Plant Physiol. (1984) 75, 596–604
0032-0889/84/75/0596/09/$01.00/0

Pea Xyloglucan and Cellulose[1]

I. MACROMOLECULAR ORGANIZATION

Received for publication December 1, 1983 and in revised form March 6, 1984

TAKAHISA HAYASHI*[2] AND GORDON MACLACHLAN
Department of Biology, McGill University, Montreal, Quebec, Canada H3A 1B1

ABSTRACT

A macromolecular complex composed of xyloglucan and cellulose was obtained from elongating regions of etiolated pea (*Pisum sativum* L. var. Alaska) stems. Xyloglucan could be solubilized by extraction of this complex with 24% KOH-0.1% $NaBH_4$ or by extended treatment with endo-1,4-β-glucanase. The polysaccharide was homogeneous by ultracentrifugal analysis and gel filtration on Sepharose CL-6B, molecular weight 330,000. The structure of pea xyloglucan was examined by fragmentation analysis of enzymic hydrolysates, methylation analysis, and precipitation tests with fucose- or galactose-binding lectins. The polysaccharide was composed of equal amounts of two subunits, a nonasaccharide (glucose/xylose/galactose/fucose, 4:3:1:1) and a heptasaccharide (glucose/xylose, 4:3), which appeared to be distributed at random, but primarily in alternating sequence. The xyloglucan:cellulose complex was examined by light microscopy using iodine staining, by radioautography after labeling with [^{3}H]fucose, by fluorescence microscopy using a fluorescein-lectin (fucose-binding) as probe, and by electron microscopy after shadowing. The techniques all demonstrated that the macromolecule was present in files of cell shapes, referred to here as cell-wall 'ghosts,' in which xyloglucan was localized both on and between the cellulose microfibrils. Since the average chain length of pea xyloglucan was many times the diameter of cellulose microfibrils, it could introduce cross-links by binding to adjacent fibrils and thereby contribute rigidity to the wall.

Xyloglucans occur widely in the primary walls of higher plant cells, where it has been proposed that they are bound in close association with cellulose microfibrils as part of the basic wall structure (1). These hemicelluloses all possess a 1,4-β-glucan backbone with 1,6-α-xylosyl residues attached to the 6-position of β-glucosyl residues. Species-specific differences occur as to the distribution of additional branching fucosyl-galactosyl residues (3, 5, 13, 20, 21). Nothing is known about the function of the branching sugars nor has evidence been published to date that xyloglucan and cellulose do in fact co-exist *in vitro* bound together in a macromolecular complex. The metabolism of these two major primary wall components is undoubtedly regulated by different processes, since xyloglucan is subject to turnover during growth (24) and different enzyme systems are probably required for synthesis of their 1,4-β-linkages (15, 16). This paper describes a xyloglucan:cellulose complex which is visualized as cell-wall 'ghosts' isolated from growing pea tissues, using techniques based on the structural characterization of pea xyloglucan by fragmentation analysis and fucose- or galactose-binding lectins as probes.

Pea epicotyl tissue has been used because of the physiological studies with peas (10, 24, 25, 36) that provided evidence for xyloglucan turnover during growth, particularly after treatment with the auxin type of hormone. Such treatment also induces the development of endo-1,4-β-glucanase activities in peas (40), and these may be responsible for the observed xyloglucan turnover. The phenomenon could contribute to the hormone-evoked 'loosening' of the wall which is believed to be required during growth. Part II of this series deals with the capacity of pea endo-1,4-β-glucanases to hydrolyze pea xyloglucan.

MATERIALS AND METHODS

Materials. L-[1-^{3}H]Fucose (1.8 Ci/mmol), $NaBT_4$ (250 mCi/mmol), and Aquasol were obtained from New England Nuclear. DEAE-Sephadex, Sepharose CL-6B, Sephadex G-50, and polysaccharide mol wt markers (blue dextran, dextrans T-500, T-70, T-40, and T-10) were obtained from Pharmacia. Bio-Gel P-6 (−400 mesh), Bio-Gel P-4 (200–400 mesh), and Bio-Gel P-2 (200–400 mesh) were from Bio-Rad. Lectins from *Ulex europeus*, *Lotus tetragonolobus*, and *Ricinus communis* were obtained from Miles Laboratories; *Pisum sativum* lectin was from Calbiochem, and concanavalin A, microcrystalline cellulose, and citrus pectin were from Sigma. Fucose-binding lectin was prepared (35) from eel serum (Waldman's Fish Co., Montreal). Fluorescein isothiocyanate was from Aldrich, and Ionagar was from Oxoid. NTB-2 emulsion, D-19 developer, and fixer were from Kodak. Arabinan and larch arabinogalactan were from Koch-Light, laminarin (5,300 mol wt) was from Nutritional Biochemicals, and carboxymethylcellulose (7LP and 7HSP) was from Hercules. Laminari-oligosaccharides and cello-oligosaccharides came from the laboratory collection. Xyloglucan-derived nonasaccharide (glucose/xylose/galactose/fucose, 4:3:1:1) and heptasaccharide (glucose/xylose, 4:3) were obtained from soybean xyloglucan as described earlier (15).

Preparations. In order to prepare fluorescein-labeled fucose-binding lectin, carbonate-bicarbonate buffer (100 μl, 0.1 M, pH 9.0) containing 0.1 mg of fluorescein isothiocyanate was added to 0.5 ml of *U. europeus* lectin (4 mg) at 4°C, and the mixture was adjusted to pH 9.0 with 0.1 N NaOH (17). Reaction was at room temperature for 1 h with stirring. After incubation, the mixture was dialyzed against five changes of 50 mM phosphate-buffered saline (pH 7.0) and applied to a Sephadex G-50 column (1.5 × 20 cm), previously equilibrated with the saline (pH 7.0). Fractions (2.0 ml) were collected and *A* at 280 and 490 nm was measured. Fractions corresponding to peaks at both wavelengths were pooled and stored at 4°C after adding NaN_3 to a final concentration of 0.02%.

A cellulase (EC 3.2.1.4) preparation from *Streptomyces griseus* (QM 814) was generously contributed by Dr. E. T. Reese (U. S.

[1] Supported by grants from the Natural Sciences and Engineering Research Council of Canada and le Programme des Formation de Chercheurs et d'Action Concertee du Quebec.

[2] Current address: ARCO Plant Cell Research Institute, Dublin CA 94566.

Army Laboratories, Natick, MA). It contains several other enzyme activities in addition to 1,4-β-glucanase (*e.g.* 1,3-β-glucanase, β-xylanase etc.). *Aspergillus oryzae* glycosidase preparation and amyloid xyloglucan from *Tamarindus indica* were kindly provided by Dr. K. Matsuda (Tohoku University, Sendai, Japan). *Aspergillus* glycosidase activity was partially purified using salicin as a substrate by fractionation through DEAE-Sephadex A-50 in order to remove carbohydrate impurities (20). This enzyme contained various polysaccharide hydrolases and glycosidases which degraded soybean xyloglucan into monosaccharides plus isoprimeverose in which all of the xylosyl residues of the polysaccharide were recovered (14).

Amyloid xyloglucan was purified by the procedure of Gaillard (9). This polysaccharide (2 g) was dissolved in 300 ml of 2 M $CaCl_2$ and centrifuged to remove precipitate. Fifty ml of 3% KI was added to the solution and the mixture was kept at 4°C for 2 h. The resulting precipitate was neutralized with 10% $Na_2S_2O_3$ solution, and dialyzed against distilled H_2O. The $[\alpha]_D$ value of the polysaccharide as purified was +71.0. This purified xyloglucan was kept in solution at a concentration of 0.5% (w/v) containing 0.02% NaN_3.

