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Dimers of a GFG hexasaccharide occur in apple fruit xyloglucan

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Abstract

Apple fruit xyloglucan is predominantly built up from XXXG, XXFG, and XLFG units (G = β-D-Glc*p*–, X = α-D-Xyl*p*-(1 → 6)-β-D-Glc*p*–, L = β-D-Gal*p*-(1 → 2)-α-D-Xyl*p*-(1 → 6)-β-D-Glc*p*–, F = α-L-Fuc*p*-(1 → 2)-β-D-Gal*p*-(1 → 2)-α-D-Xyl*p*-(1 → 6)-β-D-Glc*p*–). However, small amounts of oligosaccharides with a less heavily branched glucan backbone also occur. Structural analysis of two such oligosaccharides, isolated from a xyloglucan preparation digested with endoglucanase IV, using a combination of FAB mass spectrometry and ^{1}H NMR spectroscopy, afforded the identification of GFG and a dimer of GFG. The finding of the dodecasaccharide GFGGFG as a structural element of apple fruit xyloglucan is most unusual. © 1998 Elsevier Science Ltd

Keywords: Apple fruit xyloglucan; GFG hexasaccharide; GFGGFG dodecasaccharide; Endoglucanase IV; Structural analysis

1. Introduction

Xyloglucans play an important role in cross-linking cellulose microfibrils in the primary cell walls of plants [1]. These polysaccharides are composed of a backbone of (1 → 4)-linked β-D-Glc*p* residues to which α-D-Xyl*p*-(1 → 6)–, β-D-Gal*p*-(1 → 2)-α-D-Xyl*p*-(1 → 6)– or α-L-Fuc*p*-(1 → 2)-β-D-Gal*p*-(1 → 2)-α-D-Xyl*p*-(1 → 6)-side chains are attached [2,3]. Generally, approximately 75% of a xyloglucan backbone is branched, although lower degrees of branching occur, for instance, in rice seedlings (approximately 30% [4]) and in solanaceous plants (approximately 40% [5,6]).

The backbone of apple fruit xyloglucan is approximately 65% branched [7], which suggests that, in

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addition to the three main oligosaccharide building units, XXXG, XXFG and XLFG [8,9] (G = β-D-Glc*p*–, X = α-D-Xyl*p*-(1 → 6)-β-D-Glc*p*–, L = β-D-Gal*p*-(1 → 2)-α-D-Xyl*p*-(1 → 6)-β-D-Glc*p*–, F = α-L-Fuc*p*-(1 → 2)-β-D-Gal*p*-(1 → 2)-α-D-Xyl*p*-(1 → 6)-β-D-Glc*p*– [10]), oligosaccharides with less-substituted backbones occur also. The first indication of this was provided by Renard et al. [8] with the detection of small amounts of Glc_4Xyl_2 (exact structure unknown) in a digest of apple xyloglucan. More evidence for relatively G-rich oligosaccharides as structural elements of apple fruit xyloglucan was obtained by Vincken et al. [9], who isolated $Glc_3Xyl_1Gal_1Fuc_1$ and $Glc_6Xyl_2Gal_2Fuc_2$ from a digest of apple fruit xyloglucan. Here, the combined mass spectrometric and ^1H NMR spectroscopic characterization of these and other fragments isolated from this digest is presented, and the biological significance of the G-rich oligosaccharides is discussed.

2. Experimental

Materials.—Xyloglucan oligosaccharides were obtained after enzymatic degradation of an alkali-extracted, depectinized xyloglucan preparation derived from apple fruit material [7]. The mixture of oligosaccharides was sequentially fractionated on Bio-Gel P-2 (size-exclusion chromatography) and CarboPac PA-1 [semi-preparative high-pH anion-exchange chromatography (HPAEC)] [9]. The final fraction numbering refers to the two-step separation protocol, in which the first number refers to the fraction obtained on Bio-Gel P-2 and the second number the subfraction on CarboPac PA-1.

Endoglucanase IV (endoIV; EC 3.2.1.4) and exoglucanase I (exoI; EC 3.2.1.91) were purified from a commercial enzyme preparation from *Trichoderma viride* (Maxazyme Cl, Gistbrocades, Delft, Netherlands) as described [11].

Reduction of GFG.—GFG (approximately 100 μg) was treated with 200 μL 1.5 M ammonia containing 75 mg/mL $NaBH_4$ for 1 h at 30 °C. After neutralization with HOAc, the oligosaccharide-alditol was desalted using a mixture of Dowex 50W-X8 (H^+) and AG3-X4A (OH^-) resins (Bio-Rad, Richmond, CA, USA) in a ratio of approximately 0.6 (v/v), and the solution was concentrated in a stream of air.

Degradation of oligosaccharides by endoIV and exoI.—Approximately 20 μg GFG or GFGol was treated with exoI (approximately 100 ng protein) in 100 μL 50 mM NaOAc buffer (pH 5) for 12 h at 40 °C. The release of Glc was determined using analytical HPAEC on CarboPac PA-100 [9]. Under similar conditions, approximately 20 μg GFGGFG was treated with approximately 50 ng endoIV, and the resulting mixture was analyzed on CarboPac PA-100.

Protein content.—The protein content of enzyme preparations was determined according to [12], using BSA as a standard.

NMR spectroscopy.—Prior to ^1H NMR analysis, xyloglucan oligosaccharides were exchanged twice in deuterium oxide (99.9 at% D, MSD Isotopes) with intermediate freeze-drying. Finally, samples were dissolved in 99.96 at% D (MSD Isotopes). ^1H NMR spectra were recorded at 500 MHz on a Bruker AMX-500, or at 600 MHz on a Bruker AMX/2-600 spectrometer (Bijvoet Center, Department of NMR Spectroscopy, Utrecht University) at a probe temperature of 27 °C. Chemical shifts (δ) are expressed in ppm relative to the signal of internal acetone (δ 2.225). The 1D spectra were recorded with a spectral width of 5000 Hz at 500 MHz or 6000 Hz at 600 MHz, collecting 80–1000 free induction decays of 8K or 16K complex data points. Suppression of the HOD signal was achieved by applying a WEFT pulse sequence as described [13]. The resolution of the 1D spectra was enhanced by Lorentzian-to-Gaussian transformation and the final spectra were baseline corrected with a polynomial function when necessary.

