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Structure of Plant Cell Walls[1]

XIX. ISOLATION AND CHARACTERIZATION OF WALL POLYSACCHARIDES FROM SUSPENSION-CULTURED DOUGLAS FIR CELLS

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ABSTRACT

The partial purification and characterization of cell wall polysaccharides isolated from suspension-cultured Douglas fir (*Pseudotsuga menziesii*) cells are described. Extraction of isolated cell walls with 1.0 M LiCl solubilized pectic polysaccharides with glycosyl-linkage compositions similar to those of rhamnogalacturonans I and II, pectic polysaccharides isolated from walls of suspension-cultured sycamore cells. Treatment of LiCl-extracted Douglas fir walls with an endo-α-1,4-polygalacturonase released only small, additional amounts of pectic polysaccharide, which had a glycosyl-linkage composition similar to that of rhamnogalacturonan I. Xyloglucan oligosaccharides were released from the endo-α-1,4-polygalacturonase-treated walls by treatment with an endo-β-1,4-glucanase. These oligosaccharides included hepta- and nonasaccharides similar or identical to those released from sycamore cell walls by the same enzyme, and structurally related octa- and decasaccharides similar to those isolated from various angiosperms. Finally, additional xyloglucan and small amounts of xylan were extracted from the endo-β-1,4-glucanase-treated walls by 0.5 N NaOH. The xylan resembled that extracted by NaOH from dicot cell walls in that it contained 2,4- but not 3,4-linked xylosyl residues. In this study, a total of 15% of the cell wall was isolated as pectic material, 10% as xyloglucan, and less than 1% as xylan. The noncellulosic polysaccharides accounted for 26% of the cell walls, cellulose for 23%, protein for 34%, and ash for 5%, for a total of 88% of the cell wall. The cell walls of Douglas fir were more similar to dicot (sycamore) cell walls than to those of graminaceous monocots, because they had a predominance of xyloglucan over xylan as the principle hemicellulose and because they possessed relatively large amounts of rhamnogalacturonan-like pectic polysaccharides.

The primary cell-wall polysaccharides of gymnosperms have not been well characterized. In the earliest studies (30, 37), cells with primary walls were isolated from the cambial tissue of saplings or mature trees. Since only the glycosyl compositions of whole or fractionated cell walls were determined, little was learned of the structures of the cell-wall polysaccharides. Burke *et al.* (6) isolated the walls of suspension-cultured Douglas fir (*Pseudotsuga menziesii*) cells, and based solely on the glycosyl-linkage composition of the cell walls, concluded that they are quite different from the cell walls of six monocot species that were examined but are surprisingly similar to dicot, *e.g.* sycamore (*Acer pseudoplatanus*), cell walls. Based on the presence of 4,6-linked glucosyl, terminal and 2-linked xylosyl, and terminal galactosyl residues, it was suggested the Douglas fir cell walls contain xyloglucan. Xyloglucan is the principle hemicellulose of sycamore cell walls which also contain smaller amounts of xylan (27). On the other hand, while xylan is the principle hemicellulose of graminaceous monocots (27), no 2,4- or 3,4-linked xylosyl residues characteristic of xylans were detected in Douglas fir cell walls (6). Rhamnogalacturonan-like pectic polysaccharides account for at least 10% of sycamore cell walls (27), but Burke *et al.* (6), when examining gymnosperm cell walls, found none of the specifically linked glycosyl residues characteristic of rhamnogalacturonans, *e.g.* 2- and 2,4-linked rhamnosyl residues (28). Although no evidence of pectic polysaccharides was found (6), the seeming predominance of xyloglucan over xylan supported the conclusion that Douglas fir cell walls are more similar to dicot walls than to those of graminaceous monocots.

In the primary cell walls of the etiolated hypocotyls of another coniferous gymnosperm (*Pinus radiata*), a similar predominance of xyloglucan over xylan has been reported (24). However, no further data concerning the pine hemicelluloses have been published. Clearly, very little is known of the complex polysaccharides constituting the primary cell walls of gymnosperms.

This paper presents a survey of the wall polysaccharides of suspension-cultured Douglas fir cells that were extracted by sequential treatment of isolated cell walls with enzymes and chemical reagents. After partial purification, the glycosyl-linkage compositions of the polysaccharides were determined. The results indicated that the primary cell walls of Douglas fir contain the same types of polysaccharides that constitute the bulk of the noncellulosic polysaccharides of walls of suspension-cultured sycamore cells, including RG-I,[3] RG-II, homogalacturonan, xyloglucan, and glucuronoarabinoxylan. Differences in the detailed structures of the Douglas fir polysaccharides and their counterparts isolated from sycamore cell walls were observed.

MATERIALS AND METHODS

Cell Culture and Cell Wall Preparation. Suspension cultures of Douglas fir (*Pseudotsuga menziesii*) cells initiated from needles were grown at 26°C with aeration on gyratory shakers at 100 rpm in the dark in a medium described by Murashige and Skoog (31) that was supplemented with 2.5 mg/L thiamine, 250

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[3] Abbreviations: RG-I, rhamnogalacturonan I; RG-II, rhamnogalacturonan II; EGase, endo-β-1,4-glucanase (EC 3.2.1.4); EPGase, endo-α-1,4-polygalacturonase (EG 3.2.1.15); FAB-MS, fast-atom-bombardment mass spectrometry; KDO, 3-deoxy-*manno*-octulosonic acid.

mg/L *myo*-inositol, 30 g/L sucrose, and 1.0 mg/L 2,4-D. The cells from which walls were isolated were pale yellow in color when they were harvested.

Cell walls were isolated according to the procedure described by Talmadge *et al.* (36), except that, instead of a French pressure cell, a pressure bomb (Parr Instruments) operated at at least 1500 p.s.i. was used to rupture the cells. Cell wall preparations were treated with α-amylase to remove starch (36). The glycosyl-linkage compositions of cell walls treated with α-amylase from *Bacillus* species (type II-A, Sigma Chemical) or from porcine pancreas (type I-A: phenylmethylsulfonyl fluoride-treated, Sigma Chemical) were essentially identical (data not shown). This result indicated that the absence of noncellulosic, 3-linked glucans (see "Conclusion") in the cell wall preparations was not due to its solubilization by an endo-β-1→3,1→4-glucanase known to be present in the bacterial but absent from the porcine enzyme preparations (21). Extracellular polysaccharides were precipitated from culture filtrates in 70% ethanol (after removal of water-insoluble material by allowing it to settle at 4°C, and decanting), collected by centrifugation, dialyzed against distilled H_2O, and lyophilized.

Enzyme Purifications. Purification of EPGase from *Colletotrichum lindemuthianum* was performed as described (17). One unit of EPGase was defined as that amount of enzyme that released 1 μmol of reducing sugar per h from 0.25 μg of deesterified citrus pectin when the reaction was carried out at 25°C in 0.5 ml of 50 mM Na-acetate (pH 5.2).

EGase was purified from *Trichoderma viride* grown in the medium of Mandels and Andreotti (25) containing 7.5 g/L α-cellulose (Sigma Chemical) and 0.75 g/L Proteose Peptone (Difco). The purification procedure was a modification of that of Håkansson *et al.* (18) and Mandels and Reese (26). After filtering through Whatman GF/A glass filters, 20 L of culture filtrate was concentrated to 1 L using a Pellicon cassette system (Millipore, 10,000 mol wt cutoff). After lyophilization, the crude enzyme powder was extracted at 4°C with 50 mM ammonium acetate (pH 4.0) (100 ml buffer/10 g powder) with slow stirring for 1 h. After centrifugation (4°C, 8000*g*, 30 min), the extract was fractionated on a Bio-Gel P-30 column (8 × 56 cm) equilibrated in 50 mM ammonium acetate (pH 4.0) and the fractions containing EGase activity (assayed as described below) that eluted after a peak of β-glucosidase activity (assayed using *p*-nitrophenyl-β-D-glucoside as substrate) were pooled. The pooled fractions were chromatographed on a Sephadex G-10 column equilibrated in 20 mM K-phosphate (pH 7.0) to prepare the material for ion-exchange chromatography. The EGase-containing fractions from the G-10 column were applied to a DEAE-Sephadex A-50 column (3 × 60 cm) equilibrated in 20 mM K-phosphate (pH 7.0) and the column was washed with 0.6 L of equilibrating buffer and eluted with a 1-L, convex, logarithmic gradient of NaCl from 0 to 500 mM. Endo-β-1,4-glucanase activity eluted well after endo-β-1,3-glucanase activity (assayed by the production of reducing glycosyl residues from reduced laminaran). Fractions containing EGase activity were pooled, diaylzed against 20 mM K-phosphate (pH 7.0) using an animal membrane (Trojan Naturalamb), and lyophilized. The lyophilized enzyme was redissolved in distilled H_2O and chromatographed on a Sephadex G-10 column as described above. The enzyme-containing fractions were pooled and frozen for later use. One unit of EGase activity was defined as that amount of enzyme that released 1 μmol of reducing sugar per h from 10 μg of carboxymethyl cellulose (Hercules) when the reaction was carried out at 30°C in 0.5 ml of 50 mM Na-acetate (pH 5.2).

Extraction of Cell Wall Polysaccharides. Cell walls (15.0 g, dry weight) were suspended in 1.5 L of 1.0 M LiCl containing 0.02% thimerosal, and incubated 7 d at 25°C with magnetic stirring. The suspension was centrifuged for 15 min at 20,000*g*. The supernatant solution was passed through two layers of Whatman GF/C glass filters to remove cell wall residue, concentrated under reduced pressure at 40°C, dialyzed against distilled H_2O, and lyophilized. The pellet was resuspended and the LiCl extraction procedure repeated; the residue was suspended in 1.0 M LiCl with thimerosal a total of 15 times and incubated 7 d with magnetic stirring each time. (When extraction with 1.0 M LiCl was begun, it was not known that lengthy, repeated treatments would be necessary. Since pectic polysaccharides were extracted even after many treatments, this method cannot be recommended for general use.) After the final LiCl treatment, the wall residue was resuspended in distilled H_2O and centrifuged, and the supernatant solution was added to the final LiCl supernatant. The combined LiCl treatments extracted 24% (by weight) of the cell wall, but only 7.59 g of wall residue was recovered due to loss of material on glass filters.