Seeds of *Pisum sativum* L. var. Alaska were soaked (20 min) in 5% NaOCl, washed, allowed to imbibe water (8 h), planted in moistened vermiculite, and grown in darkness at room temperature for 1 week. The apical 10 mm of the third internode (elongating region) was used as source of pea xyloglucan. The apices (50 g) were homogenized in buffer (pH 7.0) and wall material was subjected to sequential extraction with alcohol, buffer, EDTA, and alkali (13). After extraction with 4% KOH-0.1% $NaBH_4$, pea xyloglucan (100 mg) was solubilized with 24% KOH-0.1% $NaBH_4$.

Enzymic Hydrolysis. Pea xyloglucan (5 mg) was dissolved in 0.5 ml of 50 mM acetate buffer (pH 5.0) containing 0.02% NaN_3 and incubated at 40°C with 0.4 mg of *Streptomyces* endoglucanase. The reaction was terminated by heating the mixture in a boiling water bath for 5 min. The hydrolysates collected at intervals were fractionated on a Bio-Gel P-6 (−400 mesh) column (1.5 × 100 cm) at 60°C over a 4-d period in order to separate higher oligosaccharides (18).

Partially hydrolyzed endoglucanase-treated pea xyloglucan was prepared for use in lectin-binding tests by incubation (50 mg) with *Streptomyces* endoglucanase (4 mg) at 40°C for 2 h and by fractionation on Bio-Gel P-2 column (1.5 × 100 cm). The eluates were collected in 1.5-ml fractions, and the void volume was collected as the residual endoglucanase-treated xyloglucan.

The oligosaccharide fragments formed by treatment of xyloglucan with endoglucanase were dissolved in 0.5 ml of 50 mM acetate buffer (pH 5.0) containing 0.02% NaN_3 and incubated at 40°C for 24 h with *Aspergillus* glycosidases. Hydrolysates were fractionated on a Bio-Gel P-2 column (1.5 × 100 cm) at 40°C.

Acid Hydrolysis and Sugar Linkage Analysis. Polysaccharides were hydrolyzed in sealed tubes with 2 M trifluoroacetic acid at 100°C for 5 h. The hydrolysates were evaporated to dryness. The residue was repeatedly evaporated with methanol until the acid had been completely removed. The molar ratios of neutral sugars were determined by converting them into alditol acetates, which were examined by GLC (Tracor) on a glass column (0.4 × 200 cm) of 3% SP-2340 on Supelcoport (100–120 mesh). Analysis was carried out at 210°C at a N_2 flow rate of 15 ml/min.

For mild acid hydrolysis, pea xyloglucan (50 mg) was treated with 5 ml of 10 mM trifluoroacetic acid at 60°C for 30 h. After hydrolysis, 3 volumes of ethanol were added to the mixture, and the resulting precipitate was collected by centrifugation. It was washed 4 times with 70% ethanol and evaporated to dryness.

Methylation was carried out by the method of Hakomori (12) as modified by Sandford and Conrad (33). The methylated samples were hydrolyzed and converted into the corresponding alditol acetates by the method of Lindberg (27). The partially methylated alditol acetates were analyzed as above on a column of 3% OV-225 at 170°C.

Precipitation Tests with Lectin. For the two-dimensional double-diffusion method, a Petri dish was layered with 1% Ionagar in saline containing 0.02% NaN_3. Polysaccharides (20 μg) or lectins (50 μg) were added to the center basin and/or the six peripheral wells. The plate was then incubated in a water-saturated atmosphere at room temperature.

Precipitation tests were performed by incubating 0.1 mg of *R. communis* lectin plus polysaccharides at various concentrations, 0.9% NaCl, 10 mM phosphate buffer (pH 7.0), and 0.02% NaN_3 in a total volume of 1 ml. After incubation at 37°C for 1 h, the mixtures were cooled to 4°C for 2 d. The precipitate was washed twice with ice-cold saline, and precipitated protein was determined by the Lowry method (28).

Cell Wall Ghosts. The apical 10 mm (3.6 g fresh weight) of the third internode (elongating region) of pea epicotyls was extracted three times with 70% ethanol at 70°C for 30 min. Segments were ground in a mortar with 0.1 M Tris/HCl (pH 7.0) and the mixture was separated by centrifugation. Insoluble material was extracted three times with 0.1 M EDTA (pH 7.0) at 85°C and 5 times with 4% KOH-0.1% $NaBH_4$ in an ultrasonic bath below 30°C for 3 h in order to remove much polymeric material but retain most (92%) of the xyloglucan and all cellulose fibrils. This wall residue was neutralized with 2 M acetic acid, washed 10 times with 50% ethanol, and dried. It retained chains of recognizable cell shapes, containing xyloglucan and cellulose only (weight ratio of 0.7:1.0). The empty and depleted cell walls are referred to here as ghosts.

In order to extract xyloglucan from cell wall ghosts, 3.0 mg were sonicated with 1 ml of water, 4 M urea, DMSO, 10% KOH, or 24% KOH in a sonication bath (25 to 30°C, 2 h). The mixtures were centrifuged and the supernatant extract was collected. The precipitate was again sonicated with the same solvent and centrifuged, and the extracts were combined. Aliquots were taken for determination of xyloglucan content by the iodine-sodium sulfate method (22). For extraction with H_3PO_4, 3.0 mg of cell wall ghosts were mixed with ice-cold 85% H_3PO_4 at 4°C for 12 h. The mixture was then centrifuged and the supernatant was assayed. For extraction with DMSO/paraformaldehyde (19), 3.0 mg of cell wall ghosts and 5 mg of paraformaldehyde were suspended in 1 ml of DMSO and the mixture was heated with rapid stirring at 80°C for 1 h. The mixture was then centrifuged and the supernatant was assayed.

For enzymic hydrolysis of cell wall ghosts, 10.0 mg of ghosts were incubated with 0.2 mg of *Streptomyces* endoglucanase in 2 ml of 20 mM sodium acetate buffer (pH 5.0) at 40°C for 72 or 144 h. The mixtures were centrifuged and the precipitates were washed twice with 1 ml of 0.1 N KOH by centrifugation. The treated ghosts were extracted with 2 ml of 24% KOH containing 2.5 mCi of $NaBT_4$ (40 mCi/mmol) by stirring for 12 h in order to tritiate reducing ends and solubilize xyloglucan. This procedure was repeated twice. The combined supernatants (6 ml) were neutralized with 2 N acetic acid and the extract was treated with 4 volumes of ethanol and centrifuged. The precipitate was washed with two 2-ml portions of 70% ethanol and dissolved in 500 μl of 0.1 N NaOH. The cellulose residue was washed twice with water after neutralization with acetic acid and dissolved in 72% H_2SO_4. Aliquots of the xyloglucan and cellulose solutions were assayed for radioactivity or for amount of polysaccharide by the phenol-sulfuric acid method (6). Alkali extracts were further subjected to gel filtration on a column (1.0 × 100 cm) of Sepharose CL-6B and eluted with 0.1 N NaOH.

Microscopy. Cell wall ghosts suspended in water were dried on a carbon-coated mica plate and shadowed with Pt/C at an angle of 30°. Specimens were chosen using light microscopy and

transferred onto carbon-coated Formvar films supported on metal grids and photographed with a Philips 200 electron microscope.

For radioautography, 20 tissue segments (0.5 g) were incubated with 1.0 ml of 6.25 μM [^{3}H]fucose (11.25 mCi) in the dark at 25°C for 2 h. After incubation, segments were extracted and washed with solvents required to prepare cell wall ghosts containing cellulose and labeled xyloglucan. A suspension of these ghosts in water was dried on a glass slide and coated with NTB-2 emulsion by the dipping method. After exposure for 8 weeks at 4°C, it was developed with Kodak D-19, rinsed with water (1 min), fixed with Kodak fixer at 18°C (5 min), and then washed in running water (15 min). The slide was stained with 1% fast green (30 min), washed in running water (15 min), and air-dried. It was examined with bright-field illumination and photographed.