2D NOESY experiments were carried out with a mixing time of 250 ms. 2D HOHAHA measurements were performed using an MLEV-17 mixing sequence of 100–120 ms. For 2D DQF-COSY, 2D NOESY, and 2D HOHAHA spectra 512 measurements of 2K data points were recorded. The spectral width was 4032 Hz or 4500 Hz in each dimension. 2D ROESY experiments were carried out using a spin-lock mixing pulse of 250 ms at a field strength corresponding to a 90° pulse-width between 100–110 ms. The carrier frequency was placed on the left side of the spectrum at δ 5.9 in order to minimize HOHAHA-type magnetization transfer. The spectral width was 5500 Hz in each dimension, and 512 experiments of 4K data points were recorded.

The 2D DQF–COSY, NOESY, HOHAHA, and ROESY experiments were performed using the time-proportional phase-increment method to create t_1 amplitude modulation. The HOD signal was suppressed by presaturation for 1.0 s. 2D NMR data were processed on Silicon Graphics Iris Indigo or 4D/35 stations, using Triton software (Bijvoet Center, Department of NMR Spectroscopy, Utrecht University).

Mass spectrometry.—Positive-ion mode FAB mass

spectra were obtained using MS1 of a JEOL JMS-SX/SX102A tandem mass spectrometer operated at 10 kV or 6 kV accelerating voltage (for peracetylated fraction **12.1**). The FAB gun was set at an emission current of 10 mA and generated a beam of 6 keV xenon atoms. Spectra were scanned at a speed of 30 s for the full mass range specified by the accelerating voltage used, and were recorded and averaged on a Hewlett Packard HP9000 data system running JEOL Complement software. CID mass spectra were recorded using the same instrument; after selecting the parent ion in the first mass spectrometer it was collided with helium in the third field free region collision cell, at a pressure sufficient to reduce the parent ion beam to one third of its original intensity. Daughter ion spectra were recorded by scanning the second mass spectrometer. Samples were dissolved in 10 μL aqueous 5% HOAc (native samples) or Me_2SO (peracetylated samples), and 1.5 μL aliquots of sample solution were loaded into a matrix of monothioglycerol.

Peracetylation was carried out on 1/20th of each fraction, which was dried under vacuum and then treated with 2:1 trifluoroacetic anhydride–HOAc (250 μL) [14]. The reaction proceeded for 20 min at ambient temperature and the reagents were removed under vacuum. The peracetylated samples were dissolved in $CHCl_3$ and extracted with water (three times), and the solvent finally removed under vacuum.

Nomenclature.—The xyloglucans are named according to the specific nomenclature for xyloglucans [10]. The Glc residues in the backbone of an oligosaccharide are designated by superscripts (**a** to **e**) starting from the Glc residue at the reducing end (i.e., Glc^{e}-Glc^{d}-Glc^{c}-Glc^{b}-Glc^{a}-Glc). The Xyl, Gal, and Fuc residues are denoted by the same superscript as the Glc residue to which they are attached. This is slightly different from the numbering system used by others [15] where in the larger structures the internal unbranched Glc is denoted Glc^{s}, bordered on both sides by three branched Glc residues. Since this situation does not apply here, a different notation is used.

3. Results

In a previous study [9], the degradation of apple fruit xyloglucan by endoIV was described, yielding a complex mixture of oligosaccharides which was fractionated on Bio-Gel P-2 (Fig. 1) and subfractionated on CarboPac PA-1. In that report, tentative structures based on monosaccharide analysis, mass spectrometric analysis, and degradation with Driselase were proposed for most purified oligosaccharides, and indications for the presence of new structural elements were obtained. In the present study, the proposed structures for the common oligosaccharides are confirmed. However, the emphasis of this study is the detailed characterization of the fractions **6.1** and **12.1** using FABMS and ^{1}H NMR spectroscopy.

Characterization of common xyloglucan oligosaccharides.—The current characterization of the xyloglucan fractions **5.1, 5.2, 7.4, 9.3a, 9.2,** and **10.1** has been achieved on the basis of compositional [9], mass spectrometric and ^{1}H NMR analysis. For the

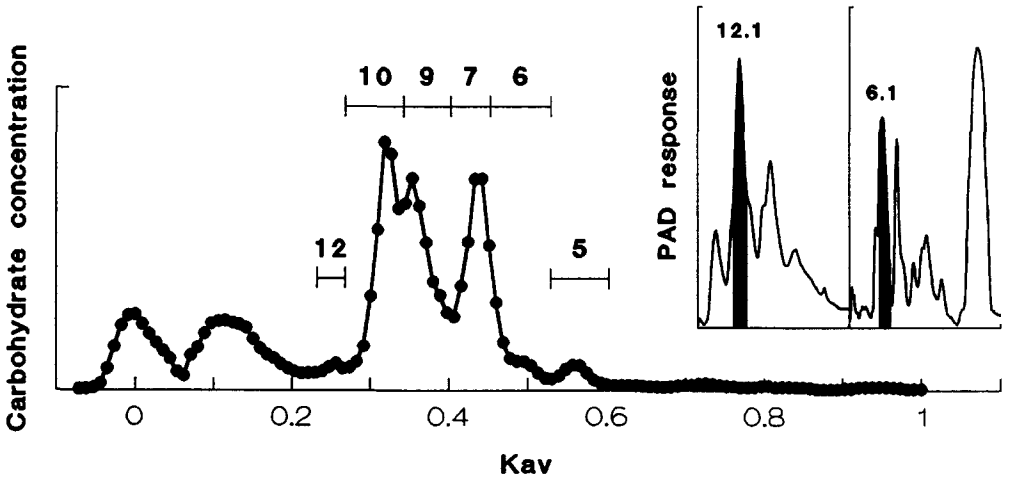

Fig. 1. Elution pattern of an apple xyloglucan digest on Bio-Gel P-2. The insets represent the elution profiles on semi-preparative HPAEC of pools **12** and **6**. The fractionation of the apple xyloglucan digest is described in more detail in [9].

