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Structural analysis of cyclamen seed xyloglucan oligosaccharides using cellulase digestion and spectroscopic methods

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Abstract

Xyloglucan polymers have been isolated from cyclamen seeds and characterized by both liquid and CP-MAS ^{13}C NMR spectroscopy. Treatment of the polysaccharides with cellulase from *Trichoderma viride* afforded XG oligomers which have been studied with both mass spectrometry and NMR spectroscopy. The repeating unit in the intact polymers and the most abundant hydrolysis product correspond to the Gal_1 Xyl_3 Glc_4 (XXLG) fragment. However, detection of notable amounts of Xyl_3 Glc_4 (XXXG) and Gal_2 Xyl_3 Glc_4 (XLLG) indicates that the galactose distribution in xyloglucan from cyclamen is irregular. FAB-MS analysis of a new derivative, prepared by forming the glycosamine of *m*-tetrafluoroethoxy aniline, has led to unambiguous sequence information for the XXLG oligomer.

Keywords: Xyloglucan; Cellulase; CP-MAS ^{13}C NMR

1. Introduction

Xyloglucan is a widely distributed cell wall polymer [1,2], either as a structural component of dicot cell walls or as storage products in some seeds. In most cases, the

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polymer is reported to contain a cellulosic-type (1-4)-β-D-glucan backbone with sidechains of α-D-xylopyranose and β-D-galactopyranosyl-(1-2)-α-D-xylopyranose (1-6)-linked to the backbone. Several minor sugars have been identified in xyloglucan from various sources. Arabinose has been detected in the xyloglucans isolated from *Nicotiana tabacum* [3] leaves, tamarind seeds [4], and suspension-cultured sycamore cells [5]. In the second species, it has been suggested that this sugar may be a constituent of contaminating pectic material whereas in the latter case, arabinose is α(1-2)-linked to the xyloglucan. No evidence for the presence of minor sugars has been reported in the case of nasturtium seeds [6]. Fucose, another minor constituent of xyloglucans, has been shown to be α(1-2)-linked to the galactose residue in the case of suspension-cultured *Rosa* [7] and sycamore cells [8] but was never detected in seed xyloglucan.

Xyloglucans, which are considered to be major load-bearing structures in primary plant cell walls by virtue of their potential to crosslink cellulose fibers, can also be thought of as a reservoir of biological signals. Indeed, specific oligosaccharides released from xyloglucan by partial digestion with cellulase are known to exert "signalling" effects on plant tissues [9]. Since every fourth glycosyl residue in the xyloglucan backbone is unbranched, the major oligosaccharide products obtained by enzymatic digestion contain a cellotetraose backbone, the three non-reducing β-D-glucosyl residues being substituted at C-6 with glycosyl sidechains. Two different effects have been reported [10]. On the one hand, xyloglucan fragments containing a terminal fucosyl residue inhibit auxin-stimulated growth whereas some fragments lacking fucose can promote growth and can activate cellulase activity. In all cases the bioassays have been conducted over long periods (18 h).

In order to investigate the early growth effects we were interested in obtaining xyloglucan fragments lacking fucose residues. It is known [11] that xyloglucans are the major storage polysaccharides of seeds such as Tropeolum, tamarind and cyclamen. Although tamarind xyloglucan, which has been thoroughly investigated, does not contain a fucose unit, the primary structure of the other species has not been established. Thus, we set out to investigate the storage polysaccharide from cyclamen seeds, in order to establish its sequence. The major goal of this study was to identify the corresponding oligosaccharide products obtained by enzymic hydrolysis.

2. Results and discussion

Structural analysis of native xyloglucan.—Three fractions, X1 (hot water soluble and copper salt precipitated [12]), X1′ (hot water soluble and aqueous copper salt soluble) and X2 (alkali soluble [13]), were obtained from the seeds of cyclamen as outlined in the Experimental section. Gel permeation chromatography (Sephacryl S 200) of all the fractions gave single peaks that were eluted in the exclusion volume indicating high molecular weight. Acid hydrolysis of these polymers followed by HPLC analysis of the products led to Gal/Glu/Xyl ratios close to that of the untreated flour, 1/3.8/3.5, with the exception of X2 (1/2.9/1.6). Small amounts of rhamnose and arabinose were also detected. As X1 and X1′ could not be distinguished on the basis of either ^{1}H or ^{13}C spectra, it would appear that the copper-salt treatment reported in the extraction

procedure for xyloglucan from tamarind [13] was superfluous in the case of cyclamen xyloglucan. These two fractions represented the bulk (75%) of the isolated polymer and only the NMR data of X1 are described below.

The 100 MHz ^{13}C spectrum of X1 which was recorded at 70°C is given in Fig. 1a and the corresponding data are collected in Table 1. The anomeric region contains three signals at 105.8, 103.5, and 100.2 ppm which could be attributed to the β-galactose, β-glucose, and α-xylose residues, respectively, by comparison with the corresponding data for methyl glycosides [14]. The attribution of the other carbons is based on results obtained for the oligomers (vide infra). Weak signals at 18.52 ppm were tentatively attributed to the methyl group of rhamnose based on the results of chemical analysis.