In order to stain cell wall ghosts with fluorescent dyes, 50 μl of fluorescein-labeled lectin (3 mg/ml) or 0.01% Calcofluor solution was added to 1 ml of ghosts (2 mg of sugar as glucose). After 30 min in the dark at room temperature, the ghosts were washed twice with 50 mM phosphate-buffered saline (pH 7.0) and mounted on slides for fluorescence microscopy. Control experiments were performed by incubation of 24% KOH-extracted cell wall ghosts with the labeled lectin. Samples were observed with a Leitz Dialus microscope equipped with an incident light Ploemopak 2.3 illuminator.

General Methods. All evaporations were carried out under diminished pressure at 35°C. Paper chromatography was performed on Whatman 3MM filter paper with the following solvent systems: 1-propanol/ethyl acetate/water, 3:2:1 (solvent A), or 1-butanol/pyridine/water, 6:4:3 (solvent B). Carbohydrate was determined by the phenol-sulfuric acid method (6), and reducing sugar was calculated as reducing power with glucose as standard (34). Xyloglucan was determined by the iodine-sodium sulfate method (22), and protein was measured according to Lowry *et al.* (28). Optical rotation was determined with a Jasco Model DIP-140 digital polarimeter. Sedimentation analysis was done with a Beckman Model E (s/n 1249) analytical ultracentrifuge.

RESULTS

Extraction and Properties of Xyloglucan from Pea Cell Walls. Table I shows the content of total carbohydrate and xyloglucan measured with the phenol-sulfuric acid method (6) and iodine-sodium sulfate method (22) in sequential fractions of pea tissue. The results indicate that 92% of total xyloglucan was concentrated in the 24% KOH extract. On acid hydrolysis, polysaccharides of this fraction yielded glucose, xylose, galactose, fucose, and arabinose (GLC) in the ratio of 100:60:18:10:1. This assay probably underestimates the fucose and xylose components. Assay of extracted xyloglucan gave a value which accounted for 98.1% of the total carbohydrate content. The fraction contained no detectable protein (Lowry method), aromatic cross-links similar to those of lignin (UV absorption), or 1,3-β-glucan (fluorescence microscopy with aniline blue). Since xyloglucan assay fully accounted for the weight of the extracted lyophilized material, it is concluded that the 4% KOH-insoluble material contained essentially only cellulose and xyloglucan.

Table I. *Solubilization of Xyloglucan and [^{3}H]Fucose-Labeled Products*

Pea segments (4.0 g) were incubated with [^{3}H]fucose as described in "Materials and Methods" and then sequentially extracted with the solvents indicated. Total sugar was assayed as glucose equivalents with the phenol-sulfuric acid method (6) and xyloglucan was determined by the iodine-sodium sulfate method (22). Purified pea xyloglucan yields an optical density in the phenol-sulfuric acid test which is 1.72 times the density of an equivalent weight of glucose.

Fraction	Total Sugar	Xyloglucan	Radioactivity
	mg Glc eq	*mg*	*dpm × 10^{-3}*
70% Ethanol	36.5	0	8,450
0.1 M Tris/HCl (pH 7.0)	20.5	0.15	720
0.1 M EDTA (pH 7.0)	17.1	0	5
4% KOH-0.1% $NaBH_4$	10.3	0.70	9
24% KOH-0.1% $NaBH_4$	14.5	8.25	23
Insoluble	11.8	0	0.8

Of various solvents tested for ability to extract xyloglucan from the 4% KOH-insoluble wall residue, only concentrated (24%) KOH was effective in dissolving xyloglucan without the cellulose. Standard reagents for breaking weak hydrogen bonds (*e.g.* 4 to 8 M urea) were almost completely ineffectual. Reagents that are excellent solvents for most hemicelluloses (*e.g.* DMSO) only extracted a small part (20%) of the xyloglucan. Reagents which merely resulted in microfibrillar swelling (*e.g.* 85% H_3PO_4) dissolved only about half of the xyloglucan and degraded part. Reagents that dissolved cellulose microfibrils (*e.g.* DMSO/paraformaldehyde) solubilized the entire residue.

The cellulose and xyloglucan complex (4% KOH-insoluble material) was incubated with *Streptomyces* endoglucanase (see "Materials and Methods") and the complex was then incubated with 24% KOH containing $NaBT_4$ in order to extract remaining xyloglucan and to tritiate accessible reducing chain ends. The amounts of polysaccharide and the incorporation of tritium were determined in the extract and the residual cellulose (Table II). The xyloglucan:cellulose weight ratio (0.7:1.0) in the original preparation was much reduced by the enzyme treatments (to 0.1:1.0) due to preferential hydrolysis and solubilization of the xyloglucan component. The xyloglucan that remained bound to the ghosts after treatment showed a major increase in number of tritiated chain ends. There was also a detectable increase in accessible chain ends of cellulose, but the numbers of new chain ends of xyloglucan were much more than those of cellulose. Evidently, the xyloglucan component of the xyloglucan:cellulose complex was much more accessible to enzymic hydrolysis than cellulose microfibrils.

Gel filtration of the bound xyloglucan before (Fig. 1A) and after 72 h (Fig. 1B) cellulose treatment confirmed that the average mol wt had been greatly reduced (about 330,000 to 70,000). The ghosts before cellulase treatment had been extracted with 4% KOH-0.1% $NaBH_4$ which would reduce accessible chain ends. Hence, subsequent extraction with 24% KOH-0.1% $NaBT_4$ did not introduce any detectable new chain ends into the zero time sample (Fig. 1A). However, after enzyme treatment (Fig. 1B), reducing chain ends in the residual bound xyloglucan were distributed throughout the mol wt profile, with more in the lower mol wt fractions, as expected. Clearly, xyloglucan remains susceptible to hydrolytic enzyme action even while tightly bound to cellulose.

Table II. *Composition of Residual Xyloglucan:Cellulose Complex after Digestion by Streptomyces Endoglucanase*

Parameter	Xyloglucan	Cellulose
Weight	*mg*	
Zero time	2.91	3.95
Cellulase		
72 h	2.05	3.75
144 h	0.30	3.60
Tritiated (reduced) chains	*dpm × 10^{-3}*	
Zero time	65	19
Cellulase		
72 h	432	42
144 h	473	120

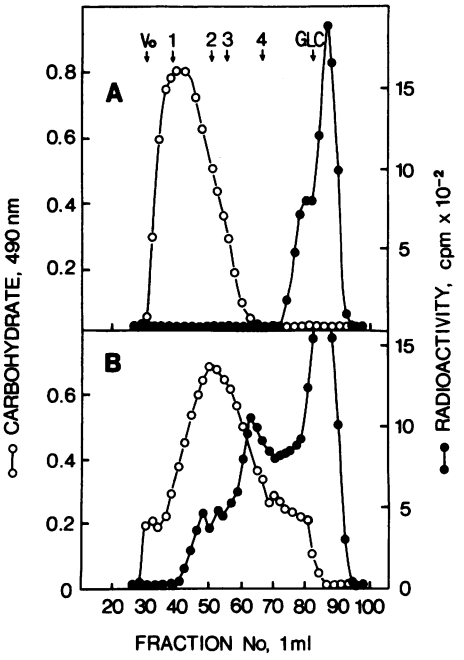

FIG. 1. Gel filtration (Sepharose CL-6B) of xyloglucan (o) extracted with 24% KOH-0.1% $NaBT_4$ from cell wall ghosts before (A) and after (B) 72-h treatment with *Streptomyces* endoglucanase. Dextran markers (mol wt): blue dextran (Vo); 1, dextran 500 (500,000); 2, dextran T-70 (70,000); 3, dextran T-40 (40,000); 4, dextran T-10 (10,400). The distribution of tritium (●) indicates that xyloglucan became labeled (reduced) only after it was hydrolyzed.

Characterization of Pea Xyloglucan. When isolated from the cell walls of pea epicotyls as the 24% KOH-extracted fraction (Table I), the hemicellulose was homogeneous by ultracentrifugal analysis. The $[\alpha]_D$ value was +46.0. In solution, it stained strongly with iodine with absorption maximum at 640 nm, a characteristic of xyloglucan (13). The gas chromatographic pattern of the partially methylated alditol acetates from the polysaccharide was identical to that from soybean xyloglucan (13).