Table 1
^{1}H NMR H-1 chemical shifts[a] for the penta- to decasaccharides derived from apple fruit xyloglucan

Residue	**5.1** FG	**5.2** XXG[b]	**7.4** XXXG[c]	**9.3a** XLXG[b]	**9.2** XXFG[c]	**10.1** XLFG[c]
Glcβ	4.667	4.662	4.661	4.661	4.665	4.665
Glcα	5.221	5.223	5.221	5.221	5.221	5.220
Glc[a]	4.513	4.561[d]	4.559[d]	4.534[d]	4.540[d]	4.548[d]
Glc[b]		4.553[d]	4.574[d]	4.565[d]	4.533[d]	4.519[d]
Glc[c]			4.550	4.534	4.547	4.533
Xyl[a]β	5.134	4.959	4.957	4.956	5.141	5.138
Xyl[a]α	5.138				5.144	5.141
Xyl[b]		4.941	4.957	5.176	4.954	5.175
Xyl[c]			4.940	4.941	4.941	4.942
Gal[a]	4.621				4.616	4.618
Gal[b]				4.558		4.555
Fuc[a]	5.286				5.272	5.275

The isolation of the different fractions has been described previously [9]. For the coding system of the mono- and oligosaccharides, see Section 2. α and β stand for the anomeric configuration of Glc.
[a]In ppm relative to the signal of internal acetone at δ 2.225.
[b]^{1}H NMR chemical shift values of the corresponding oligosaccharide-alditol have been reported [18].
[c]^{1}H NMR chemical shift values of the corresponding oligosaccharide-alditol have been reported [3].
[d]Chemical shifts may have to be interchanged; detailed assignment was not possible due to the small amounts of material.

assignment of the ^{1}H NMR spectra, the structural-reporter-group concept, as developed for xyloglucan derived oligosaccharide-alditols, have been used [3,15–18]. It should be noted that in the present study the xyloglucan oligosaccharides have not been converted into oligosaccharide-alditols [3,15–18]. The branching patterns of XLXG (fraction **9.3a**) and XXFG (fraction **9.2**) were identified using FABMS in conjunction with peracetylation (data not shown). The ^{1}H NMR H-1 chemical shift values are summarized in Table 1. The chemical shift values for Glc[a] and Glc[b], as shown in Table 1, were assigned based on comparison with their reduced homologues [3,18]. It was assumed that the differences in chemical shift values between unreduced and reduced oligosaccharides were smaller for Glc[b] than for Glc[a] (closer to the reducing end).

The fucosylated oligosaccharides XXFG (fraction **9.2**) and XLFG (fraction **10.1**) showed anomerization effects similar to those described in [19]; Xyl[a] H-1 as well as Fuc[a] CH_3 are split into two doublets. The splitting of the Fuc[a] CH_3 signal is not observed in FG. Typically, the anomerization effect seems to occur only when the backbone of FG is extended with one Glc residue at the minimum at the non-reducing side (as in GFG, Table 2).

Fraction **6.1**.—The positive ion-mode FAB mass spectrum of fraction **6.1** showed a single $[M + Na]^+$ pseudomolecular ion at *m/z* 967, corresponding to a $Hex_4Deoxyhex_1Pent_1$ hexasaccharide. The presence of a single hexasaccharide was in agreement with the intensities of the anomeric signals, as observed in the ^{1}H NMR spectrum (Fig. 2). Detailed assignments, as deduced from 2D NMR measurements are presented in Table 2. In Fig. 3 the HOHAHA spectrum of fraction **6.1** is depicted, showing the scalar coupled networks of each residue starting from the anomeric protons. The Fuc H-1 signal at δ 5.273 indicates the presence of one F element in this oligosaccharide. The chemical shifts of the anomeric signals of this F element are almost identical to those in XXFG and XLFG (compare Tables 1 and 2). The two β anomeric signals at δ 4.537 and 4.479 belong to the internal and non-reducing terminal Glc residues, respectively.

Table 2
^{1}H NMR assignments[a] of the xyloglucan fragment GFG (fraction **6.1**)

Residue	H-1	H-2	H-3	H-4	H-5	H-5_{eq}	H-6a	H-6b	CH_3
Glcβ	4.666	3.276	3.615	3.615	3.615		3.810	3.946	
Glcα	5.221	3.567	3.615	3.857[b]	3.827[b]		3.940	3.940	
Glc[a]	4.537	3.388	3.673	3.611	3.920		3.920	3.920	
Glc[b]	4.479	3.331	3.505	3.422	3.488		3.736	3.912	
Xyl[a]β	5.144	3.658	3.884	3.615[c]	3.597[c]	3.690			
Xyl[a]α	5.148								
Gal[a]	4.615	3.741	3.850	3.905	3.670		3.750	3.800	
Fuc[a]β	5.273	3.791	3.886	3.806	4.545				1.262
Fuc[a]α									1.257

For the coding system of the monosaccharides, see Section 2. α and β stand for the anomeric configuration of Glc.
[a]In ppm relative to the signal of internal acetone at δ 2.225.
[b]Chemical shifts may have to be interchanged due to strong overlap.
[c]Chemical shifts may have to be interchanged due to strong overlap.

