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Apple-fruit xyloglucans: a comparative study of enzyme digests of whole cell walls and of alkali-extracted xyloglucans

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ABSTRACT

Apple cell walls or alkali-extracted xyloglucans were digested with an endo-glucanase from *Trichoderma viride* and the resulting oligosaccharides were isolated by chromatography on Bio-Gel P-4. Three main oligosaccharides were present in similar proportions, and their structures were shown to be $[Xyl(Glc)]_3$-Glc, $[Xyl(Glc)]_2$-(FucGalXyl)Glc-Glc, and XylGlc-(GalXyP)Glc-(FucGalXyl)Glc-Glc. Each non-reducing-end Glc was 6-linked, each reducing-end Glc was 4-substituted, and each other Glc was 4,6-disubstituted. The Xyl was either terminal or 2-substituted, the Fuc was terminal, and the Gal was either terminal or 2-substituted. The ^{1}H-NMR spectra of the oligosaccharides extracted directly from the cell wall showed that they are not acetylated. Other oligosaccharides, notably $GalXyl_3Glc_4$, Xyl_2Glc_4, and Xyl_2Glc_3, were present in smaller proportions in the digest of the cell walls.

INTRODUCTION

Xyloglucans can form up to ~ 20% of the primary cell walls of dicotyledons [1,2]. The backbone of the xyloglucans is hydrogen bonded tightly and specifically to the cellulose [3] because of its cellulose-like structure and conformation [4]. Xyloglucans are thought to play a major rôle in cell-wall elongation, by controlling the "creep" of cellulose microfibrils [1] and by their oligosaccharide fragments, which can have anti-auxin activity [5,6]. Of the glucose residues, ~ 75% carry side chains attached to positions 6. The main side chains [7–9] are α-D-Xyl*p*-(1 → , β-D-Gal*p*-(1 → 2)-α-D-Xyl*p*-(1 → , and α-L-Fuc*p*-(1 → 2)-β-D-Gal*p*-(1 → 2)-α-D-Xyl*p*-(1 → . Xyloglucans of sycamore suspension-cultured cells [5,8,10–13] and legume seedlings [9,14–17] have

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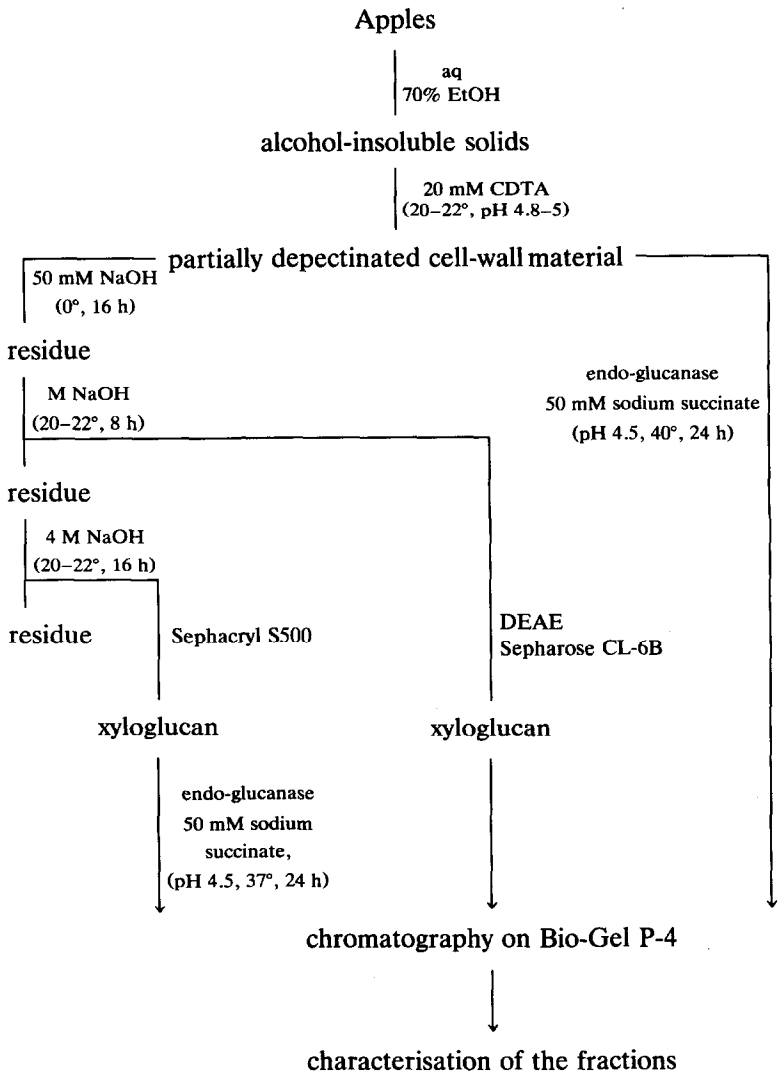
been studied extensively. Their fine structures have been studied using endo-glucanases [7,8] that split the xyloglucan backbone selectively at unbranched (1 → 4)-linked β-D-Glc residues. The Xyl side chains are not distributed randomly but form a repeating unit consisting of three XylGlc moieties followed by one unsubstituted Glc, as demonstrated [7] for tamarind xyloglucan. The presence of acetyl substituents on the Gal residues has been shown for xyloglucans from the extracellular polysaccharides [11] and cell walls [12] of sycamore. However, acetyl groups are usually lost from cell-wall xyloglucans due to the strongly alkaline conditions of extraction.

