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LOCATION OF THE *O*-ACETYL SUBSTITUENTS ON A NONASACCHARIDE REPEATING UNIT OF SYCAMORE EXTRACELLULAR XYLOGLUCAN*§

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ABSTRACT

The locations of the *O*-acetyl substituents on the major nonasaccharide repeating unit of the xyloglucan isolated from sycamore extracellular polysaccharides were determined by a combination of analytical methods, including f.a.b.-m.s. and ^{1}H-n.m.r. spectroscopy. The *O*-2-linked-β-D-galactosyl residue of the nonasaccharide was found to be the dominant site of *O*-acetyl substitution. Both mono-*O*-acetylated and di-*O*-acetylated β-D-galactosyl residues were detected. The degree of *O*-acetylation of the β-D-galactosyl residue, was estimated by ^{1}H-n.m.r. spectroscopy to be 55–60% at O-6, 15–20% at O-4, and 20–25% at O-3. ^{1}H-n.m.r. spectroscopy also indicated that ~50% of the β-D-galactosyl residues are mono-*O*-acetylated, 25–30% are di-*O*-acetylated, and 20% are not acetylated.

INTRODUCTION

Xyloglucans are hemicellulosic polysaccharides present in the cell walls of higher plants[1]. These polysaccharides consist of a backbone of 4-linked β-D-glucosyl residues, 75% of which are substituted at O-6 with an α-D-xylosyl group or residue. Some of the α-D-xylosyl residues are substituted at O-2 with β-D-galactosyl, 2-*O*-α-L-fucosyl-β-D-galactosyl, or, less often, L-arabinosyl groups. The type and degree of substitution of the D-xylosyl residues varies, depending both on the plant species and

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the tissue of origin.

Xyloglucan isolated from sycamore extracellular polysaccharides (SEPS XG)[2,3] is composed of L-arabinose, L-fucose, D-galactose, D-xylose, and D-glucose in the ratios of 0.3:1.0:1.5:6.0:8.0, and is also substituted with *O*-acetyl groups[4]. Although common substituents of polysaccharides, *O*-acetyl groups were not until recently observed in xyloglucans because the strongly alkaline conditions needed to solubilize xyloglucans from cell walls[2,5] hydrolyze acetic esters. However, SEPS is a source of soluble xyloglucans that can be isolated without using alkaline extraction[3].

The general structure of SEPS XG has been determined by characterizing oligosaccharide fragments of the polysaccharide released by a fungal endo-β-1,4-glucanase. Two major oligosaccharide fractions, the so-called "nonasaccharide" and "heptasaccharide" fractions, named for their preponderant components, were isolated by gel-permeation chromatography of the endoglucanase digestion-products. The primary structure of the main heptasaccharide was deduced by glycosyl-linkage analysis[2]. The primary structure of the major nonasaccharide product was rigorously determined[6] in 1980. Recently, the nonasaccharide fraction was further examined by both ^{1}H-n.m.r. and fast-atom-bombardment mass spectrometry (f.a.b.-m.s.), which indicated that the nonasaccharide and decasaccharide components of this fraction have one or two *O*-acetyl substituents[4].

We were motivated to examine the structures of the various *O*-acetylated forms of the SEPS XG nonasaccharide by experimental results that suggest that one or more of the components of the nonasaccharide fractions are involved in the control of auxin-induced elongation of excised pea-stems. Growth of excised pea-stem segments is enhanced by 2,4-D, and analog of the phytohormone auxin[7]. The 2,4-D-stimulated growth is inhibited[4] by the addition of SEPS nonasaccharide fraction at a concentration of 1 to 10 nM. Recently, this inhibitory effect has been independently observed in another laboratory[8].

It is likely that *O*-acetylation of cell-wall polysaccharides is an important factor in other biological processes of plants. For example, the cohesiveness of cell-wall polysaccharides might be affected by *O*-acetylation, as are the ability of certain bacterial polysaccharides to form gels[9] and the water solubility of various fungal[10] and plant[11] polysaccharides. Therefore, *O*-acetylation could play a direct role in the control of plant-cell elongation. Furthermore, the susceptibility of cell-wall polysaccharides to certain glycanases can be affected by the presence of *O*-acetyl substituents[12]. By determining the location of the *O*-acetyl substituents on the xyloglucan oligosaccharides, we may gain a better understanding of the biological roles of xyloglucans. We now report the results of experiments designed to locate the sites of *O*-acetylation of the components of the xyloglucan nonasaccharide fraction.

EXPERIMENTAL

Preparation of polysaccharides, oligosaccharides, and oligosaccharide derivatives. — Nonasaccharide- and heptasaccharide-rich fractions were obtained[2-4] by treatment of purified SEPS xyloglucan with a β-1,4-endoglucanase isolated from

Trichoderma viride, and subsequent gel-permeation chromatography on Bio-Gel P-2. SEPS XG nonasaccharide fraction (2 mg) was *O*-deacetylated by dissolving in 0.05M aqueous NaOH (1 mL) and keeping for 2 h at room temperature. Sodium hydroxide was removed from the *O*-deacetylated oligosaccharides by passing the solution through Amberlite CG-50 (H^+) cation-exchange resin.

Tamarind xyloglucan was purified by precipitation of commercial tamarind gum (Dycol Chemicals) with 70% ethanol. Tamarind xyloglucan oligosaccharides were prepared from this material by using the same techniques[2-4] that were used to obtain SEPS xyloglucan oligosaccharides. (Deuterioacetyl)ation of oligosaccharides was carried out as previously described[13].

Periodate oxidation. — Periodate oxidations[14] were performed as follows: oligosaccharides (200 μg) were dissolved in 0.04M $NaIO_4$ (150 μL), and incubated in the dark for 120 h at room temperature. Ethylene glycol (10 μL) was then added, and, after 2 h, 500 μL of $NaBD_4$ (10 mg/mL) was added, and the sample incubated overnight. An excess of glacial acetic acid was then added, and evaporated under a stream of air. Borates were removed as their methyl esters[15], and salts were removed from aqueous solutions of the oxidized-reduced oligosaccharides with Amberlite MB-3 ion-exchange resin. Alditol acetate derivatives of the surviving glycosyl residues were formed[15], separated by isothermal (220°) gas–liquid chromatography (g.l.c.) in a fused-silica capillary column (SP-2330, Supelco), and detected and quantitated by flame ionization (f.i.d.).

Glycosyl and glycosyl-linkage compositions were respectively determined according to Albersheim *et al.*[15] and Waeghe *et al.*[16], except that *O*-methylation was catalyzed by lithium methylsulfinyl carbanion prepared by the method of Blakeney and Stone[17]. Partially methylated, partially acetylated alditols were characterized by g.l.c.–m.s. (electron impact)[3] and quantitated by g.l.c. (f.i.d.) according to Sweet *et al.*[18].

Fast-atom-bombardment mass spectrometry. — F.a.b.-mass spectra were obtained by using a VG Analytical high-field ZAB 1F mass spectrometer operated at accelerating voltages of 7 kV (negative ion mode) and 8 kV (positive ion mode). The M-Scan f.a.b. gun was operated at 10 kV, and xenon was used as the bombarding gas. Spectra were recorded on u.v.-sensitive oscillographic paper and counted manually. The instrument was operated in a mass-controlled mode and linear scans were performed at a scan rate of 300 s for complete coverage of the mass range defined by the accelerating voltage used. Samples (1–10 μg) were dissolved in 5% aqueous acetic acid (underivatized sample) or methanol (derivatized sample), and loaded into 1:1 (v:v) glycerol–1-thioglycerol (1 μL) on the stainless-steel target.

