Carbohydrate Research, 197 (1990) 139–158
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

STRUCTURAL CHARACTERIZATION OF AN ARABINOSE-CONTAINING HEPTADECASACCHARIDE ENZYMICALLY ISOLATED FROM SYCAMORE EXTRACELLULAR XYLOGLUCAN*

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(Received July 21st, 1989; accepted for publication, September 3rd, 1989)

ABSTRACT

An arabinose-containing heptadecasaccharide, generated by endoglucanase digestion of the xyloglucan secreted by suspension-cultured sycamore cells, was structurally characterized. The structure of the heptadecasaccharide was unambiguously determined through combined data generated by ^{1}H-n.m.r. spectroscopic, fast-atom bombardment mass spectrometric, and glycosyl-composition and glycosyl-linkage analyses of the intact heptadecasaccharide and of oligosaccharide fragments of the heptadecasaccharide. 1D and 2D ^{1}H-n.m.r. analyses provided data for assignment of the anomeric configurations of the glycosidic linkages, as well as information about the glycosylic and linkage compositions of the heptadecasaccharide. F.a.b.-m.s. data provided the molecular weight and supplied critical information about the glycosylic composition and glycosylic sequence of the heptadecasaccharide. The heptadecasaccharide was found to be a combination of previously characterized nona- and hepta-saccharide components of xyloglucan. The nonasaccharide was shown to be glycosidically linked to the heptasaccharide through C-4 of the β-glucosyl residue nearest to the nonreducing end of the heptasaccharide component. An arabinosyl residue was glycosidically linked at C-2 of the same β-glucosyl residue at the non-reducing end of the heptasaccharide component of the heptadecasaccharide. Although the presence of arabinosyl residues in sycamore xyloglucan has been recognized since 1973, the location of the arabinosyl residues had not been ascertained.

INTRODUCTION

Xyloglucans (XGs) are hemicellulosic polysaccharides found in the primary cell walls of all the higher plants that have been examined[1]. Xyloglucans are structurally related to cellulose in that they possess a (1→4)-β-D-Glc*p* backbone,

*This article is number XXX in the series entitled The Structure of Plant Cell Walls.
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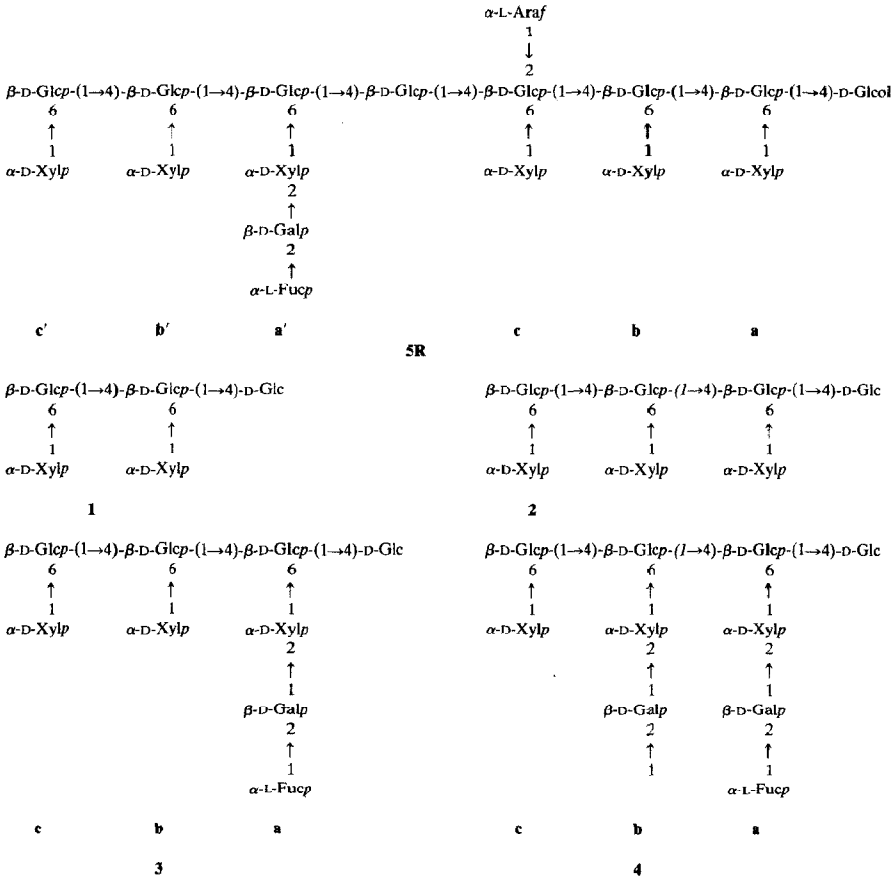

Fig. 1. Structures of sycamore xyloglucan (XG) oligosaccharides **1** through **4** and hexadecaglycosyl alditol **5R** generated by digestion of XG with a (1→4)-β-D-glucan-4-glucanohydrolase. Oligoglycosyl alditols **1R** through **4R** are prepared by converting the reducing Glc residue to Glcol by $NaBH_4$ reduction. Compound **1** is the xyloglucan pentasaccharide; **2**, the heptasaccharide; **3**, the nonasaccharide; and **4**, the decasaccharide. The letters **a**, **b**, and **c** indicate, for the residues located above them, the proximity of these residues to the reducing Glc or Glcol residue. The letters **a'**, **b'**, and **c'** in **5** and **5R** indicate, for the residues located above them, the proximity of these residues to the 4-*O*-linked glucosyl residue. These letters are used as superscript identifiers (Glc^a, $Glc^{a'}$, Xyl^a, $Xyl^{a'}$, etc.) in the text.

but they differ from cellulose in that ~75% of the β-glucosyl backbone residues are substituted at C-6 with an α-D-Xyl*p* residue[1–7]. In the XG isolated from sycamore cell walls or sycamore extracellular polysaccharides (SEPS), some of the α-D-Xyl*p* residues are substituted at C-2 with a β-D-Gal*p* residue or with the disaccharide α-L-Fuc*p*-(1→2)-β-D-Gal*p*[8]. In addition, L-arabinose has been detected in sycamore cell wall and SEPS XG[2].

The relationship between the structure of XGs and their functions in the plant cell wall has not been entirely ascertained. XGs bind strongly to cellulose through multiple hydrogen bonds and appear to coat the surface of cellulose microfibrils in

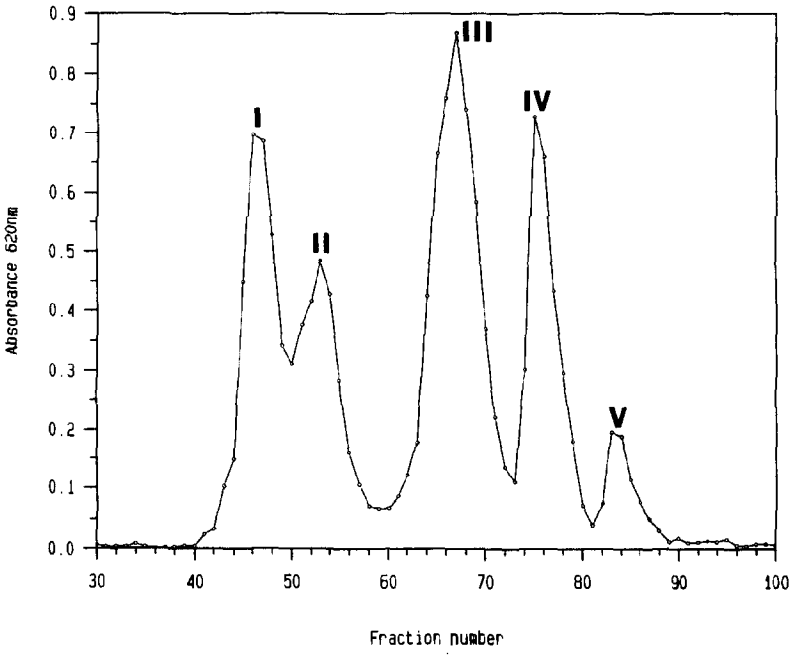

Fig. 2. BioGel P-2 chromatography of the (1→4)-β-D-glucan-4-glucanohydrolase digest of the xyloglucan isolated from sycamore extracellular polysaccharides. The hexose content of each fraction was determined colorimetrically by the anthrone assay (see ref. 32). Peak I consisted of oligosaccharides that were totally excluded by BioGel P-2. Peak III contained a mixture of decasaccharide **4** and nonasaccharide **3**. Peaks IV and V contain heptasaccharide **2** and pentasaccharide **1**, respectively. The oligosaccharide corresponding to **5R** was isolated from peak II which contains a mixture of oligosaccharides, each of which appears to consist of between 15 and 20 glycosyl residues. The structures of the other oligosaccharides in peak II have not been thoroughly analyzed.