To prepare the LiCl-residual wall material for EPGase treatments, it was twice resuspended in 50 mM Na-acetate (pH 5.2) and centrifuged. The wall residue was resuspended in 500 ml of 50 mM Na-acetate (pH 5.2) containing 0.02% thimerosal and 20,000 units of EPGase, and incubated at 25° C for 5 d with gentle magnetic stirring. After centrifugation (15 min, 20,000*g*), the supernatant solution was filtered using two layers of GF/C glass filters, concentrated under reduced pressure at 40°C, dialyzed against distilled H_2O, and lyophilized. The pellet was resuspended in the same buffer with the same amount of EPGase and incubated at 25°C for 2 d. After centrifugation, the pellet was twice resuspended in buffer and centrifuged, then resuspended once in distilled H_2O and centrifuged. All four supernatant solutions were combined after filtration through GF/C glass filters, concentrated under reduced pressure at 40°C, dialyzed against distilled H_2O, and lyophilized. Together, both EPGase treatments and subsequent washes solubilized 1.3% (by weight) of the LiCl-residual wall material or 1% of the cell wall. Only 7.35 g of wall residue was recovered after EGase treatments.

The wall residue remaining after EPGase treatment was resuspended in 500 ml of 50 mM Na-acetate (pH 5.2) containing 0.02% thimerosal and 24,000 units of EGase, and incubated at 25°C for 3 d with gentle magnetic stirring. The suspension was centrifuged (15 min, 20,000*g*), and the supernatant solution was filtered using GF/C glass filters, concentrated under reduced pressure at 40°C, passed through a column of Dowex-50W (H^+ form) to remove Na^+ ions, and lyophilized. The pellet was resuspended and treated with EGase as described above for 2 d. After centrifugation, the pellet was washed twice with buffer and once with distilled H_2O. The four supernatant solutions were combined, filtered, passed through Dowex-50W (H^+ form), and lyophilized. The two treatments with EGase released 10% (by weight) of the EPGase-residual wall material or 8% of the cell wall.

The 6.6 g of cell wall residue remaining after EGase treatment was resuspended in 1.32 L of 0.5 N NaOH containing 1 mg/ml $NaBH_4$ and incubated at 4°C for 20 h. After centrifugation (15 min, 20,000*g*, 4°C), the supernatant solution was filtered using Whatman GF/C glass filters and neutralized with acetic acid. To recover all material solubilized by NaOH and to prepare the residual material for further analysis, the pellet was twice resuspended in distilled H_2O and centrifuged, then resuspended in distilled H_2O. The pH of the suspension was adjusted to 5.5 by adding 1.0 M Na-acetate, and after centrifugation, the pellet was washed twice with 50 mM Na-acetate (pH 5.2), resuspended in distilled H_2O, dialyzed against distilled H_2O, and lyophilized. All supernatant solutions were combined, concentrated under reduced pressure and dialyzed against distilled H_2O using tubing with a nominal mol wt cutoff of 1000 (Spectrum Medical Industries). The NaOH-solubilized material was stored at 4°C prior to gel-permeation chromatography, to obviate the need to redissolve

the polysaccharides. An aliquot of the dialyzed material was lyophilized to determine how much material was extracted; a total of 13% (by weight) of the EGase-residual material, or 9% of the cell wall, was extracted in NaOH.

Deesterification and Enzyme Treatment of Cell Wall Polysaccharides. Polysaccharides to be deesterified were dissolved in 0.01 N NaOH and the pH was adjusted to 12.0 with 1.0 N NaOH. After 2 h at 4°C and pH 12.0, the pH was adjusted to 5.2 with acetic acid, and EPGase and thimerosal (final concentration of 0.02%) were added. After 24 h, deesterified and EPGase-treated polysaccharides were subjected to gel-permeation chromatography.

Enzyme Treatment of Polysaccharides in Column Fractions. Column fractions containing polysaccharides in 50 mM Na-acetate to be treated with EPGase or EGase were concentrated to 20% of their original volume under reduced pressure. Enzyme (approximately 50 units per mg of polysaccharide) was then added (activity of EPGase is higher in 250 mM Na-acetate [pH 5.2] than in 50 mM Na-acetate [pH 5.2]) (JR Thomas, unpublished observation) with thimerosal (final concentration of 0.02%).

Gel-Permeation Chromatography. Bio-Gels P-2, P-10, A-0.5m, and A-5m were obtained from Bio-Rad Laboratories. Bio-Gel P-10 (3.5 × 69 cm), A-0.5m (1.8 × 60 cm), and A-5m (3.5 × 61 and 2.3 × 46 cm) columns were equilibrated in and eluted with 50 mM Na-acetate (pH 5.2), collecting 10-, 2-, 10-, and 3.4-ml fractions, respectively. Pooled fractions from these columns were passed through Dowex-50W (H^+ form) prior to lyophilization and analysis, or concentrated under reduced pressure at 40°C prior to further gel-permeation chromatography. The Bio-Gel P-2 column (3.5 × 70 cm) was equilibrated and eluted in distilled H_2O collecting 5- or 10-ml fractions. Void and included volumes were determined by chromatographing dextran (Pharmacia) and glucose, respectively.

Analytical Techniques. The alditol acetate derivatives (1, 41) of neutral glycosyl and aceryl (35) residues were identified by GC-MS, and quantitated by GLC. A 30-m SP-2330 fused-silica GLC column (Supelco) was used isothermally at 235°C. Galactosyluronic acid, glucosyluronic acid, 4-*O*-methylglucosyluronic acid, and KDO residues were quantitated as their per-*O*-trimethylsilyl methyl glycosides, synthesized (10), and analyzed (41) as described.

Glycosyl-linkage compositions were determined by GLC and GC-MS of the partially *O*-methylated alditol acetate derivatives (4, 41) that were *O*-methylated using the procedure of Hakomori (19) as modified by Harris *et al.* (20). Methylated polysaccharides were purified (39) using Sep-Pak C_{18} cartridges (Waters Associates) and their glycosyl compositions determined by hydrolysis, reduction, and acetylation as described (41), and GLC analysis using a 30-m SP-2330 fused-silica column (Supelco). To determine the linkages of glycosyluronic acid residues, per-*O*-methylated polysaccharides were reduced with Li-triethylborodeuteride (1 M in tetrahydrofuran from Aldrich) for 1 h (41). After quenching with trifluoroacetic acid, the solvent was removed by evaporation under a stream of filtered air.

Cellulose was determined by the method of Updegraff (38). Total nitrogen and ash determinations were made by Galbraith Laboratories, Knoxville, TN. Protein content was estimated by multiplying the nitrogen content by 6.25. Carbohydrate contents were determined by combining glycosyl residue composition data, colorimetric assay responses (15), and relative response factors of different glycosyl residues in the colorimetric assay (KW Talmadge, unpublished data).

Analysis of native xyloglucan oligosaccharides using negative FAB-MS was performed by Anne Dell at Imperial College, London by dissolving samples in 5% acetic acid and loading 1 μl into a glycerol/thioglycerol mixture and using a VG Analytical ZAB-HF mass spectrometer fitted with an M-Scan fast-atom-bombardment gun. Xenon was used as the bombarding gas, and the gun was operated at 10 kV, 18 μamp. The mass spectrometer was operated at the full 8 kV accelerating voltage. Linear mass controlled scans were obtained in the 2000 to 300 mass range at a scan rate set to cover the complete instrumental mass range of 3300 mass units in 5 min. Spectra were recorded on oscillographic paper and counted manually.

RESULTS AND DISCUSSION

Glycosyl-Linkage Analysis of Douglas Fir Cell Walls. The glycosyl-linkage composition of the purified Douglas fir cell walls used in this study is presented in Table I. Also included in Table I are the glycosyl-linkage compositions of the extracellular polysaccharides released by the Douglas fir cells into their growth medium, and for comparison, the Douglas fir cell walls analyzed by Burke *et al.* (6). The glycosyl-linkage compositions of the walls used in the current study and that of Burke *et al.* (6) are very similar, except that in the latter, several components were not detected, *e.g.* 2- and 2,4-linked rhamnosyl, terminal fucosyl, and 2,4-linked xylosyl residues. The earlier investigators may have been prevented from detecting these quantitatively minor constituents because the packed GLC columns used probably did not separate them from the major constituents. The glycosyl-linkage composition of the extracellular polysaccharides secreted by suspension-cultured Douglas fir cells (Table I) suggested that the major components were arabinogalactan (11), xyloglucan (28), and xylan (28). The presence of xyloglucan in the extracellular polysaccharide was confirmed by treating the neutral fraction of the extracellular polysaccharides (separated from anionic polysaccharides by ion-exchange chromatography) with EGase; this yielded xyloglucan oligosaccharides similar to those released from cell walls by treatment with the same enzyme (M Davis, JR Thomas, AG Darvill, P Albersheim, unpublished data).

Glycosyl-Composition Analysis of Douglas Fir Cell Wall Fractions. The glycosyl-residue compositions of the material solubilized from cell walls by the four treatments used (LiCl, EPGase, EGase, and NaOH) are presented in Table II. Even though the cell walls had been treated with α-amylase, we concluded that all of the glucosyl residues in the LiCl-extracted material (23% of the carbohydrate, see Table II) was starch, because it was acid-labile (cellulose is not) and the glucosyl residues were 4- and 4,6-linked (data not shown). The amount of starch in the starting cell wall material that was extracted in LiCl was calculated. Of the starting cell wall material, 10% (by weight) was extracted in LiCl as nondialyzable carbohydrate, based on the carbohydrate content of the LiCl-extracted material (see "Materials and Methods"). Therefore, if 23% of the nondialyzable carbohydrate in the LiCl-extracted material was starch, then 2% of the starting material was extracted in LiCl as starch. This small amount of starch in the starting cell wall material did not significantly alter the percentages of cell wall accounted for by various components (given below).

The remaining 8% of the cell wall extracted in 1.0 M LiCl as nondialyzable carbohydrate was accounted for by pectic polysaccharides whose purification and characterization are described below. EPGase treatment of the residue remaining after LiCl extraction solubilized 1% (by weight) of the cell wall, all of which was carbohydrate (see "Materials and Methods"). All of the carbohydrate solubilized by EPGase was accounted for by pectic polysaccharides whose purification and characterization are described below. Thus, the LiCl and EPGase treatments together solubilized 9% of the starting wall material as pectic polysaccharides.