^{1}H-N.m.r. spectroscopy. — Spectra, at 500 MHz, of all samples (1–2-mM in D_2O) were recorded at either 27 or 75° by using a Bruker AM500 spectrometer. For high-resolution 1D experiments, a spectral width of 2500 Hz was used with 16 k data points. Up to 256 scans were averaged and the free induction decays were multiplied by a Gaussian resolution-enhancement function. Chemical shifts are reported in p.p.m. relative to 4,4-dimethyl-4-silapentane-1-sulfonate (DSS). Acetone was used

as an internal standard (δ 2.225).

The pulse sequence applied for the 2D shift correlation (COSY-90) experiments was (90°)-(t_1)-(90°)-(t_2, acquisition). Quadrature detection using 2k data points per free induction decay was used. The spectral width in both dimensions was 2250 Hz, and 256 experiments were carried out, with 32 scans each. Prior to Fourier transformation, the data were multiplied by an unshifted sine-bell window function.

Two-dimensional *J*-resolved experiments used the pulse sequence (90°)-(t_1/2)-(180°)-(t_1/2)-(t_2, acquisition). The spectral width in the t_2 dimension was set to 2000 Hz (quadrature detection with 8k data points per free induction decay). The carrier frequency was chosen such that the signals of the CH_3 protons of the α-fucosyl residue folded over into a spectral region with no other signals. Sixty-four experiments were performed, each involving 32 scans.

RESULTS AND DISCUSSION

The products of digestion of SEPS xyloglucan with β-1,4-endoglucanase were separated by gel-permeation chromatography on Bio-Gel P-2, as described[2-4], yielding nonasaccharide-rich and heptasaccharide-rich fractions. The glycosyl composition and linkage analyses of these fractions (see Tables I and II), in conjunction with their f.a.b.-mass spectra, indicated that their main components are the nonasaccharide (see Fig. 1A) described by Valent *et al.*[6] and the heptasaccharide (see Fig. 1C) described by Bauer *et al.*[2], respectively. ^{1}H-N.m.r. and f.a.b.-m.s. analysis of these fractions indicated that the components of the nonasaccharide fraction contain endogeneous *O*-acetyl substituents, whereas those of the heptasaccharide fraction do not. The most intense high-mass signals observed in the negative ion f.a.b. mass spectrum of the native (endogeneously *O*-acetylated) nonasaccharide fraction correspond to the $(M - H)^-$ quasimolecular ions for the mono- (*m/z* 1411) and di-*O*-acetylated (*m/z* 1453) nonasaccharide. Less-intense signals were observed at *m/z* 1573 and 1615, corresponding to the mono- and di-*O*-acetylated forms of a decasaccharide containing one more hexose residue than the nonasaccharide. It is likely that the decasaccharide is that (Fig. 1B) described by Kato *et*

TABLE I

GLYCOSYL COMPOSITION OF THE OLIGOSACCHARIDES

Glycosyl group or residue	*SEPS heptasaccharide*[a]	*SEPS nonasaccharide*[a]
Fucosyl	0.0	1.2
Arabinosyl	0.1	tr[b]
Xylosyl	3.0	3.1
Galactosyl	0.1	1.3
Glucosyl	4.0	4.0

[a]Fractions containing a mixture of closely related oligosaccharides. [b]tr = trace.

TABLE II

GLYCOSYL LINKAGE COMPOSITION

Linkage[a]	*SEPS heptasaccharide*[b]	*SEPS nonasaccharide*[b]
T-Fuc[c]	0.0	0.9
T-Xyl[d]	2.4	1.8
2-Xyl[d]	0.1	1.0
T-Gal	tr[e]	0.2
2-Gal	0.0	1.0
4-Glc	0.9	1.0
6-Glc	1.0	1.0
4,6-Glc	2.0	2.0

[a]Deduced linkage in intact oligosaccharide fraction: *e.g.*, T-Fuc was detected as 1,5-di-*O*-acetyl-2,3,4-tri-O-methyl-fucitol. [b]Fractions containing a mixture of related oligosaccharides. [c]T = terminal. [d]Recoveries of xylosyl derivatives are usually somewhat low and variable, because these compounds are partially degraded during hydrolysis[15]. [e]tr = trace.

al.[19,20]. A signal at *m/z* 1369 indicated that some nonasaccharide components of the fraction have no *O*-acetyl substituents. The methods that were used to locate the *O*-acetyl substituents on the XG nonasaccharide are described later.

Chemical analyses of XG nonasaccharide fraction. — Attempts to determine the location of *O*-acetyl substituents in the SEPS nonasaccharide fraction by formation of 1-methoxyethyl protecting groups and subsequent methylation[10] failed to give meaningful results. Likewise, attempts to use methyl trifluoromethane sulfonate to *O*-methylate the nonasaccharide without displacing *O*-acetyl substituents[21] were unsuccessful.

Glycosyl-composition analysis of periodate-oxidized SEPS nonasaccharide fraction indicated that some of the D-galactosyl residues in this sample had *O*-acetyl substituents. Native and *O*-deacetylated nonasaccharide samples were treated with periodic acid[14]. Over 95% of the D-xylosyl and L-furanosyl residues, and ~75% of the D-glucosyl residues in both samples were degraded by this treatment. The incomplete degradation of the D-glucosyl residues by periodic acid could be attributed to the resistance of these residues to oxidation, as the extent of their degradation did not depend on whether the nonasaccharide had been *O*-deacetylated. On the other hand, 96% of the D-galactosyl residues were decomposed if the nonasaccharide had been *O*-deacetylated prior to periodate treatment, but only 67% if the endogeneously *O*-acetylated nonasaccharide was treated with periodate. The partial protection of D-galactosyl residues from periodate oxidation when endogenous *O*-acetyl substituents were present suggests that approximately one-third of the D-galactosyl residues are acetylated at O-3 or O-4.

F.a.b.-m.s. of per(deuterioacetyl)ated nonasaccharide fraction. — In order to investigate further which glycosyl residues have *O*-acetyl substituents, the oligosaccharides of the nonasaccharide fraction were converted into their per(deuterio-

acetyl)ated derivatives, and these were examined by f.a.b.-m.s.[13]. (Deuterioacetyl)-ation of a model compound, methyl 4-*O*-acetyl-6-*O*-trityl-α-D-galactoside, and ^{1}H-n.m.r. spectroscopy of the product, were performed in order to determine whether displacement or migration of the endogenous *O*-acetyl group occurred during the (deuterioacetyl)ation procedure. No significant migration or loss of the endogenous *O*-acetyl group of the model compound was observed, as the ^{1}H-n.m.r. spectrum of the (deuterioacetyl)ated D-galactose derivative included only one major *O*-acetyl methyl signal whose integral was equal to that of the aglyconic *O*-methyl signal.

The positive ion f.a.b.-mass spectrum of the material recovered after *O*-(deuterioacetyl)ation of the native (endogenously *O*-acetylated) nonasaccharide fraction is shown in Fig. 2. The signals at *m/z* 2430 and 2427 correspond to the A^+-type[22] quasimolecular ions for the (deuterioacetyl)ated nonasaccharide containing one and two endegenous *O*-acetyl groups, respectively. The signals correspond-