the walls of growing plant cells[9]. It has been suggested that XGs may act as cell-wall stabilizing molecules that noncovalently crosslink cellulose microfibrils by hydrogen bonding to adjacent microfibrils[9]. There is evidence suggesting that oligosaccharide fragments of XG may play a role in the regulation of the rate of plant cell growth[10–13].

Endoglucanases [(1→4)-β-D-glucan-4-glucanohydrolases, EC 3.2.1.4] capable of cleaving XG into oligosaccharides have been isolated from auxin-treated pea stems[14] and from the culture fluid of various fungi[15]. The major products obtained when XG from sycamore cell walls or SEPS is treated with an endoglucanase are pentasaccharide **1**, heptasaccharide **2**, nonasaccharide **3**, and decasaccharide **4**, as shown in Fig. 1. Most of the XG nonasaccharide **3** and decasaccharide **4** fragments isolated from either sycamore cell walls or SEPS have one or two *O*-acetyl substituents on the 2-*O*-linked galactosyl residue[4,16].

We have found that about 40% of the XG isolated from the SEPS and walls of suspension-cultured sycamore cells canot be degraded by endoglucanase into oligosaccharides that contain less than ~15 glycosyl residues (Fig. 2). Terminal

L-arabinofuranosyl residues have been detected in this material[2]. This paper describes the purification and structural characterization of one of the endoglucanase-resistant XG oligosaccharides, a heptadecasaccharide. An oligosaccharide containing arabinose was chosen for our studies, as we were especially interested in determining the point of attachment of the arabinosyl residue.

RESULTS

Overview of analytical procedures. — An arabinose-containing SEPS XG oligoglycosyl alditol **5R** was purified and structurally characterized (Fig. 1, see below for nomenclature). This structure was determined using a variety of techniques, including glycosyl-residue and glycosyl-linkage composition analyses, ^{1}H-n.m.r. spectroscopy, and f.a.b.-mass spectrometry of **5R** and fragments of **5R**. The arabinosyl residue of **5R** was selectively removed, and the resulting pentadecaglycosyl alditol was treated with endoglucanase. The digestion products were characterized by f.a.b.-m.s. and ^{1}H-n.m.r. spectroscopy. The combined data allowed us to unambiguously determine the structure of **5R**. The following sections describe the results of these analyses.

Nomenclature for identification of the glycosylic residues of XG oligosaccharides and their oligoglycosyl alditols. — The glycosyl residues of **1**, **1R**, **2**, **2R**, **3**, **3R**, **4**, **4R**, and **5R**, where "R" indicates that the reducing glucose (Glc) has been converted to "glucitol" (Glcol), are distinguished by a superscript a, b, c, a′, b′, or c′. The superscripts a, b, and c represent increasing distance from the reducing glucose or the glucitol, and the superscripts a′, b′, and c′ in **5** and **5R** represent increasing distance from the 4-*O*-linked-glucose (Fig. 1). For example, the glucosyl residue linked directly to the glucitol is referred to as Glc^a. Residues that are part of a sidechain attached to a particular glucosyl residue are referred to by the superscript of that glucosyl residue. For example, the 2-*O*-linked-α-xylosyl residue and the adjacent terminal α-xylosyl residue of **3** are referred to as Xyl^a and Xyl^b, respectively.

Isolation of **5R**. — XG isolated from the extracellular polysaccharides of suspension-cultured sycamore cells (SEPS)[2] was digested with *Trichoderma viride* (1→4)-β-D-endoglucanase to selectively hydrolyze unbranched 4-*O*-linked-β-glucosyl residues. Digestion of the XG with endoglucanase and chromatography of the cleavage products on a column of BioGel P-2 resulted in the separation of five major carbohydrate-containing peaks (Fig. 2). A mixture of compounds **3** and **4** is eluted as peak III[4], while compounds **1** and **2** are eluted as peaks IV and V, respectively[4].

The oligosaccharides in peaks I and II previously had not been structurally characterized. Oligosaccharides that were totally excluded by BioGel P-2[4] eluted as peak I, while those containing 15–20 glycosyl residues eluted as peak II. The oligosaccharides in peak II were *O*-deacetylated with aqueous alkali, treated with endoglucanase, and finally converted to oligoglycosyl alditols with $NaBH_4$. Endo-

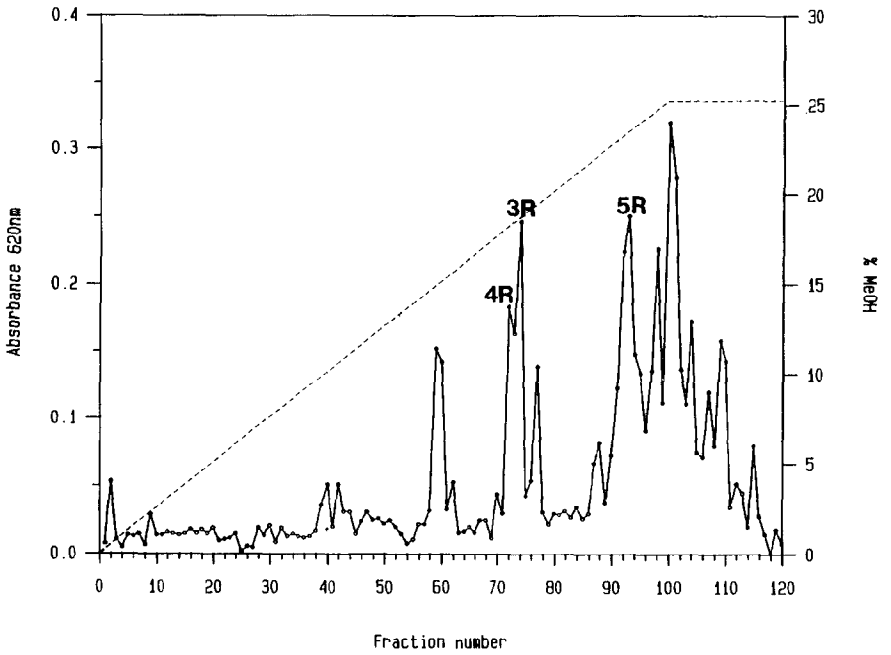

Fig. 3. Reverse-phase C-18 h.p.l.c. chromatography of the oligoglycosyl alditols prepared by deacetylation, endoglucanase digestion, and $NaBH_4$ reduction of the oligosaccharides that were eluted from the BioGel P-2 column in peak II (Fig. 2). The oligoglycosyl alditols were eluted using a gradient of 0–25% aqueous MeOH over 30 min, followed by isocratic elution with 25% MeOH in H_2O for an additional 10 min. Compound **5R** was further purified by a second chromatography on this column eluting with 14–25% MeOH in H_2O.