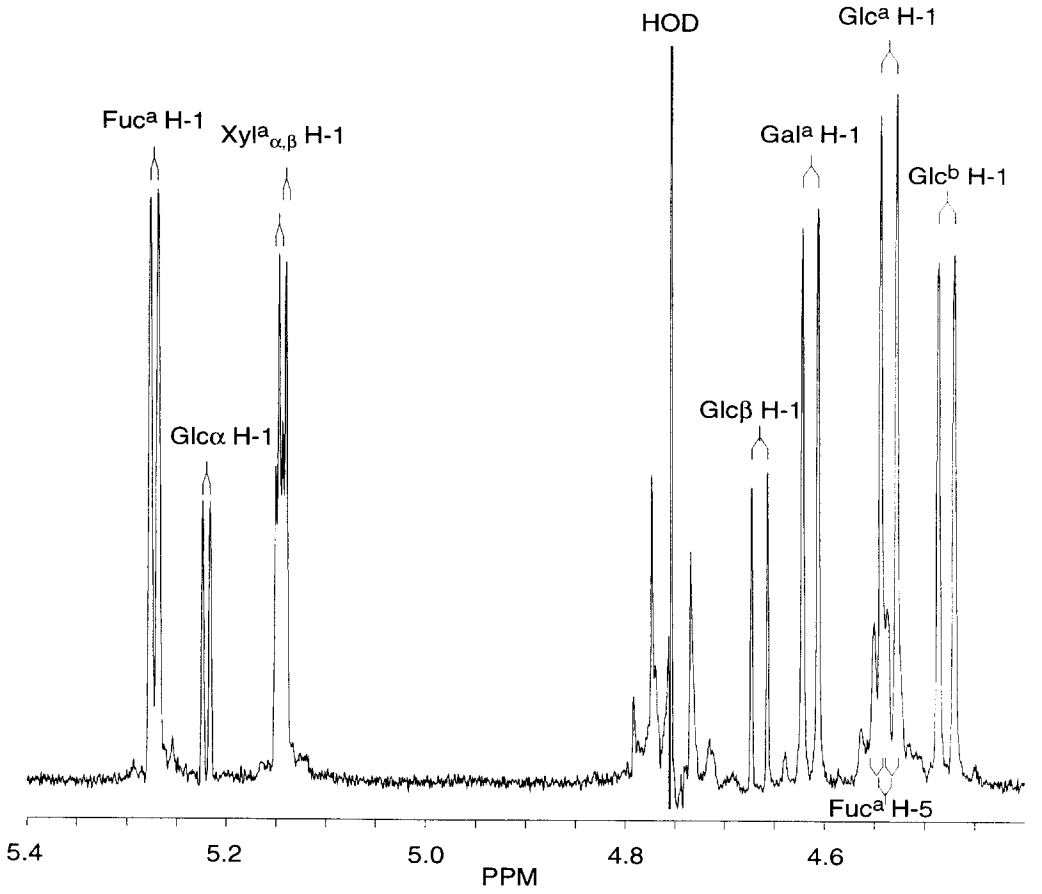

Fig. 2. Resolution-enhanced 500-MHz ^{1}H NMR spectrum of GFG.

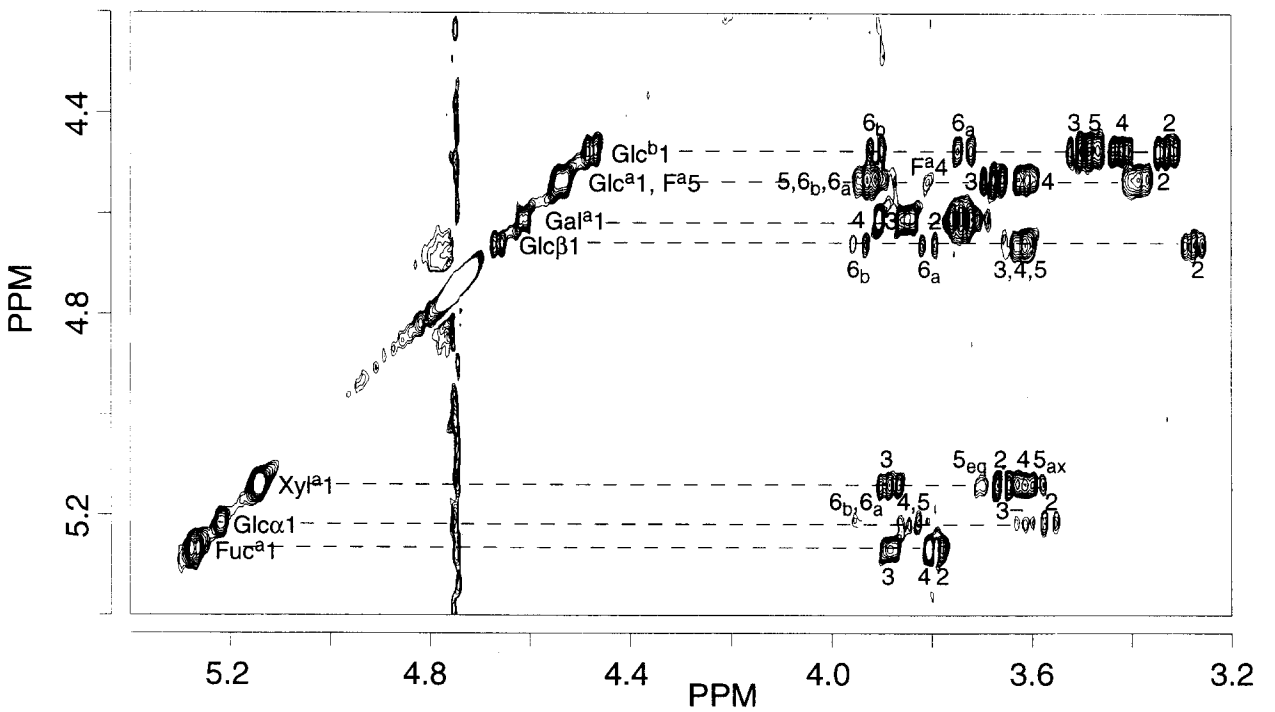

Fig. 3. Partial 500-MHz 2D HOHAHA spectrum (δ 3.2–5.4) of GFG. The diagonal peaks of the protons in the anomeric region are indicated. The numbers near cross-peaks refer to the protons of the scalar coupling network belonging to a diagonal peak.