EGase treatment of the EPGase-residual wall material released an additional 8% (by weight) of the cell wall, all of which was carbohydrate (see "Materials and Methods"). Of the glycosyl

Table I. *Glycosyl-Linkage Composition of Douglas Fir Cell Walls, Cell-Wall Residue, and Extracellular Polysaccharides (Normalized to 100)*

Cell walls were isolated from suspension-cultured cells essentially as described by Talmadge *et al.* (36). Extracellular polysaccharides were precipitated from culture filtrates in 70% ethanol, dialyzed, and lyophilized prior to analysis. Cell-wall residue consisted of material that remained after LiCl, EPGase, EGase, and NaOH treatments of isolated cell walls. Permethylated alditol acetates were prepared, identified, and quantified as described in "Materials and Methods." For comparison, the data of Burke *et al.* (6) are included.

Glycosyl Residue	Linkage[a]	Cell Walls of Burke *et al.*[b]	Cell Walls	Cell-Wall Residue	Extracellular Polysaccharides
			mol %		
Glucosyl	Terminal	0	2.2	0.8	0
	4-Linked	37.0	24.0	41.0	6.8
	4,6-	7.0	5.6	13.0	16.0
Galactosyl	Terminal	7.6	12.0	6.3	5.9
	2-Linked	0	2.2	1.2	2.7
	3-	0	1.6	0.7	2.2
	4-	4.8	5.9	2.3	0
	6-	+[c]	0.8	0.4	2.7
	3,4-	0	1.1	0.5	0
	3,6-	0	+	+	9.8
	4,6-	0	0.9	3.8	0
Xylosyl	Terminal	4.5	6.0	5.3	12.0
	2-Linked[d]	8.4	3.4	2.4	11.0
	4-[d]	4.2	3.4	2.4	11.0
	2,3-[d]	0	0.7	0.3	0.6
	2,4-[d]	0	1.4	0.7	1.4
	3,4-[d]	0	0.7	0.3	0.6
Arabinosyl	Terminal	4.5	6.3	4.8	9.4
	3-Linked	5.4	4.5	2.4	1.9
	5-	12.6	6.2	4.5	2.9
	2,5-	0	2.2	0.7	0
	3,5-	+	3.0	1.0	0
Fucosyl	Terminal	0	2.8	2.4	2.7
Rhamnosyl	Terminal	0	0	0	1.0
	2-Linked	0	1.5	1.1	0
	2,4-	0	2.0	1.8	0

[a] Where ambiguous, hexosyl, deoxyhexosyl, and xylosyl residues were assumed to be in the pyranose ring form and arabinosyl residues in the furanose ring form. [b] See Burke *et al.* (6). [c] Amounts were not determined, but derivatives were present. [d] Amounts were estimated by mass spectrometric analysis.

Table II. *Glycosyl-Residue Composition of Material Extracted from Douglas Fir Cell Walls by Various Treatments (Normalized to 100)*

Isolated cell walls were treated sequentially with the reagents and enzymes listed below. After dialysis (in the case of LiCl-, EPGase-, NaOH-extracted material) or passage through Dowex-50W (H^+ form) (in the case of EGase-extracted material), glycosyl-residue compositions of extracted material were determined by GLC of per-*O*-trimethylsilyl methyl glycoside derivatives.

Glycosyl Residue	Treatment			
	LiCl	EPGase	EGase	NaOH
		normalized wt %		
Rhamnosyl	7	12	2	6
Fucosyl	3	3	4	5
Arabinosyl	22	27	8	21
Xylosyl	3	5	18	15
Galactosyl	18	22	15	22
Glucosyl	23	7	46	15
GalUA	25	24	7	16

residues in the EGase-solubilized material (Table II), all of the rhamnosyl, arabinosyl, and galactosyluronic acid residues and about half of the galactosyl residues were accounted for by pectic polysaccharides (described below). Therefore, 25% of the EGase-solubilized material was pectic polysaccharide(s); the remaining 75% was xyloglucan. Thus, the xyloglucan and pectic polysaccharides solubilized by EGase accounted for 6 and 2%, respectively, of the cell wall.

Sodium hydroxide (0.5 N) solubilized another 9% (by weight) of the cell wall from the EGase-treated residue all of which was carbohydrate (see "Materials and Methods"). The NaOH-solubilized material contained pectic polysaccharides and hemicelluloses (described below). By attributing all of the rhamnosyl and galactosyluronic acid residues and half of the arabinosyl and galactosyl residues in the NaOH-solubilized material (Table II) to pectic polysaccharides, it was calculated that pectic polysaccharides comprised 44% and hemicellulose 56% of the NaOH-solubilized material (4 and 5%, respectively, of the cell wall). It was difficult to determine the ratio of xyloglucan to xylan in the NaOH-extracted hemicelluloses because they were never fully separated from one another, but it was estimated that no more than a fifth of the NaOH-extracted hemicellulose (1% of the cell wall) was xylan. To summarize, a total of 26% of the

cell wall was extracted by the combined treatments, 15% as pectic polysaccharides, 10% as xyloglucan, and no more than 1% as xylan.

Cellulose accounted for 23% of the cell wall, protein for 34% (calculated from a nitrogen content of 5.4%, see "Materials and Methods"), and ash (inorganic material) for 5%. Thus, with the 26% noncellulosic polysaccharides that were extracted, a total of 88% of the cell wall was accounted for. Since the residue remaining after the combined treatments still contained all of the glycosyl linkages that were present in the starting material (Table I), it is likely that some of the noncellulosic polysaccharides remained in the residue, a situation that has been described by other workers (see Chambat *et al.* (8) and references therein). Although noncellulosic polysaccharides probably remained in the residue after the four extraction procedures, those that were removed and characterized were likely to have been representative of the total noncellulosic polysaccharides, since 88% of the starting material was accounted for and since the glycosyl-linkage composition of the residue was much the same as that of the starting wall material (Table I). Although the walls used in this study were light chocolate brown in color, no evidence of lignin was found; the walls did not stain red with phloroglucinol (saturated solution in 2.4 N HCl), which would have indicated the presence of lignin, and no residue remained after extraction of walls with 24 N H_2SO_4 (38). Browning of the cell walls may have been due to condensation of tannins during isolation; alternative methods of isolation were not attempted. It is unclear what effect the browning had on extractions of polysaccharides.

Purification of Polysaccharides Isolated from Douglas Fir Cell Walls. Each of the pectic and hemicellulosic polysaccharides that have been shown to be widely distributed in the primary cell walls of angiosperms, both monocots and dicots, were isolated from Douglas fir cell walls. The scheme of purification by gel-permeation chromatography of polysaccharides isolated from Douglas fir cell walls is shown in Figures 1 and 2. Below the labels of the purified fractions in Figures 1 and 2 are given the identities of the polysaccharide(s) in the fractions that were deduced from the glycosyl and glycosyl-linkage compositions (discussed below). Polysaccharides similar to the pectic polysaccharide RG-I isolated from sycamore cell walls (28) were isolated from Douglas fir cell walls by each of the four treatments (LiCl, EPGase, EGase, and NaOH) used in this study (Figs. 1 and 2). Treatment with LiCl also extracted polysaccharides (in fractions L-2, L-3, and L-4; Fig. 1) closely resembling RG-II of sycamore cell walls (12). Both EGase and NaOH extracted xyloglucan from Douglas fir cells that was similar to that isolated from many plant sources (27) (Fig. 2). The NaOH-extracted material contained a small amount of xylan similar to dicot xylan (14) (Fig. 2). A detailed description of the gel-permeation chromatography summarized in Figures 1 and 2 is given below, followed by a discussion of the glycosyl and glycosyl-linkage compositions of the purified oligo- and polysaccharides.

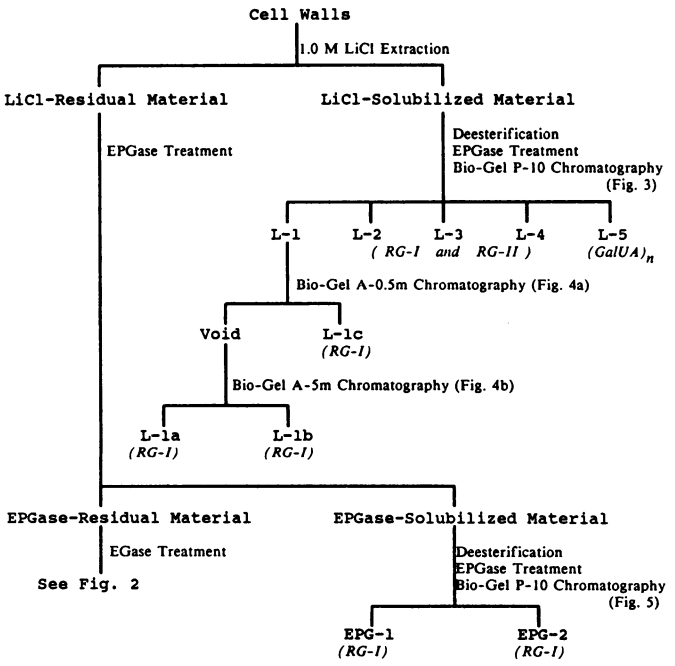

FIG. 1. Scheme for the fractionation of pectic polysaccharides solubilized from Douglas fir cell walls with 1.0 M LiCl and EPGase.