glucanase treatment of *O*-deacetylated peak II oligosaccharides was designed to hydrolyze any glycosyl residues that were resistant to endoglucanase hydrolysis, because the glycosidic linkage had been sterically hindered by an *O*-acetyl group. The reducing residues of the resulting oligosaccharides were converted to alditols in order to eliminate the possible separation, during subsequent h.p.l.c., of the α and β anomers of otherwise identical oligosaccharides. The oligoglycosyl alditols derived from peak II (Fig. 2) *O*-deacetylation and retreatment with endoglucanase were subjected to reverse-phase chromatography on an RP-18 h.p.l.c. column using a gradient of 0–25% MeOH in H_2O (Fig. 3). The h.p.l.c. chromatogram revealed that the *O*-deacetylated, endoglucanase-treated peak II mixture contained at least eight oligoglycosyl alditols. Oligoglycosyl alditols **3R** and **4R** (Fig. 3), which were identified by 1D ^{1}H-n.m.r. spectroscopy, were clearly generated by endoglucanase treatment of the *O*-deacetylated oligosaccharides. Glycosyl-composition analysis of the other major carbohydrate-containing peaks that eluted from the h.p.l.c. column (Fig. 3) indicated that column fractions 92 and 93 contained arabinose. Fractions 92 and 93 were combined and rechromatographed on the RP-18 h.p.l.c. column using a gradient of 14–25% MeOH in H_2O. The major component eluting

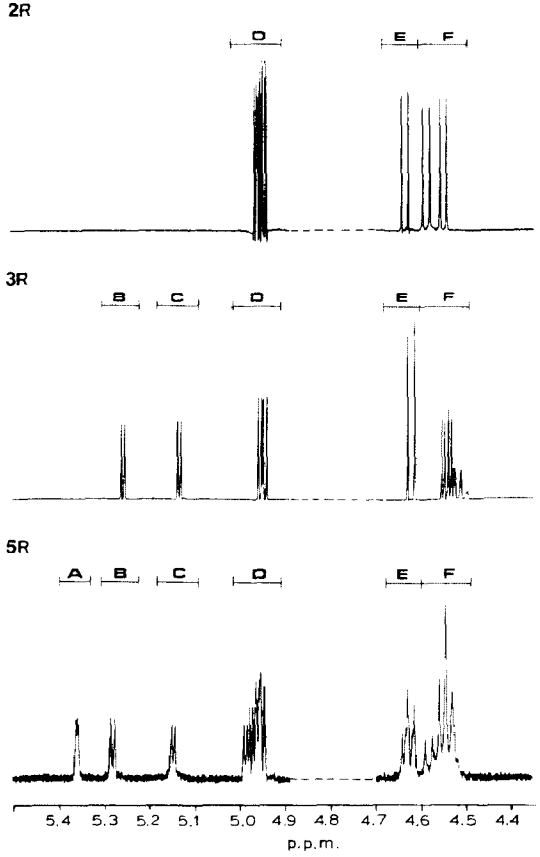

Fig. 4. Anomeric regions of the 1D ^{1}H-n.m.r. spectra of oligoglycosyl alditols **2R**, **3R**, and **5R**. The HDO signal at δ 4.75 has been deleted for clarity (dashed lines). Six subregions of the ^{1}H-n.m.r. spectra are indicated by bars in the figure. These subregions encompass the following signals: (A) H-1 of α-arabinosyl residues; (B) H-1 of α-fucosyl residues; (C) H-1 of α-xylosyl residues with an α-Fuc-(1→2)-β-Gal substituent at C-2; (D) H-1 of terminal, nonreducing α-xylosyl residues; (E) H-1 of 4,6-*O*-linked β-glucosyl residues linked to the alditol, H-1 of 2-*O*-linked β-galactosyl residues, and H-1 of 2,4,6-*O*-linked β-glucosyl residues; (F) H-1 of 4,6-*O*-linked β-glucosyl residues not linked to the alditol, H-1 of 4-*O*-linked β-glucosyl residues, H-1 of 6-*O*-linked β-glucosyl residues, and H-5 of α-fucosyl residues.

from this column is **5R**. The structural characterization of **5R**, the major oligoglycosyl alditol in column fractions 92 and 93, will now be described.

^{1}H-n.m.r. analysis of **5R**. — Much of the structure of **5R** was deduced by comparing the ^{1}H-n.m.r. spectrum of **5R** to the previously assigned[8] ^{1}H-n.m.r. spectra of compounds **1R**–**4R**. The XG oligosaccharides, whose ^{1}H-n.m.r. spectra had previously been assigned, did not contain arabinosyl residues. Therefore, H-1 and H-2 of the L-arabinofuranosyl residue were assigned by comparing published chemical shifts and vicinal coupling constants for methyl α- and β-L-arabinofuranosides[17–19] to those obtained from the COSY spectrum of **5R**.

The anomeric regions of the ^{1}H-n.m.r. spectra of **2R**, **3R**, and **5R** were

divided into subregions[8] A–F as indicated in Fig. 4. A COSY experiment performed on **5R** reflected the connectivity of adjacent protons within each glycosylic residues. These data allowed the fucosyl H-5 and most of the other H-1 and H-2 resonances in the spectrum of **5R** to be assigned. Lists of the chemical shifts and coupling constants of H-1 signals are shown in Table I, and those of the H-2 signals are presented in Table II.

The doublet in subregion A of the ^{1}H-n.m.r. spectrum of **5R** was not present in the spectra of either **2R** or **3R**. The chemical shift (δ 5.355) and vicinal coupling constant ($J_{1,2}$ = 1.8 Hz) of this resonance identified it as H-1 of the terminal L-arabinofuranosyl residue of **5R**, which was shown to be present by glycosyl-linkage composition analysis (see Tables III and IV). The signal at δ 4.18 ($J_{2,3}$ = 3.6 Hz) was identified as H-2 of the L-arabinofuranosyl residue by virtue of its connectivity in the COSY spectrum of **5R** to the doublet at δ 5.355. The H-1 and H-2 chemical shifts (δ 5.355 and δ 4.18) and vicinal coupling constants ($J_{1,2}$ = 1.8 Hz and $J_{2,3}$ = 3.6 Hz) of the L-arabinofuranosyl residue of **5R**, confirmed that it is the α-anomer[17–19].

Integration of the signals contained in subregions A through F in the ^{1}H-n.m.r. spectrum of **5R**, yielded the following normalized area ratios 0.9:1.0:1.1:5.0:2.8:6.2, respectively. These ratios agree with the predicted values (1:1:1:5:3:6) for a hexadecaglycosyl alditol consisting of an arabinosyl residue and oligosaccharides **2** and **3**, with one of the latter present as an oligoglycosyl alditol. This calculation includes the contribution made by the H-5 resonance from α-fucosyl residues that are present in subregion F[8,20] (see legend to Fig. 4). The agreement between the calculated and predicted areas for the anomeric and fucosyl H-5 signals in the spectrum of **5R** provides evidence that **5R** accounts for 90% or more of the components in h.p.l.c. fractions 92 and 93. This conclusion was supported by the results of the f.a.b.–m.s. analysis (see below).

Glycosyl-residue and glycosyl-linkage composition analyses of **5R**. — Quantitative recovery of derivatized glycosyl residues in glycosyl-composition and glycosyl-linkage analyses is difficult to achieve. This is due in part to the different susceptibilities of the various glycosidic linkages to acid hydrolysis, and it is further complicated by the different susceptibilities of the resulting glycoses to acid-catalyzed degradation. Undermethylation of selected glycosyl residues and a proclivity of some residues to base-catalyzed degradation during methylation are additional problems associated with glycosyl-linkage analyses. Although the less-than-quantitative nature of the glycosyl-residue and glycosyl-linkage composition data obtained for compound **5R** is evident (Tables III and IV), these analyses correlate well with the ^{1}H-n.m.r. data described above.