Quantitative analysis of the "visible" proportion of the xyloglucan polysaccharides with inverse-gated spectra in the presence of an internal standard (Me-α-glucoside) showed that roughly 60–65% of the polymers were sufficiently mobile to be detected by

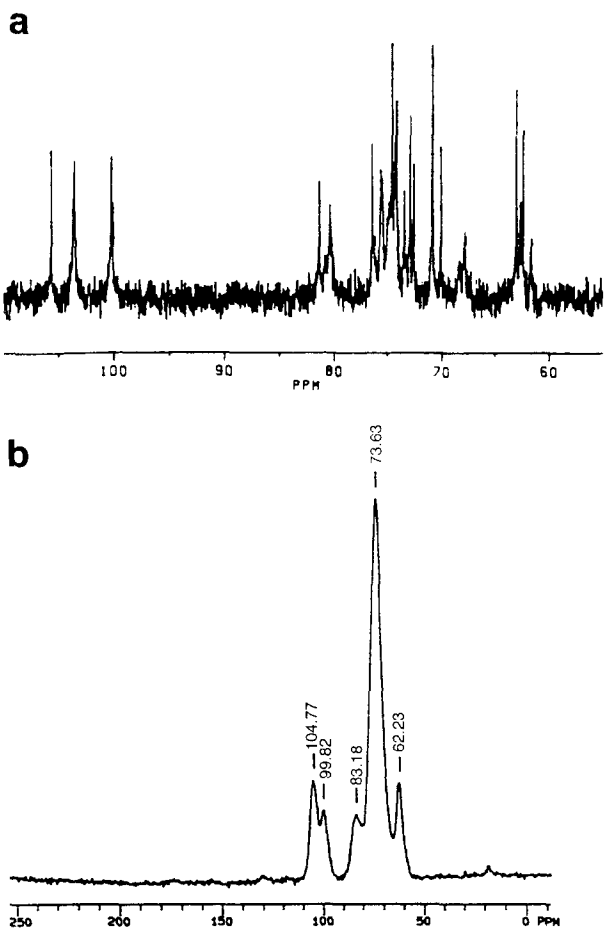

Fig. 1. ^{13}C 100 MHz (a) and 75 MHz CP-MAS (b) spectra of xyloglucan from cyclamen seeds.

Table 1
100 MHz high-resolution [a] and 75.43 MHz CP-MAS ^{13}C spectra of xyloglucan from cyclamen

Carbon	100 MHz	CP MAS
1 Gal*p*	105.81	104.77
1 Glc*p*	103.52	to
1 Xyl	100.20	99.82
2 Xyl*b*	81.33	83.18
4 Glc*p*	79.48–	
	80.79	
5 Gal*p*	76.36	
5 Glc*p*	75.40–	
3 Glc*p*	75.76	
3 Xyl*t*	74.53	
3 Gal*p*		
2 Glc*p*	74.13	
3 Xyl*b*	73.40	73.63
2 Xyl*t*	72.88	
2 Gal*p*	72.51	
4 Xyl	70.82	
4 Gal*p*	69.98	
6 Glc*b*	67.77	
5 Xyl*t*	62.95	
5 Xyl*b*	62.57	62.23
6 Gal*p*	62.33	
6 Glc	61.57	
6 Rha*p*	very weak	

[a] Assignments were made by comparison with the signals of the oligosaccharides obtained by enzymic hydrolysis. Key: Glc*b*, 6-substituted (1-4)-linked glucose, Xyl*t* = terminal xylose, Xyl*b* = branched xylose. In D_2O at 70°C, $\delta_{DMSO} = 39.5$ ppm.

liquid-state NMR. This indicated that the NMR data were representative of the polymer as a whole and precluded the coexistence of extensive ordered regions (gel formation). The η_{Gal}, η_{Xyl} and η_{Glc} values of 1.3, 1.2 and 0.8, respectively, which were determined for X1, reflect the decreasing mobility of the various residues in these visible regions of the polymer. This local mobility was compatible with a glucosyl backbone substituted by xylose sidechains occasionally bearing terminal galactose residues.

The CP-MAS spectrum, Fig. 1b, is similar to that in the liquid-state except that the β-glucosyl C-4 resonances (82–85 ppm) are displaced a few ppm downfield and broadened in the solid state. This indicates that the glucan chain adopts a 2_1 helical conformation like cellulose in the solid state, as would be expected [15,16], but the degree of order is not high. The spectrum for the seed flour (not shown) is very similar to the isolated xyloglucan sample X1, which suggests that the solid-state conformations in vivo in the seed and in vitro are similar. An additional weak signal at 18.51 ppm is compatible with the presence of a 6-deoxy sugar.

The spectral dispersion of the proton signals of these polymers was optimum at 70°C. In the anomeric region, the resonances at 4.59, 4.97, and 5.16 ppm could be assigned to the β-glycosyl (β-glucose and β-galactose) residues, terminal α-xylose sugars (Xyl*t*), and branched α-xylose (Xyl*b*) units respectively by analogy with the assignments

Table 2
400 MHz ^{1}H NMR chemical shift data [a] of a 2% w/v solution of the hot water-soluble copper salt-precipitated polysaccharide, X1, in D_2O at 70°C

Residue	H-1	H-2	H-3	H-4	H-5	H-6
β-Glc*p*	4.59	3.44	3.72	3.71 [b]	3.85 [b]	3.94, 4.02
α-Xyl*t*	4.97	3.57	3.76	n.a	n.a	
α-Xyl*b*	5.16	3.68	3.94	n.a	n.a	
β-Gal*p*	4.60	3.64	3.67 [b]	3.97 [b]	3.70 [c]	3.81 [c], n.a

[a] Xyl*t* = terminal xylose, Xyl*b* = branched xylose; n.a indicates a resonance that is not assigned. [b] From NOESY spectrum. [c] From the assignments of the oligosaccharides.

obtained for the enzymic hydrolysis products [17]. The relative intensities of these signals (β-Glycosyl/Xyl*t*/Xyl*b*, 5.3/2.2/1) corroborate the ^{13}C data. A signal at 5.35 ppm which corresponds to the chemical shift of α-L-Ara*f* (which was detected in small amounts by chemical analysis) and certain high-field peaks are observed. The latter resonances may stem from the small amount of protein shown to be present from chemical analysis [18]. The resonance at 1.35 ppm can be tentatively attributed to Me-6 of a rhamnose residue again based on the results of chemical analysis.