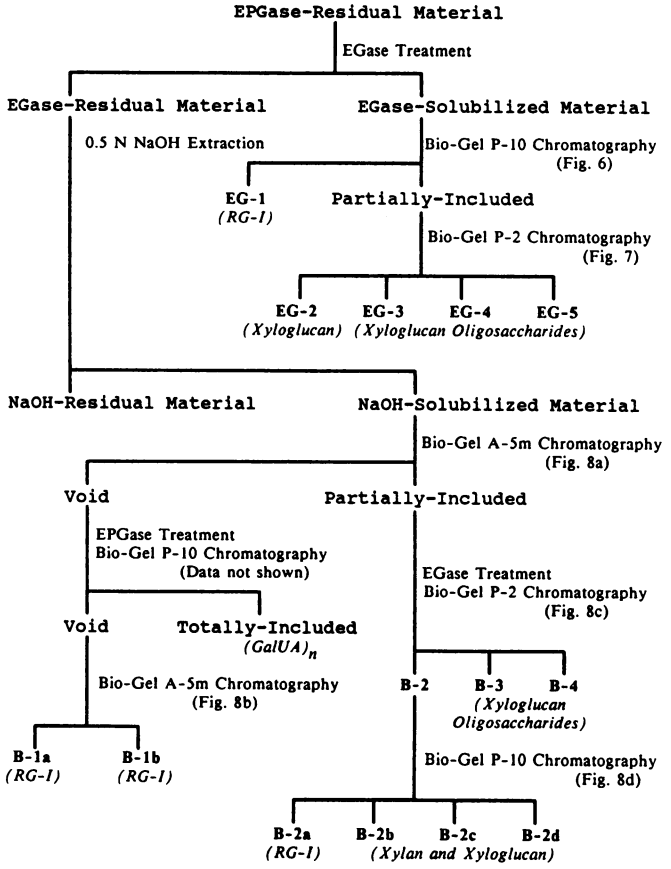

FIG. 2. Scheme for the fractionation of pectic and hemicellulosic polysaccharides solubilized from Douglas fir cell walls with EGase and 0.5 N NaOH.

Gel-Permeation Chromatography of LiCl-Extracted Polysaccharides. Starch and the pectic polysaccharides in the LiCl-extracted material were separated from one another by gel-permeation chromatography on a Bio-Gel A-5m column (data not shown). The fractions from the Bio-Gel A-5m column that contained pectic polysaccharides were pooled and fractionated as summarized in Figure 1. An amount of the pectic material in the pooled Bio-Gel A-5m fractions suitable for the capacity of the column used (156 mg) was deesterified, treated with EPGase, and chromatographed on a Bio-Gel P-10 column giving fractions L-1 to L-5 (Fig. 3). Fraction L-5 which eluted in the totally-included volume from the Bio-Gel P-10 column contained small oligomers of galactosyluronic acid (data not shown) hydrolyzed from pectic polysaccharides by the EPGase; the anthrone response of these fractions (Fig. 3) was accounted for by interference in the colorimetric assay by the glycosyluronic acid residues in the fractions. Fractions L-2, L-3, and L-4 that eluted from the

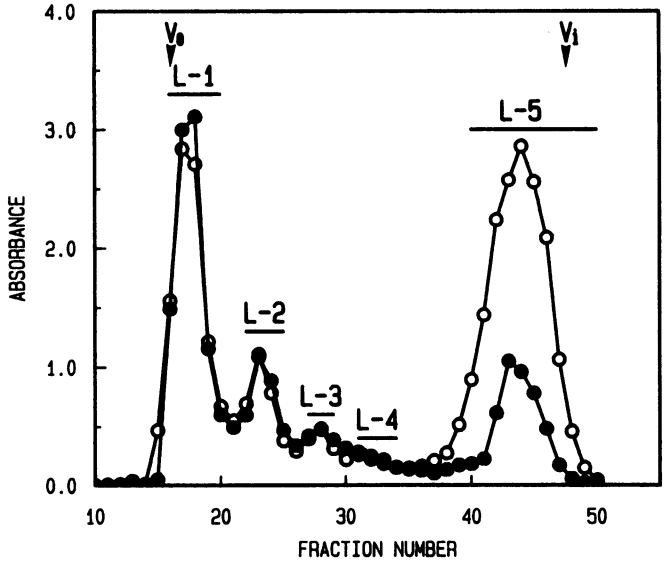

FIG. 3. Bio-Gel P-10 fractionation of the pectic polysaccharides extracted in 1.0 M LiCl (Fig. 1). Pectic polysaccharides (156 mg) were separated from starch by gel-permeation chromatography on Bio-Gel A-5m (see "Results and Discussion"), deesterified, treated with EPGase, and chromatographed on Bio-Gel P-10. Column fractions were assayed for neutral glycosyl residues by the anthrone method (15) (A_{620}, ●) and for glycosyluronic acid residues by the *m*-hydroxybiphenyl method of Blumenkrantz and Asboe-Hansen (5) (A_{520}, ○). Column fractions 16 to 20 were combined as fraction L-1, 22 to 25 as fraction L-2, 27 to 29 as fraction L-3, 31 to 34 as fraction L-4, and 40 to 50 as fraction L-5.

Bio-Gel P-10 column in the partially-included volume (Fig. 3) were each retreated with EPGase and rechromatographed on the same Bio-Gel P-10 column until no additional glycosyluronic acid residues could be detected in the totally included fractions (a total of three EPGase treatments of each fraction was necessary).

Fraction L-1, that eluted in the void volume from the Bio-Gel P-10 column (Fig. 3), was chromatographed on a Bio-Gel A-0.5m column (Fig. 4A). The elution profile was the same regardless of whether the material had been retreated with EPGase. Fraction L-1c that eluted in the partially-included volume from the Bio-Gel A-0.5m column (Fig. 4A) was not purified further, while the material that eluted in the void volume (fractions 17–19, Fig. 4A) was chromatographed on a Bio-Gel A-5m column (Fig. 4B). The material that eluted in the void volume from the Bio-Gel A-5m column (fractions 17 to 20, Fig. 4B) had a neutral-glycosyl composition (primarily rhamnosyl, arabinosyl, and galactosyl residues, data not shown) indicative of pectic polysaccharides, but it was not characterized further due to lack of material; the major peak of carbohydrate-containing material (fraction L-1a) and the shoulder (fraction L-1b) were separately rechromatographed on the same Bio-Gel A-5m column (data not shown) prior to characterization.

Gel-Permeation Chromatography of EPGase-Solubilized Polysaccharides. EPGase-solubilized material was fractionated as summarized in Figure 1. Material released from Douglas fir walls by EPGase (97 mg) was deesterified, treated with EPGase, and chromatographed on a Bio-Gel P-10 column (Fig. 5). Fraction EPG-2 was characterized without further chromatography, while fraction EPG-1 that eluted in the void volume from the Bio-Gel P-10 column (Fig. 5) was first chromatographed on a Bio-Gel A-5m column from which it eluted as a single, broad peak in the partially-included volume (data not shown).

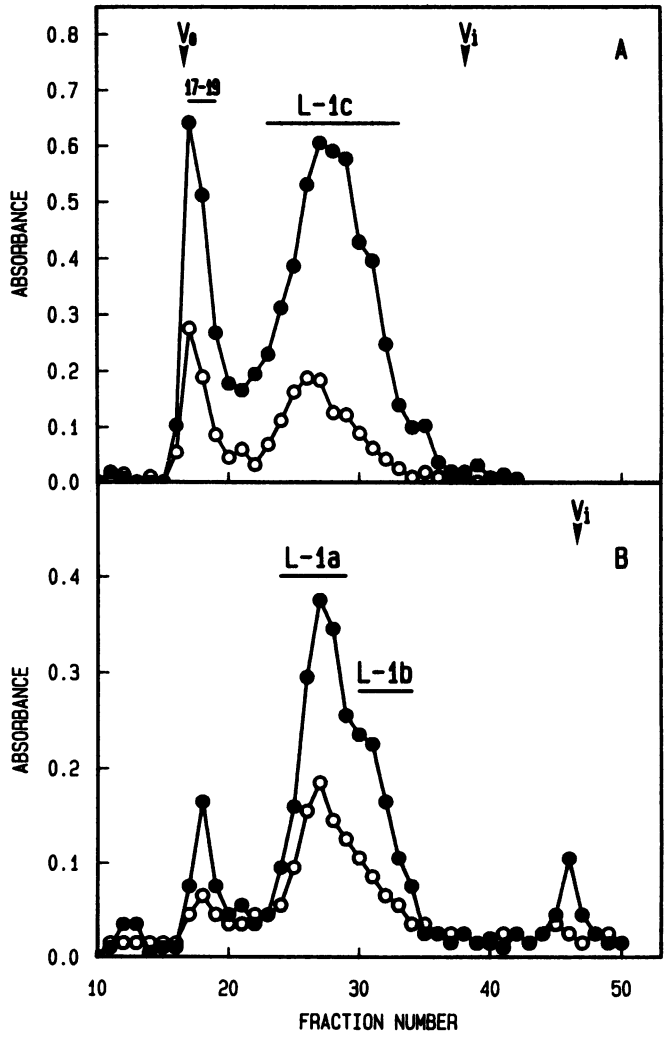

FIG. 4. A, Bio-Gel A-0.5m fractionation of material in fraction L-1 from the Bio-Gel P-10 column (Fig. 3) (symbols as in Fig. 3). Column fractions 17 to 19 were combined and chromatographed on Bio-Gel A-5m (see Fig. 4B), and fractions 23 to 33 were combined as fraction L-1c. B, Bio-Gel A-5m fractionation (2.3 × 46 cm column) of material in fractions 17 to 19 from the Bio-Gel A-0.5m column (Fig. 4A) (symbols as in Fig. 3). Column fractions 17 to 20 were combined and analyzed for netural-glycosyl residues by GLC. Column fractions 24 to 29 were combined as fraction L-1a and 30 to 34 as fraction L-1b.

Gel-Permeation Chromatography of EGase-Solubilized Polysaccharides. The fractionation of EGase-solubilized material is summarized in Figure 2. Of the material released from Douglas fir walls by EGase, an amount suitable for the size of the column used (100 mg) was chromatographed first on a Bio-Gel P-10 column (Fig. 6). Fraction EG-1 that eluted in the void volume from the Bio-Gel P-10 column (Fig. 6) was characterized without further chromatography, while the material that eluted from the column in the partially-included volume (fractions 34–44, Fig. 6) was chromatographed on a Bio-Gel P-2 column (Fig. 7). Fractions EG-2 to EG-5 eluted in the partially-included volume from the Bio-Gel P-2 column (Fig. 7) and were characterized without further chromatography.