Relatively short hydrolysis times were chosen for the glycosyl-residue analyses in order to minimize the degradation of acid-labile arabinose, fucose, and xylose. These conditions may have resulted in incomplete release of the β-glucosyl residues which would account for the lower-than-expected recovery of glucose (Table IV). Recovery of the other sugars in **5R** agreed with the proposed structure of **5R**.

TABLE I

^{1}H-N.M.R. H-1 CHEMICAL SHIFTS AND COUPLING CONSTANTS[1] IN SUBREGIONS A-F OF THE SPECTRA OF OLIGOGLYCOSYL ALDITOLS **2R**, **3R**, AND **5R**, AND DIONEX SAMPLES **X** AND **Y**

Residue	**2R**	**X**	**3R**	**Y**	**5R**
Ara	—	—	—	—	5.355 (1.8)
Fuc	—	—	5.257 (4.0)	5.257 (4.0)	5.275 (3.8)
Xyl^a	4.951 (3.6)	4.951 (3.5)	5.132 (3.5)	5.131 (3.5)	4.941–4.981[2] (3.5–3.7)
Xyl^b	4.956 (3.7)	4.958 (3.7)	4.952 (3.6)	4.950 (3.6)	4.941–4.981[2] (3.5–3.7)
Xyl^c	4.939 (3.4)	4.939 (3.5)	4.941 (3.6)	4.939 (3.6)	4.941–4.981[2] (3.5–3.7)
Xyl$^{a'}$	—	—	—	—	5.141 (3.5)
Xyl$^{b'}$	—	—	—	—	4.941–4.981[2] (3.5–3.7)
Xyl$^{c'}$	—	—	—	—	4.941–4.981[2] (3.5–3.7)
Gal	—	—	4.621 (7.8)	4.620 (7.7)	4.617 (8)[3]
Glc^a	4.631 (7.9)	4.633 (8.0)	4.621 (7.8)	4.620 (7.9)	4.633 (8)[3]
Glc^b	4.586 (8.0)	4.585 (8.0)	4.539 (8.0)	4.538 (7.9)	4.531–4.576[2] (8)[3]
Glc^c	4.548 (8.1)	4.547 (8.0)	4.546 (7.9)	4.543 (8.0)	4.617 (8)[3]
Glc$^{a'}$	—	—	—	—	4.531–4.576[2] (8)[3]
Glc$^{b'}$	—	—	—	—	4.531–4.576[2] (8)[2]
Glc$^{c'}$	—	—	—	—	4.548 (8)[3]
4 Glc	—	—	—	—	4.531–4.576[2] (8)[3]

[1]($J_{1,2}$) coupling constants are depicted in parentheses beneath the corresponding H-1 chemical shift. [2]The chemical shifts and coupling constants are within this range, but the signals could not be distinguished from one another by the techniques used. [3]The coupling constant could not be measured more accurately due to overlapping signals.

The conditions selected for hydrolysis of per-*O*-methylated **5R** during glycosyl-linkage analysis were also a compromise between complete release of β-glucosyl residues and degradation of acid-labile residues; the nonstoichiometric recoveries of the various partially methylated alditol acetates reflect this compromise. Two different methods were used to methylate **5R**. The method of Ciucanu and Kerek[21] resulted in a higher-than-expected recovery of the derivative of 2,3,6-tri-*O*-methyl-D-glucose. The modified[22] method of Hakomori[23] resulted in a higher-than-expected recovery of the derivative of 2,3,4-tri-*O*-methyl-D-fucose (Table IV). Side reactions can result[24] in the formation of unexpected derivatives

TABLE II

^{1}H-N.M.R. H-2 CHEMICAL SHIFTS IN THE SPECTRA OF OLIGOGLYCOSYL ALDITOLS **2R**, **3R**, AND **5R**

Residue	**2R**	**3R**	**5R**
Ara	—	—	4.18 (3.6)[1]
Fuc	—	3.796	3.80
Xyl[a]	3.547	3.674	(3.54–3.55)[2]
Xyl[b]	3.547	3.546	(3.54–3.55)[2]
Xyl[c]	3.542	3.546	(3.54–3.55)[2]
Xyl[a']	—	—	3.66
Xyl[b']	—	—	(3.54–3.55)[2]
Xyl[c']	—	—	(3.54–3.55)[2]
Gal	—	3.715	3.73
Glc[a]	3.413	3.418	3.42
Glc[b]	3.403	3.409	(3.41–3.42)[2]
Glc[c]	3.338	3.334	3.56
Glc[a']	—	—	(3.41–3.42)[2]
Glc[b']	—	—	(3.41–3.42)[2]
Glc[c']	—	—	3.35
4 Glc	—	—	(3.41–3.42)[2]

[1]The ($J_{2,3}$) coupling constant for the arabinosyl residue is given in the parentheses beneath its H-2 chemical shift. [2]The chemical shifts are within this range, but the signals could not be distinguished from one another by the techniques used.

TABLE III

GLYCOSYL-RESIDUE COMPOSITION[a] OF **5R**

Glycosyl residue	*Molar ratio*
Fuc	1.0
Ara	1.0
Xyl	6.0
Gal	1.0
Glc	7.5[b]

[a]The hydrolysis was done in 2N TFA for 20 min at 121°. [b]The less stringent-than-normal hydrolysis conditions were chosen in order to minimize degradation of acid-labile arabinosyl, fucosyl, and xylosyl residues. These conditions might have resulted in the incomplete release of β-glucosyl residues, thereby accounting for the less-than-expected recovery of D-glucose.

when methylation is performed using the method of Ciucanu and Kerek. Nevertheless, the glycosyl-residue and glycosyl-linkage composition data for **5R** were consistent with a structure consisting of a combination of nonasaccharide **3**, heptasaccharide **2** and an arabinofuranosyl residue at C-2 of one of the 4,6-*O*-linked-glucosyl residues.

Experiments involving mild acid treatment of **5R**. — Heptadecasaccharide **5** was determined to consist of nonasaccharide **3**, heptasaccharide **2**, and an arabinofuranosyl residue by two experiments involving mild acid treatment of **5R**. Oligo-

TABLE IV

GLYCOSYL-LINKAGE COMPOSITION[a] OF **5R**

Parent residue	*Positions methylated*[b]	*Deduced linkage*	*Ciucanu and Kerek*[c]	*Hakomori*[d]	*Predicted molar ratio*
Fuc	2,3,4	T Fuc*p*	0.8	1.6	1
Ara	2,3,5	T Ara*f*	1.1	1.3	1
Xyl	3,4	2 Xyl*p*	1.3	1.2	1
Xyl	2,3,4	T Xyl*p*	4.8	5.1	5
Gal	3,4,6	2 Gal*p*	1.0	0.9	1
Glc	1,2,3,5,6	4 Glcol	0.4	0.4	1
Glc	2,3,6	4 Glc*p*	1.9	1.3	1
Glc	2,3,4	6 Glc*p*	0.9	1.0	1
Glc	2,3	4,6 Glc*p*	4.1	3.4	4
Glc	3	2,4,6 Glc*p*	0.8	0.8	1

[a]This data was generated by using hydrolysis conditions (2N TFA, 1 h, 121°) that were a compromise between complete release of the β-glucosyl residues and degradation of the acid-labile residues. Therefore, the ratios of the glycosyl residues are not always integers. [b]The partially methylated glycosyl residues generated following acid hydrolysis of permethylated **5R** were converted to alditols, and the free hydroxyls were then *O*-acetylated. [c]Methylation of **5R** was performed by the method of Ciucanu and Kerek (ref. 21). [d]Methylation of **5R** was performed by the method of Hakomori (ref. 23).

glycosyl alditol **5R** was first subjected to controlled acid hydrolysis conditions that resulted in the cleavage of 67% of the arabinosidic linkages, 16% of the fucosidic linkages, and no detectable cleavage of other glycosidic residues. A glycosyl-linkage analysis was performed on **5R** both before and after the acid-catalyzed selective cleavage of the arabinofuranosyl residue. The recovery of the derivative of 2,4,6-*O*-linked-glucopyranose was 54% lower for the acid-treated **5R** than for the untreated **5R**. This difference observed between the glycosyl-linkage composition of **5R**, which was determined before and after mild acid treatment, strongly suggested that the arabinosyl residue was attached to C-2 of one of the five 4,6-di-*O*-linked-glucosyl residues. This conclusion was confirmed by the results described below.