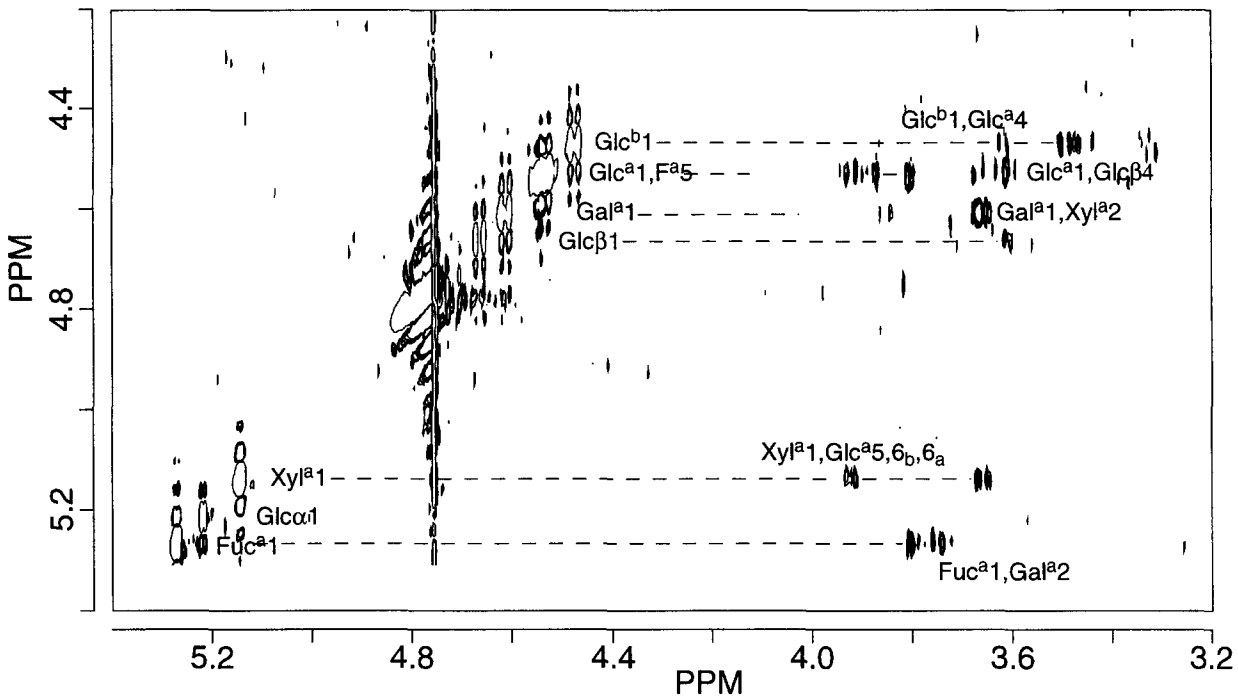

Fig. 4. 500-MHz 2D ROESY spectrum (δ 3.2–5.4) of GFG. Solely the interresidual ROE connectivities along the H-1 tracks are denoted. Fuc^{a}1, Gal^{a}2 means a ROE cross-peak between Fuc^a H-1 and Gal^a H-2.

The sequence of these Glc residues (Glc^a H-1, δ 4.537; Glc^b H-1, δ 4.479) has been identified on the basis of cross-peaks observed in the ROESY spectrum (Fig. 4). Other evidence for the assignment of Glc^b can be found in the fact that the chemical shift values of the ring protons are in agreement with those of the Glc unit at the 'non-reducing' end of methyl β-cellobioside [20]. The ^{1}H NMR data are consistent with the component in fraction **6.1** corresponding to GFG.

β-D-Glc*p*-(1→4)-β-D-Glc*p*-(1→4)-D-Glc*p*
|
α-L-Fuc*p*-(1→2)-β-D-Gal*p*-(1→2)-α-D-Xyl*p*-(1→6)

(6.1)

In order to demonstrate the position of the extended side chain at Glc^a, fraction **6.1** was also examined using positive-ion mode FABMS after peracetylation. An $[M + H]^+$ pseudomolecular ion can be identified at *m/z* 1701, corresponding to the fully acetylated hexasaccharide. Abundant oxonium ions are present at *m/z* 273 (Fuc$^+$), 331 (Glc$^+$), 561 (Fuc-Gal$^+$), 777 (Fuc-Gal-Xyl$^+$), 1353 (GF$^+$), and 1641 (GFG$^+$), defining the branching pattern of the hexasaccharide, in which the extended side chain is attached to the internal Glc residue in the backbone. CID tandem MS analysis of the first oxonium ion at *m/z* 1641 yields the same abundant oxonium fragments (see Fig. 5A) and confirms the assignment.

Finally, fraction **6.1** was treated with exoI, and the products formed co-eluted with Glc and FG on CarboPac PA-100. Upon reduction of fraction **6.1** and subsequent degradation by exoI, Glc, but no glucitol was detected in the reaction mixture. These data are consistent with the results that the Glc residue containing the fucosylated side chain is flanked on both sides by an unbranched Glc residue.