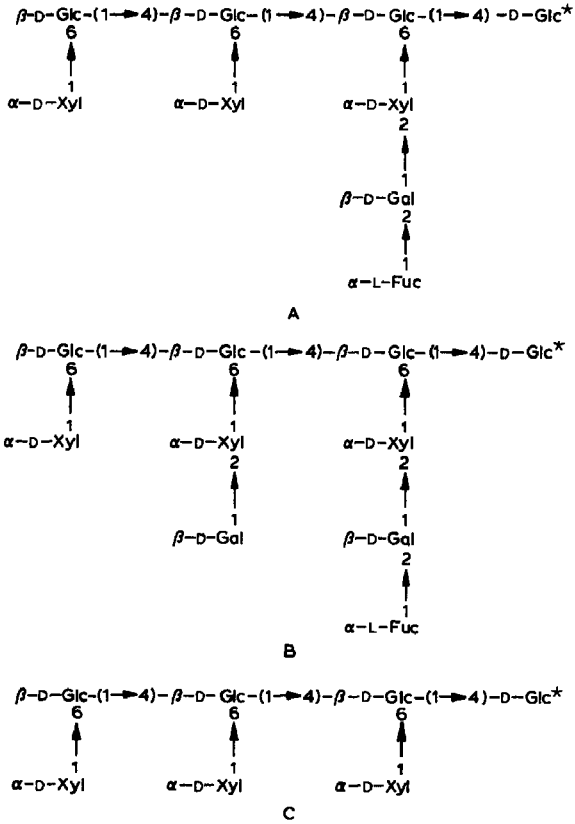

Fig. 1. Structures of the major oligosaccharide components of Bio-Gel P-2 fractions obtained after β-1,4-endoglucanase digestion of SEPS xyloglucan. (A) Predominant component of the nonasaccharide fraction. This fraction also contains ~15% of a decasaccharide (B). (C) Predominant component of the heptasaccharide fraction. The Tamarind XG oligosaccharides (also used in this study) are composed of this heptasaccharide with β-Gal residues at O-2 of some of the α-Xyl residues. The asterisk (*) indicates the reducing residue.

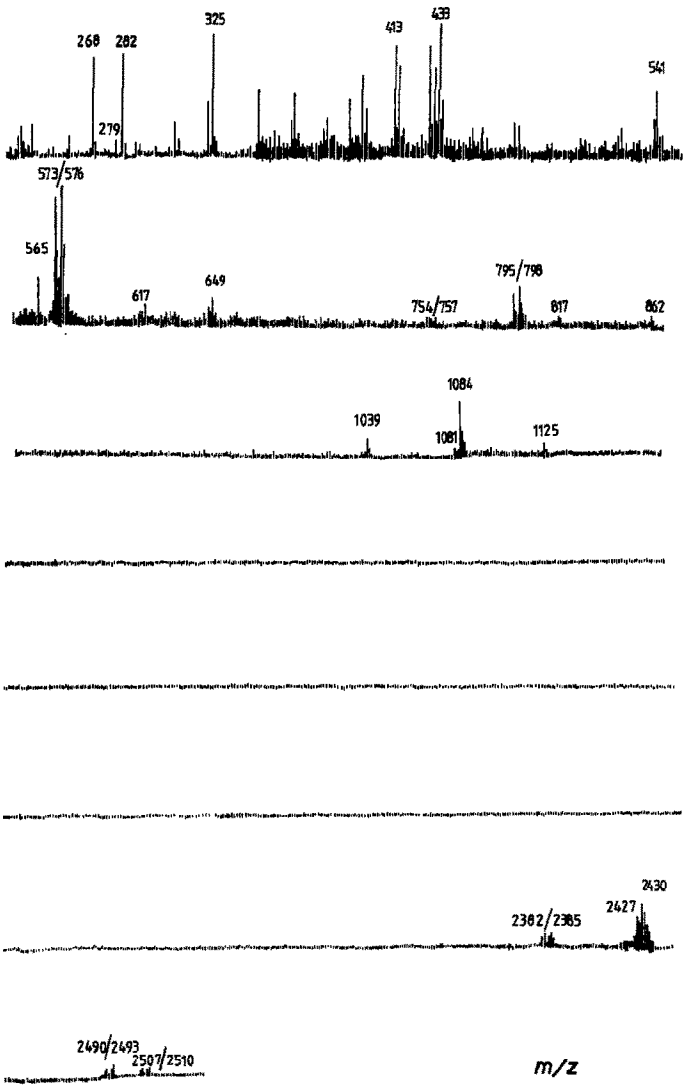

Fig. 2. Positive f.a.b.-mass spectrum of the material recovered after per-*O*-(deuterioacetyl)ation of the native (*i.e.*, endogenously *O*-acetylated) nonasaccharide fraction.

ing to the $[M + H]^+$ (*m/z* 2493 and 2490) and $[M + NH_4]^+$ (*m/z* 2510 and 2507) quasimolecular ions are of very low intensity. The dominance of A^+-type ions in the molecular-ion region of the f.a.b.-mass spectra of acetylated oligosaccharides had been previously reported[23]. Other A^+-type fragment ions derived from nonreducing termini were observed, allowing glycosyl residues bearing endogenous *O*-acetyl groups to be distinguished from those fully substituted with *O*-(deuterioacetyl) groups. The ion at *m/z* 282 arises from $[Fuc]^+$ (no acetate), whereas those at *m/z* 573 and 576 correspond to $[(Fuc\text{-}Gal)Ac_2]^+$ and $[(Fuc\text{-}Gal)Ac]^+$, respectively. The

intensity of the signal at *m/z* 282 is considerably greater than that of the signals at *m/z* 279 (potentially $[(Fuc)Ac]^+$, see later) and *m/z* 579 (*i.e.*, $[Fuc\text{-}Gal]^+$). Therefore, the quantitatively dominant *O*-acetylation site is the D-galactosyl residue, with one or two *O*-acetyl substituents. The minor signal observed at *m/z* 279, corresponding in mass to the fragment ion $[(Fuc)Ac]^+$ expected if the fucosyl residue is endogenously *O*-acetylated, suggests that a small proportion of the fucosyl residues are mono-*O*-acetylated. However, an ion of undetermined structure is routinely observed at *m/z* 279 in positive mode f.a.b.-mass spectra of samples that have been *O*-(deuterioacetyl)ated under acidic conditions[13,24]. This ion is observed when various different matrices are used to obtain spectra, even when the sample contains no carbohydrate. Furthermore, in successive scans of the *O*-(deuterioacetyl)ated SEPS nonasaccharide fraction, the signal intensity at *m/z* 279 increased relative to the intensities of known carbohydrate signals. This suggests that the ion at *m/z* 279 is due to the reagent impurity rather than to the carbohydrate sample. This interpretation is supported by ^{1}H-n.m.r. data, presented later, which indicate that the L-fucosyl group is not significantly *O*-acetylated.

Analysis by f.a.b.-m.s. also demonstrated that the D-xylosyl residue to which the D-galactosyl residue is attached does not bear any endogenous *O*-acetyl groups. The presence of endogenous *O*-acetyl substituents on this D-xylosyl residue would be expected to cause the ratio of the $[(Fuc\text{-}Gal\text{-}Xyl)Ac]^+$ signal (*m/z* 798) to the $[(Fuc\text{-}Gal\text{-}Xyl)Ac_2]^+$ signal (*m/z* 795) to differ from the ratio of the $[(Fuc\text{-}Gal)Ac]^+$ signal (*m/z* 576) to the $[(Fuc\text{-}Gal)Ac_2]^+$ signal (*m/z* 573). However, the ratios of these signals are not significantly different, indicating that the 2-linked D-xylosyl residue is not *O*-acetylated.

The f.a.b.-m.s. data indicate that the nonasaccharide might have another, quantitatively minor *O*-acetylation site. A small but reproducible signal was observed at *m/z* 1081, corresponding to a nonreducing $[(Xyl_2Glc_2)Ac]^+$ fragment.

We conclude from the f.a.b.-m.s. data that the major site of *O*-acetyl substitution of the SEPS XG nonasaccharide is the D-galactosyl residue, which may be either mono- or di-*O*-acetylated. Signal intensities in the f.a.b.-mass spectrum (see Fig. 2) indicate that the ratio of mono- to di-*O*-acetylated D-galactosyl residues is ~1.3:1.0, and that small proportions of non-acetylated D-galactosyl residues are also present.

