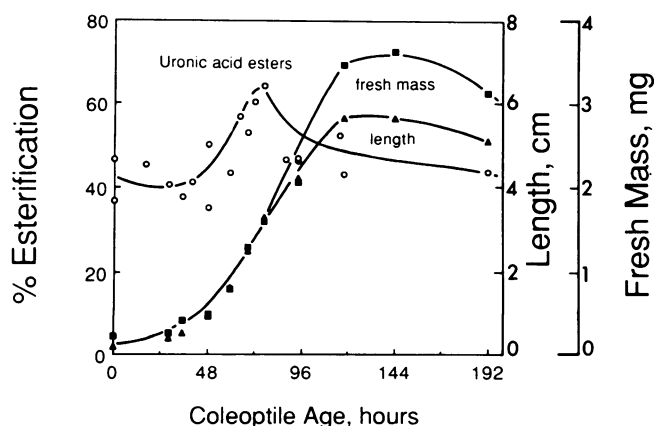

Figure 3. Changes in the proportion of esterified uronosyl acids in the coleoptile wall during elongation and accumulation of wall mass. The percentage of esterification was calculated from total uronic acid content determined colorimetrically (12) before and after reduction with NaBD₄ alone.

GalA deduced from MS was consistent with that estimated colorimetrically. The calculated percentage of esterification of GalA increased from about 65% to about 80% during the most rapid phase of elongation and decreased to about 60% when elongation had ceased (Fig. 6). The GalA and GlcA were initially 7 and 4.5% of the cell wall mass, respectively. The wall mass increased 14-fold during elongation (Fig. 3), and during this time GalA increased to about 10% of the wall, whereas GlcA decreased slightly to only 2.5% (Fig. 7B). The changes in mol% of the major neutral sugars during this time are consistent with the known changes in polymer synthesis and alteration in the grass coleoptile walls during elongation (3). The marked increase and subsequent decrease in the relative amounts of glucose are a result of the accumulation of the mixed-linkage (1→3),(1→4)-β-D-glucan specifically during rapid elongation and hydrolysis of the glucan at the end of elongation (Fig. 7A). Amounts of xylose also increase relative to arabinose during elongation, consistent with the observation of loss of substitution of arabinose from GAX to form more unsubstituted xylans (3, 4).
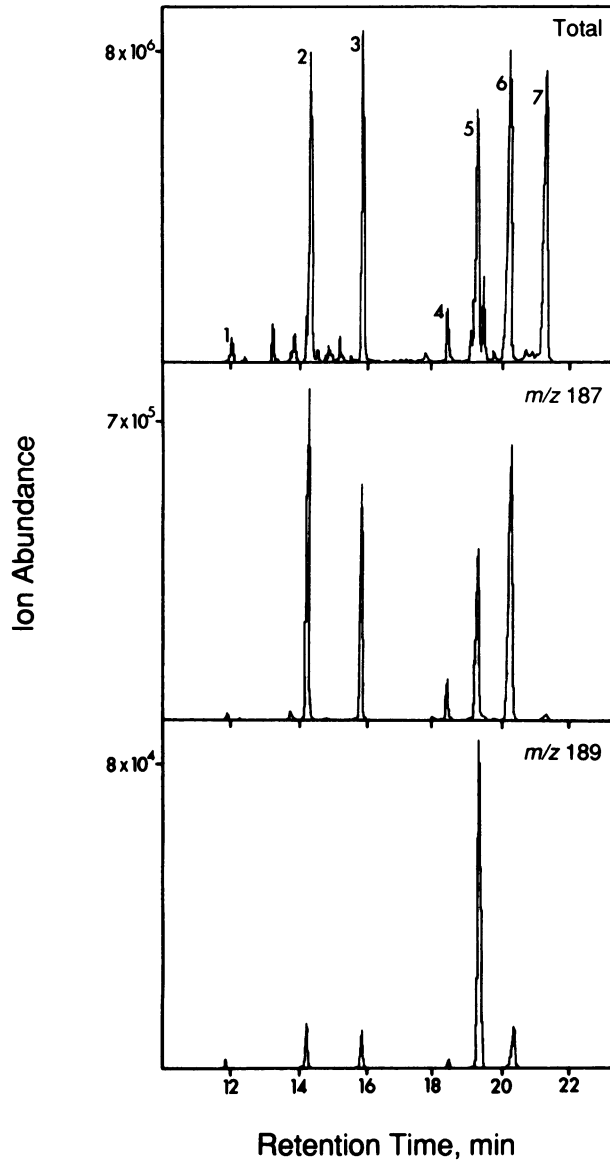
Localization of the Galactosyluronic Acid Esters