During work on the structure of cell walls of apple fruit [18–22], we have become interested in the fucogalactoxyloglucan which is their main hemicellulose [19–25]. There have been few studies of the structure of this polysaccharide in spite of its importance. Methylation analyses on the more-or-less purified polysaccharide [23–25] have been reported, as has a degradation by endo-glucanase [25] but without characterisation of the products. As apple fruit contains mature, primary-like, cell walls, the structure of the xyloglucan might differ from that of rapidly growing tissues (e.g., bean seedlings) or suspension-cultured cells. Apple xyloglucan has been extracted [21] by M and 4 M sodium hydroxide or by digestion of the cell wall with an endo-glucanase [22]. The alkali extracted xyloglucans proteins (M NaOH extract), low molecular weight mannans (4 M NaOH extract), and traces of residual pectic material. Treatment with the endo-glucanase solubilised two distinct fractions, namely, high molecular weight pectic material and xyloglucan oligosaccharides. The fractions corresponding to the xyloglucan or fragments thereof had similar sugar compositions [21,22].

We now report on the nature of the linkages and the structure of the subunits of apple xyloglucans.

RESULTS

Two approaches were used to obtain oligosaccharides (Scheme 1): (*a*) the xyloglucans were extracted with strong alkali, purified, then digested with an endo-glucanase [(1 → 4)-β-D-glucan glucanohydrolase; EC 3.2.1.4] from *Trichoderma viride* [26], or (*b*) the endo-glucanase was applied directly to the cell-wall preparation. Procedure (*b*) preserves the ester linkages which would be hydrolysed [12] by procedure (*a*). Cell walls were isolated from fresh apples as alcohol-insoluble solids and the soluble pectins were extracted first from the cell wall by a mild procedure (20 mM cyclohexanediaminetetra-acetic acid at 20° and pH 4.8–5) in order to facilitate access of the endo-glucanase with minimal alterations of the cell-wall structure (partially depectinated cell-wall material or CDTA-IR) [18]. The fractional extraction was continued with 0.05 M NaOH to eliminate pectins before extraction of the hemicelluloses [21] by M and 4 M NaOH. The M-NaOH extract represented 4.3% of the CDTA-IR and contained ~ 30% of xyloglucan which could be isolated by ion-exchange chromatography. The 4 M-



Scheme 1. Isolation of the xyloglucan oligosaccharides.

NaOH extract represented 10.5% of the CDTA-IR and comprised ~75% of xyloglucan. A fraction enriched in xyloglucan was obtained by chromatography on Sephacryl S-500. Incubation with endo-glucanase solubilised 12% of the CDTA-IR, of which ~40% corresponded to a pectin fraction (also extracted in the blank) and 60% to xyloglucan fragments freed by the enzyme [22].

Fractionation of the endo-glucanase digests.—The digests of the purified xyloglucans and the cell wall were subjected to chromatography on Bio-Gel P-4 (Fig. 1) that gave material in the void volume and a series of oligosaccharides with K_{av} values corresponding to those of malto-oligosaccharides with dp 9–13.

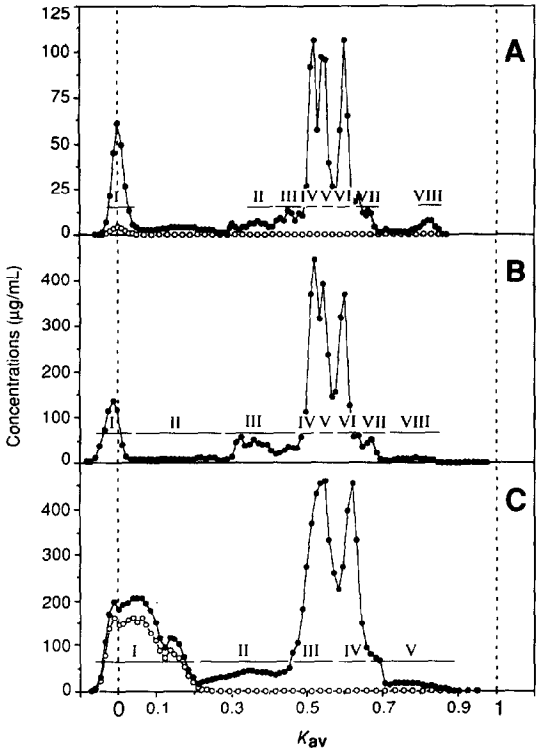


Fig. 1. Chromatography on Bio-Gel P-4 of the endo-glucanase digests: A, xyloglucan extracted by M NaOH; B, xyloglucan extracted by 4 M NaOH; C, partially depectinated cell wall; •, neutral sugars; ○, uronic acids.

The digests from the xyloglucans extracted by concentrated alkali were qualitatively almost identical (Fig. 1, A and B) and contained three major peaks with K_{av} 0.52 (A-IV and B-IV), 0.54 (A-V and B-V), and 0.60 (A-VI and B-VI) in almost identical relative proportions. Two minor components (A-VII and B-II) were present with $K_{av} \sim 0.70$, whereas the digest from the M-NaOH xyloglucan contained an additional peak with K_{av} 0.82 (A-VIII), corresponding to glucose. Material of higher molecular weight was also present, including oligosaccharides that contained fucose, galactose, xylose, and glucose (up to $K_{av} \sim 0.3$) and residual non-xyloglucan contaminants as the material at the void volume (Tables I and II). The three major oligosaccharides of each digest were purified further on Bio-Gel P-4 (Fig. 2, A and B). Each peak consisted of one major component with minor impurities. The two digests appeared to be similar with respect to the three major oligosaccharides (Table I and II) and the minor components, where these could be determined. Some of the minor components, e.g., A-IVc, B-IVc, or B-Va, came from the neighbouring main oligosaccharides. Others, e.g., A-IVa and B-IVa or B-IVc and B-Va, were original oligosaccharides present in low proportions in the endo-glucanase digests.