^{1}H-N.m.r. spectroscopy of SEPS XG oligosaccharide fractions. — ^{1}H-N.m.r. spectroscopy can be used to determine the position of *O*-acetyl substituents on carbohydrates, as these substituents strongly influence the chemical shifts of nearby protons. Specifically, protons that are attached to carbon atoms bearing an *O*-acetyl group are deshielded by ~0.5 to 1.5 p.p.m. relative to the corresponding protons in the unsubstituted alcohol[25]. The signals arising from such protons are often observed in the anomeric region of the ^{1}H-n.m.r. spectrum (δ 4.2 to 5.5)[25]. The locations of *O*-acetyl substituents on the components of the native nonasaccharide fraction were determined by assigning all of the signals between δ 4.2 and 5.5 in the ^{1}H-n.m.r. spectrum of the native nonasaccharide fraction, and determining which

of these signals were shifted out of this region by *O*-deacetylation.

^{1}H-N.m.r. (500 MHz) spectra of native and *O*-deacetylated SEPS XG nonasaccharide fraction (see Figs. 3 and 4) in D_2O were recorded at 27 and 75°. The signal assignments were based on comparison of these spectra with those of closely related xyloglucan oligosaccharides, and to published spectra of oligosaccharides containing similar substructures, such as the human blood-group H antigen [α-L-Fuc*p*-(1→2)-β-D-Gal*p*].

Most of the spectra presented herein were recorded at 75° because, at this temperature, the HDO signal was shifted out of the anomeric region. Also, considerably better resolution of certain complex groups of signals was obtained at 75° than at 27°. However, it was observed that significant *O*-deacetylation occurred during prolonged exposure of the native nonasaccharide fraction to the higher temperature. The chemical shifts of some of the signals exhibited slight temperature-dependence, but those of others did not. Thus, examination of the spectra recorded at 27° allowed differentiation spin of systems that gave rise to overlapping signals in the spectra at 75°. The conclusion regarding the sites of *O*-acetylation in the native XG nonasaccharide fraction were reached by combining the results obtained at 75° and 27°.

^{1}H-N.m.r. spectroscopy of the O*-deacetylated SEPS XG nonasaccharide fraction.* — The anomeric-proton signals of the *O*-deacetylated SEPS XG nonasaccharide fraction (see Fig. 3B, and Table III) were assigned as follows. The doublets at δ

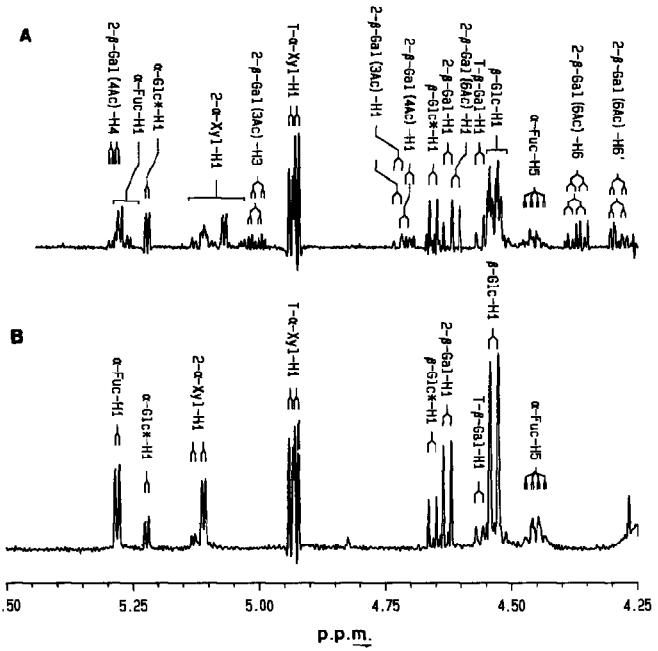

Fig. 3. Anomeric region of the resolution-enhanced 500 MHz ^{1}H-n.m.r. spectra (D_2O, 75°) of (A) the native nonasaccharide fraction and (B) the *O*-deacetylated nonasaccharide fraction. The asterisk (*) indicates a reducing residue.

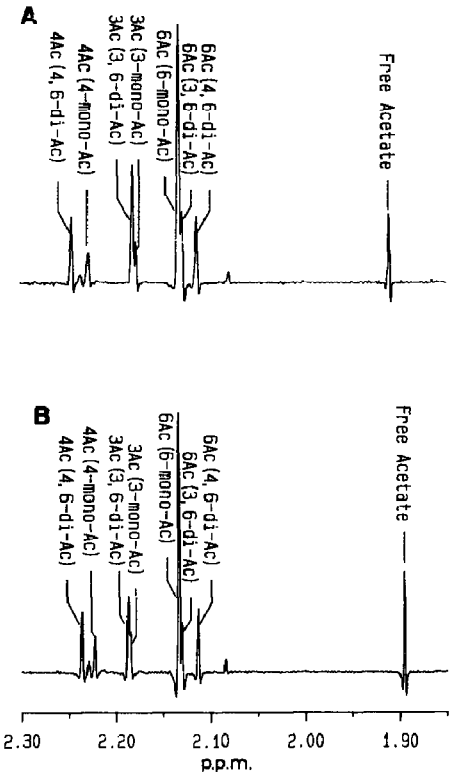

Fig. 4. Resolution-enhanced, partial, 500-MHz ^{1}H-n.m.r. spectra of native nonasaccharide fraction at 25° (A) and 75° (B). [All *O*-acetyl methyl proton signals are assigned as *O*-acetyl substituents of the 2-linked β-D-galactosyl residue. The location of the *O*-acetyl substituent is indicated outside the parentheses, and the substitution pattern for the residue is indicated in parentheses. For example, the methyl proton signal of the acetyl group at O-6 of the 4,6-di-*O*-acetyl β-D-galactosyl residue is labelled 6-Ac(4,6-di-Ac).]

5.224 ($J_{1,2}$ 4.0 Hz) and 4.659 ($J_{1,2}$ 7.9 Hz) were respectively assigned to H-1 of the α and β anomers of the reducing D-glucose residue, based on their chemical shifts and coupling constants[26], and the observation that these signals disappear upon reduction of the oligosaccharide with $NaBH_4$. The doublets at δ 4.927 and 4.939 ($J_{1,2}$ 3.6 Hz) were assigned to H-1 of the terminal α-D-xylosyl groups, based on the observation of signals with nearly identical coupling constants and chemical shifts in the spectrum of a closely related heptasaccharide (see Fig. 1C, and Table III) from SEPS XG, which contains terminal α-D-xylosyl groups as its only α-linked components[2,27]. Similarly, the doublet at δ 4.536 ($J_{1,2}$ 7.9 Hz) was assigned to H-1 of the nonreducing β-D-glucose residues. The signal at δ 5.131 ($J_{1,2}$ 3.6 Hz) was assigned to H-1 of the 2-linked α-D-xylosyl residue, based on the observation of signals with similar chemical shifts and coupling constants in the spectra of related oligosaccharides obtained from Tamarind XG. The Tamarind XG oligosaccharides consist of the heptasaccharide structure (see Fig. 1C) with terminal β-D-galactosyl residues