Gel-Permeation Chromatography of NaOH-Extracted Polysaccharides. Material extracted in 0.5 N NaOH was fractionated as summarized in Figure 2. An amount of the NaOH-extracted material suitable for the size of the column used (123 mg) was chromatographed on a Bio-Gel A-5m column (Fig. 8A). The material in fractions 17 to 23 that contained glycosyluronic acid

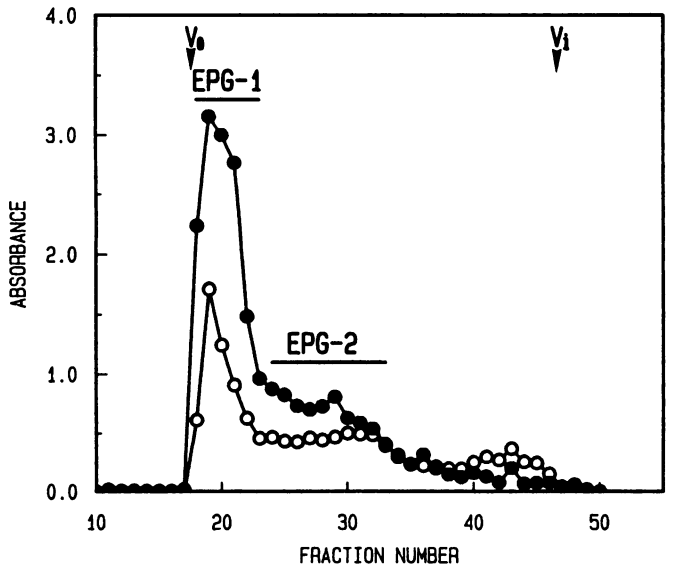

FIG. 5. Bio-Gel P-10 fractionation of EPGase-solubilized cell wall material. The EPGase-solubilized material (97 mg) was deesterified, treated with EPGase, and chromatographed as described (symbols as in Fig. 3). Column fractions 18 to 23 were combined as fraction EPG-1 and 24 to 33 as fraction EPG-2.

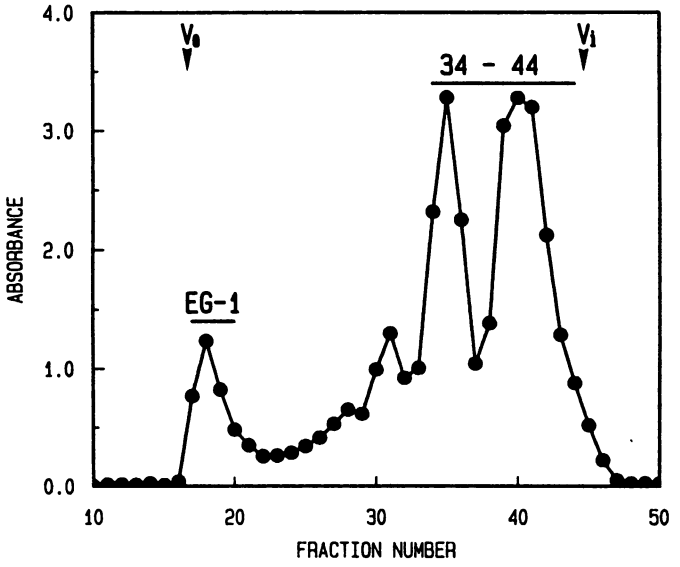

FIG. 6. Bio-Gel P-10 fractionation of EGase-solubilized cell wall material (100 mg) (symbols as in Fig. 3). Column fractions 17 to 20 were combined as fraction EG-1 and 34 to 44 were combined for further chromatography (see Fig. 7).

residues was treated with EPGase and chromatographed on a Bio-Gel P-10 column (data not shown) to separate polysaccharides from EPGase-generated oligosaccharides of galactosyluronic acid; the material that eluted in the void volume of the column contained all of the neutral glycosyl residues in the eluate, while a glycosyluronic acid-rich fraction eluted in the totally included volume. The neutral-glycosyl-containing material from the Bio-Gel P-10 column was chromatographed on a Bio-Gel A-5m column (Fig. 8B). Both halves of the later-eluting peak from this Bio-Gel A-5m column (fractions B-1a and B-1b, Fig. 8B) were characterized without further chromatography.

Comparison of the elution volumes of the bulk of the carbohydrate from the Bio-Gel A-5m column before and after EPGase

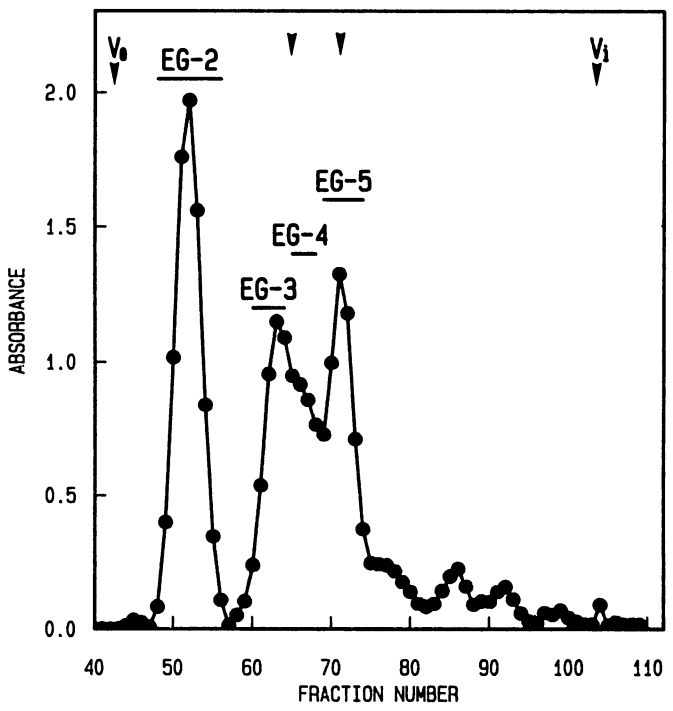

FIG. 7. Bio-Gel P-2 fractionation of EGase-solubilized material in fractions 34 to 44 from the Bio-Gel P-10 column (Fig. 6). Fractions (5-ml) were collected and assayed using the anthrone method (15) (A_{620}, ●). Column fractions 48 to 56 were combined as fraction EG-2, 60 to 64 as fraction EG-3, 65 to 68 as fraction EG-4, and 69 to 74 as fraction EG-5. Nona- and heptasaccharide components of sycamore xyloglucan (see McNeil *et al.* [29] and references therein) eluted in fractions 65 and 72, respectively (arrowheads).

treatment (compare elution volume and glycosyluronic acid content, A_{520}, of fractions 17 to 23 in Fig. 8A to that of the later-eluting peak in Fig. 8B) showed that EPGase treatment greatly reduced the mol wt of the polysaccharides extracted in NaOH. It is unclear why EPGase did not digest *in muro* the polysaccharides susceptible to digestion by EPGase that were solubilized from the cell wall by NaOH treatment. It is possible that if the particle size of the cell walls were decreased, for example by ball milling, the yield of pectic polysaccharides obtained by EPGase treatment might have increased.

The NaOH-extracted material that eluted in the partially-included volume from the first Bio-Gel A-5m column (fractions 31 to 43, Fig. 8A) was chromatographed on a Bio-Gel P-2 column after treatment with EGase (Fig. 8C). Fractions B-3 and B-4 that eluted in the partially-included volume from the Bio-Gel P-2 column were analyzed without further chromatography, while the material that eluted in the void volume (fraction B-2, Fig. 8C) was chromatographed on a Bio-Gel P-10 column (Fig. 8D) to yield fractions B-2a, B-2b, B-2c, and B-2d. Without EGase treatment, all of the carbohydrate in fractions 31 to 43 from the Bio-Gel A-5m column (Fig. 8A) voided a Bio-Gel P-10 column (data not shown) indicating that the mol wt of the carbohydrate in fractions B-2b, B-2c, and B-2d, that eluted in the partially-included volume from a Bio-Gel P-10 column (Fig. 8D), and in fractions B-3 and B-4, that eluted in the partially included volume from a Bio-Gel P-2 column (Fig. 8C), were greatly decreased by EGase treatment.

Characterization of Pectic Polysaccharides. *Rhamnogalacturonan I.* A pectic polysaccharide, with a glycosyl-linkage composition similar to that of RG-I of sycamore cell walls, was extracted from Douglas fir walls by each of the four treatments (LiCl, EPGase, EGase, and NaOH) used in this study (Table III).