Table 3
^{1}H NMR H-1 chemical shifts[a] for GFG (fraction **6.1**) and GFGGFG (fraction **12.1**)

Residue	GFG	GFGGFG
Glcβ	4.666	4.666
Glcα	5.221	5.221
Glc^a	4.537	4.536
Glc^b	4.479	4.536
Glc^c		4.536
Glc^d		4.536
Glc^e		4.475
Xyl^{a}β	5.144	5.144
Xyl^{a}α	5.148	
Xyl^d		5.144
Gal^a	4.615	4.615
Gal^d		4.615
Fuc^a	5.273	5.273
Fuc^d		5.273

For the coding system of the monosaccharides, see Section 2. α and β stand for the anomeric configuration of Glc.
[a]In ppm relative to the signal of internal acetone at δ 2.225.

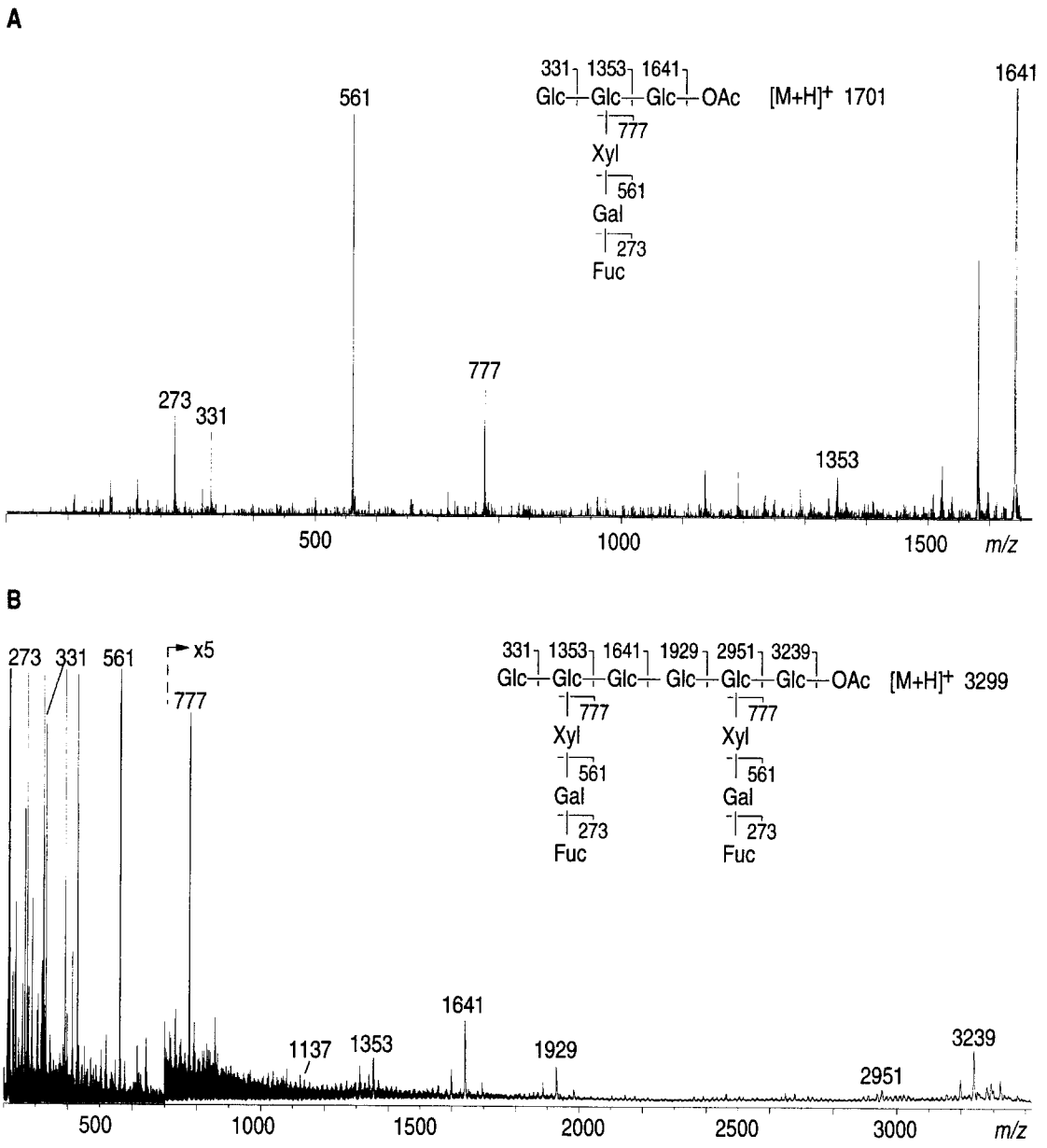

Fig. 5. CID mass spectrum of the oxonium ion at *m/z* 1641 of peracetylated fraction **6.1**, with fragmentation scheme (A); FAB mass spectrum of peracetylated fraction **12.1**, with fragmentation scheme (B).

Fraction **12.1**.—Comparing the ^{1}H NMR spectrum of fraction **12.1** with that of GFG (fraction **6.1**) shows that the two spectra are very similar except for the different peak intensity ratios (Fig. 6). The resonances of the anomeric signals are summarized in Table 3. The 2D NMR spectra of fraction **12.1** are also very similar to those of GFG.

The positive ion-mode FAB mass spectrum of the peracetylated derivative contains a low abundance signal corresponding to the $[M + H]^+$ pseudomolecular ion at *m/z* 3299, consistent with a composition of $Hex_8Deoxyhex_2Pent_2$ (Fig. 5B). The low-mass region in the spectrum is very similar to that of GFG (Fig. 5A). Additionally, signals corresponding to oxonium ions are present at *m/z* 1929 ($GFGG^+$), 2951 ($GFGGF^+$), and 3239 ($GFGGFG^+$), defining the branching pattern (Fig. 5B). No signals are present to suggest a difference in structure between the two side chains, or to indicate minor components with differences in the branching pattern. It can thus be concluded that the structure of the compound present in fraction **12.1** is GFGGFG, a dimer of the structure in fraction **6.1**. This is the first observation of two neighbouring internal unbranched Glc residues to be present in a fucosylated xyloglucan oligosaccharide produced by endoIV digestion. To determine whether GFGGFG is endoIV-resistant, this oligosaccharide was re-incubated with endoIV. Analysis of the digest

on CarboPac PA-100 showed that GFGGFG was completely degraded to GFG. From this it was concluded that GFGGFG is not endoIV-resistant, but that cleavage of GFG↓GFG is more difficult for endoIV than that of, for instance, XXFG↓XXXG.