TABLE III

ASSIGNMENT OF SIGNALS[a] IN THE ANOMERIC REGION OF THE ^{1}H-N.M.R. SPECTRA OF SEPS XG OLIGOSACCHARIDE FRACTIONS

Assignment	*Heptasaccharide fraction*	*O-Deacetylated nonasaccharide fraction*	*Native nonasaccharide fraction*
4-*O*-Ac-β-Gal[b] H-4			5.283/5.295 (3.6,1.2)
α-Fuc H-1		5.282 (4.0)	5.259–5.284 (4.0)
Reducing α-Glc H-1	5.223 (3.8)	5.224 (4.0)	5.222 (3.8)
2-Linked[c] α-Xyl H-1		5.131 (3.6)	5.127 (3.6)
2-Linked[d] α-Xyl H-1		5.111 (3.6)	5.030–5.116 (3.3)–(3.6)
3-*O*-Ac-β-Gal[b] H-3			5.003/5.009 (3.3,10.0)
T-α-Xyl H-1	4.927/4.941 (3.6)	4.927–4.939 (3.6)	4.927/4.939 (3.6)
3-*O*-Ac-β-Gal[b] H-1			4.726/4.727 (7.5)/(7.7)
4-*O*-Ac-β-Gal[b] H-1			4.703/4.714 (7.7)
Reducing β-Glc H-1	4.654 (7.9)	4.659 (7.9)	4.657 (8.0)
2-Linked β-Gal H-1		4.629 (7.6)	4.628 (7.9)
6-Mono-*O*-Ac-β-Gal[b] H-1			4.612 (8.2)
T-β-Gal H-1		4.564 (7.0)	4.564 (7.1)
β-Glc H-1	4.538/4.563 (7.9)/(8.0)	4.536 (7.9)	4.531–4.549 (7.8)–(8.0)
α-Fuc H-5		4.453 (6.4,2.1)	4.456 (6.4,2.0)
6-*O*-Ac-β-Gal[b] H-6			4.369/4.375 (8.0,11.7)

TABLE III *(continued)*

6-*O*-Ac-β-Gal[b]	4.288/4.290
H-6′	(4.4,11.8)

[a]Chemical shifts are given to three decimal places. Coupling constants are in parentheses, separated by a comma when more than one coupling constant applies to a given signal. A hyphen (-) between values indicates that a range of values is given. A virgule (slash, /) separates discrete values when more than one is given. [b]"3-*O*-Ac-β-Gal", "4-*O*-Ac-β-Gal", and "6-*O*-Ac-β-Gal" refer to β-Gal residues having an acetyl group at O-3, -4, -6, respectively, regardless of whether the residue has a second *O*-acetyl substituent, or not. However, H-1 of 6-*mono*-*O*-acetyl-β-Gal is specifically assigned (δ 4.612). [c]α-Xyl with terminal β-Gal attached at O-2 (decasaccharide, see text). [d]α-Xyl with α-Fuc-(1→2)-β-Gal attached at O-2.

attached to O-2 of some of the α-D-xylosyl groups[27]. The anomeric protons of the 2-linked α-D-xylosyl residues give rise to doublets at positions ranging from δ 5.11 to 5.15 ($J_{1,2}$ ~4 Hz). On the basis of reported assignments of the ^{1}H-n.m.r. spectra of mammalian glycoprotein oligosaccharides containing the 2-*O*-α-L-fucosyl-β-D-galactosyl moiety[28], the doublet (δ 5.282, $J_{1,2}$ 4.0 Hz) and the multiplet (δ 4.453, $J_{4,5}$ 1, $J_{5,6}$ 6.4 Hz) were respectively assigned to H-1 and to H-5 of the α-L-fucosyl residue. Similarly, the doublet (δ 4.629, $J_{1,2}$ 7.6 Hz) was assigned to H-1 of the 2-linked-β-D-galactosyl residue.

The *O*-deacetylated SEPS nonasaccharide fraction contains ~15% of a decasaccharide[19,20] (see Fig. 1B, f.a.b.-m.s. section, and Tables I and II). The low intensity signals at δ 5.131 ($J_{1,2}$ 3.6 Hz) and δ 4.564 ($J_{1,2}$ 7.0 Hz) can be attributed to the anomeric protons of the second 2-linked α-D-xylosyl residue and the terminal β-D-galactosyl group, respectively, of this decasaccharide.

^{1}H-N.m.r. spectroscopy of the native SEPS XG nonasaccharide fraction. — The ^{1}H-n.m.r. spectra of the native SEPS XG nonasaccharide fraction were recorded at 27° and 75° (see Figs. 3A and 4). The degree of *O*-acetylation of the oligosaccharides was estimated by comparing (see Table IV) the signal integrals of the *O*-acetyl methyl protons (δ 2.07 to δ 2.25) to those of the methyl protons of the α-L-fucosyl residue (δ 1.26). The 27° spectrum (see Fig. 4A) of a sample that had not previously been exposed to high temperature was recorded, and the 75° spectrum (see Fig. 4B) of the same sample was subsequently recorded. The slight increase in the intensity of the free acetate peak (δ 1.9) indicated that some *O*-deacetylation (~10%) had occurred during exposure to high temperature. The estimated number of *O*-acetyl groups per oligosaccharide decreased from 1.1 to 1.0 upon heating. However, the same *O*-acetyl methyl signals were still present after heating, in approximately the same proportions. The degree of *O*-acetylation of the nonasaccharide fraction varies slightly from preparation to preparation, but is typically from 1.0 to 1.2 *O*-acetyl groups per molecule.

The large number of *O*-acetyl methyl singlets having non-stoichiometric intensity ratios indicates significant heterogeneity in the native SEPS nonasaccharide fraction. Multiple signals for H-1 of the 2-linked β-D-galactosyl residue (bearing

TABLE IV

INTEGRALS AND ASSIGNMENTS FOR ^{1}H-N.M.R. SIGNALS DIAGNOSTIC FOR THE *O*-ACETYLATION PATTERN IN THE NATIVE SEPS XG NONASACCHARIDE FRACTION

Assignment	*δ*	*Integral*[c]
4,6-di-*O*-Ac-Gal[a] 6-*O*-Ac-methyl protons[b]	2.113	0.30
3,6-di-*O*-Ac-Gal 6-*O*-Ac methyl protons	2.131	1.50[d]
6-mono-*O*-Ac-Gal 6-*O*-Ac-methyl protons	2.134	
3-mono-*O*-Ac-Gal 3-*O*-Ac methyl protons	2.185	0.67[d]
3,6-di-*O*-Ac-Gal 3-*O*-Ac-methyl protons	2.188	
4-mono-*O*-Ac-Gal 4-*O*-Ac methyl protons	2.223	0.17
4,6-di-*O*-Ac-Gal 4-*O*-Ac methyl protons	2.236	0.27
6-*O*-Ac-Gal (all)[e] H-6	4.288–4.290[f]	0.57 (0.60)[g]
6-*O*-Ac-Gal (all)[e] H-6′	4.369–4.375[f]	0.55 (0.60)[g]
3-*O*-Ac-Gal (all)[e] H-3	5.003/5.009[f]	0.21 (0.22)[g]
4-*O*-Ac-Gal (all)[e] H-4	5.283/5.295[f]	0.15[h] (0.15)[g]

[a]Molecular species. [b]Location of assigned proton. [c]Integrals relative to the signal of the methyl protons of the α-L-fucosyl residue (δ 1.26), taken as 3.0. [d]Certain groups of signals (indicated by the brackets) were not sufficiently resolved to permit individual determination of their integrals. The value given is the total integral for the group of signals. [e]The wordt 'all' in parentheses indicates that the assigned signals arise from all of the 2-linked β-Gal residues having an *O*-acetyl group at the indicated position, including both mono- and di-*O*-acetylated residues. [f]A hyphen (-) between values indicates that a range of values is given. A virgule (slash, /) separates discrete values when more than one is given. [g]Theoretical values for the integral, calculated from the integrals of the corresponding *O*-acetyl methyl protons, are in parentheses. [h]The signal corresponding to H-4 of 4-*O*-acetyl- and 4,6-di-*O*-acetyl-D-galactosyl residues was obscured by H-1 of the α-L-fucosyl group in the 75° spectrum of the nonasaccharide fraction. Therefore, the signal integral for this proton was calculated from the 27° spectrum, where the signal was resolved. The other signal integrals in this Table were taken from the 75° spectrum, as more signals were resolved at this temperature.