NOESY spectra of X1 were acquired for mixing times of 0.1 and 0.5 s (Fig. 2). Weak direct interresidue effects (H-1Glc*p*/H-4Glc*p*, H-1Xyl*p*/H-6Glc*p*) were observed with the shorter mixing time while both strong direct interresidue cross-peaks (**A** H-1Glc*p*/H-4Glc*p*, **B** H-1Xyl*t*/H-6Glc*p* and **C** H-1Xyl*b*/H-6Glc*p*) and strong indirect interresidue effects (for example **E** H-1Xyl*p*/H-5Glc*p* via H-6Glc*p* and **F** H-1Xyl*t*/H-1Glc*p* via H-6Glc*p* and H-5Glc*p*) are detected for the longer mixing time. The majority of the ^{1}H resonances in the spectrum of X1 could be assigned based on both the phase-sensitive NOESY and DQFCOSY[δ-δ, ^{1}H-^{1}H] experiments and these data are collected in Table 2. Both ^{1}H and ^{13}C data suggest that the average octasaccharide repeating unit is Gal_1 Xyl_3 Glu_4.

Structural analysis of XG oligosaccharides.—Cyclamen xyloglucan was digested with cellulase from *Trichoderma viride* and then separated into five successive fractions, F1 (bed volume of the column, 4%), F2 (19%) F3 (65%), F4 (10%) and F5 (2%, a mixture of disaccharides) by Bio-Gel P-2 chromatography [19]. The two major fractions, F2 and F3, were subsequently chromatographed a second time on Bio-Gel P2 and afforded three fractions each (F2-1, 31%; F2-2, 17%; F2-3, 52%; F3-1, 31%; F3-2, 40% and F3-3, 29%) (HPLC). The retention times on the HPLC column (which was previously calibrated with dextran oligosaccharides) suggested that the degree of polymerisation (DP) of the oligosaccharides was between 7 and 8 (Fig. 3) with the exception of F2-1 which was expected to contain longer XG oligomers.

The results of analysis of F2-1, F2-3, F3-1 and F3-2 by matrix-assisted laser desorption/ionization mass spectrometry [20] (MALDI-MS) are collected in Table 3. It is possible to distinguish oligosaccharides with the same DP but different proportions of hexoses and pentoses (i.e. XXXG and GXLG of Fig. 4a). As expected from separation by gel permeation chromatography, the larger oligomers exhibit the shorter retention