TABLE I

Neutral-sugar (NS) composition of the Bio-Gel P-4 fractions of the M-NaOH xyloglucan endo-glucanase digest (A) (mol%)

Fraction	K_{av}	NS	Rha	Fuc	Ara	Xyl	Man	Gal	Glc
I	−0.05–0.05	15	5.4		54.8	8.7	2.6	24.7	3.8
II	0.34–0.41	3		10.4	0.5	25.6	4.4	15.3	43.9
III	0.42–0.49	6		13.3		19.7	2.6	17.1	47.3
IV	0.50–0.53	21							
a				13.0		23.5		19.9	43.7
B				9.4		33.9		17.6	39.1
c				11.7		36.6	0.6	12.8	38.3
V	0.54–0.57	19							
A				12.8		35.6		12.6	39.0
VI	0.58–0.61	18							
a				6.6	0.5	35.3		14.2	43.4
B				2.5		45.2		2.7	49.6
VII	0.62–0.67	8		11.1		23.7	0.4	13.1	51.7
VIII	0.81–0.84	2				4.2			95.8

The chromatogram from the cell-wall digest showed less sharply defined peaks (Fig. 1C) and two major oligosaccharide peaks with K_{av} 0.55 (C-III) and 0.62 (C-IV). The peak with K_{av} 0.55 was asymmetric, indicative of heterogeneity. The pectic material was eluted as a wide peak in the void volume. The materials in peaks C-III and C-IV were each rechromatographed on Bio-Gel P-4 (Fig. 2C): C-III then gave two main components, and finally three fractions C-IIIC, C-IIID,

TABLE II

Neutral-sugar (NS) composition of the Bio-Gel P-4 fractions of the 4 M-NaOH xyloglucan endo-glucanase digest (B) (mol%)

Fraction	K_{av}	NS	Rha	Fuc	Ara	Xyl	Man	Gal	Glc
I	−0.07–0.02	11		4.8	38.2	28.5	6.1	18.0	4.4
II	0.03–0.28	4		3.4	8.7	44.7	15.7	9.6	18.0
III	0.29–0.46	12		11.3	0.5	27.5	0.8	18.9	41.0
IV	0.47–0.52	22							
a				15.8		25.2		18.0	40.9
B				12.3		32.6		19.7	35.4
c				12.6		33.7		13.1	40.6
V	0.53–0.57	23							
a				12.0		31.5		19.8	36.7
B				12.1		36.5		11.2	39.5
c				3.3		37.1		12.7	47.0
VI	0.58–0.64	23							
a				3.3		45.1		11.9	39.8
B				1.0		43.4		1.2	54.5
c				15.2		22.2		15.3	47.4
VII	0.64–0.71	4		8.8	0.5	33.0	0.4	9.1	48.2
VIII	0.71–0.82	2		0.6		41.5	1.4	0.7	55.7

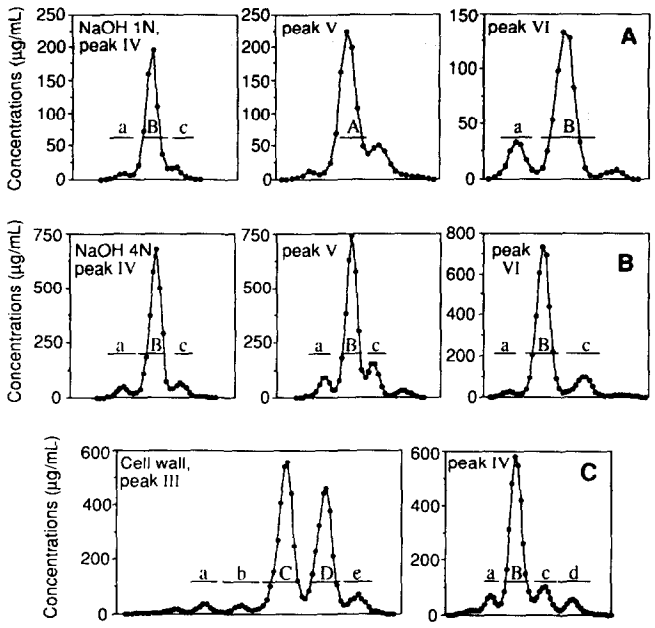


Fig. 2. Purification of the xyloglucan oligosaccharides from Fig. 1 on Bio-Gel P-4: A, xyloglucan extracted by M NaOH; B, xyloglucan extracted by 4 M NaOH; C, partially depectinated cell-wall; •, neutral sugars.

and C-IVB were obtained. Their sugar compositions were similar to those obtained for the digests from the extracted xyloglucans (Table III).

Each of the fractionations of the endo-glucanase digests revealed three major

TABLE III

Neutral-sugar (NS) composition of the Bio-Gel P-4 fractions of the cell-wall endo-gluctanase digest (C) (mol%)

Fraction	K_{av}	NS	Rha	Fuc	Ara	Xyl	Man	Gal	Glc
I	−0.02–0.20	33	10.2	1.4	57.5	10.6		18.5	1.8
II	0.21–0.43	8	3.2	7.4	5.2	38.7	1.0	15.6	28.9
III	0.44–0.57	34							
a				15.9	0.5	21.4		16.2	46.0
b			0.8	13.3	0.6	24.8	0.7	18.0	41.8
C				12.1		32.8		19.5	35.7
D				12.2		34.2		12.2	41.5
e				3.4	0.8	34.0		14.2	47.8
IV	0.58–0.66	20							
a				1.9	0.8	35.3		13.4	48.8
B				0.0		46.5		1.5	52.1
c				12.6	0.4	22.8		15.3	48.8
d				6.5	0.7	33.5	1.0	7.3	51.5
V	0.67–0.87	5		3.7	1.5	28.1	0.7	4.5	61.5