Oligoglycosyl alditol **5R** was next subjected to another mild acid treatment that resulted in the hydrolysis of 49% of the arabinosidic linkages, 10% of the fucosidic linkages, and no detectable hydrolysis of other glycosyl residues. The mild-acid-treated sample was then incubated with excess endoglucanase. The digestion products were reduced with $NaBD_4$ and separated by high-pH anion-exchange chromatography on a Dionex CarboPack-1 h.p.l.c. column. Anthrone-positive material eluted from the Dionex column in three major peaks at about 14, 16, and 42 min in the approximate ratios of 1:1:2, respectively. The column fractions containing these peaks were separately pooled, then desalted by ion-exchange chromatography. These fractions, in order of their elution from the Dionex column, will be referred to as X, Y, and Z.

1D ^{1}H-n.m.r. spectroscopy confirmed that samples X and Y consisted of the oligoglycosyl alditols **2R** and **3R**, respectively[8]. The H-1 chemical shfits and

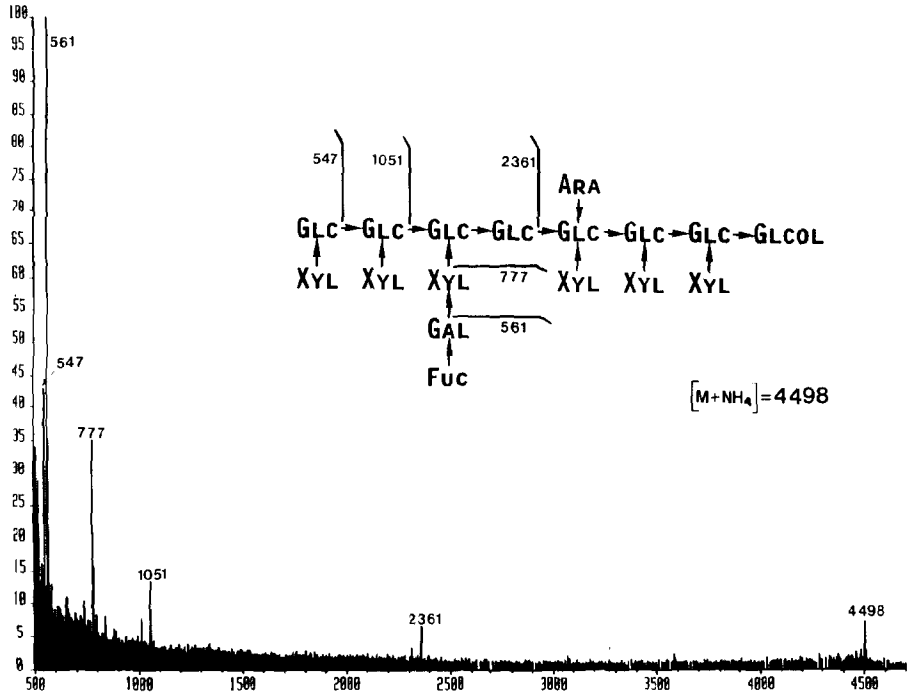

Fig. 5. Positive-ion f.a.b.-mass spectrum of per-*O*-acetylated oligoglycosyl alditol **5R**. The ion at *m/z* 4498 is the $[M + NH_4]^+$ pseudomolecular ion. The remaining ions are A^+ type fragments[8,26,28] that were generated during ionization of per-*O*-acetylated **5R**. Ions are labelled with the nominal mass of the isotopomer containing only ^{12}C, ^{1}H, and ^{16}O atoms.

coupling constants ($J_{1,2}$) of the components in samples X and Y are compared in Table I to those of the previously assigned[8] H-1 chemical shifts and coupling constants ($J_{1,2}$) of the glycosyl residues of **2R** and **3R**. The ^{1}H-n.m.r. spectrum of sample Z suggested that it consisted of a mixture of intact **5R** (80–90%) and **5R** in which the terminal fucosyl residue was missing. Removal of the arabinosyl residue from **5R** rendered it susceptible to endoglucanase. This fact established that heptadecasaccharide **5** consists of oligosaccharides **2** and **3** and an arabinosyl residue, and that the presence of the arabinosyl residue prevents **5** from being cleaved by endoglucanase.

The endoglucanase digestion products obtained after selective removal of the arabinosyl residue of **5R** were reduced with $NaBD_4$ in order to label the oligosaccharide subunit at the nonreducing end of **5R** with a deuterium atom. The deuterium-labelled oligoglycosyl alditol was identified by negative-ion f.a.b.-m.s. analysis of fractions X and Y, using 1-amino-2,3-dihydroxypropane (*i.e.*, 1-aminoglycerol) as the ionization matrix[25]. The negative-ion f.a.b.-mass spectrum of X indicated the presence of hydrogen-reduced **2R** ($[M - H]^-$ nominal mass 1063). The negative-ion f.a.b.-mass spectrum of Y indicated the presence of deuterium-

reduced **3R** ($[M - H]^-$ nominal mass 1372). We therefore concluded that **5R** contains the nonasaccharide **1** glycosidically linked to C-4 of Glc^c of the reduced heptasaccharide **2R**. The glucosidic linkage connecting **3R** and **2R** is susceptible to (1→4)-β-D-endoglucanase c leavage when the α-arabinofuranosyl residue of **5R** is removed.

Identification of the specific 2,4,6-O-linked-glucosyl residue of **5R** *to which the arabinosyl residue is attached.* — The inability of (1→4)-β-D-endoglucanase to cleave **5R** was attributed to the presence of the α-arabinofuranosyl residue at C-2 of a 2,4,6-*O*-linked-β-glucosyl residue (see in the foregoing section). This suggested that the α-arabinofuranosyl residue was located near the 4-*O*-linked-glucosyl residue that is otherwise susceptible to endoglucanase digestion. In order to determine to which glucosyl residue the arabinosyl residue is attached, positive- and negative-ion f.a.b.-m.s. were performed on per-*O*-acetylated and underivatized **5R**, respectively (see Tables V and VI).

A positive-ion f.a.b.-mass spectrum of per-*O*-acetylated **5R**[26], recorded using thioglycerol as a matrix, yielded intense signals corresponding to $[M + NH_4]^+$ pseudomolecular ions[27] and A^+ type[28] (nonreducing end) fragment ions (Fig. 5, Table V). The ion at *m/z* 4498 was the $[M + NH_4]^+$ pseudomolecular ion for **5R**. The strong A^+ type ion at *m/z* 2361, corresponding to ($FucGalXyl_3Glc_4$) provided evidence that the arabinosyl residue was not located on either of the 4,6-*O*-linked glucosyl residues of the nonasaccharide portion of **5R**.

A negative-ion f.a.b.-mass spectrum of underivatized **5R** was recorded using 1-aminoglycerol as the ionization matrix. Eight scans were digitally summed to increase the signal-to-noise ratio. Negative-ion f.a.b.-m.s. of **5R** generated a spectrum that contained a strong $[M - H]^-$ pseudomolecular ion and less intense fragment ions derived from the reducing end of the oligoglycosyl alditol (Fig. 6, Table VI)[8,26]. These fragment ions were observed at *m/z* values corresponding to cleavage of glycosidic bonds between C-1 and O-1, and release of an anionic fragment consisting of the alditol end of **5R**.