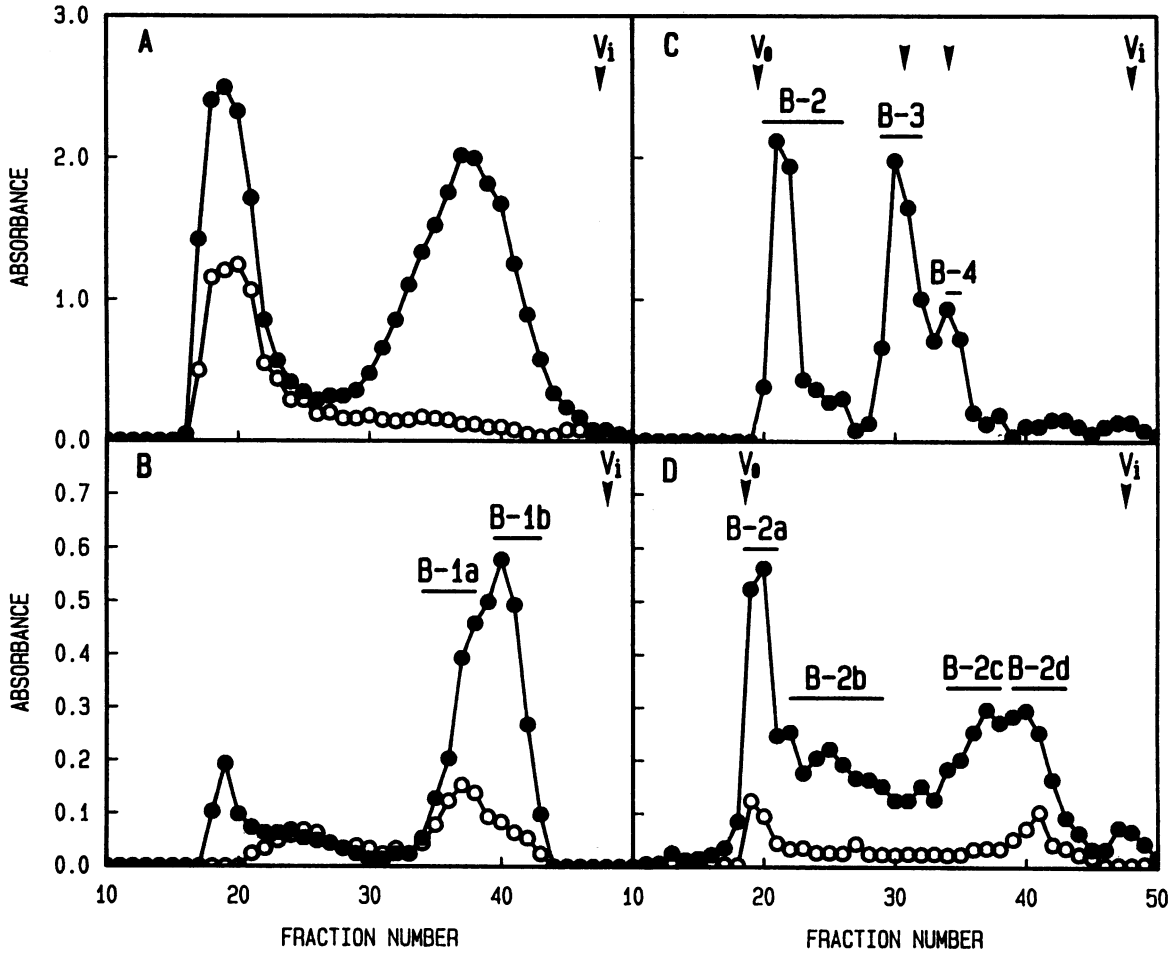

FIG. 8. A, Bio-Gel A-5m fractionation (3.5 × 61 cm column) of 123 mg (14%) of the NaOH-extracted cell wall material (symbols as in Fig. 3). B, Rechromatography of material in fractions 17 to 23 from the Bio-Gel A-5m column (Fig. 8A) after EPGase treatment. The material in fractions 17 to 23 was treated with EPGase and first chromatographed using a Bio-Gel P-10 column to separate polysaccharides from EPGase-generated oligosaccharides of galactosyluronic acid (data not shown); the void-volume material from the Bio-Gel P-10 column was chromatographed on the Bio-Gel A-5m column (3.5 × 61 cm column) (symbols as in Fig. 3). Column fractions 35 to 38 were combined as fraction B-1a and 40 to 43 as fractions B-1b. C, Bio-Gel P-2 fractionation of EGase-treated material in fractions 31 to 43 from the Bio-Gel A-5m column (Fig. 8A). Column fractions (10 ml) were assayed using the anthrone method (15) (A_{620}, ●). Column fractions 20 to 26 were combined as fraction B-2, 29 to 32 as fraction B-3, and 34 to 35 as fraction B-4. Nona- and heptasaccharide components of sycamore xyloglucan (see McNeil *et al.* [29] and references therein) eluted in fractions 31 and 34, respectively (arrowheads). D, Bio-Gel P-10 fractionation of the material in fraction B-2 from the Bio-Gel P-2 column (Fig. 8C) (symbols as in Fig. 3). Column fractions 19 to 21 were combined as fraction B-2a, 22 to 29 as fraction B-2b, 34 to 38 as fraction B-2c, and 39 to 43 as fraction B-2d.

The Douglas fir polysaccharide (Table III) contained the 2- and 2,4-linked rhamnosyl, terminal and 5-linked arabinosyl, and terminal, 4- and 6-linked galactosyl residues characteristic of sycamore RG-I (28). In addition, as in sycamore cell walls (28), most of the galactosyluronic acid residues in Douglas fir RG-I (*e.g.* in fractions L-1a, L-1c, EPG-1, B-1a, and B-2a) were 4-linked (data not shown). The galactosyluronic acid compositions of the fractions listed in Table III ranged from 4% in fraction EPG-1 to 22% in fraction EPG-2; sycamore RG-I contains 18% galactosyluronic acid (28).

Although the glycosyl-linkage compositions of the Douglas fir wall fractions (Table III) were similar to that of sycamore RG-I, differences were evident. The only specifically linked glycosyl residues present in sycamore RG-I that were not detected in Douglas fir RG-I were 2,6-linked galactosyl residues (1.5 mol % of sycamore RG-I, Table III). Another difference between the RG-I polysaccharides of Douglas fir and sycamore cell walls was evident in the relative amounts of the various rhamnosyl linkages (Table III). Sycamore RG-I contains nearly equimolar amounts of 2- and 2,4-linked rhamnosyl residues, a small amount of terminal rhamnosyl residues, and a very small amount of triply-branched (2,3,4,-linked) rhamnosyl residues (28). In comparison, Douglas fir fraction L-1a was rich in 2-linked rhamnosyl residues and fractions EPG-2 and EG-1 were rich in 2,4-linked rhamnosyl residues, while fraction L-1a was rich in triply-branched (2,3,4-linked) rather than 2,4-linked rhamnosyl residues. Fractions EPG-2, EG-1, and B-1 all contained significant amounts of 2,3-linked rhamnosyl residues, a mode of linkage that was not detected in sycamore RG-I (28). In every case, the RG-I-like Douglas fir polysaccharides contained more triply-branched than terminal rhamnosyl residues; the opposite is true of sycamore RG-I (28). Another difference in the glycosyl-linkage compositions of the RG-I-like polysaccharides of Douglas fir and sycamore RG-I was the relatively large amounts of 3-linked arabinosyl residues found in the Douglas fir polysaccharides (Table III). Also, Douglas fir fraction L-1b had a high content of 3,6-linked galactosyl residues (Table III), which is very suggestive of the presence of an arabinogalactan (11). Despite such differences,

Table III. *Glycosyl-Linkage Composition of Polysaccharides Isolated from Douglas Fir Walls Similar to Sycamore Rhamnogalacturonan I (Normalized to 100)*

Polysaccharides extracted from isolated cell walls by treatment with LiCl, EPGase, EGase, and NaOH (designated below by the prefixes L-, EPG-, EG-, and B-, respectively) were partially purified using gel-permeation chromatography (see text). Permethylated alditol acetates of polysaccharides in the pooled column fractions listed below were prepared, identified, and quantified as described in "Materials and Methods." For comparison, the glycosyl-linkage composition of sycamore RG-I is included.

Glycosyl Residue and Linkage[a]	Douglas Fir Wall Fraction									Sycamore RG-I[d]
	L-1a	L-1b	L-1c	EPG-1	EPG-2	EG-1	B-1a	B-1b	B-2a	
	mol %									
Glucosyl										
Terminal	0	+[b]	+	0.5	+	0	0	0	+	0
4-	3.0	1.6	1.6	5.6	1.1	1.5	0.8	+	10.7	0
4,6-	7.0	0	+	5.0	0.5	1.3	+	+	2.5	0
Galactosyl										
Terminal	5.2	7.6	15.1	13.0	16.9	20.4	15.1	6.1	8.4	7.7
2-	0	0	0.8	1.4	0.6	0.7	1.9	0	0	0.7
3-	17.0	9.4	4.2	2.6	4.6	2.7	3.0	1.9	3.1	3.3
4-	3.6	2.0	4.9	3.5	12.7	13.5	3.8	2.1	4.9	10.3
6-	5.6	6.0	1.0	+	1.4	2.5	1.7	+	2.2	9.1
2,3-	0	0	0	0	2.2	0	0	0	0	0
2,4-	0	0	+	+	0.6	+	+	+	+	0.6
3,4-	0	0	0	1.2	2.2	2.0	0	0	1.6	0
2,6-	0	0	0	0	0	0	0	0	0	1.5
3,6-	+	17.9	1.1	0.5	+	+	+	+	+	1.5
4,6-	+	2.1	1.1	0.8	0.6	1.5	0.5	5.6	+	2.9
3,4,6-	0	4.9	+	0	+	0	0	1.6	0	0
Xylosyl										
Terminal	2.9	+	+	3.9	+	1.2	+	+	+	2.4
2-[c]	+	0	+	1.8	+	0.5	0	0	0	0
4-[c]	+	0	+	1.8	+	0.5	0	0	0	0
Arabinosyl										
Terminal	16.4	19.3	13.4	13.2	13.1	9.1	22.9	16.2	23.4	11.6
2-	+	+	+	+	+	+	+	+	+	2.7
3-	3.9	9.3	17.2	13.0	4.8	5.4	22.3	18.9	9.4	2.7
5-	3.9	8.2	19.8	16.7	18.2	10.0	14.4	23.2	11.8	13.6
2,5-	3.2	1.0	6.8	4.6	0.6	2.9	2.7	8.1	3.6	1.2
3,5-	3.5	3.0	7.4	5.3	4.4	3.4	6.9	13.1	4.1	4.3
Rhamnosyl										
Terminal	1.1	1.9	+	+	+	+	0	0	+	2.2
2-	6.8	1.2	1.1	0.8	0.7	3.0	1.7	0.6	4.0	9.5
2,3-	0	0	+	+	1.0	0.6	1.0	0	0	0
2,4-	1.0	1.5	2.8	2.0	11.1	12.4	1.7	1.4	4.6	9.7
2,3,4-	8.9	2.4	0.5	+	1.5	1.8	1.1	0.5	1.9	0.7
Fucosyl										
Terminal	1.1	+	+	1.6	+	+	+	+	0	1.7
3,4-	0	0	0.5	0	0	0	0	0	0	0

[a] Where ambiguous, all hexosyl, deoxyhexosyl, and xylosyl residues were assumed to be in the pyranose ring form and arabinosyl residues in the furanose ring form. [b] Derivatives present in amounts less than 0.5 mol %. [c] Amounts of these derivatives were estimated by mass spectrometric analysis. [d] See McNeil *et al.* (28) and York *et al.* (41).

the glycosyl-linkage compositions of the pectic polysaccharides isolated from Douglas fir walls strongly resembled sycamore RG-I. Pectic polysaccharides resembling RG-I, but with different ratios of the various components, have also been isolated from monocots and dicot species other than sycamore (see McNeil *et al.* (29) and references therein).

Rhamnogalacturonan II. Several of the LiCl-extracted Douglas-fir cell-wall pectic polysaccharide fractions contained glycosyl residues characteristics of RG-II, another well-characterized pectic polysaccharide present in sycamore (and other dicot) cell walls (12, 40). Fractions L-2, L-3, and L-4 all contained significant amounts of 2-*O*-methylfucosyl, 2-*O*-methylxylosyl, apiosyl, aceryl, and KDO residues (each residue accounting for 2–5% by weight of the glycosyl residues), all of which are diagnostic of RG-II (12, 35, 40). In fact, apiose is the only one of these five sugars reported to be present in any wall polysaccharide other than RG-II (that of a monocot [see Darvill *et al.* (14) and references therein]) and RG-II is the only known biological source of aceric acid (35). There is evidence for the presence of RG-II in monocot cell walls (12, 22, 40), and polysaccharides containing the diagnostic glycosyl residues of RG-II listed above have been isolated from the walls of suspension-cultured rice cells (JR Thomas, AG Darvill, P Albersheim, unpublished data).