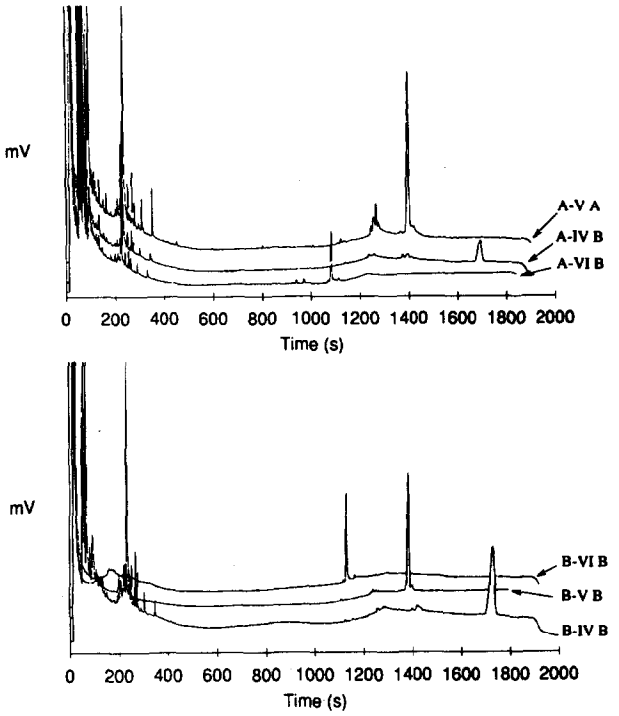


Fig. 3. High-temperature GLC of the methylated oligosaccharide-alditols purified from the M-NaOH xyloglucan (A) and the 4 M-NaOH xyloglucan (B) digests. For coding of the traces, see Fig. 2.

(and some minor) oligosaccharides with similar sugar compositions, whether the starting material was a xyloglucan extracted by alkali or was the cell wall itself.

High-temperature GLC.—The three series of xyloglucan oligosaccharides and the cell-wall digest were each reduced with sodium borodeuteride then methylated, and analysed by high-temperature GLC. Fig. 3 shows the chromatograms of the methylated oligosaccharide-alditols obtained from the digests of the M-NaOH (A) and 4 M-NaOH xyloglucan (B), which confirmed the purity of the oligosaccharides isolated from the latter digest. However, some oligosaccharides with different molecular weights were still present in the oligosaccharides isolated from the former digest, notably in A-VA. The peaks at ~ 200 s are due to contaminants from the methylation reaction.

Fig. 4 shows the chromatograms of the three oligosaccharides from the endo-glucanase digest and of the unfractionated endo-glucanase digest, reduced and methylated after elimination of the pectins by ion-exchange chromatography on Dowex 50W-X4. By comparison with the elution pattern C in Fig. 1, a better separation was obtained, and there were additional smaller peaks that reflected the presence of other members of the same homologous series. Comparison of the three series of chromatograms indicated that the same major oligosaccharides were present in all three digests.

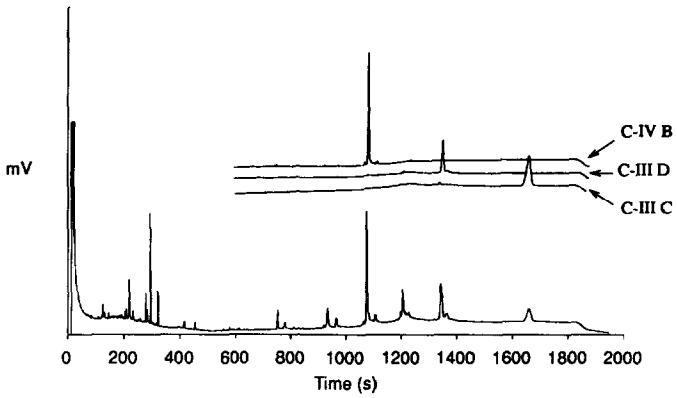


Fig. 4. High-temperature GLC of the methylated oligosaccharide-alditols of the whole cell-wall digest and of its purified oligosaccharides (for coding, see Fig. 2).

Linkage analysis of the oligosaccharides.—Methylation analysis of the oligosaccharides from the 4 M-NaOH xyloglucan digest is shown in Table IV. Each contained a 4-linked glucose residue at the reducing end (which gave 4-*O*-acetyl-1,2,3,5,6-penta-*O*-methylglucitol), and a 6-substituted glucose residue at the non-reducing end (which gave 1,6-di-*O*-acetyl-2,3,4-tri-*O*-methylglucitol). The simplest oligosaccharide was B-VIB, where all the side chains were single xylose residues. In B-VB, terminal fucose, 2-substituted galactose, and 2-substituted xylose were present in similar proportions, showing the presence of Fuc*p*-(1 → 2)-Gal*p*-(1 → 2)-Xyl*p*-1 → as a side chain. The largest oligosaccharide (B-IVB) contained three

TABLE IV

Linkages composition [a] of the xyloglucan oligosaccharides isolated from the 4 M-NaOH xyloglucan digest

Residue	Methylated positions	Deduced linkage	Peak B-IV B	Peak B-V B	Peak B-VI B
Xyl	2,3,4	T Xyl	1.13	1.85	3.04
Xyl	2,3	4 Xyl	1.73	0.95	0.09
Fuc	2,3,4	T Fuc	0.80	0.80	
Gal	2,3,4,6	T Gal	0.82	0.13	
Gal	3,4,6	2 Gal	0.56	0.62	
Glc	1,2,3,5,6	Reducing end	0.47	0.50	0.54
Glc	2,3,4,6	T Glc	0.15	0.07	0.08
Glc	2,3,4	6 Glc	1.00	1.00	1.00
Glc	2,3,6	4 Glc	0.08	0.03	0.10
Glc	2,3	4,6 Glc	1.69	1.79	1.56

[a] In molar ratios relative to 1,5,6-tri-*O*-acetyl 2,3,4-tri-*O*-methylglucitol, using the appropriate molar responses.