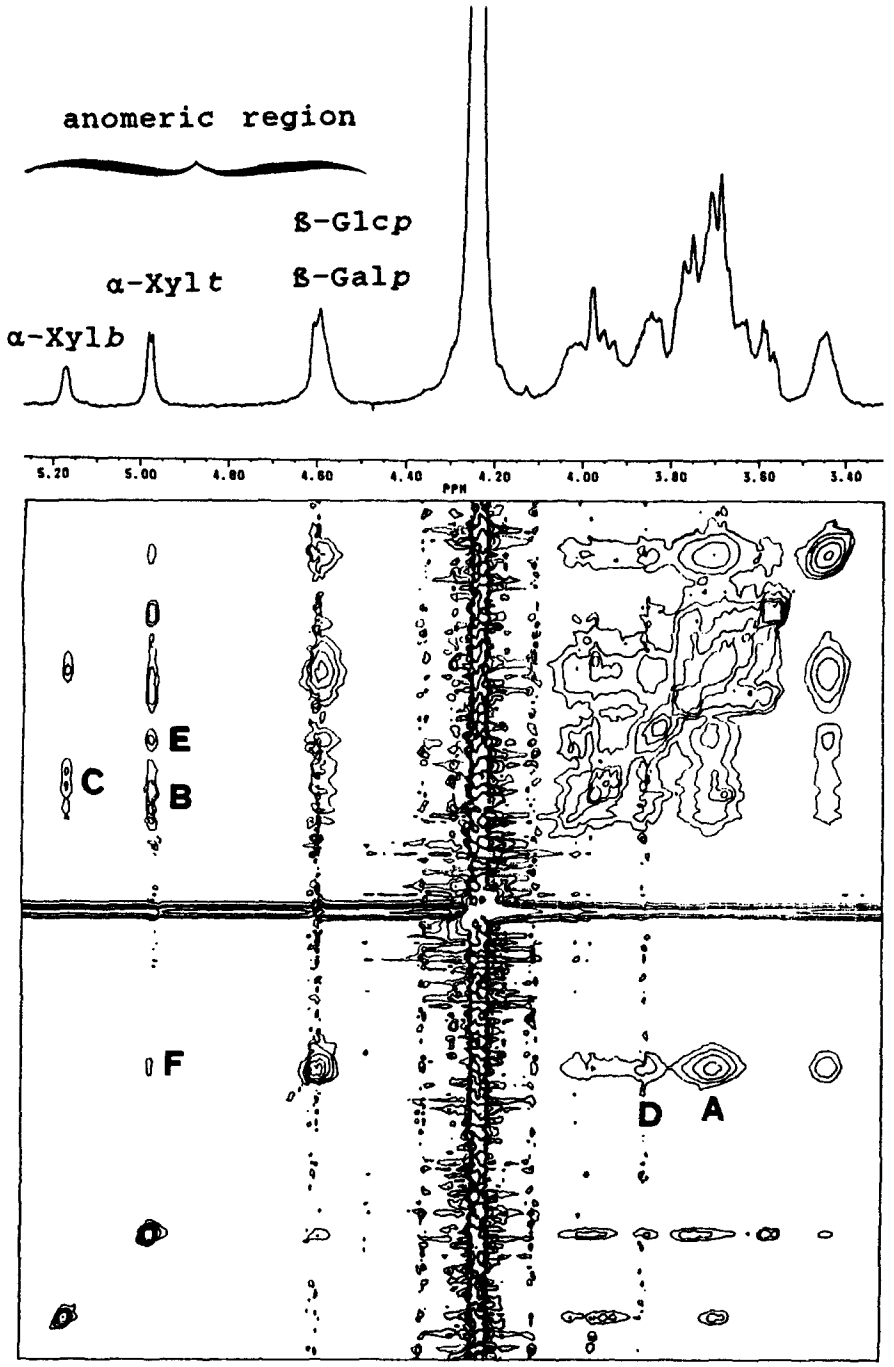

Fig. 2. 400 MHz NOESY spectrum of a 2% w/v solution of the hot water-soluble copper salt-precipitated xyloglucan, X1, in D_2O at 70°C acquired with a mixing time of 0.5 s and the corresponding 1D spectrum. The following cross-peaks are labelled: (a) Direct effects. Interresidue — **A** H1-Glc*p*/H4-Glc*p*, **B** H1-Xyl*t*/H6-Glc*p*, **C** H1-Xyl*b*/H6-Glc*p* and Intraresidue — **D** H1-Glc*p*/H5-Glc*p*. (b) Indirect effects. Interresidue — **E** H1-Xyl*p*/H5-Glc*p* via H6-Glc*p* and **F** H1-Xyl*t*/H1-Glc*p* via H6-Glc*p* and H5-Glc*p*.

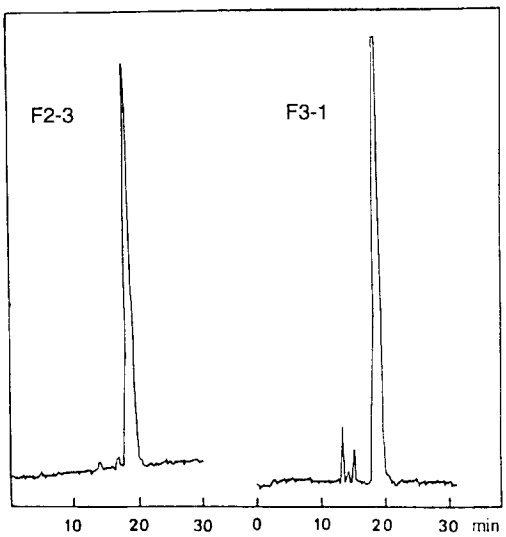

Fig. 3. HPLC chromatography of fractions F2-3 and F3-1.

times. It has not been established previously that the peak intensity in MALDI spectra of oligosaccharides corresponds to actual abundance in the sample (unlike for peptides where this clearly is not the case), but the relative abundance of the oligosaccharide species in the spectra did correlate with the abundance estimates based on the NMR data (see below). Whether this observation is general for carbohydrates or not will require further investigation. Thus, based on signal intensity in the MALDI spectra, the major component of F2-1 was nonasaccharide (56%) whereas that of F3-1 was octasaccharide (71%). In contrast, F3-2 contained almost equal amounts of heptamers (45%) and octamers (40%). Globally, an octasaccharide was the most abundant product in agreement with the repeating unit deduced from NMR analysis of the intact polysaccharide. Incomplete separation can be attributed to the fact that enzymic hydrolysis afforded an almost equimolar mixture of heptamers and octamers.

As in the case of the xyloglucan polymers, the relative amounts of the different types of residues in these fractions can be determined by integrating the anomeric region of the proton NMR spectra which contains five distinct signals. Weak signals for the anomeric protons of the α and β reducing residues (in a 1/2 ratio), which are absent in the spectra of the native polysaccharides, are detected at 5.27 and 4.69 ppm, respectively. A doublet at 5.17 and a pair of doublets at 4.97 ppm with small coupling constants characteristic of the anomeric signals of protons of residues with the α orientation are assigned to branched and terminal xylose, respectively, on the basis of chemical shift values [17,21]. Finally, a broad resonance at 4.60 ppm in the anomeric region of β-glycosyl units is observed for the non-reducing glucose and the galactose residues. The intensities of these anomeric signals relative to the total integrated intensity of the reducing resonances is given in Table 4. The corresponding values from the MALDI-MS spectra are also indicated (values in parentheses). Although the

Table 3
Percentage of oligomers [a] detected in the MALDI-MS spectra of F2 [b] and F3 [c]

Fraction	DP7		DP8	DP9
	XXXG	GXLG [d]	XXLG [d]	XLLG [d] GXXLG [d]
F2-1			35.3	56.3
F2-3	29.6	3.9	58.7	7.8
F3-1	15.9	3.4	70.9	9.8
F3-2	44.7	7.3	40.3	

[a] The structures corresponding to the oligomers are given in Fig. 4a. [b] Weak amount (< 9%) of larger oligomers (DP10) were detected in F2-1. [c] Weak amount (< 7.7%) of another DP8 was detected in F3-2. [d] The position of the L fragment was determined by FAB-MS.