Pectins of dicots are of two general groups depending on how they are held into the wall matrix (16). First, those held only by Ca^{2+} in junction zones are solubilized by chelating agents. Second, those held by ester linkages, e.g. by diferulic acids attached to galactosyl or arabinosyl units of neutral sugar side chains of neighboring RGs (13), are removed subsequently by dilute alkali, presumably by cleavage of ester bonds (16). Wall structure in grasses is very different from dicots (5, 7). Pectins constitute a small portion of the cell wall and are thought to be removed mostly by chelating agents (6). Grass pectins also contain highly substituted GAXs, but most of these GAXs are held by ester linkages and removed by dilute alkali (3, 4, 11). Some PGA and RG may be retained by the wall after extraction with ammonium oxalate but the extent has never been documented fully. The dilute alkali used in our previous extractions also contained 3 mg/ml NaBH₄. Some of the GalA may have been reduced to Gal, although Maness et al. (20) indicated that the concentration used was probably too low to reduce significant amounts of esterified uronic acid.

The chelator-soluble pectins were further resolved into two fractions by ion-exchange chromatography (Fig. 8), similar to the pectins of a proso millet cell culture (6, 9). Fraction I comprised an association of PGA with GAX and type II arabinogalactan(protein)s and fraction II enriched in PGA and RG with associated arabinans and type I arabinogalactan(protein)s (6). These chelator-soluble pectins and those remaining attached to the cell wall were each subjected to double reduction. After primary reduction of depectinated
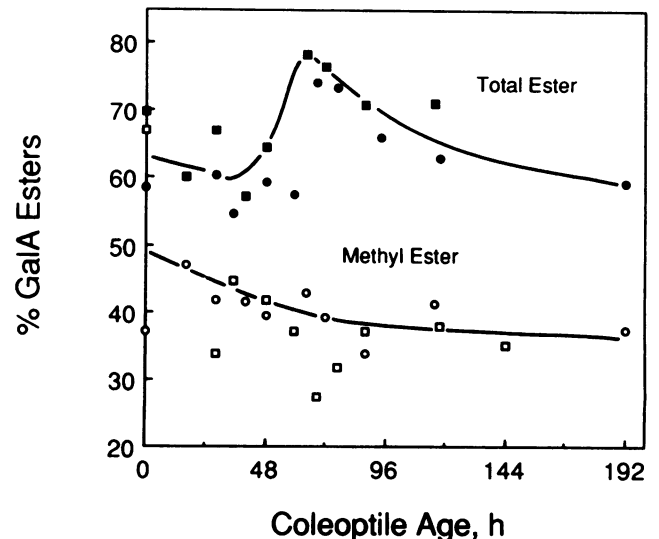

Figure 5. Reconstructed ion chromatograms of alditol acetate derivatives and esterified uronic acids reduced with NaBD₄. After reduction, the remaining uronic acids were carboxyl activated and reduced with NaBH₄. Top, Reconstructed chromatogram from total ions. Sugars identified are: 1, Rhm; 2, Ara; 3, Xyl; 4, Man; 5, Gal; 6, Glc; and 7, myo-inositol (internal standard). Middle, Reconstructed chromatogram from m/z 187 showing that amounts are proportional to total ions in each sugar. Bottom, Reconstructed chromatogram from m/z 189. Amounts of m/z 189 in Ara, Xyl, Man, and Glc are equal to that expected from ¹³C alone; only Gal contained m/z 189 arising from introduction of deuterium.