After treatment of this wall component with *Streptomyces* endoglucanase, the degree of hydrolysis was determined by assaying for reducing groups (34) and iodine-staining residual xyloglucan (22). Kinetics of the hydrolysis showed that reducing sugar continued to be produced at a linear rate for at least 48 h under the conditions used, while the xyloglucan-iodine complex disappeared by 3 h, *i.e.* typical of endohydrolysis. The gel filtration pattern (Bio-Gel P-6) of the hydrolysates (Fig. 2) indicated that pea xyloglucan was constructed of two different oligosaccharide repeating units, a nonasaccharide which contained glucose/xylose/galactose/fucose, 4:3:1:1, and a heptasaccharide of glucose/xylose, 4:3. The two units were present in the polymer in a molar ratio close to 1:1. These units were the first major products to appear after enzyme treatment and they accumulated with time until they were essentially the only products. They did not undergo further hydrolysis by this particular endoglucanase preparation, even when the reaction time was prolonged to 96 h or when higher enzyme concentrations were used. The $[\alpha]_D$ values were +51.8 for nonasaccharide and +85.3 for heptasaccharide, which are identical to values obtained for such hydrolysis products of other xyloglucans (15, 21, 23). Linkages appear to be the same as those of soybean xyloglucan, but equal amounts of nona- and heptasaccharides were present in pea xyloglucan rather than in a ratio of 2:1 (13).

Partial enzymic hydrolysis of pea xyloglucan also yielded dimers of the oligosaccharide units which were mainly composed of 16 saccharides plus small amounts of 18 and 14 saccharides

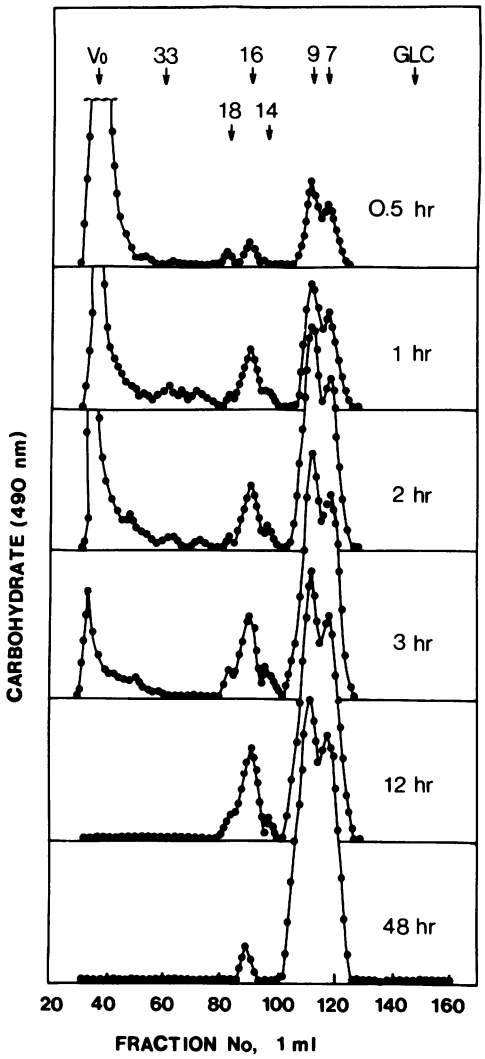

FIG. 2. Fractionation on Bio-Gel P-6 columns of products formed from pea xyloglucan following treatment with *Streptomyces* endoglucanase. The degree of polymerization of standards is indicated, *i.e.* 33, laminarin; 9, soybean xyloglucan-derived nonasaccharide; 7, heptasaccharide. The identity of higher oligosaccharides, *i.e.* 18 (9→9), 16 (9→7), 14 (7→7), was confirmed by elution, exhaustive endoglucanase hydrolysis, and rechromatography on a Bio-Gel P-4 gel.

(Fig. 2). The degree of polymerization of the dimers was estimated by calibration with authentic xyloglucan-derived nonasaccharide and heptasaccharide from soybean and with laminarin. It was confirmed by measuring the ratio of sugar content to reducing sugar terminals. The dimer containing 16 saccharides was reduced with $NaBT_4$ and digested with *Streptomyces* endoglucanase, and the hydrolysate was subjected to gel filtration on Bio-Gel P-4. The radioactivity of the hydrolysate was derived from heptasaccharide only, indicating that the structure of this dimer was nonasaccharide → heptasaccharide. Since the nona- and heptasaccharides accumulated in a molar ratio of 1:1 at all stages of hydrolysis, and the main dimer contained the two oligosaccharides (Fig. 2), it appears that pea xyloglucan contains these units distributed at random, but primarily in alternating sequence.

When pea xyloglucan and its nonasaccharide and heptasaccharide were isolated and treated with *Aspergillus* glycosidases, the gel filtration patterns of these second hydrolysates (Fig. 3) indicated that each sample produced monosaccharides and a disaccharide. The latter was indistinguishable from authentic

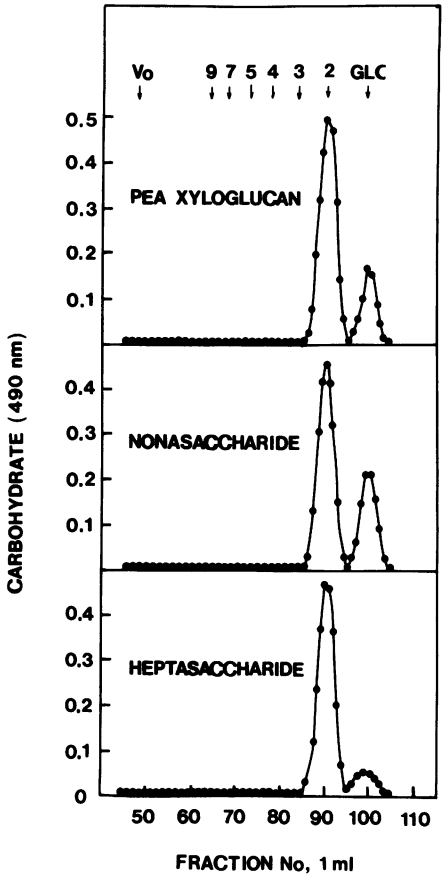

FIG. 3. Fractionation on Bio-Gel P-2 columns of the hydrolysates obtained from pea xyloglucan and pea xyloglucan-derived nona- and heptasaccharides with *Aspergillus* glycosidases. Standard markers: 9, nonasaccharide; 7, heptasaccharide; 5 to 2, laminari-oligosaccharide series.

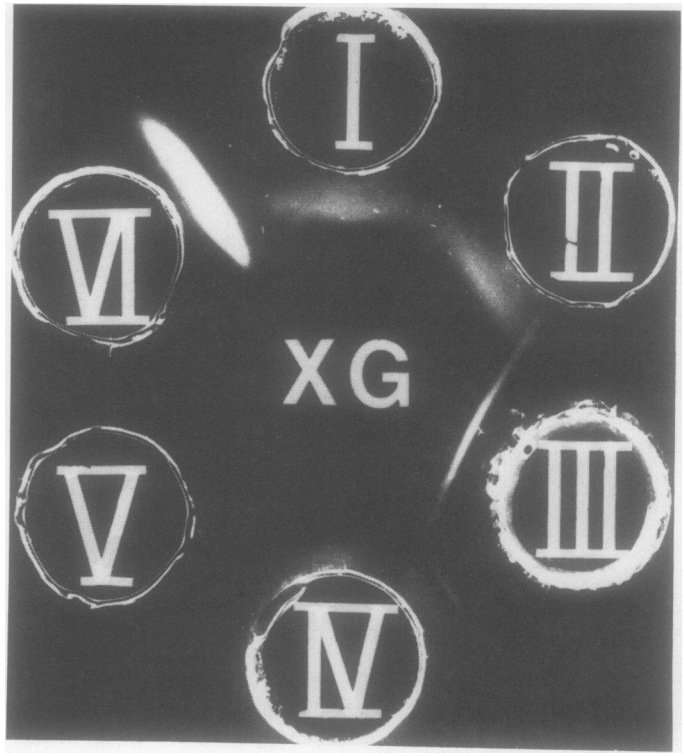

FIG. 4. Reactions of pea xyloglucan with various lectins compared by diffusion precipitation. Pea xyloglucan (XG) was in the center well and peripheral wells contained fucose-binding lectins from *U. europeus* (I), *L. tetragonolobus* (II), and eel serum (III), β-galactose-binding lectin from *R. communis* (IV), and α-glucose-binding lectins from *P. sativum* (V) and concanavalin A (IV). Note that some of these lectins are glycoproteins and mutually interact.

isoprimeverose (6-*O*-α-D-xylopyranosyl-D-glucopyranose) on paper chromatography with solvents A and B. All of the xylosyl residues of the polysaccharide and the oligosaccharides were recovered in the disaccharide, *i.e.* none was present in the monosaccharide peak which contained glucose, galactose, and fucose.