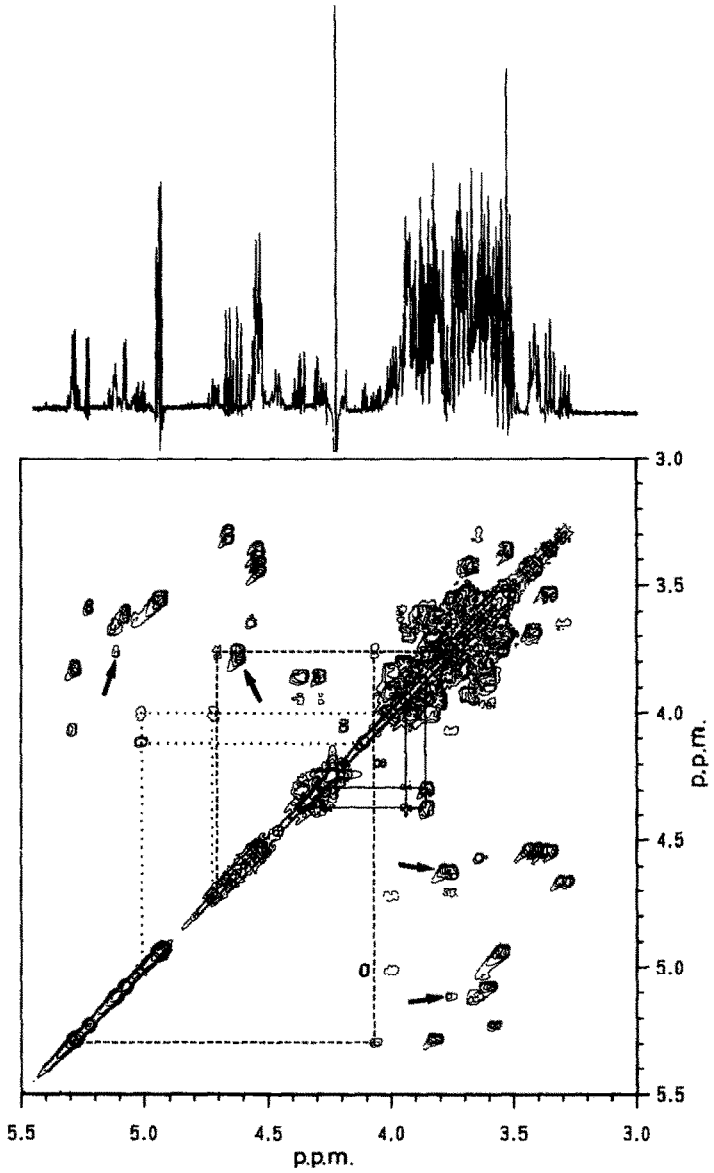

Fig. 5. Partial 2-D $\{^1H^1H\}$ COSY spectrum and resolution-enhanced 1-D spectrum of the native SEPS XG nonasaccharide fraction recorded for a solution in D_2O at 75°. [The connectivities for the 4-*O*-acetylated β-Gal systems are depicted with dashed lines (----), those for the 3-*O*-acetylated β-Gal systems with dotted lines (····), and those for the 6-*O*-acetylated β-Gal systems with solid lines (——). The off-diagonal signals indicated by arrows were not assigned as part of these spin systems, because they were shifted differentially at the two temperatures at which COSY spectra were recorded. Off-diagonal resonances due to $J_{4,5}$ in β-Gal residues were not observed under the experimental conditions used. See text for details.]

O-acetyl substituents), and for H-1 of the adjacent α-L-fucosyl group and 2-linked α-D-xylosyl residue were also observed (see Fig. 3A). The assignment of the ano-

meric signals of the α-L-fucosyl group and 2-linked α-D-xylosyl residue was based on the assignment of the corresponding signals in the spectrum of the *O*-deacetylated nonasaccharide fraction (see Fig. 3 and Table III). The observation of characteristic coupling patterns (*i.e.*, doublets with $J_{1,2}$ ~4 Hz) in the *J*-resolved spectrum, and characteristic connectivities in the COSY spectrum (see Fig. 5) of the native nonasaccharide fraction confirmed these assignments. The assignment of the signals arising from the various *O*-acetylated, 2-linked β-D-galactosyl residues is described later.

Several non-anomeric signals were observed (see Fig. 3A) in the anomeric region (δ 4.2 to 5.5) of the spectrum of the native nonasaccharide fraction. Only anomeric protons and H-5 of the L-fucosyl group were observed in this region of the spectrum of the *O*-deacetylated nonasaccharide fraction (see Fig. 3B). The origins of the non-anomeric signals were assigned as follows. Two doublets of doublets at δ 4.37 ($J_{6,6'}$ 11.7 $J_{5,6}$ 8.0 Hz) and δ 4.30 ($J_{6,6'}$ 11.8, $J_{5,6'}$ 4.4 Hz) were assigned to H-6 and H-6′ of 6-*O*-acetyl-β-D-galactosyl residues, based on the coupling patterns of these signals and the establishment by f.a.b.-m.s. that the β-D-galactosyl residue is the dominant site of *O*-acetyl substitution (*vide supra*). The 2-D $\{^1H^1H\}$ COSY spectrum of the native SEPS XG nonasaccharide (see Fig. 5) indicated that these two signals are *J*-coupled to each other ($J_{6,6'}$ ~12 Hz), as expected for the geminal H-6 and H-6′ of aldohexoses. Inspection of these signals in the resolution-enhanced 1D spectrum (see Fig. 3A) showed that they are each composed of at least two multiplets. The COSY spectrum shows that the high-intensity β-D-galactosyl H-6 and H-6′ signals (δ 4.368 and 4.288) are *J*-coupled to a proton resonant at δ 3.85, while the low-intensity β-D-galactosyl H-6 and H-6′ signals (δ 4.375 and 4.290) are *J*-coupled to a proton resonant at δ 3.93. These resonances at δ 3.85 and 3.93 were therefore assigned to H-5 of the 6-*O*-acetyl-β-D-galactosyl residues. The detection of two distinct resonances for H-5 of the 6-*O*-acetyl-β-D-galactosyl residues can be explained by assuming that some of the 6-*O*-acetyl-β-D-galactosyl residues also have a second *O*-acetyl substituent, strongly affecting the resonance of H-5 but only weakly affecting the resonances of H-6 and H-6′. Other signals in the ^{1}H-n.m.r. spectra (see later) confirmed that the native nonasaccharide fraction contains components having 4,6-di-*O*-acetyl-D-galactosyl and 3,6-di-*O*-acetyl-D-galactosyl residues.

Another complex group of non-anomeric signals appears, at δ 5.01, in the ^{1}H-n.m.r. spectrum of the native SEPS nonasaccharide fraction. These signals, assigned to β-D-galactosyl H-3 atoms, were shifted downfield due to acetylation at O-3. Two-dimensional *J*-resolved spectroscopy (data not shown) revealed that this group of signals is composed of two doublets of doublets at δ 5.003 and 5.009 (each with $J_{3,4}$ 3.3 Hz and $J_{2,3}$ 10.0 Hz). Off-diagonal signals in the COSY spectrum (see Fig. 5) indicated that these signals are *J*-coupled to signals at δ 4.11 and δ 4.00 (H-4 and H-2, respectively, of 3-*O*-acetyl-β-D-galactosyl residues). In addition, the signal(s) at δ 4.00 (H-2) show a connectivity to doublets at δ 4.72 ($J_{1,2}$ 7.7 Hz). These doublets (δ 4.727 and 4.714) could, by virtue of their indirect connectivity to the

multiplets at δ 5.01 and the coupling patterns of the ring protons of this system, be assigned to the anomeric protons of 3-*O*-acetylated β-D-galactosyl residues. Thus, ^{1}H-n.m.r. analysis showed that ~22% (see Table IV) of the β-D-galactopyranosyl residue of the native XG nonasaccharide fraction are acetylated at O-3.