α-L-Fuc*p*-(1→2)-β-D-Gal*p*-(1→2)-α-D-Xyl*p*-(1→6)
|
β-D-Glc*p*-(1→4)-β-D-Glc*p*-(1→4)-β-D-Glc*p*-(1→4)-β-D-Glc*p*-(1→4)-β-D-Glc*p*-(1→4)-D-Glc*p* (12.1)
|
α-L-Fuc*p*-(1→2)-β-D-Gal*p*-(1→2)-α-D-Xyl*p*-(1→6)

4. Discussion

Apple fruit xyloglucan is predominantly built up from XXXG, XXFG and XLFG, but oligosaccharides with a less heavily branched glucan backbone can also occur [8,9]. In this investigation two such oligosaccharides were identified using MS and ^{1}H NMR analyses. Their structures were shown unambiguously

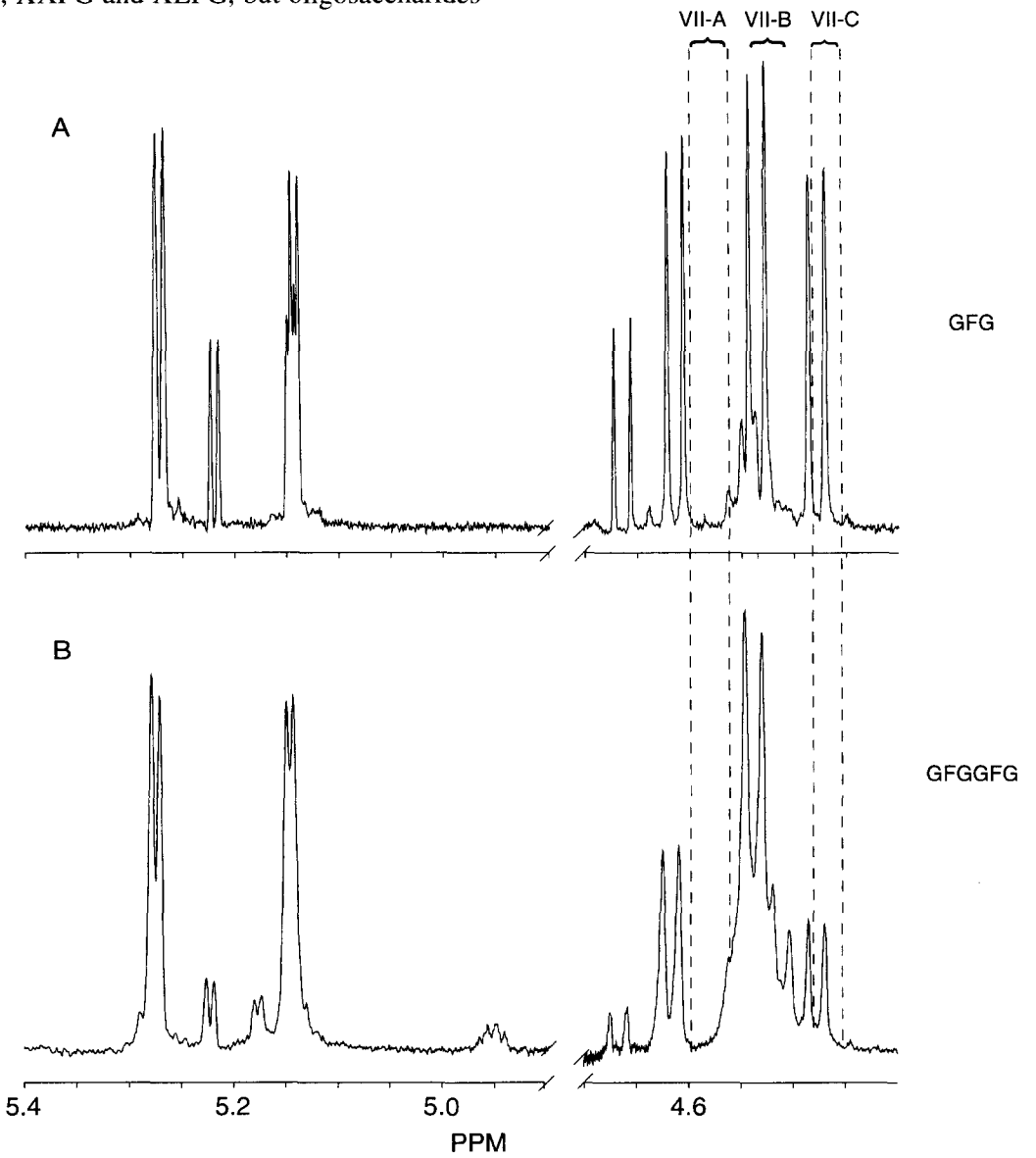

Fig. 6. Partial 500-MHz ^{1}H NMR spectra (δ 4.4–5.4) of (A) GFG and (B) GFGGFG. The subclasses of region VII are diagnostic for different structural features of the xyloglucans, as described in the text. The chemical shift range corresponds to the centre of the multiplet, and does not necessarily include all of the lines in the multiplet.

to correspond to GFG (recently also described in [21]) and GFGGFG.