The intense $[M - H]^-$ pseudomolecular ion at *m/z* 2547 in the negative-ion

TABLE V

POSITIVE-ION FAST-ATOM BOMBARDMENT MASS SPECTRUM OF PER-*O*-ACETYL **5R**

Nominal mass of ion	*Ion type*[a]	*Ion composition*
547	A^+	XylGlc
561	A^+	FucGal
777	A^+	FucGalXyl
1051	A^+	Xyl_2Glc_2
2361	A^+	$FucGalXyl_3Glc_4$
4498	$[M + NH_4]^+$	$FucGalXyl_6AraGlc_7Glcol$

[a]Ions labelled A are derived from the nonreducing end of **5R** as described by Kochetkov and Chisov (ref. 28).

TABLE VI

NEGATIVE-ION FAST-ATOM BOMBARDMENT MASS SPECTRUM OF UNDERIVATIZED **5R**

Nominal mass of ion	*Ion type*[a]	*Ion composition*
475	B	XylGlcGlcol
637	B*	$XylGlc_2Glcol$
769	B	Xyl_2Glc_2Glcol
1063	B*	Xyl_3Glc_3Glcol
1195	B	$AraXyl_3Glc_3Glcol$
1226	B*	Xyl_3Glc_4Glcol
1357	B	$AraXyl_3Glc_4Glcol$
1827	B*	$FucGalXyl_4Glc_5Glcol$
1959	B	$AraFucGalXyl_4Glc_5Glcol$
1975	B*	Xyl_5Glc_7Glcol
2107	B	$AraXyl_5Glc_7Glcol$
2107	B*	Xyl_6Glc_7Glcol
2121	B*	$FucGalXyl_5Glc_6Glcol$
2239	B	$AraXyl_6Glc_7Glcol$
2253	B	$AraFucGalXyl_5Glc_6Glcol$
2283	B*	$FucGalXyl_5Glc_7Glcol$
2401	B	$GalXyl_6Glc_7Glcol$
2415	B	$FucGalXyl_5Glc_7Glcol$
2547	[M − H]	$FucGalXyl_6Glc_7Glcol$
2569	[M − 2H + Na]	$FucGalXyl_6Glc_7Glcol$

[a]Ions labelled B arise from the reducing end of **5R** by a single-cleavage event. Ions labelled B* also arise from the reducing end, but by a double-cleavage event. These ions correspond to the "Pathway B" ions described by Dell (ref. 26).

f.a.b.-mass spectra of **5R**, which corresponded in mass to [$AraFucGalXyl_6Glc_7Glcol$], confirmed the glycosylic composition of **5R**. More importantly, fragment ions, which corresponded in mass to structures that arose from **5R** by a single or double cleavage, allowed for unambiguous identification of the glucosyl residue to which the arabinosyl residue was attached. The ion at *m/z* 1195 established that the arabinosyl residue was located on Glc^a, Glc^b, or Glc^c of **5R**. The absence of an ion at *m/z* 901 [$Pent^3Glc^2Glcol$] and the presence of an ion at *m/z* 769 [$Pent_2Glc_2Glcol$] indicated that the arabinosyl residue was not attached to either Glc^a or Glc^b (see Fig. 1). Therefore, the arabinofuranosyl residue in **5R** had to be attached to Glc^c of **5R** (Fig. 1).

Low-abundance negative ions that were formed by fragmentation of **5R** at two different sites are denoted by asterisks in Fig. 6 and Table VI. These double-cleavage ions[26], which contained the glucitol residue, appeared to arise from **5R** by cleavage of the backbone and one of the pentosyl sidechains of **5R**. The ion at *m/z* 1195, corresponding in mass to [$AraXyl_3Glc_3Glcol$] is a single-cleavage ion. This ion esablished that the arabinosyl residue was located on the **2R**-portion of **5R** because the fragment ion contained four pentoses and only three hexoses. The strong ion at *m/z* 1063, corresponding in mass to [Xyl_3Glc_3Glcol] or [$AraXyl_2Glc_3Glcol$], could only have resulted from a double cleavage of **5R**, that

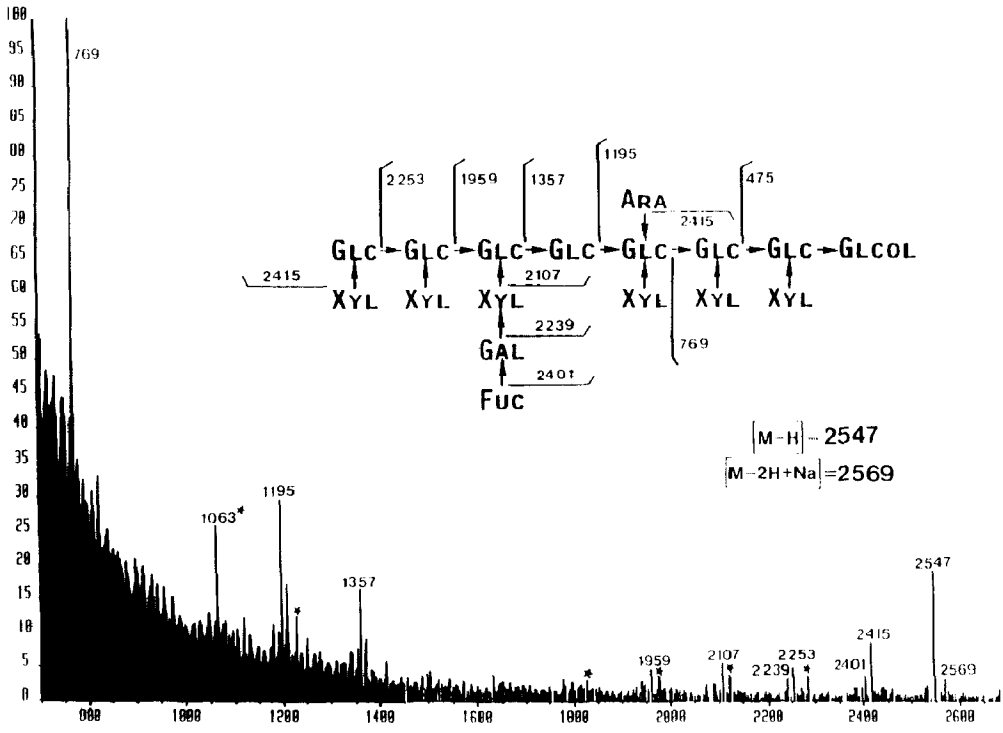

Fig. 6. Negative-ion f.a.b.–mass spectrum of oligoglycosyl alditol **5R** in which eight scans were digitally summed to increase the signal-to-noise ratio. Fragment ions were assigned as having formed by cleavage of the C-1–O-1 bond, releasing the alditol-containing fragment as an oxyanion. The ions that cannot be formed from **5R** by a single cleavage event were assigned as "double-cleavage" ions[26], formed by the cleavage of the backbone and one of the pentosyl sidechains of **5R**. Double-cleavage ions were detected in the mass spectrum of **5R**, 132 mass units below each of the ions with nominal masses of 769 or greater. These double-cleavage ions are denoted by asterisks in the figure. The intense ion at *m/z* 2547 is the $[M - H]^-$ pseudomolecular ion for **5R**. The fragment ion at *m/z* 1195 localized the position of the arabinosyl residue to the **2R**-portion of **5R**. The presence of an ion at *m/z* 769 and the complete absence of an ion at *m/z* 901 established that the arabinosyl residue is linked to Glc^c (see Fig. 1). Ions are labelled with the nominal mass of the isotopomer containing only ^{12}C, ^{1}H, and ^{16}O atoms.

is, by cleavage of a xylosyl or an arabinosyl residue from the ion at *m/z* 1195. Every primary fragment ion of **5R** that contains an arabinosyl residue (*i.e.*, all ions with an *m/z* of 1195 or greater) has associated with it a less intense double-cleavage ion at 132 amu lower mass. We believe that these double-cleavage ions result from preferential cleavage of the arabinofuranosyl residues from the primary fragmentation ions.