The presence of two similar but distinct multiplets at δ5.01 suggested that some of the 3-*O*-acetyl-β-D-galactosyl residues have a second *O*-acetyl substituent, which, when present, shifts the position of the H-3 signal by 0.006 p.p.m. It was concluded that O-6 is the principal site of the second acetyl substituent on the 3-*O*-acetyl-D-galactosyl residues, because significant acetoxylation of the β-D-galactopyranosyl residues occurred at C-6, and some of the 6-*O*-acetylated D-galactopyranosyl residues appear to contain a second *O*-acetyl substituent (*vide supra*). In addition, no signals were detected that could be assigned to the ring protons of 3,4-di-*O*-acetylated β-D-galactosyl residues. Therefore, the two multiplets at δ 5.009 and 5.003 arise from H-3 of 3-*O*-acetyl- and 3,6-di-*O*-acetyl-β-D-galactopyranosyl residues present in the native XG nonasaccharide fraction.

The only other possible site of *O*-acetyl substitution of the 2-linked β-D-galactosyl residues is O-4. A chemical shift between δ 3.85 and 4.0 for H-4 in nonacetylated 2-linked β-D-galactosyl residues was expected[28]. It was therefore expected that the signal of H-4 of 4-*O*-acetylated β-D-galactosyl residues would exhibit a chemical shift between δ 4.8 and 5.5, due to the α-effect of *O*-acetylation[25] (*i.e.*, deshielding by some 1.0 to 1.5 p.p.m. for protons attached to *O*-acetylated ring-carbon atoms). It was also expected that the signal arising from H-4 of β-D-galactosyl residues would be a doublet of doublets with coupling constants of ~3 to 4 Hz ($J_{3,4}$) and 1 Hz ($J_{4,5}$). Signals that could be assigned to H-4 of 4-*O*-acetylated β-D-galactosyl residues, although quite weak, were observed at δ 5.295 and 5.283 in the 75° spectrum of the native SEPS XG nonasaccharide fraction. These signals, partially obscured by signals arising from H-1 of the α-L-fucosyl groups, were detected because their connectivity in the COSY spectrum does not match that of the α-L-fucosyl H-1 signals. Specifically, low-intensity signals at δ ~5.29 (H-4) are *J*-coupled, *via* signals at δ 4.06 (H-3) and 3.76 (H-2), to low-intensity signals at δ 4.70 (H-1). The chemical shifts (δ 4.70) and coupling constants ($J_{1,2}$ 7.7 Hz) of these signals indicated that they arise from the anomeric protons of β-pyranosyl residues. Thus, the pattern of *J*-connectivity evident in the COSY spectrum indicates that the β-anomeric protons at δ 4.70 are on the same residues as protons that are deshielded by acetylation at O-4. The H-4 signals (δ 5.295 and 5.283) are partially obscured by the signals of the anomeric proton of the α-L-fucosyl group, and so were examined by 2D *J*-resolved spectroscopy. The coupling constants of these multiplets ($J_{3,4}$ 3.6, $J_{4,5}$ 1.2 Hz) make them difficult to distinguish from the adjacent α-L-fucosyl H-1 signals, but are consistent with their assignment as H-4 of β-D-galactosyl residues. For the reasons already described (*i.e.*, multiple signals for H-4 of 4-*O*-acetyl-β-D-galactopyranosyl residues, the presence of 6-*O*-acetyl-β-D-galactopyranosyl residues having a second *O*-acetyl substituent, and the lack of signals that could be associated with 3,4-di-*O*-acetyl-β-D-galactopyranosyl residues), it was concluded that the native

XG nonasaccharide fraction contains both 4-*O*-acetyl- and 4,6-di-*O*-acetyl-β-D-galactopyranosyl residues.

The f.a.b.-m.s. analysis (*vide supra*) indicated that the major site of *O*-acetylation on the native XG nonasaccharide fraction is the β-D-galactosyl residue, but does not preclude the possibility that the terminal α-L-fucosyl group also bears some *O*-acetyl substituents. The n.m.r. data indicated that there are few, if any, *O*-acetyl groups on the L-fucosyl group. The coupling pattern and chemical shift of the signals at δ 4.29 and 4.37 in the spectrum of the native nonasaccharide indicated that these signals arise from H-6 of a hexosyl residue acetylated at O-6. These signals obviously cannot be due to H-6 of the L-fucosyl (6-deoxy-L-galactosyl) group. The only other signals of significant intensity that are shifted into the anomeric region of the spectrum by *O*-acetylation were assigned to H-3 and H-4 of *O*-acetylated D-galactosyl residues rather than L-fucosyl groups. This assignment was made because the COSY spectra (at 27° and 75°) of the native nonasaccharide fraction shows indirect connectivity between these signals and β-anomeric proton signals ($J_{1,2}$ 7.7 Hz), ruling out the possibility that the signals arise from the fucosyl group, which is α-linked. The integrals of the signals assigned to protons shifted into the anomeric region by *O*-acetylation correlate well with those of the *O*-acetyl methyl protons (ratios of 3:1; see later and Table IV); therefore, all of the significant *O*-acetylation sites have been accounted for. It was concluded that *O*-acetyl substitution of the L-fucosyl group is not quantitatively significant.

Assignment of the O*-acetyl methyl protons of the native SEPS XG nonasaccharide fraction.* — The signals arising from the methyl protons of *O*-acetyl substituents were assigned on the basis of the following criteria. (*1*) The signal integral for each proton attached to an acetoxylated carbon atom is one-third that of the signal for the methyl protons of the corresponding *O*-acetyl group. (*2*) The signal integrals for the methyl protons of the two *O*-acetyl groups on a particular di-*O*-acetyl-D-galactosyl residue are equal. (*3*) When two *O*-acetyl substituents are present on the same residue, the positions of signals arising from the *O*-acetyl methyl protons may be shifted, relative to the positions of the corresponding signals arising from the mono-*O*-acetylated residue. Because the *O*-acetyl substituents of 4,6-di-*O*-acetyl-D-galactopyranosyl residues are closer together in space than those of 3,6-di-*O*-acetyl-D-galactopyranosyl residues, this shift should be of greater magnitude for 4,6-di-*O*-acetyl- than for 3,6-di-*O*-acetyl-D-galactosyl residues.

The most intense *O*-acetyl methyl signal in the resolution-enhanced 75° spectrum of the native nonasaccharide (see Fig. 4B) is the singlet at δ 2.134. Therefore, this signal was assigned to the methyl protons of the *O*-acetyl group on a 6-mono-*O*-acetyl-D-galactopyranosyl residue (see Table IV). The adjacent signals, at δ 2.131 and 2.113, were assigned to the methyl protons of the acetyl group on O-6 of 3,6-di-*O*-acetyl- and 4,6-di-*O*-acetyl-D-galactopyranosyl residues, respectively. As expected, the 6-*O*-acetyl methyl signal is shifted more by the presence of the nearby 4-*O*-acetyl group of the 4,6-di-*O*-acetylated residue than by the relatively distant 3-*O*-acetyl group of the 3,6-di-*O*-acetylated residue. The total integral for the signals

assigned to H-6 of the various 6-*O*-acetylated β-D-galactosyl residues (see Fig. 3A and Table IV) is, within the accuracy of the measurements, one-third that of the total integral of the signals assigned to the methyl protons of the acetyl groups at O-6 of the D-galactosyl residues (see Table IV). Also, the integrals of the signals assigned to the 4-*O*-acetyl methyl protons and 6-*O*-acetyl methyl protons of the 4,6-di-*O*-acetyl-D-galactosyl residues are equal (see Table IV). Thus, the criteria already described for the assignment of the *O*-acetyl methyl proton signals are fulfilled for the acetyl substituents at O-6 of the β-D-galactosyl residues. Similar reasoning was used to assign the signals of the methyl protons of other *O*-acetyl groups in the sample. In order to confirm the signal assignments for the *O*-acetyl methyl protons, the 1-D spectrum of a SEPS XG nonasaccharide subfraction that was enriched in di-*O*-acetylated components was also examined (data not shown). The ratio of signal intensities in this spectrum was also found consistent with the assignments already given.