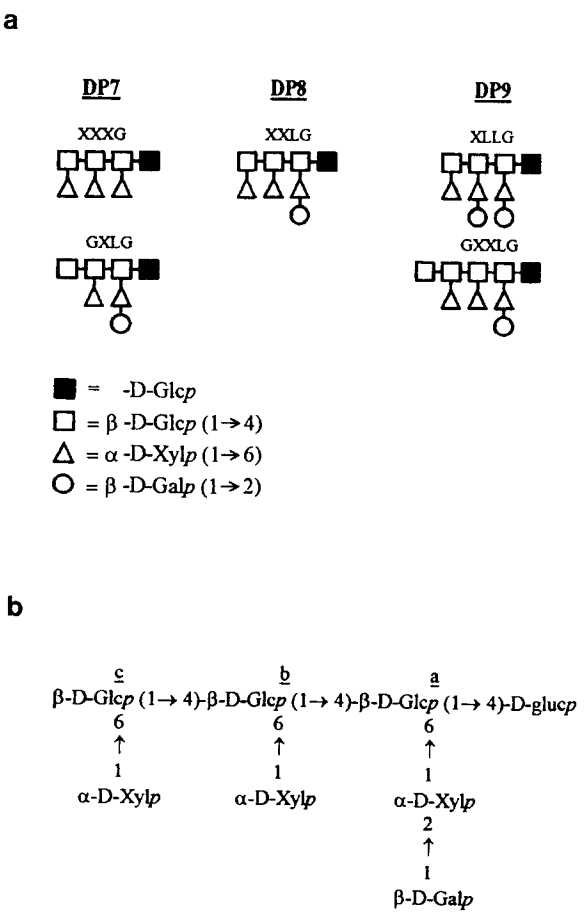

Fig. 4. Molecular formulae of the xyloglucan oligomers from cyclamen: (a) proposed on the basis of MALDI data and (b) determined for XXLG from FAB-MS analysis (labels for the residues of XXLG are also given).

Table 4
Estimation of the proportion of residue types in F2 and F3 from ^{1}H NMR of the anomeric region (MALDI-MS)

Fraction	Glc *p*-OH	α-Xyl *b*	α-Xyl *t*	β-Glc *p*, β-Gal *p*
F1	1	3.7	3	10.0
F2	1	1.5	2.3	5.0
F2-3 [b]	1	1.4	2.2	4.3
(XLLG)	(1)	(0.8)	(2.2)	(3.8)
(GXXLG)	(1)	(0.7)	(2.3)	(3.8)
F3 [a]	1	0.9	2.4	3.7
F3-1	1	1.3	2.4	4.6
(XLLG)	(1)	(0.9)	(2.0)	(3.9)
(GXXLG)	(1)	(0.8)	(2.1)	(3.9)
F3-2	1	0.9	2.4	3.8
	(1)	(0.6)	(2.2)	(3.6)
F3-3	1	0.6	2.0	2.6

[a] A 1/1/1 ratio of F3-1, F3-2 and F3-3 leads to a similar proportion of residue types as for F3.
[b] Proportion evaluated from the MALDI-MS experiments with both possible structures XLLG and GXXLG for DP9.

intensities of the ^{1}H NMR signals for the branched xylose residues are systematically too strong, suggesting the presence of some contaminating material, on the whole, the two analytical methods are in good agreement.

An unexplained transformation of the multiplets corresponding to the anomeric protons of the glucose residues was observed during this study. Upon dissolving the samples in D_2O and recording a spectrum at 296 K, two well-resolved doublets were observed for the α and β reducing anomeric protons as shown for the xyloglucan fraction (Fig. 5a). However, after performing measurements at 343 K (again the spectral dispersion was optimal at this temperature), these doublets were gradually transformed into multiple signals (Fig. 5b). The transformation was irreversible, and affected only the

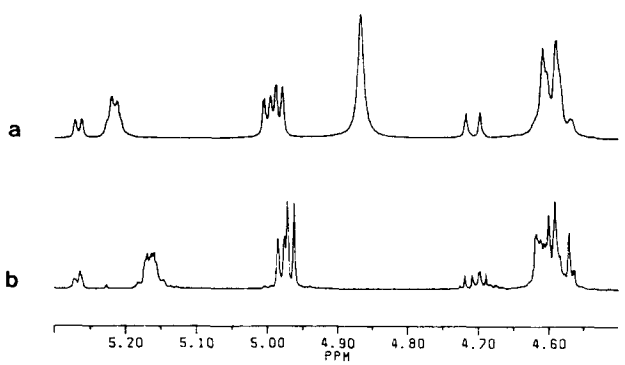

Fig. 5. Anomeric region of the ^{1}H spectra of F2 (a) immediately after sample preparation at 296 K and (b) several weeks later after several prolonged measurements at 343 K.