Figure 6. Comparison of total and methyl esters of galactosyluronic acid units of cell wall polymers during coleoptile growth. Total ester was determined from both diminution of total uronic acid after reduction with NaBD₄ alone and from GLC-MS of the alditol acetates. Methyl ester was determined from colorimetric analysis of methanol after saponification (26) and total uronic acid (12). In both determinations, the contribution of GalA to total uronic acid was determined by GLC-MS. Circles and squares represent data from two separate experiments.
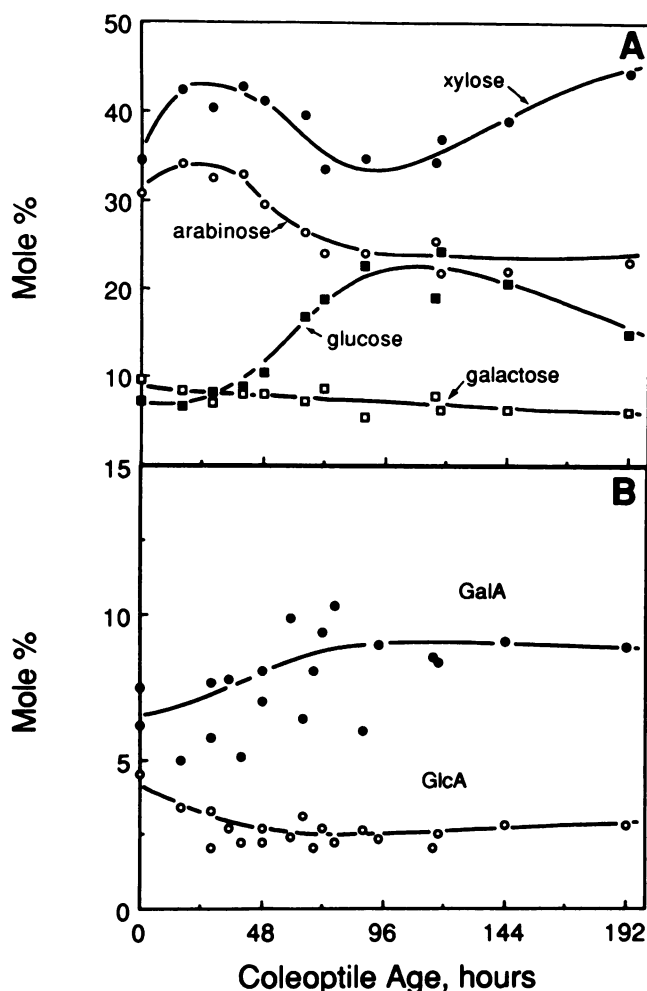
Figure 7. Changes in proportions of neutral sugars and uronic acids in cell wall polymers during elongation. A, Neutral sugars determined by GLC-MS of alditol acetates (after correction for galactosyl and glucosyluronic acid). B, Uronic acids were determined from samples doubly reduced with NaBD₄ and GLC-MS of their alditol acetates (calculated from the proportion of respective uronic acid as their 6,6-dideuterioacetate derivatives).

walls, newly soluble polymers and those extracted sequentially with 0.1 and 1.0 M NaOH were each subjected to the carbodiimide-activated secondary reduction. Methanol determinations after saponification were also made in duplicate samples, and together, these methods were used to determine free uronic acid, methyl-esterified uronic acid, and total esterified uronic acid in polymers extracted with ammonium oxalate and those remaining attached to the cell wall. Considerable amounts of GalA were in the ammonium oxalate extracts, but most of the GalA was in polymers that remained attached to the cell wall (Table I). Methanol accounted for most, but not all, of the esterified GalA in the ammonium oxalate extract and the two fractions derived from it. However, methanol released from the wall after ammonium oxalate extraction accounted for only about 30% of the total GalA, whereas double reduction demonstrated that >70% of the GalA was apparently esterified (Table I). These data indicate that the

novel esters are specifically in polymers tightly associated with the wall matrix. However, reduction of the esters of the galactosyluronic acid-rich polymers solubilized only about 12% of the total uronic acid remaining in the wall after extraction with ammonium oxalate (Table I). A majority of the remaining galactosyluronic acid-rich polymers needed 0.1 or 1.0 M NaOH to extract them, indicating that the ester linkages alone do not immobilize the polymers in the wall matrix.