Hydrolysis of pea xyloglucan with mild acid (0.01 N trifluoroacetic acid at 60°C for 30 h) completely removed fucose and a trace of xylose from the polysaccharide. The product, on complete acid hydrolysis, yielded only glucose, xylose, and galactose. This indicates that fucosyl residues are probably linked as acid-labile terminals, *i.e.* typical of α1,2-linkages (32).

Xyloglucans which contain terminal α-L-fucosyl-(1→2)-β-D-galactosyl residues as side chains (3, 5, 13, 21) would be expected to complex with α-L-fucose-binding lectins, *e.g.* from *U. europeus* (31), *L. tetragonolobus* (30), and eel serum (35), but not to lectins that are specific to terminal β-galactose, *e.g.* from *R. communis* (37), or α-D-glucose, *e.g.* from *P. sativum* (39), or concanavalin A (11). In Ouchterlony-style precipitation tests (Fig. 4), pea xyloglucan clearly reacted strongly with all of the fucose-binding lectins but not those with other specificities. There was no detectable reaction, in particular, with galactose-binding lectins, indicating that pea xyloglucan galactose residues are essentially all fucosylated, unlike those in amyloid xyloglucan (23).

Pea xyloglucan which was partially degraded (to 20,000 mol wt) by fungal endoglucanase treatment slightly reacted with fucose-binding lectin (Fig. 5A) but, after mild acid treatment, it lost this property along with fucose and became reactive only with galactose-binding lectin (Fig. 5B). Amyloid xyloglucan, which contains no fucose, also reacted with galactose-binding lectin (38) (Fig. 5B). In quantitative precipitin tests, mild acid-treated pea xyloglucan yielded a precipitin curve with *R. communis* lectin which was virtually identical to that obtained with amyloid xyloglucan (data not shown). Evidently, mild acid-treated pea xyloglucan, like amyloid xyloglucan, contains terminal galactosyl residues with a β-linkage and had lost fucose as chemical data indicated. It is assumed that like soybean xyloglucan and amyloid xyloglucan, the galactose was attached to the 2-position of xylosyl residues by the β-linkage implied by *R. communis* lectin binding.

A summary of this analysis of pea xyloglucan structure is shown in Figure 6.

Visualization of Xyloglucan:Cellulose Association. Extracted xyloglucan binds iodine to produce a distinctive green color (λ_{max} = 640 nm) which is the basis for the iodine-sodium sulfate method for estimating this product. When xyloglucan remains firmly associated with cellulose, as in the 4% KOH-insoluble material of cell walls, addition of iodine results in an absorption spectrum which includes material adsorbing at 640 nm, but added to it is strong absorption between 500 and 600 nm due to iodine binding to the cellulose component. This could be stained with iodine and seen by light microscopy to contain recognizable cell shapes and files of cell walls. These cell wall ghosts were diffusely stained a green-brown color and little fine structure could be discerned.

Electron microscopy of shadowed preparations of the cell wall ghosts showed that the surface was indeed amorphous (Fig. 7A). However, during sequential extraction with alkali (10 to 24% KOH), cellulose fibrils became increasingly evident (Fig. 7, B and C). Vertically oriented fibrils run in ribs on the outer surfaces

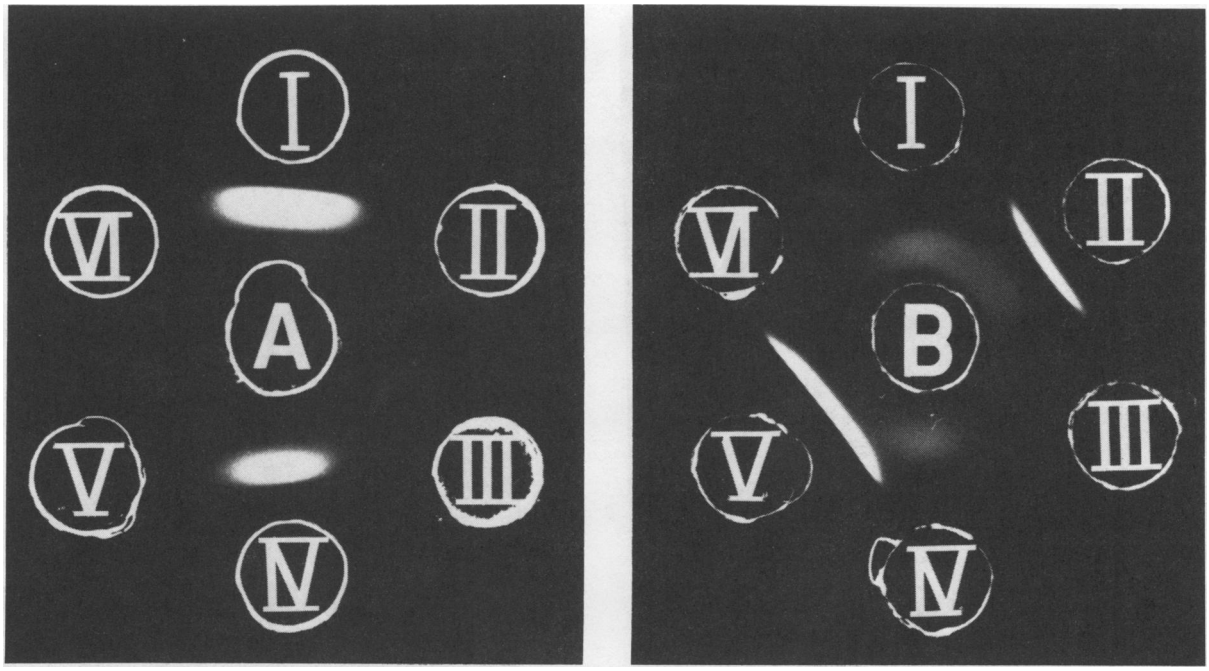

FIG. 5. Reactions of various polysaccharides with lectins from *U. europeus* (A) and *R. communis* (B). I, pea xyloglucan; II, mild acid-treated pea xyloglucan; III, partially endoglucanase-treated pea xyloglucan; IV, soybean xyloglucan; V, amyloid xyloglucan; VI, carboxymethylcellulose.