The neutral glycosyl-linkage compositions of fractions L-2 and L-3 (Table IV) confirmed the presence of RG-II in Douglas fir cell walls. Both fractions contained the 3′-linked apiosyl, 3,4-linked fucosyl, and 3-linked rhamnosyl residues characteristic of RG-II (12). The only neutral glycosyl linkages known to be a

Table IV. *Glycosyl-Linkage Composition of Polysaccharides Isolated from Douglas Fir Walls Similar to Sycamore Rhamnogalacturonans I and II (Normalized to 100)*

Polysaccharides extracted from isolated cell walls in 1.0 M LiCl were partially purified using gel-permeation chromatography (see text). Permethylated alditol acetates of polysaccharides in fractions L-2 and L-3, denoted in Figure 3, were prepared, identified, and quantified as described in "Materials and Methods." For comparison, the glycosyl-linkage compositions of sycamore RG-I and RG-II are included.

Glycosyl Residue	Linkage[a]	Douglas Fir Wall Fraction		Sycamore Wall Fraction[c]	
		L-2	L-3	RG-I	RG-II
		mol %			
Glucosyl	Terminal	+[b]	+	0	0
	4-	2.2	2.5	0	5.8
	4,6-	+	+	0	0
Galactosyl	Terminal	9.2	14.5	7.7	5.5
	2-	+	+	0.7	0
	3-	1.5	2.9	3.3	2.5
	4-	5.9	6.4	10.3	0
	6-	2.4	+	9.1	0
	2,4-	3.7	2.0	0.6	12.5
	3,4-	1.2	2.1	0	0
	2,6-	0	0	1.5	0
	3,6-	+	+	1.5	0
	4,6-	+	+	2.9	0
Xylosyl[c]	Terminal	5.6	3.3	2.4	2.7
Arabinosyl	Terminal	13.2	16.8	11.6	4.2
	2-(furanose)	+	+	2.7	0
	2-(pyanose)	1.8	1.8	0	10.6
	3-	1.4	1.4	2.7	0
	5-	6.5	8.3	13.6	0
	2,5-	+	+	1.2	0
	3,5-	1.1	1.2	4.3	0
Rhamnosyl	Terminal	5.0	2.8	2.2	11.1
	2-	4.5	4.1	9.5	0
	3-	5.2	1.3	0	10.0
	2,3-	1.0	+	0	0
	2,4-	3.6	7.7	9.7	2.7
	3,4-	0	0	0	2.4
	2,3,4-	5.9	3.3	0.7	2.2
Fucosyl[c]	Terminal	5.8	5.5	1.7	7.5
	3-	0	0	0	6.4
	3,4-	5.1	1.7	0	3.9
Apiosyl[d]	3'-	5.3	5.0	0	10.3

[a] Where ambiguous, hexosyl, deoxyhexosyl, and xylosyl residues were assumed to be in the pyranose ring form and arabinosyl residues in the furanose ring form. [b] Derivatives were present in amounts less than 1.0 mol %. [c] Includes endogenously methylated 2-*O*-methyl derivatives. [d] Identity confirmed by comparison to sycamore RG-II component. [e] See Darvill *et al.* (12), McNeil *et al.* (28), and York *et al.* (41).

part of sycamore RG-II that were not detected in fractions L-2 and L-3 were 3,4 linked rhamnosyl and 3-linked fucosyl residues (Table IV), but there were enough 2,3,4-linked rhamnosyl residues in Douglas fir cell walls to account for all of the 3,4- and 2,3,4-linked rhamnosyl residues of sycamore RG-II. The neutral glycosyl-linkage composition of fraction L-4 was similar to that of fraction L-3 (data not shown). Fractions L-2 and L-3 also contained glycosyl linkages characteristic of RG-I (Table IV). Particularly indicative of RG-I were the 2- and 2,4-linked rhamnosyl and 4-linked galactosyl residues; a 2,6-linked galactosyl residue was the only component of RG-I that was not detected in fractions L-2 and L-3. Except for the 2,3-linked rhamnosyl and 3,4-linked galactosyl residues, every glycosyl linkage detected in fractions L-2 and L-3 is known to be present in sycamore RG-I and/or RG-II (12, 28).

The acidic, as well as neutral, glycosyl-linkage composition of fraction L-2 was indicative of sycamore pectic polysaccharides RG-I and RG-II. Fractions L-2, L-3, and L-4 contained about 45% galactosyluronic acid and 5% glucosyluronic acid. The linkages of the glycosyluronic acid residues in fraction L-2 were the same as those present in RG-I and RG-II (12, 28); the residues were terminal, 4-, and 3,4-linked galactosyluronic acid and 2-linked glucosyluronic acid in a molar ratio of 2.7:4.4:1.5:1.0 (absolute amounts were not obtained due to the incomplete reduction of C-6 of glycosyluronic acid residues by Li-triethylborodeuteride). It is unclear whether the variously linked glycosyl residues characteristic of sycamore RG-I and RG-II were present in a single or in different molecules of Douglas fir that coeluted in fractions L-2, L-3, and L-4. If RG-I and RG-II of Douglas fir are covalently interconnected, such a molecule could be unique, since sycamore RG-I and RG-II do not coelute from a Bio-Gel P-10 column after EPGase treatment (12, 28), although evidence that some of these molecules can be obtained covalently connected from sycamore cell walls has been obtained (unpublished data of this laboratory).

Homogalacturonan. Homogalacturonan was a significant portion of the pectic polysaccharides isolated from Douglas fir walls. Its presence was indicated by the release of mono- and oligosaccharides of galactosyluronic acid from pectic polysaccharides by treatment with EPGase, for example, during the formation of fractions L-2, L-3, L-4, B-1a, and B-1b described above. About half of the glycosyluronic acid in the LiCl-extracted material was homogalacturonan (compare glycosyluronic acid contents of the fractions in Fig. 3) which accounts for about 12% of the carbohydrate of the LiCl-extracted material (Table II) and therefore about 1% of the cell wall, since 10% of the cell wall was extracted in LiCl as carbohydrate (see above). More homogalacturonan was probably hydrolyzed during the EPGase treatment of LiCl-extracted wall residue, but the galactosyluronic acid products would have been lost during dialysis of the solubilized polysaccharides. A homogalacturonan accounting for 5% of the wall has been isolated from *Rosa* cells (9). Also, a pectic fraction containing 91% galactosyluronic acid and accounting for less than 1% of the wall (estimated from the data presented) has been purified from a monocot cell wall (34). Sycamore cell walls contain 6% homogalacturonan, based on the release of galactosyluronic acid oligosaccharides by EPGase treatment (27).

Characterization of Hemicelluloses. *Xyloglucan.* Polysaccharides were extracted with both EGase and NaOH from Douglas fir walls that had glycosyl-linkage compositions (Table V) characteristic of the xyloglucans isolated from many plant sources (27). Included for comparison in Table V is the glycosyl-linkage composition of the xyloglucan from sycamore extracellular polysaccharides (41). In the analysis of sycamore xyloglucan, the amount of 6-linked glucosyl residues is smaller, and that of 4,6-linked glucosyl residues is larger, than in the Douglas fir xyloglucan, because the sycamore xyloglucan had not been subjected to EGase digestion that converts nearly one-third of the branched (4,6-linked) glucosyl residues into 6-linked glucosyl residues; the Douglas fir wall fractions were treated with EGase.

The elution volumes from a Bio-Gel P-2 column (Figs. 7 and 8C) and the glycosyl-linkage compositions (Table V) of the oligosaccharides in Douglas fir fractions EG-5 and B-4 were characteristic of the xyloglucan heptasaccharide isolated from sycamore cell walls; that is, it did not contain terminal fucosyl or terminal or 2-linked galactosyl residues (see McNeil *et al.* (27)

Table V. *Glycosyl-Linkage Composition of Xyloglucan Oligosaccharides Isolated from Douglas Fir Walls (Normalized to 100)*

Permethylated alditol acetates of polysaccharides in the fractions denoted in Figures 7 and 8B (except fraction B-2) were prepared, identified, and quantitated as described in "Materials and Methods." For comparison, the glycosyl-linkage composition of xyloglucan isolated from sycamore extracellular polysaccharides is included.

Glycosyl Residue and Linkage[a]	Douglas Fir Wall Fraction						Sycamore Xyloglucan[b]
	EG-2	EG-3	EG-4	EG-5	B-3	B-4	
	mol %						
Glucosyl							
4-Linked	10.8	8.9	10.3	14.0	8.6	13.4	13.4
6-	5.9	10.6	12.4	13.9	7.8	15.8	0.5
4,6-	29.4	21.8	25.4	27.5	21.8	36.3	31.5
Galactosyl							
Terminal	7.9	9.9	7.8	2.2	7.4	0.6	1.8
2-Linked	8.5	11.0	6.7	0	12.6	0	5.6
Xylosyl							
Terminal	17.1	9.9	18.3	38.8	12.0	27.5	27.8
2-Linked	14.0	18.8	13.2	3.6	16.8	3.7	8.3
2,4-	0	0	0	0	2.8	1.9	0.6
Fucosyl							
Terminal	6.4	9.1	5.8	0	8.7	0	5.3
Arabinosyl							
Terminal	0	0	0	0	1.4	0	1.0

[a] Where ambiguous, hexosyl, deoxyhexosyl, and xylosyl residues were assumed to be in the pyranose ring form and arabinosyl residues in the furanose ring form. [b] Neutral fraction of sycamore extracellular polysaccharides before EGase treatment (see York *et al.* [41]); also contains 1.3 mol % of 2,4,6-linked glucosyl residues.

and references therein). This identification was supported by the results of FAB-MS analysis of fraction EG-5. The major component of fraction EG-5 was an oligosaccharide with a mol wt (1062) equivalent to that of the sycamore heptasaccharide. A minor component of fraction EG-5 was an oligosaccharide with a mol wt (1224) equivalent to that of an octasaccharide (heptasaccharide with an additional hexosyl residue). The presence of small amounts of terminal galactosyl and 2-linked xylosyl residues in fraction EG-5 (Table V) suggested that, to about 25% of the heptasaccharides, an additional galactosyl residue was linked through *O*-2 of one of the xylosyl residues to form an octasaccharide that is a reported component of xyloglucan (see McNeil *et al.* (29) and references therein).