DISCUSSION

The structure of sycamore SEPS xyloglucan hexadecaglycosyl alditol **5R** (Fig. 1) was unambiguously determined by combining data generated by glycosyl-residue

and glycosyl-linkage composition, ^{1}H-n.m.r. spectroscopic, and f.a.b.-mass spectrometric analyses of intact **5R** and of oligosaccharide fragments of **5R**. **5R** is a combination of the previously characterized xyloglucan components, nonasaccharide **3** and hexaglycosyl alditol **2R**, which are linked glycosidically through C-4 of Glc^c of **2R**, and an α-L-arabinofuranosyl residue glycosidically linked to C-2 of Glc^c of the **2R**-portion of **5R** (Fig. 1).

Intact **5R** is resistant to endoglucanase degradation, while **5R** modified by removal of the arabinofuranosyl residue is cleaved by endoglucanase to **3** and **2R**. Chesson *et al.*[29] reported that the presence of arabinosyl residues in xylan is inhibitory to its enzymic breakdown, and Greve *et al.*[30] provided evidence that a bacterial arabinosidase enhances the ability of hemicellulolytic and pectinolytic enzymes to cleave plant cell-wall polysaccharides.

The presence of the arabinosyl residues on XG appears to enhance its solubility. It has been shown that, when the arabinosyl residues of tobacco arabinoxyloglucan are removed, the polysaccharide becomes insoluble in water[31]. This observation suggests that the presence of arabinosyl substituents on hemicelluloses plays an important role in the biological functions of these plant polysaccharides.

The large XG oligosaccharides that eluted in Peak II of the BioGel P-2 column (Fig. 3) are also resistant to further endoglucanase digestion, although most of the oligosaccharides do not contain arabinose. We detected 2,4,6-tri-*O*-linked-glucose in one of the large endoglucanase-resistant oligosaccharides that does not contain arabinose. It is possible that a xylosyl residue glycosidically attached to C-2 of a 4,6-di-*O*-linked-glucose residue is preventing further endoglucanase digestion of the oligosaccharide. The resistance of the larger XG oligosaccharides to endoglucanase degradation is interesting in light of the apparent role of the smaller oligosaccharides in control of plant cell growth[10–13]. Indeed, the large XG oligosaccharides may also be biologically active.

The structures of the other BioGel P-2 peak II oligosaccharides could consist of various combinations of the deca-, nona-, and hepta-saccharide components of XG, perhaps with an arabinosyl or xylosyl residue glycosidically attached to C-2 of a 4,6-di-*O*-linked-glucosyl residue. Characterizing the structures of these endoglucanase-resistant XG oligosaccharides is the next logical step towards understanding the structure of polymeric SEPS XG.

MATERIALS AND METHODS

Enzymes. — An endoglucanase [(1→4)-β-D-glucan 4-glucanohydrolase, EC 3.2.1.4], that cleaves only the unbranched 4-*O*-linked glucosyl residues of XGs, was isolated from cultures of *T. viride* as previously described[2].

Colorimetric assays. — Hexose-containing carbohydrates were detected by the anthrone method[32] and were quantitated and reported relative to the response of 1 mg of XG (*i.e.*, as anthrone-mg equivalents of XG).

Purification of XG. — XG from SEPS (sycamore extracellular poly-

saccharides) was prepared as described from the culture filtrate of suspension-cultured *Acer pseudoplatanus* cells[2].

Endoglucanase digestion of xyloglucan. — Purified SEPS (100–150 mg), which was suspended (4–5 mg/mL) in 50mM sodium acetate, pH 5.2, containing 0.02% thimerosol, was incubated (48 h, 25°) with 3.33 units of *T. viride* endoglucanase. (One unit of the *T. viride* endoglucanase was defined as that amount that produces 1 μmole of reducing glucose per min, using carboxymethylcellulose as the substrate.) The digestion mixture was then desalted on a column of Sephadex G-15. The carbohydrate-containing fractions that were eluted from the desalting column were concentrated and separated into size classes by chromatography in water on a BioGel P-2 column.

Sephadex G-15 chromatography. — Endoglucanase-digested SEPS was desalted by chromatography on a Sephadex G-15 column (1.6 × 16 cm) eluted with water at 0.5 mL/min, collecting 1.5-mL fractions.

Purification of XG oligosaccharides by BioGel P-2 chromatography. — BioGel P-2 chromatography resulted in the separation of five major carbohydrate containing fractions. Two BioGel P-2 columns (−400 mesh, 1.6 × 95 cm, Amicon) were connected in series. Samples (100–150 mg) in water were loaded onto the columns with a Rheodyne low-pressure LC injector, and the oligosaccharides were eluted with water (10 mL/h, delivered with a peristaltic pump), collecting 3-mL fractions. Fractions containing oligosaccharides consisting of 15–20 glycosyl residues [peak II, (Fig. 2)] were combined and lyophilized.

Deesterification and endoglucanase treatment of XG oligosaccharides. — The XG oligosaccharides (30 mg) contained in peak II from the BioGel P-2 column were dissolved in deionized water (5 mL), and the pH was adjusted to pH 11.5 with 0.1M NaOH. The pH was kept at pH 11.5 for 1 h by adding 0.1M NaOH at 15-min intervals as required, and then the pH was adjusted to pH 5.2 with 0.1M acetic acid (final concentration of 30–50mM NaOAc). Thimerosol (final concentration of 0.02%) and two units of *T. viride* endoglucanase were added to the deesterified oligosaccharides, and the reaction was incubated for 48 h at 25°. The digestion mixture was then lyophilized.

Reduction of XG oligosaccharides. — The lyophilized products of the endoglucanase digestion of deesterified XG oligosaccharides were dissolved in a 1M NH_4OH solution (10 mg/mL) containing $NaBH_4$ (10 mg/mL). After 1 h the reduction mixture was placed in an ice bath, and acetone was added (150 μL/mg of $NaBH_4$). Acetic acid (5M) was used to adjust the pH to pH 5.0 to facilitate the separation of borate from the oligosaccharides on a Sephadex G-15 desalting column. An alternative method for the removal of borate was used when reducing less than 1 mg of oligosaccharide. In such cases, after the addition of acetone, the solution was passed through a Dowex 50 [H^+] column (0.5 mL) that had just been washed with ice-cold deionized water. The eluant was dried under a stream of filtered air, and borate was removed as trimethyl borate by codistillation with methanol[33].

Purification of **5R**. — The deesterified, endoglucanase-treated oligoglycosyl alditols (prepared from peak II oligosaccharides as described above) were further purified by reverse-phase chromatography on a Hibar Lichrosorb RP-18 semi-preparative h.p.l.c. column (10 × 250 mm). The oligoglycosyl alditols (~10 mg) were eluted (5 mL/min) using a 30-min gradient of 0–25% methanol in H_2O, followed by a 10-min isocratic elution with 25% methanol in H_2O. Fractions were collected at 0.3-min intervals. The glycosyl compositions of the carbohydrate eluting from the column were determined. Column fractions 92 and 93 (Fig. 3) contained the most arabinose. Column fractions 92 and 93 were pooled and analyzed by ^{1}H-n.m.r. spectroscopy. The same fractions obtained from each of two other identical chromatography procedures on the reverse-phase column were pooled and analyzed by ^{1}H-n.m.r. spectroscopy to ascertain that they contained identical material. Fractions 92 and 93 from each of the three chromatography procedures were combined. The combined fractions, which contained 2 mg of anthrone XG equivalents, were analyzed by 1D and 2D ^{1}H-n.m.r. spectroscopy. The 1D ^{1}H-n.m.r. spectrum of combined fractions 92 and 93 made it apparent that the degree of purity was less than 90%. Therefore, the oligoglycosyl alditols in these fractions were rechromatographed on the RP-18 h.p.l.c. column as described in the foregoing, except that a gradient of 14–25% methanol in H_2O was used as eluant. Analysis by 1D ^{1}H-n.m.r. spectroscopy of the oligoglycosyl alditols that eluted in the major peak from this h.p.l.c. column revealed a homogeneity of greater than 90%, and accounted for 1.3 mg anthrone XG equivalents. This material, the major component contained in column fractions 92 and 93, was **5R**.