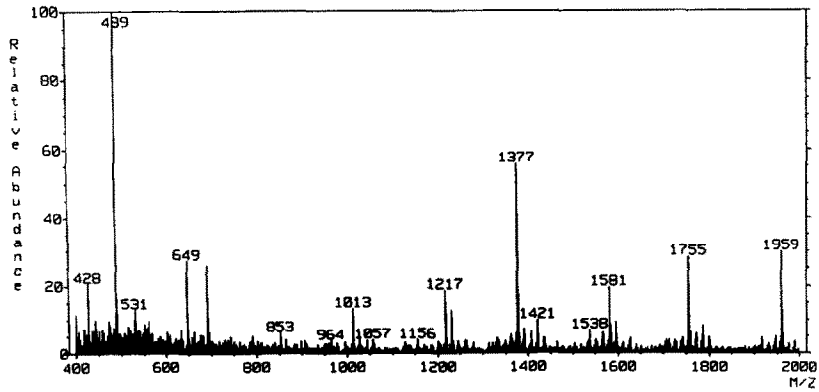


Fig. 5. DCI-mass spectrum of the methylated oligosaccharide-alditols from the whole cell-wall digest.

different side chains, namely, Xyl*p*-1 →, Gal*p*-(1 → 2)-Xyl*p*-1 →, and Fuc*p*(1 → 2)-Gal*p*-(1 → 2)-Xyl*p*-1 →.

The results of methylation analysis are only semi-quantitative, due to the different susceptibilities of the linkages to acid hydrolysis and to the high volatility of some of the partially methylated alditol acetates (notably from the reducing-end glucose). However, the molar ratios indicate the identification of B-VIB as Xyl_3Glc_4, B-VB as $FucGalXyl_3Glc_4$, and B-IVB as $FucGal_2Xyl_3Glc_4$.

DCI-mass spectrometry.—The DCI-mass spectra of the methylated, deuterated oligosaccharide-alditols gave intense pseudo-molecular ions ($[M + NH_4]^+$), with odd-numbered *m/z* values which allowed the parent oligosaccharides to be identified as the heptasaccharide XG7 (methylated Xyl_3Glc_3Glcol at *m/z* 1377) for fractions A-VIB, B-VIB, and C-IVB; the nonasaccharide XG9 (methylated $FucGalXyl_3Glc_3Glcol$ at *m/z* 1755) for fractions A-VA, B-VB, and C-IIID; and the decasaccharide XG10 (methylated $FucGal_2Xyl_3Glc_3Glcol$ at *m/z* 1959) for fractions A-IVB, B-IVB, and C-IIIC.

The in-source desorption in ammonia was sufficiently mild to allow direct analysis of the methylated oligosaccharide-alditols of the cell-wall digest. Fig. 5 shows the spectrum of this digest, which consists of the pseudo-molecular ions of a series of xyloglucan fragments ranging from G_2 to XG10. XG7 was the main component (*m/z* 1377), which is consistent with the peak intensity distribution observed in the gas chromatogram of Fig. 4. Other ions correspond to XG10 (*m/z* 1959), XG9 (*m/z* 1755), XG8 (*m/z* 1581, $GalXyl_3Glc_3Glcol$), XG6 (*m/z* 1217, tentatively identified as Xyl_2Glc_3Glcol), and XG5 (*m/z* 1013, Xyl_2Glc_2Glcol). Several smaller blocks from the xyloglucan chain were also observed: *m/z* 853 ($XylGlc_2Glcol$, XG4?), 649 ($XylGlc_2Glcol$), 693 (Glc2Glcol), and 489 (GlcGlcol). These compounds are being studied further.

A more detailed study of the desorption profiles of the purified XG7, XG9, and XG10 showed a predominance of their pseudo-molecular ions at the "cold" end of

the total ion current. The appearance of these ions coincided with the appearance of series of fragment ions with odd m/z values, which contained the deuterated alditol moiety. These A(Pditol)-type ions have been rationalised [27,28] in studies of reduced methylated maltotetraose. At higher temperatures of desorption, even ions generated from the non-reducing end (Z) were also observed. Structures could be assigned to the A- and Z-type ions on the basis of the fragmentation pattern observed in the unbranched model compound. The glucose main chain is designated G3–G2–G1–G0, in which G0 is the deuterated glucitol moiety.

The interpretation of the spectrum of XG7 ($[M + NH_4]^+$, m/z 1377) shown in Fig. 6 was straightforward (Scheme 2). The Z-type ions at m/z 192 and 208 are derived from the Xyl side chains. The ions at m/z 412 (and 414), 776 (and 778), and 1142 (and 1144) are from cleavages between G3 and G2, between G2 and G1 [loss of XylG3-(Xyl)G2], and between G1 and G0, respectively. The ions at m/z 617 and 1217 are A-type ions corresponding to cleavage between G2 and G1, and to loss of one Xyl residue. Most of the diagnostic ions can be rationalised and confirm the XylGlc-(Xyl)Glc-(Xyl)Glc-Glcol structure.