Fractions EG-3 and EG-4 also were analyzed using FAB-MS to obtain information on their oligosaccharide compositions. The major component of fraction EG-3 was an oligosaccharide with a mol wt (1532) equivalent to that of a decasaccharide composed of the nonasaccharide present in sycamore xyloglucan (27) with an additional terminal galactosyl residue. The glycosyl-linkage data (Table V) suggest that the additional terminal galactosyl residue was attached through *O*-2 of a xylosyl residue. Evidence for this decasaccharide in sycamore (A Dell, WS York, AG Darvill, P Albersheim, unpublished data) and bean (23, 32) xyloglucan has been obtained. An oligosaccharide with a mol wt (1370) the same as that of the sycamore nonasaccharide was a minor component of fraction EG-3. The glycosyl-linkage composition of fraction EG-3 (Table V) was exactly what would be expected if the fraction contained predominantly decasaccharide. Fraction EG-4 contained primarily oligosaccharides of a mol wt equivalent to that of an octasaccharide (1224) and nonasaccharide (1370) together with small amounts of an oligosaccharide with a mol wt (1532) equivalent to the decasaccharide. Fraction EG-2 probably contained larger oligosaccharides of xyloglucan similar to those proposed to be components of sycamore xyloglucan (3).

No evidence was found of xyloglucan oligosaccharides substituted with *O*-acetyl groups such as those that have been isolated from the xyloglucan in sycamore extracellular polysaccharides (WS York, AG Darvill, M McNeil, P Albersheim, A Dell, unpublished data) (29). Although fraction B-3 contained a small amount of terminal arabinosyl residues (Table V), no evidence was obtained for oligosaccharides with terminal arabinosyl residues linked to *O*-2 of xylosyl residues, such as those present in small amounts in xyloglucan isolated from potato (33), tobacco (16), and bean (32). It is not known why the xyloglucan in fractions B-3 and B-4 was not hydrolyzed to oligosaccharides and solubilized from the cell walls during EGase treatment of the EPGase-residual material.

Xylan. Evidence of the presence of xylans in Douglas fir walls was obtained by the glycosyl-linkage-composition analyses of fractions B-2b, B-2c, and B-2d (Table VI). Particularly diagnostic of xylans were the high amounts of 4- and 2,4-linked xylosyl residues (13, 29). Although all three fractions lacked the 2-linked arabinosyl residues present in the side chains of graminaceous monocot xylans (13, 29) and in sycamore glucuronoarabinoxylan (14) (see Table VI), Douglas fir xylan was rich in 3-linked arabinosyl residues (absent in sycamore). Fraction B-2d lacked, but fractions B-2b and B-2c contained, terminal arabinosyl residues, which are the most common side chain of graminaceous monocot xylans (13) and are also present in sycamore glucuronoarabinoxylan (Table VI). Fraction B-2d did contain terminal, endogenously methylated 4-*O*-methylglucosyluronic acid (about 10% of the glycosyl residues), a residue that is a prevalent side chain of graminaceous monocot xylans (13, 29), and one that accounts for 5% of the glycosyl residues of sycamore glucuronoarabinoxylan (14). Interestingly, fraction B-2d also contained 2-linked glucosyluronic acid residues (data not shown) that are not present in any other xylans that have been examined (27).

Table VI. *Glycosyl-Linkage Composition of Xylan-Containing Fractions Isolated from Douglas Fir Walls (Normalized to 100)*

Permethylated alditol acetates of polysaccharides in the fractions denoted in Figure 8D (except fraction B-2a) were prepared, identified, and quantified as described in "Materials and Methods." For comparison, the glycosyl-linkage composition of sycamore glucuronoarabinoyxlan is included.

Glycosyl Residue and Linkage[a]	Douglas Fir Wall Fraction			Sycamore Glucurono-arabinoxylan[b]
	B-2b	B-2c	B-2d	
			mol %	
Glucosyl				
4-Linked	8.4	4.1	5.0	0
6-	0	0	4.8	0
3,4-	2.1	0.5	0	0
4,6-	1.4	11.6	9.3	0
Galactosyl				
Terminal	7.0	7.0	5.4	0
2-Linked	0	2.9	3.5	0
3-	0.5	0.9	0.3	0
4-	0	2.4	0	0
6-	0	2.9	2.1	0
3,4-	1.1	0.3	0	0
Xylosyl				
Terminal	10.4	16.8	16.1	11.6
2-Linked[c]	3.8	5.9	5.7	0
3-	0	0	0.9	0
4-[c]	22.4	17.7	22.9	51.5
2,4-	12.3	9.3	17.5	27.2
Arabinosyl				
Terminal	12.9	7.6	0	5.4
2-Linked	0	0	0.3	3.8
3-	11.9	5.3	2.7	0
5-	2.1	1.7	0	0
2,5-	1.3	0	0	0
3,5-	1.8	0	0	0
Fucosyl				
Terminal	0.3	2.7	2.4	0

[a] Where ambiguous, hexosyl, deoxyhexosyl, and xylosyl residues were assumed to be in the pyranose ring form and arabinosyl residues in the furanose ring form. [b] Glucuronoarabinoxylan isolated from sycamore cell walls by extraction in 0.5 N NaOH; terminal glucosyluronic acid and terminal 4-*O*-methyl glucosyluronic acid residues not included (see Refs. 14 and 41). [c] Amounts of these derivatives were estimated by mass spectrometric analysis.

Except for the lack of 2-linked arabinosyl and terminal glucosyluronic acid and the presence of 3-linked arabinosyl residues, fraction B-2d appeared to contain a xylan similar to sycamore glucuronoarabinoxylan (Table VI). The xylan in fractions B-2b, B-2c, and B-2d was more similar to dicot xylan than to graminaceous monocot xylan, because in monocots the xylan side chains are attached through *O*-2 of some xylosyl residues and *O*-3 of the majority (29) while in dicot (sycamore) and Douglas fir xylans the side chains are attached only through *O*-2 of xylosyl residues (see Table VI).

In addition to xylan, fractions B-2b, B-2c, and B-2d contained xyloglucan as evidenced by the presence of terminal fucosyl, terminal and 2-linked galactosyl, terminal and 2-linked xylosyl, and 4-, 6-, and 4,6-linked glucosyl residues (Table VI). The fact that the polysaccharides in fractions B-2b, B-2c, and B-2d eluted in the partially included volume from a Bio-Gel P-10 column after, but not before, treatment with EGase is further evidence that these three fractions contained xyloglucan. It is interesting that the elution volume from a Bio-Gel P-10 column of the xylan in fractions B-2b, B-2c, and B-2d was changed by EGase treatment. This result indicated that xyloglucan and xylan solubilized from Douglas fir walls by NaOH were interacting with and/or covalently attached to each other. (The EGase does not contain xylanase activity.)

Table VII. *Summary of the Polysaccharide Compositions of Primary Cell Walls of Dicots, Graminaceous[a] Monocots, and Douglas Fir*

Wall Polysaccharide	Dicot[b]	Monocot	Douglas Fir
		wt % of cell wall	
Pectic polysaccharides	34	5–10[c]	22[d,e]
Hemicelluloses (Ratio of xyloglucan to xylan)	24 (4:1)	37[f] (1:17)[f]	16[e] (10:1)
Cellulose	23	11[g]	23
Mixed-linked glucan	0	15[h]	0

[a] The cell walls of the Gramineae have been studied far more extensively than those of other monocots. While some non-graminaceous monocots appear to have primary cell walls similar to dicots, others do not (see Bacic and Harris [2] and references therein). [b] Values for walls of suspension-cultured sycamore cells given in Darvill *et al.* (13). [c] Estimated using published values of galactosyluronic acid contents of monocot cell walls (see Ref. 13). Evidence of RG-I and RG-II in monocot cell walls has been published. For example, small amounts of glycosyl residues characteristic of RG-II (2-*O*-methylxylosyl, 2-*O*-methylfucosyl, apiosyl, aceryl, and KDO residues) have been detected in monocot cell walls (12, 40). Furthermore, polysaccharides with glycosyl linkages characteristic of RG-I and RG-II have been isolated from monocot cell walls (see McNeil *et al.* [29] and references therein) and RG-I has been solubilized from walls of suspension-cultured maize cells by EPGase treatment (JR Thomas, AG Darvill, P ALbersheim, unpublished data). However, the amounts of RG-I and RG-II in monocot cell walls have not been determined. [d] This value does not include homogalacturonan solubilized during EPGase treatment but lost during dialysis of the solubilized material. [e] Estimated by assuming noncellulosic polysaccharides in the NaOH-residual wall material (12% of the cell wall) are composed of the same polysaccharides in the same proportions as those that were isolated (see text). [f] Estimate based on data given in Refs. 7 and 29. [g] Average of values for six monocot species given in Ref. 6. [h] Estimated using data given in Ref. 7. Mixed-linked glucans are limited to the cell walls of grasses (6, 13). Furthermore, the levels of mixed-linked glucans can vary greatly depending on the stage of development of the cell wall (see Bacic and Harris [2] and references therein).

CONCLUSION

The results presented here support the conclusion of Burke *et al.* (6) that the primary cell walls of Douglas fir are more closely related to those of dicots than to graminaceous monocot cell walls. A comparison of the polysaccharide compositions of the primary cell walls of dicots, graminaceous monocots, and Douglas fir is presented in Table VII. Like dicots and unlike graminaceous monocots, Douglas fir cell walls are composed of greater than 20% pectic polysaccharides (RG-I, RG-II, and homogalacturonan), and the predominant hemicellulose is xyloglucan, not xylan. Furthermore, the cellulosic contents of dicot and Douglas fir cell walls are twice that of the cell walls of graminaceous monocots, and no β-(1→3, 1→4)-linked glucan, resembling that isolated from the cell walls of the Gramineae (see McNeil *et al.* (27) and references therein), was detected in Douglas fir cell walls.

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