Table 5
400 MHz ^{1}H NMR chemical shift data [a] of a solution of F3 in D_2O at 70°C

	H1	H2	H3	H4	H5	H5′	H6	H6′
α-Glc*p*	5.27	3.63	3.91					
	(3.9)							
β-Glc*p*	4.69	3.35	3.68	(3.52) [b]				
	(7.8)							
Glc [a]	4.60	3.45	3.71		3.85		3.99	
					(6.9, 2.3)		(−11.4)	
Glc [b]	4.60	3.45	3.71	n.a.	3.87 [c]		3.97 [c],	
					(5.7)		4.03 [c]	
Glc [c]	4.58	3.39	3.57	3.52	3.74		3.96,	
							3.87	
	(7.8)	**(9.3)**	(9.5)	(9.1)	(6.9, 2.3)		(−11.4)	
Xyl [a]	5.17	3.69	3.94		3.60 [d]			
	(3.9)							
Xyl [b]	4.97	3.58	3.77 [f]		3.61 [d]			
	(3.9)	(9.3)						
Xyl [c]	4.96	3.57	3.77 [e]					
	(3.9)	(9.3)						
Gal	4.61	3.67	3.69	3.98 [b]	3.71 [d]		3.81 −	
							3.86 [d]	
		(9.1)	(4.6)					

[a] δ in ppm ($J_{x,x+1}$ in Hz; boldface values were extracted from the 1D spectrum ±0.5 Hz/pt and the others from the COSYDQF spectrum ±2.3 Hz/pt). A superscript a, b, or c indicates the position of the residue in the oligomer. The order of the backbone residues is **Glc** [a]-**Glc** [b]-**Glc** [c]-**Glc***p*OH. Residues (e.g. **Xyl** [a]) in the side chain attached to **Glc** [a] are designated by a superscript "a", etc. H6 is the methylene proton with a large $J_{5,6}$ value and H6′ is the methylene proton with a small $J_{5,6'}$ value. n.a. indicates a resonance that is not assigned. [b] From relayed-COSY spectrum. [c] Assignments were based on the F1 spectrum in which correlations with both H6 and H5 are observed for H6′. The methylene proton assignments may be reversed. The assignments are also corroborated by comparison with the data for X1 (NOESY). [d] From heteronuclear COSY spectrum. [e] No correlation with C3 in the heteronuclear COSY spectrum.

signals of the reducing sugar (particularly the anomeric proton of the β anomer) while the relative integrated intensity of the multiple doublets was analogous to that of the original doublets. All the *m/z* analyses were conducted on samples with multiple signals for the reducing sugars.

The ^{1}H and ^{13}C NMR spectra of the various fractions are very similar (except as regards the relative intensities of certain signals, vide infra) and the corresponding data for the F3 sample are collected in Tables 5 and 6, respectively. The assignments were based on COSYDQF, relayed-COSY and heteronuclear COSY spectra. It is to be noted that the carbon signals of the Glc^a and Glc^b units (see Fig. 4b for the labelling) were attributed by comparison with literature values [17,21] and these assignments may be reversed. A slight upfield shift for the anomeric signal of the Xyl^c with respect to Xyl^b residue has been described [17,21] for related XG oligomers. This convention has been adopted in Tables 5 and 6. To the best of the authors' knowledge no unambiguous proof for these assignments has been given and the chemical shifts of Xyl^b and Xyl^c may be reversed.

Table 6
100 MHz ^{13}C NMR chemical shift data [a] of a solution of F3 in D_2O at 70°C

	C1	C2	C3	C4	C5	C6
α-Glc*p*	94.02					62.67
	(0.40)					
β-Glc*p*	98.01					62.67
	(0.60)					
Glc [ab]	104.36	75.04	76.38 [c]	81.67	75.43 [d]	68.69
	(0.43)	(0.47) [e]	(0.42)	(0.38)		(0.19)
Glc [bb]	104.53	75.04	76.38 [c]	81.47	75.43 [d]	69.11
	(0.33)	(0.47) [e]	(0.42)			
Glc [c]	104.72	75.34	78.01	72.06	76.64 [c]	68.79
	(0.41)		(0.48)	(0.42)	(0.43)	(0.23)
Xyl [a]	100.95	82.21	74.30	72.27	63.48 [g]	
	(0.41)	(0.36)		(0.75) [f]		
Xyl [b]	101.11	73.79	75.43 [c]	71.73	63.85 [b]	
	(0.64)	(0.69)	(0.74)	(0.75) [f]	(0.41)	
Xyl [c]	100.64	73.79	75.43 [c]	71.73	63.62 [b]	
	(0.74)	(0.69)	(0.74)	(0.75) [f]	(0.42)	
Gal	106.47	73.43	75.04 [h,g]	70.96	77.23 [h,g]	63.19
	(0.68)	(0.60)	(0.47) [e]	(0.52)	(0.56)	(0.51)

[a] The residue labels and the abbreviations are the same as in Table 5. δ in ppm (T_1 in s). [b] Assignment may be reversed. [c] From the carbon T_1 values. [d] Attribution is ambiguous as the heteronuclear COSY crosspeak was very weak. [e] Average value for C3 Gal and C2 **Glc** [b]. [f] Average value for the three C4 of **Xyl** [a,b,c]. [g] From the carbon spectrum of F1 in which this signal is enhanced. [h] The C3 and C5 resonances were assigned according to ref. [22].