The DCI-mass spectrum of XG9 ($[M + NH_4]^+$, m/z 1755) does not contain the A-type ion at m/z 617, but the Z-type ions at m/z 412 (and 414) and 776 (and 778) persist (Fig. 7), demonstrating that the Fuc and Gal are carried by the Xyl attached to G1. The presence and position of the FucGalXyl side chain is confirmed by the Z-type ion at m/z 588, produced by a cleavage between the Xyl and the main chain Glc, and the A-type ion at m/z 995, produced by a cleavage between G2 and G1. The Z-type ions at m/z 206 and 222 point to terminal Fuc; no ion indicative of the FucGal constituent is evident in the spectrum. Again, the ions at m/z 192 and 208, and m/z 412 (and 414) point to a terminal Xyl and XylGlc at G3, respectively. Diagnostic A-type ions at higher masses are m/z 1581 (loss of Fuc), 1377 (loss of Fuc and Gal), and 1217 (loss of Fuc, Gal, and Xyl). These data are consistent with a XylGlc-(Xyl)Glc-(FucGalXyl)Glc-Glcol structure.

The spectrum of XG10 ($[M + NH_4]^+$, m/z 1959) is shown in Fig. 8. Diagnostic A-type ions indicative of the FucGalXyl side chain are observed at m/z 1785 (loss of Fuc), 1581 (loss of Fuc and Gal), and 1421 (loss of Fuc, Gal, and Xyl). The strong ion at m/z 588 confirms the presence of this side chain. The terminal position of the Fuc is evident from the ions at m/z 206 and 222. The ions at m/z 192 and 208, and that at m/z 412 (and 414) point to a terminal Xyl and a XylGlc, respectively, at the non-reducing end of the molecule. The presence of a GalXyl side chain is indicated by the ions at m/z 1755 (loss of Gal) and 1595 (loss of Gal and Xyl). The position of the GalXyl and FucGalXyl side chains can be determined from the A-type ions derived from cleavages between G2 and G1 and between G3 and G2. Indeed, FucGalXyl attached to G1 gives rise to the ion at m/z 995. The position of the FucGalXyl side chain is confirmed by the Z-type ions from cleavage between G3 and G2 at m/z 980 and 982. Consequently, the structure of the XG10 is XylGlc-(GalXyl)Glc-(FucGalXyl)Glc-Glcol. However, there are several A- and Z-type ions in this spectrum which could not be

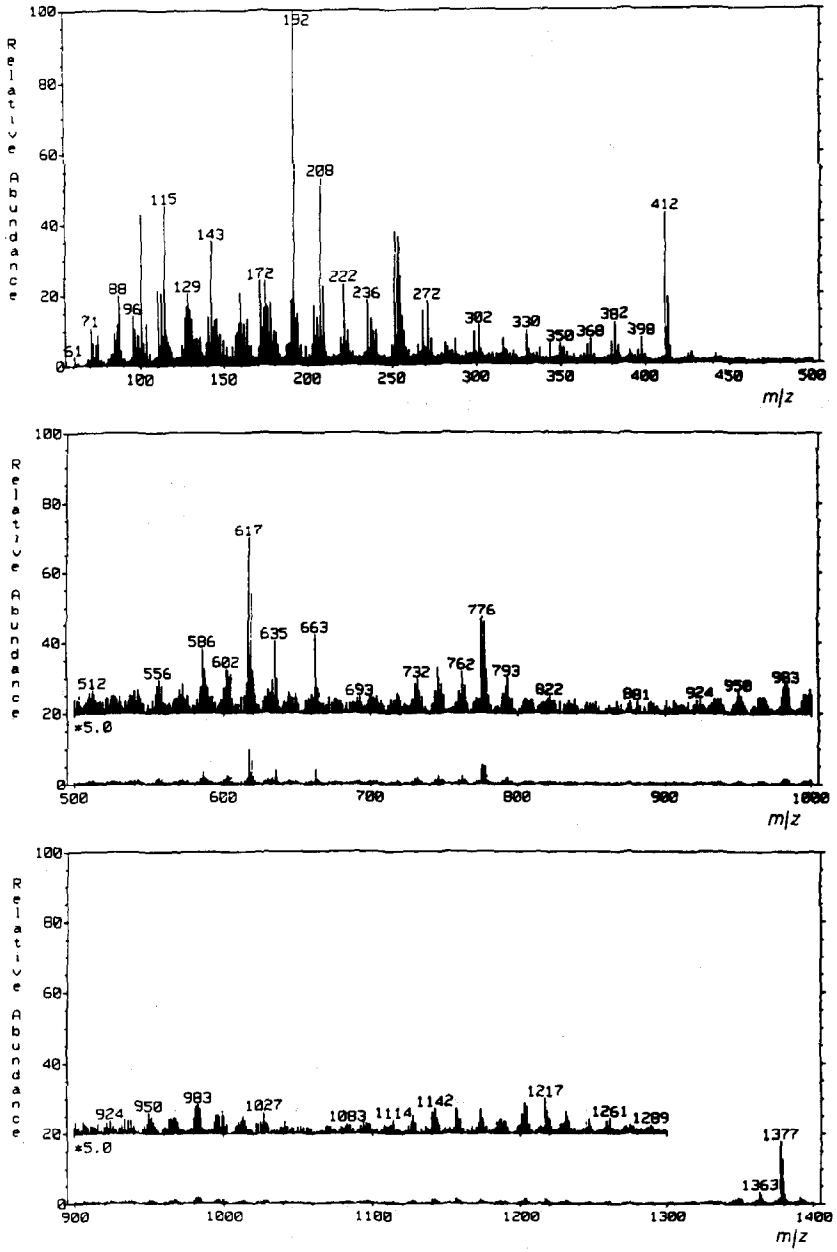
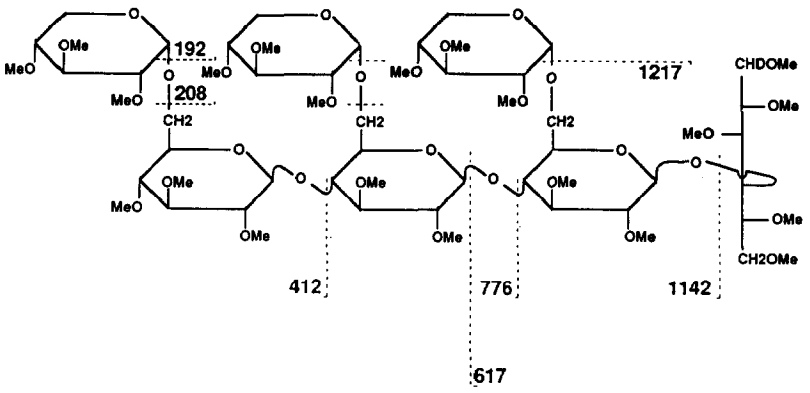


Fig. 6. DCI-mass spectrum of the methylated alditol of XG7 (C-IVB).

explained, and the possibility of minor proportions of structural isomers cannot be excluded.