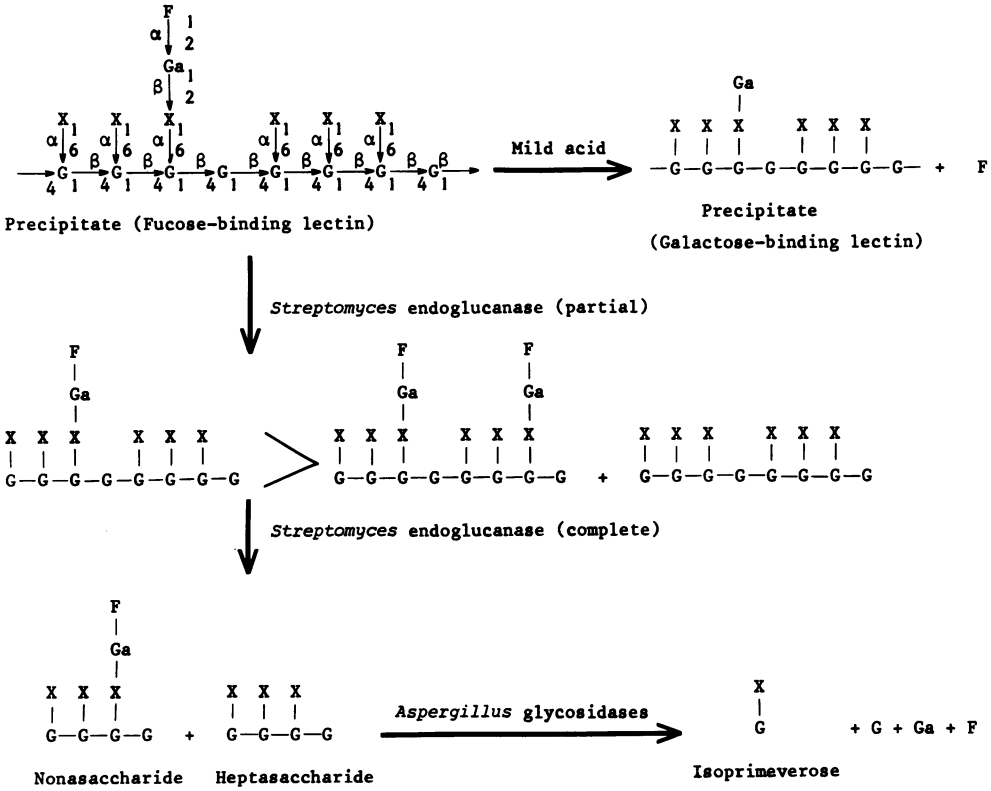

FIG. 6. Scheme used for the characterization of pea xyloglucan. F, fucose; Ga, galactose; G, glucose; X, xylose.

of the walls and there is a predominantly transverse arrangement of fibrils on the inner surface (29). The latter are disturbed in their transverse order by gaps which are presumably loci where intercellular pits occur in living cells. None of this structure is visible when xyloglucan is present in the ghosts, which implies that xyloglucan occurs both on and between the cellulose microfibrils.

The location of xyloglucan was also examined by radioautography and by binding of fluorescent lectin. Pea xyloglucan was labeled by supplying [^{3}H]fucose to living tissues and 4% KOH-insoluble material was prepared as in Table I. Radioautographs of the resulting ghosts (Fig. 8A) showed that silver grains were distributed over the entire ghost surface, with no discernable localization at the light microscope level. The fucose in these ghosts was present entirely in xyloglucan, *i.e.* on extraction with 24% KOH and gel filtration, the label distribution exactly paralleled the profile of iodine-reactive material (data not shown).

When a fluorescein-labeled lectin which specifically binds fu-

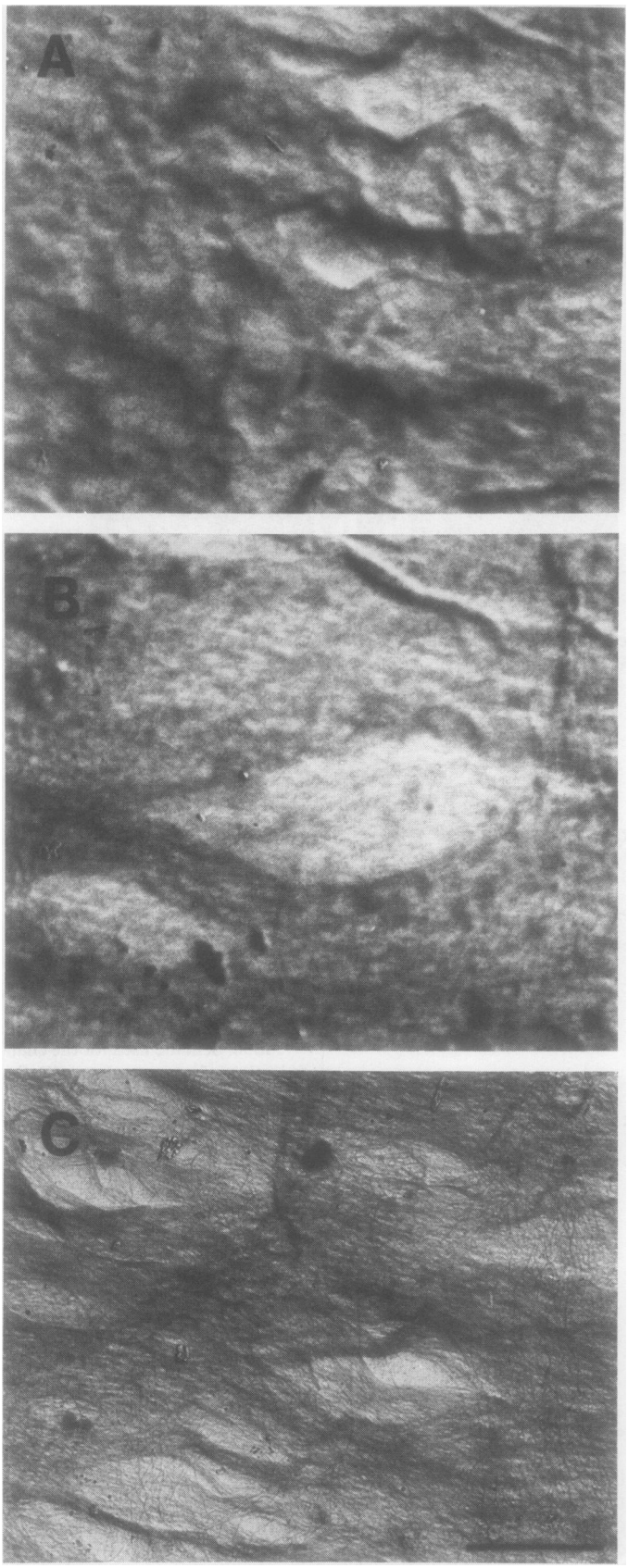

FIG. 7. Electron micrographs of shadowed preparations of cell wall ghosts. A, extracted with 4% KOH (composed of xyloglucan and cellulose, 0.7:1.0); B, extracted with 10% KOH (which removed 60% of xyloglucan); C, extracted with 24% KOH (composed of cellulose only). Magnification bar is 5 μm.