The ^{13}C assignments were also confirmed by relaxation measurements. The carbon longitudinal relaxation times, T_1, of the XG oligomers, which are a sensitive probe of the local mobility of the various residues [23], are also collected in Table 6 (values in parentheses). The overall tumbling (τ_c) time of F3-2 at 70°C (evaluated from the average T_1 value of the glucose methine carbons) is indicated in the plot of T_1, versus the effective correlation time [24], τ_c, at 100 MHz (Fig. 6). The correlation time which leads to vanishingly small crosspeaks ($\omega\tau_c = 1.12$) in the corresponding homonuclear NOESY spectrum is also labelled. Indeed, such a τ_c value was corroborated by the paucity of crosspeaks in the NOESY spectrum of F2 acquired with a 0.5-s mixing time. The weak negative H1/H4-Glc crosspeaks (the only ones which were clearly visible) were compatible with a τ_c value just barely in the small-molecule region (left) of the curve.

The main-chain glucose residues are expected to have the most restricted internal motion which is corroborated by T_1 values of roughly 0.4 s for the corresponding methine carbons. In contrast, the Xyl*t* residues which are attached to the main chain through flexible 6-1 linkages present methine T_1 values of 0.7 s. The galactosyl carbone T_1 values are slightly lower (0.5–0.6 s) in keeping with the more restricted 1-2 linkage. Finally, the relaxation times of the methine carbons of the Xyl*b* residues are similar to those of glucose.

In the course of FABMS analysis of the oligomers several derivatives were tested which did not give satisfactory results (permethylation, transformation into amino acid

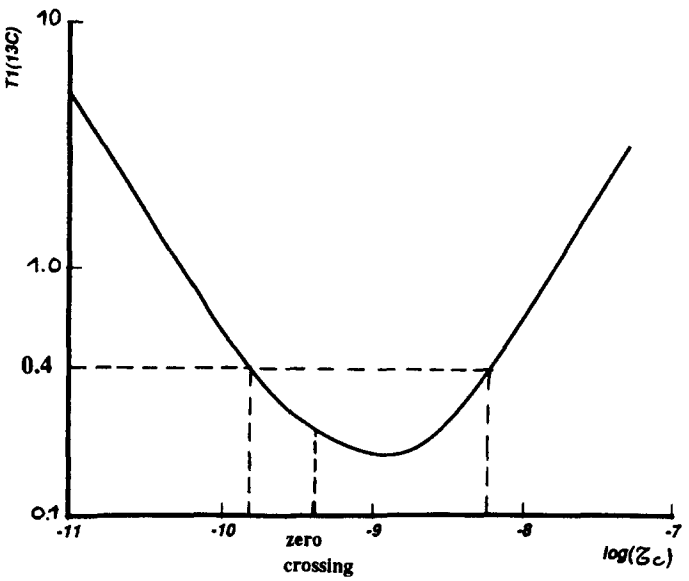

Fig. 6. Plot of the relation between the carbon longitudinal relaxation time, T_1, versus the effective correlation time, τ_c, at 100 MHz.

ethyl ester reductive amination product, etc.). However, a new derivative, prepared by forming a glycosylamine of *m*-tetrafluoroethoxyaniline [25] (see Experimental), led to quite clear sequence information for XXXG and XXLG in F3-2 and F2-3, respectively. The fragmentation data, in particular the loss of a hexose from the 828 ion, indicated that the galactose residue was linked to Xyl^a in XXLG (Fig. 7). In the case of the F2-1 fraction, several structures were compatible with the positive ion FAB-MS but the XLLG structure could be assigned to the major species from the NMR data which indicated the presence of two branched xylose sugars and two galactose residues. Detection of a weak signal in the anomeric region of the ^{13}C spectrum in the vicinity of the three peaks of the non-reducing glucose residues could indicate the presence of

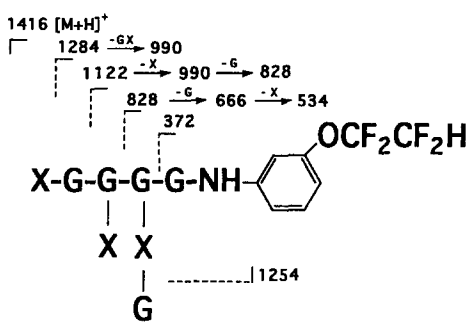

Fig. 7. Fragmentation of F2-3 derivatized as *m*-tetrafluoroethoxyanilinoglycosylamine (TFEA).

GXXLG and or XLGXG. However, no supplementary peak is observed in the region of the unbranched C-6 carbons in comparison with the F3 spectrum.

Detection of the three oligomers, XXXG, XXLG, and XLLG, suggests that the distribution of galactose in xyloglucan from cyclamen is fairly irregular and that this species would be a rich source of XG oligosaccharides. The biological activity of the oligosaccharides from cyclamen is currently under investigation.

3. Experimental

Isolation of polysaccharides from cyclamen.—2000 dry cyclamen seeds (nearly 20 g) were ground in a ball-bearing mixer. The resulting flour was extracted with boiling water (3 × 1 h). The suspension was then filtered through blutex. The filtrate was concentrated and mixed with an equal volume of Fehling solution [12]. The blue precipitate was collected by centrifugation (40 000 *g* × 10 min), decomplexed by adding 3 N HCl, precipitated with alcohol (4/1 volumes) and recovered by centrifugation (40 000 *g* × 10 min). Alcoholic precipitation was repeated 3 times. The final precipitate (X1) was ground in liquid nitrogen, freeze-dried and weighed (3.2 g). Water-soluble polymer, not complexed with copper (X1′), was recovered by adding ethanol to the supernatant of the Fehling treatment. Similar treatment as for X1 afforded 3.72 g of X1′. Lastly, the water-insoluble residue was submitted to an alkali extraction in the presence of sodium borohydride according to Edwards et al. [13] and 2.28 g of an alkali-soluble water-insoluble polymer (X2) was also recovered. Samples (4.5 mg) of X1, X1′, and X2 were hydrolyzed in 3 N H_2SO_4 (1 h, 100°C). The solutions were neutralized with $SrCO_3$, filtered through millipore filters (0.45 mm), reduced to a small volume and analyzed by HPLC as previously described [26].