^{1}H-NMR spectroscopy.—No differences were observed between the ^{1}H-NMR spectra of the oligosaccharides purified from the 4 M-NaOH xyloglucan and from



Scheme 2. Fragmentation pattern of methylated alditol of XG7.

the cell-wall digests (Fig. 9) and there were no signals for *O*-acetyl groups [11], implying that the xyloglucan oligosaccharides obtained directly from apple cell wall are not acetylated.

DISCUSSION

Apple xyloglucans consist of three main repeating units, namely, XG7 [XylGlc-(Xyl)Glc-(Xyl)Glc-Glc], XG9 [XylGlc-(Xyl)Glc-(FucGalXyl)Glc-Glc], and XG10 [XylGlc-(GalXyl)Glc-(FucGalXyl)Glc-Glc]. Their structures are identical to those reported from tamarind xyloglucan (XG7) [7], sycamore extracellular polysaccharides (XG9) [29], and mung-bean xyloglucan (XG10) [30]. In contrast to the general situation (sycamore cell walls and extracellular polysaccharides [8], red-kidney bean [31], mung-bean hypocotyls [15], pea epicotyls [16], soybean suspension-cultured cells [32]), where XG7 and XG9 preponderate, XG7, XG9, and XG10 were present in nearly identical proportions in the xyloglucan from apple cell walls. Minor proportions of other units, notably XG8, but also smaller units (XG6 and XG5), were detected. Low proportions of glucose and glucodisaccharide were present in the digests, indicating that (*a*) there are no extensive unsubstituted regions in the xyloglucan molecules and (*b*) the endo-glucanase is unable to degrade cellulose, which represents ~ 30% of the cell-wall material, to oligomers. The xyloglucan oligosaccharides in the cell-wall digest were not acetylated. The lack of acetylation in the xyloglucan from apple contrasts with that from sycamore [11,12].

DCI-MS gave excellent information on sequence and required very small amounts of material (1–2 μg per DCI-mass spectrum, and 5–10 μg per high-temperature GLC). These methods can be applied to unfractionated enzyme digests and provide a rapid method for investigating the nature of the structural units of xyloglucan. For apple xyloglucan, these methods revealed minor oligosaccharides which are neglected when lengthy purification procedures are used to obtain mg quantities for characterisation. A fast and sensitive procedure is now available that

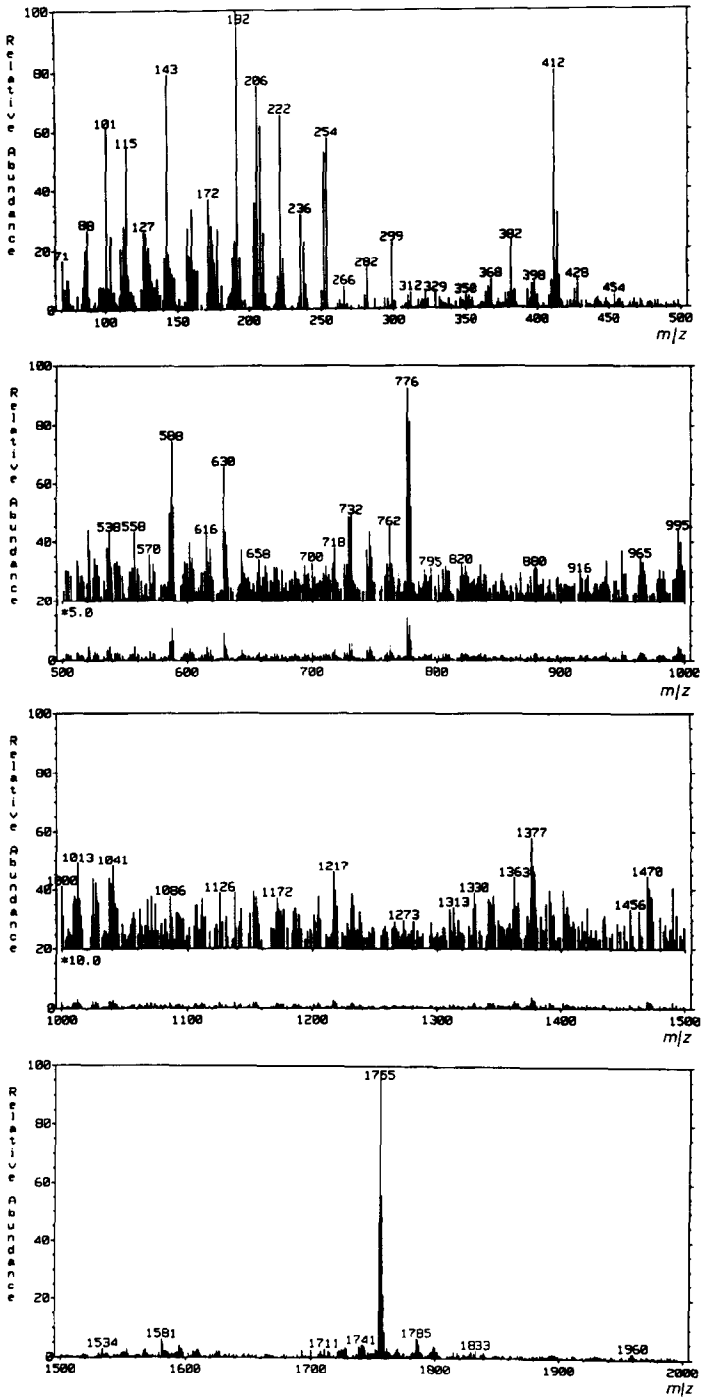


Fig. 7. DCI-mass spectrum of the methylated alditol of XG9 (C-IIID).