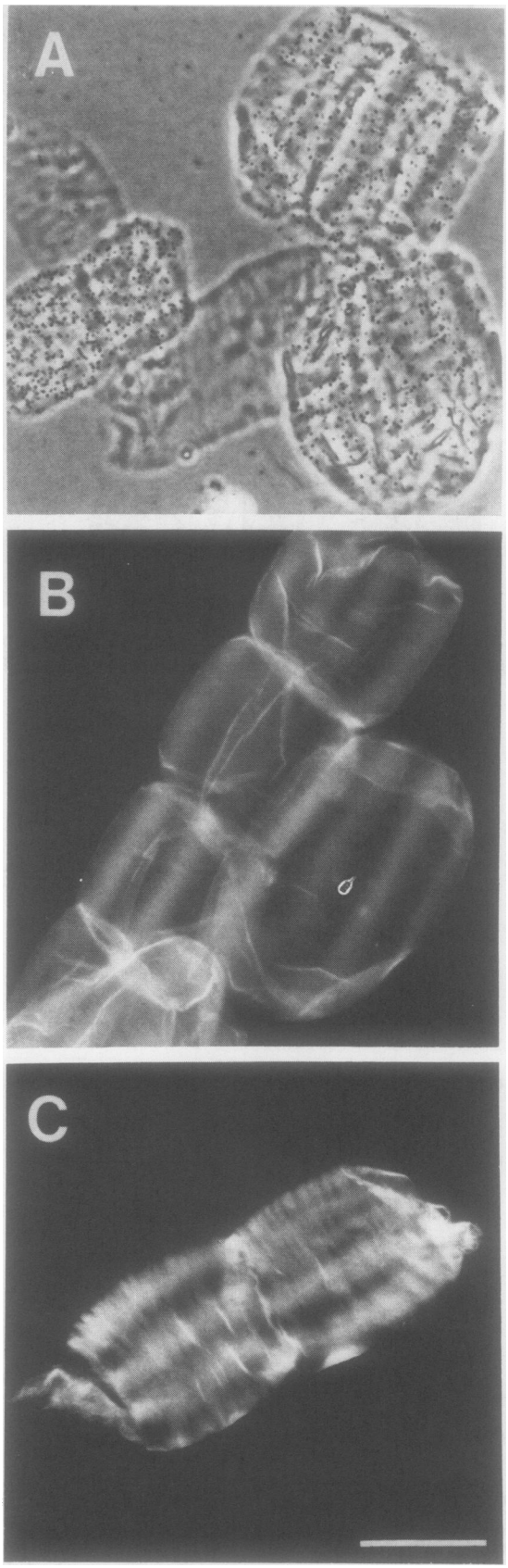

FIG. 8. Radioautograph of [^{3}H]fucose-labeled cell wall ghosts (A), fluorescence microscopy of ghosts labeled with fucose-binding lectin (B), and fluorescence microscopy of *Streptomyces* endoglucanase-treated ghosts (xyloglucan/cellulose, 0.08:1.0) stained with Calcofluor (C). Magnification bar is 20 μm.

Table III. *Saturation Binding between Pea Xyloglucan, Cell Wall Ghosts, and Cellulose Derivatives*

Ligand (30 mg)	Adsorption of Additive		
	Pea xyloglucan	Cellopentaose	CM-cellulose
		mg	
Microcrystalline cellulose	0.6	0.84	0
Pea cellulose (24% KOH-insoluble)	1.67	0.87	0.15
Cell wall ghosts	0	0	1.23

cose was incubated with ghosts, fluorescence (Fig. 8B) was most intense in bands that traversed the ghosts in a vertical direction in relation to the cell axis and in the junctions between cells. These vertical bands were not characterized by longitudinally oriented striations and did not correspond with the cellulose microfibrils in ribs (Fig. 8C) (29). Rather, the bands had a transverse striation which reflected the microfibrillar orientation of the most inner fibrils in the interrib regions. The removal of most xyloglucan by enzymic hydrolysis had clearly extracted xyloglucan from between the fibrils of cellulose, particularly from the inner wall surface and from the fibrillar gaps seen in electron micrographs (Figs. 7C and 8C).

Binding Studies *In Vitro*. Several cell wall polysaccharides, *e.g.* arabinan, citrus pectin, and larch arabinogalactan, showed no detectable binding to pea cell wall ghosts by estimates based on carbohydrate determination with the phenol-sulfuric acid method. Likewise, extracted pea xyloglucan and cellopentaose, which bound readily *in vitro* to pea and microcrystalline cellulose, did not bind to cell wall ghosts (Table III). This suggests that cellulose microfibrils in the ghosts were totally masked (saturated) with endogenous bound xyloglucan. On the other hand, CM-cellulose bound effectively to the cell wall ghosts, presumably because there was interaction with xyloglucan strands in the ghosts. Interaction between CM-cellulose and pea xyloglucan can also be observed viscometrically, *i.e.* the viscosity of solutions of mixtures is higher than the sum of viscosities of separate solutions of these materials.

DISCUSSION

Specific lectins were particularly useful in this study to confirm the fine structure of xyloglucan side groups (Figs. 4 and 5) and provide evidence for its distribution in the wall (Fig. 8B). A lectin:xyloglucan:cellulose complex was visualizable in cell wall ghosts which extended over the whole wall surface, with particularly high concentrations between microfibrils on the inner wall surfaces and at junctions between cells in files.

The disposition of cellulose microfibrils in the primary walls of growing pea epicotyl cells appears to be identical whether matrix materials are removed by "scouring" with concentrated alkali (Fig. 7C) or by prolonged treatment with endo-1,4-glucanase (Fig. 8C). In both cases, fibrils are oriented primarily in transverse directions on the inner surfaces of walls, and some change direction to travel in longitudinally oriented "ribs" on the outer surfaces. Cell wall ghosts containing only cellulose often remain attached in files, with the ribs in adjacent cells continuing in the same relative positions. In fact, rib microfibrils appear to extend across from cell to cell (Fig. 8, B and C) (29), which is not surprising in view of the fact that files of young cells are all formed by cell division from a single apical initial cell. It seems clear that wall matrix material, and xyloglucan in particular, was not required to maintain the shape and integrity of the basic fibrillar wall network.

Xyloglucan in cell wall ghosts, as visualized by electron microscopy (Fig. 7A), radioautography (Fig. 8A), or fluorescence with a fucose-binding lectin (Fig. 8B), was clearly present both on and between the cellulose microfibrils. It is presumably the interfibrillar component of xyloglucan which was free to bind to iodine and particularly accessible to hydrolysis by endoglucanase (Table II). However, few of the individual xyloglucan molecules can have been completely free from any association with cellulose microfibrils because very little (1.7%) was extractable by homogenization in buffers (Table I). These results, together with the fact that the alkali-insoluble pea xyloglucan:cellulose complex contains no detectable protein (26) or aromatic cross-links (8), imply that xyloglucan is strongly bound to cellulose microfibrils by hydrogen bonds, as proposed by Albersheim (1).

With an average mol wt of 330,000, pea xyloglucan molecules contain a backbone of about 1,100 contiguous 1,4-linked glucose units, which represents a maximum chain length of 550 nm. This is many times the diameter of cellulose microfibrils (7) and quite sufficient to cross-link adjacent fibrils by hydrogen bonding, even if some free sectors assumed a nonlinear configuration. Indeed, it is several times the thickness of the entire primary wall. The whole surface area of microfibrils appears to have been bound to xyloglucan, as proposed by Albersheim (1), because no further xyloglucan would bind to the isolated ghosts (Table III). Thus, it seems legitimate to regard the complex of xyloglucan and cellulose in these walls as a naturally occurring multimolecular cross-linked structure in which microfibril surfaces are totally masked and saturated with xyloglucan.

It should be noted that the maximum amount of pea xyloglucan which bound to cellulose microfibrils when the two were added together *in vitro* was never more than 5 to 6% of the weight of cellulose (Table III). This represents a value comparable to the surface:volume ratio of microfibrils with diameters (35 to 40 Å) such as those observed in primary walls (7). The implication is that when microfibril surfaces became coated with xyloglucan in such reconstitution tests, no further xyloglucan could be adsorbed. In cell wall ghosts, in contrast, the weight ratio of xyloglucan:cellulose was approximtely 70% (Table II), and much stronger alkali was required to remove it than the xyloglucan from reconstituted complexes (2, 5). This indicates that the natural xyloglucan:cellulose complex was not fully reassembled merely by combining the two polysaccharides *in vitro*. Presumably there are controls *in vivo* over the deposition of xyloglucan in relation to cellulose that were not duplicated *in vitro*.

These observations support proposals (1) that xyloglucan in primary walls could be a major component that contributes to wall rigidity. It is highly susceptible to hydrolysis by endoglucanase and such enzymes are known to be regulated by growth hormones in many plants. In pea stems, two endo-β-1,4-glucanases are induced by auxin treatment (40) and one of these (buffer-insoluble) is localized on the inner wall surface (4) where xyloglucan appears to be particularly concentrated. The implication is that xyloglucan is the more "natural" substrate for endogenous plant "cellulases," a possibility that is examined in Part II of this study.