Enzymatic hydrolysis of xyloglucan [19].—Cellulase from *Trichoderma viride*, Sigma Chemical Co., was previously de-salted by filtration on a Amicon YM 10 membrane and dissolved in 4.5 mL of 0.1 M acetate buffer (pH 4.7). 500 mg of X1 was added and the solution was stirred at 20°C for 3 h. The resulting mixture was centrifuged for 15 min to remove insoluble material and an aliquot of the supernatant was analyzed for sugar content (anthrone). Gel permeation was conducted on a Biogel P2 (22 × 1.8 cm; flow rate 1 L/20 h) column with an aqueous acetate buffer (0.1 M, pH 4.2) and fractions were collected in 2.2 mL tubes. The two main anthrone-reactive fractions which followed the bed-volume (F2 and F3) were chromatographed individually a second time on the Biogel P2 column using water as the eluent. Both F2 and F3 were then analyzed by HPLC on a Spectra Physics SP8750 Chromatograph equipped with a Showex SB800 (5 mm × 50) precolumn and a Showex SB802 (OHpack 8 mm × 300) column using water as the eluent (flow rate 1 mL/min). The retention times were calibrated with the following authentic dextran oligomers: G3, G7, T10 + T70 (Pharmacia).

Preparation of glycosamines for FAB-MS analysis.—The derivatization was carried out by heating the oligosaccharide with an excess of *m*-tetrafluoroethoxyaniline (Aldrich) in aqueous MeOH at 60°C for 1 h. The reaction mixture was evaporated under vacuum and used directly for FAB-MS analysis without further purification.

Mass spectrometry.—MALDI-MS was performed on a Vestec LaserTec Research instrument (Vestec Corp., Texas), equipped with a nitrogen laser (337 nm, 3 ns pulse). The accelerating voltage was 28 kV. Samples were prepared for analysis by mixing 1 μL of the oligosaccharide solution with 5 μL of the matrix solution (saturated alpha-cyano-4-hydroxycinnamic acid in 1/1 H_2O/CH_3CN containing 10 pmol/μL insulin and insulin B-chain as internal standards on a stainless steel probe tip. The solution was air dried prior to introduction into the ionization source. FAB-MS was performed in the positive ion mode on a JEOL HX-110 double focussing mass spectrometer (JEOL, USA). The accelerating voltage was 10 kV and ionization was effected by bombardment with a 6 keV beam of xenon atoms. Resolution was set at 3000. Derivatized samples were dissolved in H_2O/MeOH (4 : 1); 1 mL of this solution mixed on the probe tip with 2μL of a matrix (1 : 1 glycerol/thioglycerol or 1 : 1 glycerol/thioglycerol saturated with dimercaptosuccinic acid).

CP MAS spectra.—CP MAS spectra were recorded at ambient temperature on a Varian VXR-300 spectrometer operating at 75.43 MHz for ^{13}C, as described previously [27]. Contact time was 1 ms with pulse width 90°, acquisition time 19.2 ms. Relaxation delay was normally 1 s. Spin rates were between 4.0 and 5.0 kHz.

High-resolution spectra.—A Bruker AM-400 spectrometer operating in the FT mode at 400.13 MHz for ^1H and 100.57 MHz for ^{13}C was used. Samples were dissolved in D_2O (10–25 mg in 0.5 mL). Me_2SO was the internal reference (δ_C 39.5, δ_H 2.72). The spectral window for the ^1H NMR spectra was 10 ppm for 16 k data points with a pulse width of 8 μs (45°), an 8 s delay between each scan, and an acquisition time of 2 s. ^{13}C NMR spectra were recorded with complete proton decoupling unless stated otherwise. The pulse width was 8 μs (60°) and the acquisition time was 1.11 s with a 7 s delay between each scan. Heteronuclear NOEs were determined by comparing the relative intensities broad-band and inverse-gated spectra.

Double-quantum-filtered phase-sensitive COSY experiments [28] were performed using a (90°)–(t_1)–(90°)—(90°)–(FID, t_2) sequence while phase-sensitive NOESY experiments [29] were recorded with a (90°)–(t_1)–(90°)–τ_m–(90°)–(FID, t_2) sequence. The spectral width was optimized for the COSY spectra while this parameter was doubled in the case of NOESY spectra in order to avoid quadrature images. The number of data points in F2 was 1024, and 512 increments were recorded. The 90° pulse was 21 μs (8.5–11) and the total acquisition time was 16 (24–38) h for the COSY (NOESY) experiments. In the case of the NOESY experiments, the first data file was halved to reduce t_1 ridges. Before Fourier transformation, the data matrices were zero-filled to 1K × 1K and multiplied with a $\pi/2$-shifted squared sine bell. The tau-values [30] in the double relayed-COSY experiment were 70 (t_1) and 30 (t_2) ms respectively.

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Apperley on the SERC spectrometer at Durham, UK. Mass spectra were acquired at the MSU-NIH Mass Spectrometry Facility, which is supported in part by a grant (RR00480) from NIH, NCRR.

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