DISCUSSION

The double-reduction technique provides an alternative to determination of total esterified uronic acids. Normally, methyl esters of uronic acids are deduced from measurements of total uronic acid and methanol released by saponification. This new technique revealed that other kinds of esters, or ester-like configurations, may be present and, perhaps, related to covalent linkage of galactosyluronic acid-rich polymers to the cell wall. The chemical nature of these esters remains unknown. Binding of Ca^{2+} to the GalA of PGA was not strong enough to induce reduction by NaBD₄ alone (Fig. 2, inset). The novel esters were found mostly retained in the cell wall after extraction with chelators. Some of the polymers were rendered soluble after primary reduction with NaBD₄ alone, but most were solubilized by 0.1 or 1.0 M NaOH. These fractions extracted with dilute alkali are also enriched in GAX (2–4), and the chelator-soluble material also contains a fraction resolved by ion-exchange chromatography that contains a galacturonic acid-rich polymer associated with GAX (6). These data indicate that GAX may be the specific polymer to which the galactosyluronic acid-rich polymers are attached.
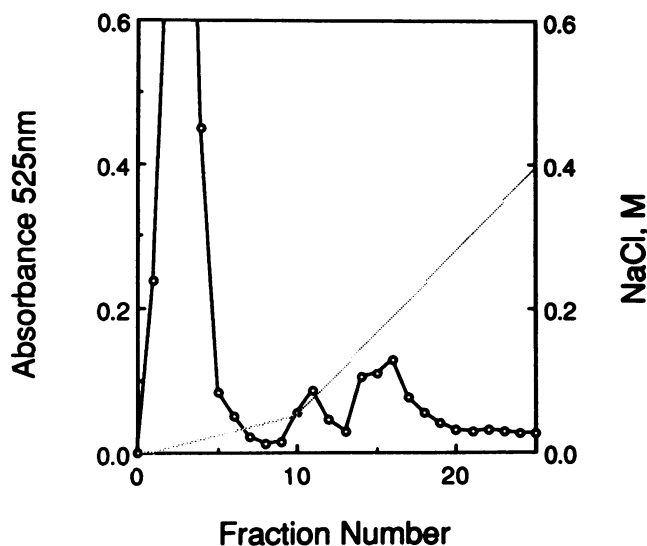

Fraction Number

Figure 8. Separation of chelator-soluble polymers by ion-exchange chromatography. Material extracted by ammonium oxalate was dissolved in 10 mM Na citrate, pH 5.5, and eluted first in a 40-mL linear gradient to 50 mM NaCl and then a 100-mL linear gradient to 0.6 M NaCl. Fractions of 4 mL were collected and assayed for uronic acid (12). Fractions I and II described in Table I are fractions 2 through 5 and 10 through 18, respectively.

Table I. *Comparison of Methyl-Esterified and Other Esters in Wall Fractions*

Fraction	Total GalA[a]	Total GlcA[a]	GalA	
			Methyl ester[b]	Total ester[c]
		mol%		
Total cell wall (177)[d]	9.1	2.6	40.0	68.2
Ammonium oxalate (58)	41.2	0.6	54.3	64.7
Fraction I	49.4	0.4	61.7	84.3
Fraction II	55.0	0.9	40.7	51.5
Fraction III			Not done	27.0
Cell wall minus ammonium oxalate (121)	7.1	2.3	28.6	72.8
Reduction soluble (14)	15.9	0.7	–[e]	79.8
0.1 M NaOH (29)	5.5	5.0	–	70.9
1.0 M NaOH (45)	7.4	1.8	–	71.7