Acknowledgments—We thank Dr. S. Saka (Pulp and Paper Research Institute, Quebec) for assistance in the preparation of shadowed samples and Dr. J. Marsden (Biology Dept, McGill University) for help in performing radioautography. We also thank Dr. E. T. Reese (United States Army Laboratories) for the gift of cellulase preparation from *S. griseus* and Dr. K. Matsuda (Tohoku University, Japan) for the gift of *A. oryzae* glycosidase preparation and *T. indica* xyloglucan.

LITERATURE CITED

1. ALBERSHEIM P 1976 The primary cell wall. *In* J Bonner, JE Varner, eds, Plant Biochemistry. Academic Press, N Y, pp 225–274
2. ASPINALL GO, JA MOLLOY, JWT CRAIG 1969 Extracellular polysaccharides from suspension-cultured sycamore cells. Can J Biochem 47: 1063–1070
3. ASPINALL GO, TN KRISHNAMURTHY, KG ROSELL 1977 A fucogalactoxyloglucan from rapeseed hulls. Carbohydr Res 55: 11–19
4. BAL AK, DPS VERMA, H BYRNE, GA MACLACHLAN 1976 Subcellular localization of cellulases in auxin-treated pea. J Cell Biol 69: 97–105
5. BAUER WD, KW TALMADGE, K KEEGSTRA, P ALBERSHEIM 1973 The structure of plant cell walls. II. The hemicellulose of the walls of suspension-cultured sycamore cells. Plant Physiol 51: 174–187
6. DUBOIS M, KA GILLES, JK HAMILTON, PA REBERS, F SMITH 1956 Colorimetric method for determination of sugars and related substances. Anal Chem 28: 350–356
7. FREY-WYSSLING A 1969 The ultrastructure and biogenesis of native cellulose. Fortschr Chem Organ Naturstoffe 27: 1–30
8. FRY, SC 1982 Isodityrosine, a new cross-linking amino acid from plant cell-wall glycoprotein. Biochem J 204: 449–455
9. GAILLARD BDE 1961 Separation of liner from branched polysaccharides by precipitation as iodine complexes. Nature 191: 1295–1296
10. GILKES NR, MA HALL 1977 The hormonal control of cell wall turnover in *Pisum sativum* L. New Phytol 78: 1–15
11. GOLDSTEIN IJ, CE HOLLERMAN, EE SMITH 1965 Protein-carbohydrate interaction. II. Inhibition studies on the interaction of concanavalin A with polysaccharides. Biochemistry 4: 876–883
12. HAKOMORI S 1964 A rapid permethylation of glycolipid, and polysaccharide catalyzed by methylsulfinyl carbanion in dimethyl sulfoxide. J Biochem 55: 205–208
13. HAYASHI T, Y KATO, K MATSUDA 1980 Xyloglucan from suspension-cultured soybean cells. Plant Cell Physiol 21: 1405–1418
14. HAYASHI T, Y KATO, K MATSUDA 1981 Biosynthesis of xyloglucan in suspension-cultured soybean cells. An assay method for xyloglucan xylosyltransferase and attempted synthesis xyloglucan from UDP-xylose. J Biochem 89: 325–328
15. HAYASHI T, K MATSUDA 1981 Biosynthesis of xyloglucan in suspension-cultured soybean cells. Occurrence and some properties of xyloglucan 4-β-D-glucosyltransferase and 6-α-D-xylosyltransferase. J Biol Chem 256: 11117–11122
16. HAYASHI T, K MATSUDA 1981 Biosynthesis of xyloglucan in suspension-cultured soybean cells. Evidence that the enzyme system of xyloglucan synthesis does not contain β-1,4-glucan 4-β-D-glucosyltransferase activity (EC 2.4.1.12). Plant Cell Physiol 22: 1571–1584
17. HURN BL, SM CHANTLER 1980 Production of reagent antibodies. Methods Enzymol 70: 104–142
18. JOHN M, SCHMIDT J, WANDREY C, SAHM H 1982 Gel chromatography of oligosaccharides up to DP 60. J Chromatogr 247: 281–288
19. JOHNSON DC, MD NICHOLSON, FC HAIGH 1976 Dimethyl sulfoxide/paraformaldehyde: a nondegrading solvent for cellulose. Appl Polym Symp 28: 931–943
20. KATO Y 1978 Studies on the structure and functional role of xyloglucan in immature *Leguminosae*. PhD thesis, Tohoku University, Sendai, Japan
21. KATO Y, K MATSUDA 1980 Structure of oligosaccharides obtained by hydrolysis of mung bean xyloglucan with *Trichoderma viride* cellulase. Agric Biol Chem 44: 1759–1766
22. KOOIMAN P 1960 A method for the determination of amyloid in plant seeds. Recl Trav Chim Pays-Bas 79: 675–678
23. KOOIMAN P 1961 The constitution of *Tamarindus*-amyloid. Recl Trav Chim Pays-Bas 80: 849–865
24. LABAVITCH JM, PM RAY 1974 Turnover of cell wall polysaccharides in elongating pea stem segments. Plant Physiol 53: 669–673
25. LABAVITCH JM, PM RAY 1974 Relationship between promotion of xyloglucan metabolism and induction of elongation by indoleacetic acid. Plant Physiol 54: 499–502
26. LAMPORT DTA, L EPSTEIN 1983 A primary cell wall cellulose "warp" extension "weft" model. Plant Physiol 72: S-60
27. LINDBERG B 1972 Methylation analysis of polysaccharides. Methods Enzymol 28: 178–195
28. LOWRY OH, NJ ROSEBROUGH, AL FARR, RJ RANDALL 1951 Protein measurement with the Folin phenol reagent. J Biol Chem 193: 265–275
29. MACLACHLAN GA 1976 A potential role for endo-cellulase in cellulose biosynthesis. Appl Polym Symp 28: 645–658
30. PEREIRA MEA, EA KABAT 1974 Specificity of purified hemagglutinin (lectin) from *Lotus tetragonolobus*. Biochemistry 13: 3184–3192
31. PEREIRA MEA, EC KISAILUS, F GRUEZO, EA KABAT 1978 Immunochemical studies on the combining site of the blood group H-specific lectin 1 from *Ulex europeus* seeds. Arch Biochem Biophys 185: 108–115
32. REGE VP, PAINTER TJ, WM WATKINS, WTJ MORGAN 1964 Isolation of serologically active fucose-containing oligosaccharides from human blood-group H substance. Nature 203: 360–363
33. SANDFORD PA, HE CONRAD 1966 The structure of the *Aerobacter aerogenes* A3(S1) polysaccharide. I. A reexamination using improved procedures for methylation analysis. Biochemistry 5: 1508–1517
34. SOMOGYI M 1952 Notes on sugar determination. J Biol Chem 195: 19–23
35. SPRINGER GF, T TAKAHASHI, PR DESAI, BJ KOLECKI 1965 Cross reactive human blood-group H(O) specific polysaccharide from *Sassafras albidum* and characterization of its hapten. Biochemistry 4: 2099–2113
36. TERRY ME, RL JONES, BA BONNER 1981 Soluble cell wall polysaccharides released from pea stems by centrifugation. I. Effect of auxin. Plant Physiol 68: 531–537
37. VAN WAUWE JP, FG LOONTIENS, CK DE BRUYNE 1973 The interaction of *Ricinus communis* hemagglutin with polysaccharides and low molecular weight carbohydrates. Biochim Biophys Acta 313: 99–105
38. VAN WAUWE JP, FG LOONTIENS, CK DE BRUYNE 1974 On the interaction of *Ricinus communis* lectin with plant amyloids. Biochim Biophys Acta 354: 148–151
39. VAN WAUWE JP, FG LOONTIENS, CK DE BRUYNE 1975 Carbohydrate binding specificity of the lectin from the pea (*Pisum sativum*). Biochim Biophys Acta 379: 456–461
40. VERMA DPS, GA MACLACHLAN, H BYRNE, D EWINGS 1975 Regulation and *in vitro* translation of messenger ribonucleic acid for cellulase from auxin-treated pea epicotyls. J Biol Chem 250: 1019–1026