Mass spectrometry is excellently suited for identifying xyloglucan oligosaccharides because of its sensitivity and applicability to the analysis of mixtures. Since the monosaccharide composition and the linkage types for xyloglucan oligosaccharides are well-established, determination of the length and heterogeneity as well as the distribution of side chains along the backbone is generally the most important information required. This information is most sensitively obtained using MS of peracetylated species, when the branching pattern and heterogeneity may be readily defined, even for fractions containing mixtures of components.

The ^{1}H NMR spectroscopic data of the free (novel) xyloglucan oligosaccharides provide an extension to the databank of structural-reporter-groups, established so far for xyloglucan oligosaccharide-alditols [3,15–17]. The present data show that the anomeric signals of non-reducing terminal unbranched Glc residues, flanked by a Glc residue containing a fucosylated side chain appear in a very narrow region. Two examples of such residues are included in this study, viz. Glc^b of GFG and Glc^e of GFGGFG with chemical shift values of δ 4.479 and 4.475, respectively. In the literature a chemical shift value of δ 4.483 for Glc^b of GFGol has been reported [21]. Apparently, the distance from the reducing end of the carbohydrate chain (compare GFG, GFGGFG, and GFGol) does not affect the chemical shift values of these Glc residues much.

Previously, three regions (VI, VII-A, and VII-B) were distinguished (Fig. 6), which encompass all chemical shift values of different Glc residues present in the backbone of xyloglucan molecules [15]. Region VI (δ 4.600–4.660) includes H-1 signals of 4,6-linked β-Glc attached to C-4 of Glc-ol (e.g., XXFG), and H-1 signals of 2,4,6-linked β-Glc (e.g., XXFGAXXG) (A = α-L-Ara*f*-(1 → 2)-[α-D-Xyl*p*-(1 → 6)]-β-D-Glc*p*–. Region VII-A (δ 4.560–4.600) includes H-1 signals of 4,6-linked β-Glc^b when no α-Fuc-(1 → 2)-β-Gal-(1 → 2)-moiety is present on Xyl^a (e.g., XXFGAXXG). Region VII-B (δ 4.480–4.560) represents H-1 signals of 4-linked, 6-linked, and 4,6-linked β-Glc not assigned in regions VI or VII-A (e.g., XXFGAXFG). Based on our results and a recent report from others [21], the structural-reporter-group regions for xyloglucans can be extended with a new region on the upfield side of region VII-B. This new region VII-C (δ 4.475–4.485) includes the H-1 signals of non-reducing terminal unbranched Glc residues that are flanked by an F element (e.g., GFG). Our results show further that next to the H-1 signal of a single internal unbranched Glc residue (e.g., XXFGAXFG), the H-1 signals of two vicinal internal unbranched Glc residues (GFGGFG) are included in region VII-B.

It is an intriguing question as to whether structures such as GFG and GFGGFG are synthesized as such or originate from post-depositional modifications in the cell wall. Unlike cellulose, xyloglucans are assembled entirely in the plant cell before being transported to, and secreted into the cell wall. The biosynthesis of xyloglucan involves an alternate transfer of Glc and Xyl to a nascent xyloglucan acceptor by xyloglucan 4-β-D-glucosyltransferase and a 6-α-D-xylosyltransferase, respectively [22–24]. This chain elongation, which is confined to the Golgi cisternae [25,26], depends on the presence of neither Gal nor Fuc [27]. Decoration of the xyloglucan (with Gal, Fuc) occurs predominantly in the *trans* Golgi network or dictyosomes [25,26]. The UDP-Xyl concentration plays an important role in the branching pattern of the glucan backbone. However, the exact mechanism for leaving unsubstituted Glc residues is still a matter of speculation. The structure of xyloglucan suggests that these residues are introduced at regular intervals [28]. In apple fruit xyloglucan every fourth Glc residue in the backbone remains unbranched (XXXG-like structural elements).

Structural deviations from the basic apple fruit xyloglucan structure may be a result of the action of endogenous glycosidases. A complete set of xyloglucan-modifying enzymes has been described in the literature, including β-glucosidase [29], α-xylosidase [30,31], β-galactosidase [32] and α-fucosidase [33]. It is expected that similar enzymes are also present in apple, and these could be responsible for generating XG, FG, XXG, and GFG building blocks at the non-reducing end of a xyloglucan molecule. For instance, the GFG block may be formed from XXFG by the consecutive action of α-xylosidase, β-glucosidase, and α-xylosidase (XXFG → GXFG → XFG → GFG). The occurrence of GFGGFG is more difficult to explain because no α-xylosidases catalyzing a GFGXFG → GFGGFG conversion have been reported to date.

Xyloglucan endotransglycosylase (XET) [34] cleaves a xyloglucan molecule (donor), and transfers the non-reducing portion of the donor molecule to the non-reducing end of an acceptor molecule. Thus, in principle, XET action could be responsible for the eventual presence of XG, FG, XXG, and GFG in a

mid-chain position, if these oligosaccharides are originally present as the non-reducing building units of the acceptor molecule. However, not all oligosaccharides can be incorporated in this way. It appears that the acceptor molecule should meet certain requirements; two vicinal X elements (e.g., as in XXG) are required for XET action [35], so that FG, GXG, and XGG are not suitable as acceptor substrates. So far GFG has not been tested although it is expected that XET will not recognize this oligosaccharide as an acceptor, since it lacks vicinal X elements at the non-reducing end. However, it has been shown that XET can utilize GXXGXXXG as an acceptor substrate [36]. This suggests that the requirement for Xyl substitution of the non-reducing Glc residue is not as critical for larger acceptor molecules (e.g., GXXGXXXG) as it is for smaller ones (e.g., XXG). Therefore, a role for XET in forming GFGGFG can not be excluded. With the current state of knowledge of biosynthesis and enzymatic modification of xyloglucans the adequate explanation for the occurrence of GFGGFG in apple fruit xyloglucan requires further study.

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