[a] Determined from amount of 6,6-deuteriogalactose and 6,6-deuterioglucose in total sugar by GLC-EIMs of alditol acetates after reduction of all uronic acids with NaBD$_4$.   [b] Determined by paired colorimetric assays of total uronic acid (12) and methanol after saponification (26), with proportions of GalA and GlcA from GLC-EIMS of alditol acetates.   [c] Determined from ratios of the proportions of 6,6-dideuteriogalactose in galactose after carbodiimide-activated reduction with NaBH$_4$ and NaBD$_4$, respectively.   [d] Values in parentheses are total μg of uronic acid per coleoptile harvested at 66 h.   [e] –, Unable to determine methanol after reduction with borohydride or extraction with NaOH. Polymers extracted with ammonium oxalate were separated into two fractions by anion-exchange chromatography; these fractions and the cell wall remaining after extraction with ammonium oxalate were subjected to the double reduction. The cell wall was then separated into polymers rendered soluble by reduction and those extracted sequentially with 0.1 and 1.0 M NaOH.

Studies using both ^{1}H and ^{13}C NMR spectroscopy have shown that methyl groups are stoichiometric with the esterified uronic acid in plant pectins (15, 23). Maness *et al.* (20) also reported good agreement for the percentage of pectin methyl ester determined colorimetrically and by reduction with NaBH$_4$. The chelator-soluble pectin from grasses also contains a high proportion of methyl ester that accounts for much of the esterified uronic acid deduced by NaBH$_4$ reduction (Table I). We also examined the chelator-soluble pectins by ^{13}C-NMR spectroscopy and found resonances at 54 ppm from methyl groups, but there were no significant signals from ethyl or other small hydrocarbons or alcohols that could account for the novel esters (L.O. Sillerud, N.L. Fink, and N.C. Carpita, unpublished results). The novel esters are mostly in polymers resistant to extraction by chelators, however, and subsequent extraction of hemicellulosic polymers by alkali would have destroyed these esters. NMR spectroscopy often provides confirmation of structures deduced by other means; therefore, a critical reexamination of the possible cross-linking of GalA to sugars or hydroxycinnamic acid components should ensue. Lamport (19) suggested this possibility several years ago, but the lability of the linkage perhaps has made detection of these esters difficult. Brown and Fry (1) chemically synthesized some possible GalA-sugar esters, but, heretofore, formation of such bonds has not been demonstrated in plants. Dicots contain much more pectin than grasses; yet, these novel esters have not been reported. The alternative technique by Maness *et al.* (20) and our modification using NaBD$_4$ should now make possible these investigations. Our findings certainly warrant a closer look at the organization of pectic substances in dicotyledonous plants.

LITERATURE CITED

1. **Brown JA, Fry SC** (1989) The preparation and properties of novel uronoyl-sugar esters, model compounds for possible cross-links in the plant cell wall. *In* SC Fry, CT Brett, JSG Reid, eds, Proceedings of the Fifth Cell Wall Meeting, abstract No. 156. Scottish Cell Wall Group, Edinburgh, UK
2. **Carpita NC** (1983) Hemicellulosic polymers of cell walls of *Zea* coleoptiles. Plant Physiol 72: 515–521
3. **Carpita NC** (1984) Cell wall development in maize coleoptiles. Plant Physiol 76: 205–212
4. **Carpita NC** (1984) Fractionation of hemicellulose from maize cell walls with increasing concentrations of alkali. Phytochemistry 23: 1089–1093
5. **Carpita NC** (1987) Biochemistry of the "growing" cell wall. *In* DJ Cosgrove, DP Knievel, eds, The Physiology of Cell Expansion during Plant Growth. American Society of Plant Physiologists, Rockville, MD, pp 28–45
6. **Carpita NC** (1989) Pectic polysaccharides of maize coleoptiles and proso millet cells in liquid culture. Phytochemistry 28: 121–125
7. **Carpita NC, Gibeaut DM** (1988) Biosynthesis and secretion of plant cell wall polysaccharides. *In* DD Randall, DG Blevins, WH Campbell, eds, Current Topics in Plant Biochemistry and Physiology, Vol 7. University of Missouri, Columbia, MO, pp 112–133
8. **Carpita NC, Kanabus J** (1987) Extraction of starch with dimethylsulfoxide and quantitation by enzymic assay. Anal Biochem 161: 132–139