The assignment of the *O*-acetyl methyl proton signals allows estimation of the proportion of the various *O*-acetylated species present in the native SEPS XG nonasaccharide fraction (see Table IV). The main site of *O*-acetyl substitution of the SEPS nonasaccharide is O-6 of the β-galactosyl residue (55–60%), with lesser amounts of acetylation at O-3 (20–25%) and at O-4 (15%) of the D-galactosyl residue. Of the oligosaccharides in the SEPS XG nonasaccharide fraction used for this study, ~50–55% have one *O*-acetyl substituent, 25–30% have two *O*-acetyl substituents, and 15–20% are not acetylated. This is in rough agreement with the estimates for the total degree of acetylation arrived at by f.a.b.-m.s. analysis (see earlier).

Interestingly, although 6-mono-*O*-acetyl-D-galactosyl residues are abundant in the nonasaccharide fraction, the fraction contains fewer 3-mono-*O*-acetyl-D-galactosyl residues than 3,6-di-*O*-acetyl-D-galactosyl residues, and fewer 4-mono-*O*-acetyl-D-galactosyl residues than 4,6-di-*O*-acetyl-D-galactosyl residues (see Fig. 4). The changes in signal intensity observed upon heating the sample to 75° suggest that the acetyl group at O-6 of the D-galactosyl residue is the most easily hydrolyzed (*i.e.*, the ratio of 4,6-di-*O*-acetyl to 4-mono-*O*-acetyl signals, and the ratio of 3,6-di-*O*-acetyl to 3-mono-*O*-acetyl signals, both decreased). If significant *O*-deacetylation at O-6 of the D-galactosyl residue occurred during sample preparation, it is possible that the 3-mono- and 4-mono-*O*-acetyl-D-galactosyl residues were derived from residues originally acetylated at O-6 as well. That is, the polysaccharide produced by the plant cells might not have significant levels of 3-mono-*O*-acetyl- and 4-mono-*O*-acetyl-D-galactosyl residues.

The heterogeneity with respect to the *O*-acetylation pattern of the SEPS XG nonasaccharide fraction could also result from migration of *O*-acetyl groups on the oligosaccharides. *O*-Acetyl groups have been known to migrate on a given glycosyl residue under physiological conditions[29]. Therefore, it is possible that the native XG nonasaccharide fraction may contain an equilibrium mixture of the various *O*-acetylated forms.

The results presented herein demonstrate that a combination of f.a.b.-m.s. and ^{1}H-n.m.r. analyses can be used to locate and quantitate the sites of *O*-acetyl substitution in a complex mixture of closely related oligosaccharides. The ability to carry out such analyses is important because in some cases it may be impractical, due to the migration of *O*-acetyl groups, to isolate unique *O*-acetylated species for chemical study.

Analysis by ^{1}H-n.m.r. and f.a.b.-m.s. of the oligosaccharides derived from SEPS xyloglucan has shown that xyloglucans can exhibit more structural complexity than previously imagined. During the course of this study, heterogeneity in the glycosyl sequence of various XG oligosaccharides was also observed.

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REFERENCES

1. M. McNeil, A. G. Darvill, S. C. Fry, and P. Albersheim, *Annu. Rev. Biochem.*, 53 (1984) 625–663.
2. W. D. Bauer, K. W. Talmadge, K. Keegstra, and P. Albersheim, *Plant Physiol.*, 51 (1973) 174–187.
3. W. S. York, A. G. Darvill, M. McNeil, T. T. Stevenson, and P. Albersheim, *Methods Enzymol.*, 118 (1986) 3–40.
4. W. S. York, A. G. Darvill, and P. Albersheim, *Plant Physiol.*, 75 (1984) 295–297.
5. T. Hayashi and G. Maclachlan, *Plant Physiol.*, 75 (1984) 596–604.
6. B. S. Valent, A. G. Darvill, M. McNeil, B. K. Robertsen, and P. Albersheim, *Carbohydr. Res.*, 79 (1980) 165–192.
7. J. Labavitch and P. Ray, *Plant Physiol.*, 53 (1974) 669–673.
8. G. J. McDougall and S. C. Fry, personal communication.
9. M. A. O'Neill, V. J. Morris, R. R. Selvendran, I. W. Sutherland, and I. T. Taylor, *Carbohydr. Res.*, 148 (1986) 63–69.
10. C. Hara, T. Kiho, and S. Ukai, *Carbohydr. Res.*, 111 (1982) 143–150.
11. T. Matsuo and T. Mizuno, *Agric. Biol. Chem.*, 38 (1974) 465–466.
12. P. Biely, C. R. MacKenzie, J. Puls, and H. Schneider, *Bio/Technology*, 4 (1986) 731–733.
13. A. Dell and P. R. Tiller, *Biochem. Biophys. Res. Commun.*, 135 (1986) 1126–1134.
14. I. J. Goldstein, G. W. Hay, B. A. Lewis, and F. Smith, *Methods Carbohydr. Chem.*, 5 (1965) 361–370.
15. P. Albersheim, D. J. Nevins, P. D. English, and A. Karr, *Carbohydr. Res.*, 5 (1967) 340–345.
16. T. J. Waeghe, A. G. Darvill, M. McNeil, and P. Albersheim, *Carbohydr. Res.*, 123 (1983) 281–304.
17. A. B. Blakeney and B. A. Stone, *Carbohydr. Res.*, 140 (1985) 319–324.
18. D. P. Sweet, R. H. Shapiro, and P. Albersheim, *Carbohydr. Res.*, 40 (1975) 217–225.
19. Y. Kato and K. Matsuda, *Agric. Biol. Chem.*, 44 (1980) 1759–1766.
20. Y. Kato, J. Matsushita, T. Kubodera, and K. Matsuda, *J. Biochem. (Tokyo)*, 97 (1985) 801–810.
21. P. Prehm, *Carbohydr. Res.*, 78 (1980) 372–374.
22. N. K. Kochetkov and O. S. Chizhov, *Adv. Carbohydr. Chem.*, 21 (1966) 39–92.

23. P.-K. TSAI, A. DELL, AND C. E. BALLOU, *Proc. Natl. Acad. Sci. U.S.A.*, 83 (1986) 4119–4123.
24. J. E. OATES, unpublished results.
25. J. HAVERKAMP, H. VAN HALBEEK, L. DORLAND, AND J. F. G. VLIEGENTHART, *Eur. J. Biochem.*, 122 (1982) 305–311.
26. J. P. KAMERLING, L. DORLAND, H. VAN HALBEEK, J. F. G. VLIEGENTHART, M. MESSER, AND R. SCHAUER, *Carbohydr. Res.*, 100 (1982) 331–340.
27. P. KOOIMAN, Recl. *Trav. Chim. Pays-Bas*, 80 (1961) 849–865.
28. H. VAN HALBEEK, L. DORLAND, J. F. G. VLIEGENTHART, N. K. KOCHETKOV, N. P. ARBATSKY, AND V. A. DEREVITSKAYA, *Eur. J. Biochem.*, 127 (1982) 21–29.
29. J. P. KAMERLING, R. SCHAUER, A. K. SHUKLA, A. STOLL, H. VAN HALBEEK, AND J. F. G. VLIEGENTHART, *Eur. J. Biochem.*, in press.