Chemical analysis of oligoglycosyl alditols. — Glycosyl-composition analyses were determined by the formation of alditol acetate derivatives which were quantitated by isothermal (220°) g.l.c. on an SP-2330 fused-silica column (30 m, Supelco)[33]. Glycosyl-linkage analyses were determined by g.l.c. and by g.c.–m.s. of the partially *O*-methylated alditol acetate derivatives[22] that were *O*-methylated by two separate procedures. In the method of Ciucanu and Kerek[21], methylation was catalyzed by solid NaOH in Me_2SO. In the modified[22] method of Hakomori[23], methylation was catalyzed by methyl sulfinyl carbanion in Me_2SO. Methylated oligoglycosyl alditols (100 μg) were purified by $CH_2Cl_2 \cdot H_2O$ extractions and converted to partially methylated alditol acetates (PMAAs) as follows. Hydrolysis was carried out in 250 μL of 2M CF_3CO_2H (1 h, 121°). The resulting partially methylated aldoses were converted to partially methylated alditols by reduction with $NaBD_4$. The latter were acetylated by heating for 3 h at 121° in Ac_2O (100 μL). When the methylation was catalyzed by solid NaOH in Me_2SO, the residual NaOAc acted as a catalyst. In the cases where methylsulfinyl carbanion was used as a catalyst in the methylation reaction, acetylation was done using pyridine (200 μL) and Ac_2O (200 μL, 20 min, 121°). The PMAAs were identified by g.l.c. retention times using a SP-2330 fused-silica column (30 m, Supelco), and by g.l.c.–m.s.[22].

Fast-atom bombardment mass spectrometry. — F.a.b.-mass spectra were recorded with a VG ZAB SE instrument operating at low resolution (1:1000) with

an accelerating voltage of 8 kV. Oligoglycosyl alditols were per-*O*-acetylated (50 μg) for positive-ion f.a.b.-m.s. using a 2:1 solution[34] of trifluoroacetic anhydride and glacial acetic acid (250 μL). The per-*O*-acetylated oligoglycosyl alditols were purified by $CH_2Cl_2 \cdot H_2O$ extractions and then dissolved in methanol (10 mg/mL). One μL of this solution was mixed on the mass spectrometer probe tip with thioglycerol (2 μL). The mass spectrometer was calibrated by first recording the spectrum of CsI. Isotopomeric ions of high-mass ion clusters were not resolved under the low-resolution conditions used. Therefore, the VG software was used to determine the centroid *m/z* values corresponding to the average mass of the ion clusters. The centroid *m/z* values were readily converted into the nominal masses of the isotopomers containing only ^{12}C, ^{1}H, and ^{16}O atoms using the CARBOMASS[35] software developed in this laboratory.

Negative-ion f.a.b.-mass spectra of underivatized oligoglycosyl alditols were obtained by dissolving the oligoglycosyl alditols in H_2O (10–30 μg/μL) and mixing this solution (1 μL) on the probe tip with 1-amino-2,3-dihydroxypropane (2 μL). Eight scans were digitally summed to provide spectra with an increased signal-to-noise ratio.

Nuclear magnetic resonance spectroscopy. — The salt content of samples that had been eluted from the Dionex column was reduced by passage through a small cation-exchange column (Dionex, OnGuard-H) prior to ^{1}H-n.m.r. spectroscopic analysis. The exchangeable protons of all samples were replaced with deuterons before the samples were dissolved (0.5–5mM) in D_2O (99.96% D, Aldrich). ^{1}H-n.m.r. spectra were recorded at 27° with a Bruker AM-500 n.m.r. spectrometer. For 1D experiments a spectral width of 3000 Hz was used with 32k data points, and up to 512 scans were averaged. Chemical shifts are reported in δ-units (p.p.m.) relative to 4,4-dimethyl-4-silapentane-1-sulfonate (DSS). Acetone was used as an internal standard (δ 2.225). Fourier transformation was performed without resolution enhancement when integrals of signals were calculated. Alternatively, free-induction decays were multiplied by a Gaussian resolution-enhancement function and Fourier transformed. Double-quantum filtered (^{1}H, ^{1}H) COSY spectra[36] were recorded in the phase-sensitive mode using a spectral width of 1428 Hz. The evolution time (t_1) was incremented in steps of 350 μs to obtain 512 free-induction decays (f.i.d.s.), each consisting of the sum of 96 transients acquired in 2k data points. Quadrature detection in both dimensions was accomplished by the time-proportional phase-increment (TPPI) method[37].

Partial acid hydrolysis of **5R**. — Selective removal of arabinosyl residues was accomplished by dissolving **5R** (0.1–1 mg) in M CF_3CO_2H (0.5 μg/μL) for 75 or 96 h at 25°. An equal volume of 2-propanol was added, and the sample was dried with a stream of filtered air. Additional 2-propanol was added to aid in the removal of traces of CF_3CO_2H, and the sample was again blown to dryness.

Those glycosyl residues liberated by the partial hydrolysis were quantitated as follows. To an aliquot (100 μg) of the acid-treated **5R**, *myo*-inositol (6 μg, internal standard) was added, and half of the sample was transferred to a separate

tube. The contents of tube no. 1 were completely hydrolyzed using 2M CF_3CO_2H (100 μL, 30 min, 121°) and blown to dryness with 2-propanol as described for the removal of M CF_3CO_2H (see in the foregoing paragraph). The contents of tubes no. 1 and no. 2 were then reduced with $NaBH_4$, acetylated, analyzed by g.c.–m.s. and quantitated by g.l.c. as described for the chemical analysis of oligoglycosyl alditols[33]. Comparison of the amounts of each derivative recovered from tubes no. 1 and no. 2 provided an estimation of the percent hydrolysis of terminal glycosyl residues.

Incubation of mild-acid-treated **5R** *with* T. viride *endoglucanase.* — **5R** (0.85 mg), which had been treated for 75 h at 25° in M CF_3CO_2H, was incubated with 0.27 units of *T. viride* endoglucanase (50mM NaOAc, pH 5.2, 48 h, 25°). The digestion products were reduced with $NaBD_4$ and separated by Dionex h.p.l.c.

Chromatography on Dionex h.p.l.c. — The $NaBD_4$-reduced products of endoglucanase-digested mild-acid-treated **5R** (0.95 mg) were dissolved in water (220 μL) and chromatographed (1 mL/min, collecting fractions at 0.3-min intervals) on a Dionex CarboPack-1 (0.46 × 25 cm) h.p.l.c. column using pulsed amperometric detection[38]. Column fractions were subsequently assayed for anthrone-positive material. The oligoglycosyl alditols were eluted from the Dionex column with 100mM NaOH containing increasing concentrations of NaOAc. The NaOAc gradient used was as follows: time 0–2.5 min, no NaOAc (isocratic); time 2.5–5.0 min, linear gradient of 0–60mM NaOAc; time 5.0–30 min, 60mM NaOAc (isocratic); time 30–35 min, linear gradient of 60–200mM NaOAc; time 35–50 min, 200mM NaOAc (isocratic).

ACKNOWLEDGMENTS

This work was supported in part by U.S. Department of Energy grant DE-FG09-85-ER13426 and Department of Energy grant DE-FG09-87-ER13810 as part of the USDA-DOE-NSF Plant Science Centers Program. The authors thank M. O'Neill for assistance in performing high-pH anion-exchange chromatography and J. Thomas for the supply of purified (1→4)-β-D-glucan-4-glucanohydrolase from *T. viride*.

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