9. Carpita NC, Mulligan JA, Heyser JW (1985) Hemicelluloses of the cell walls of a proso millet cell suspension culture. Plant Physiol 79: 480–484

10. Carpita NC, Shea EM (1989) Linkage structure of carbohydrates by gas chromatography-mass spectrometry (GC-MS) of partially methylated alditol acetates. In CJ Biermann, GD MacGinnis, eds, Analysis of Carbohydrates by GLC and MS. CRC Press, Boca Raton, FL, pp 157–216

11. Carpita NC, Whittern D (1986) A highly substituted glucuronoarabinoxylan from developing maize coleoptiles. Carbohydr Res 146: 129–140

12. Filisetti-Cozzi TMCC, Carpita NC (1991) Measurement of uronic acids without interference from neutral sugars. Anal Biochem 197: 157–162

13. Fry SC (1986) Cross-linking of matrix polymers in the growing cell walls of angiosperms. Annu Rev Plant Physiol 37: 165–186

14. Galambos JT (1967) The reaction of carbazole with carbohydrates. I. Effect of borate and sulfamate on the carbazole color of sugars. Anal Biochem 19: 119–132

15. Grasdalen H, Bakøy OE, Larsen B (1988) Determination of the degree of esterification and the distribution of methylated and free carboxyl groups in pectins by ^{1}H-N.M.R. spectroscopy. Carbohydr Res 184: 183–191

16. Jarvis MC (1984) Structure and properties of pectin gels in plant cell walls. Plant Cell Environ 7: 153–164

17. Kauss H, Hassid WZ (1967) Enzymic introduction of the methyl ester groups of pectin. J Biol Chem 242: 3449–3453

18. Koch JL, Nevins DJ (1989) Tomato fruit cell wall. I. Use of purified tomato polygalacturonase and pectinmethylesterase to identify developmental changes in pectins. Plant Physiol 91: 816–822

19. Lamport DTA (1970) Cell wall metabolism. Annu Rev Plant Physiol 21: 235–270

20. Maness NO, Ryan JD, Mort AJ (1990) Determination of the degree of methyl esterification of pectins in small samples by selective reduction of esterified galacturonic acid to galactose. Anal Biochem 185: 346–352

21. Moustacas A-M, Nari J, Diamantidis, Noat G, Crasnier M, Borel M, Ricard J (1986) Electrostatic effects and the dynamics of enzymic reactions at the surface of plant cells. 2. The role of pectin methylesterase in the modulation of electrostatic effects in soybean cell walls. Eur J Biochem 155: 191–197

22. Nari J, Noat G, Diamantidis G, Woudstra M, Ricard J (1986) Electrostatic effects and the dynamics of enzymic reactions at the surface of plant cells. 3. Interplay between limited cell-wall autolysis, pectin methylesterase activity and electrostatic effects in soybean cell walls. Eur J Biochem 155: 199–202

23. Pfeffer PE, Doner LW, Hoagland PD, McDonald GG (1981) Molecular interactions with dietary fiber components. Investigation of the possible association of pectin and bile acids. J Agric Food Chem 29: 455–461

24. Powell DA, Morris ER, Gidley MG, Reese DA (1982) Conformations and interactions of pectins. II. Influence of residue sequence on chain association in calcium pectate gels. J Mol Biol 155: 517–531

25. Taylor RL, Conrad HE (1972) Stoichiometric depolymerization of polyuronides and glycosaminoglycuronans to monosaccharides following reduction of their carbodiimide-activated carboxyl groups. Biochemistry 11: 1383–1388

26. Wood PJ, Siddiqui IR (1971) Determination of methanol and its application to measurement of pectin methyl ester content and pectin methylesterase activity. Anal Biochem 39: 418–428