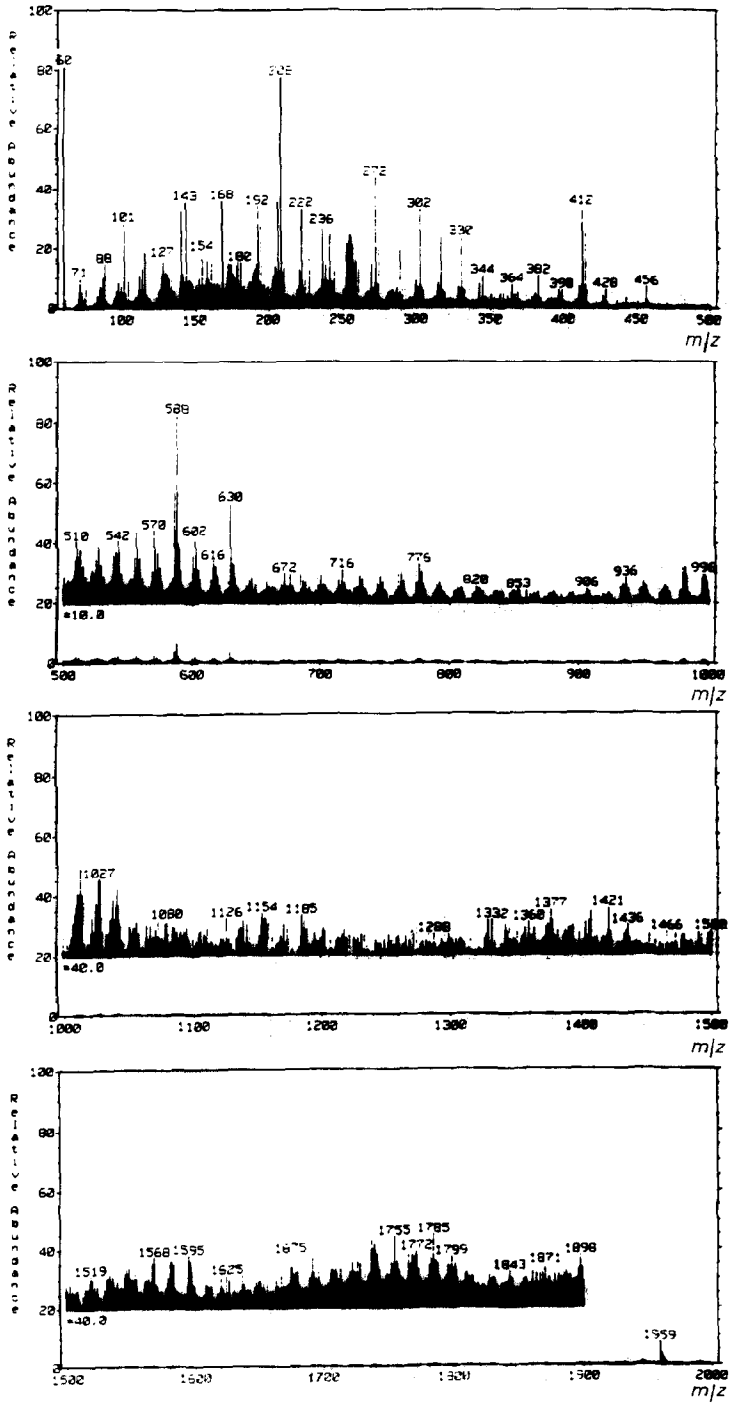


Fig. 8. DCI-mass spectrum of the methylated alditol of XG10 (C-IIIC).

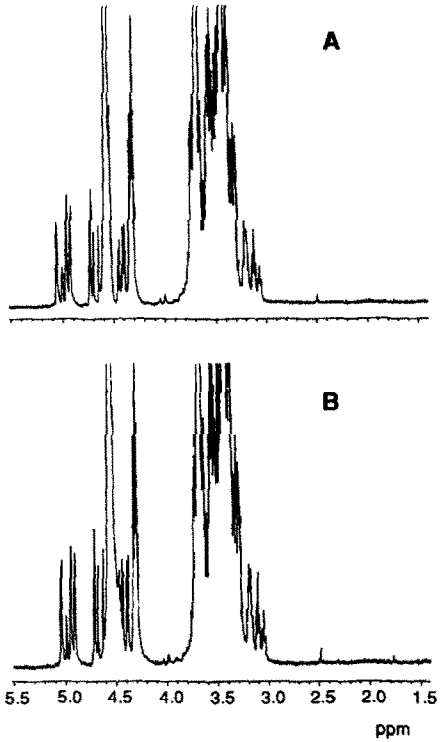


Fig. 9. ^{1}H-NMR spectra of the oligosaccharides purified from the 4 M-NaOH xyloglucan and the cell-wall digests: a, XG10 from the 4 M-NaOH xyloglucan digest (B IVB); b, XG10 from the cell-wall digest (C IIIC).

should enable investigation of the fine structure of xyloglucans in cell walls of different plants, of different tissues of a plant, and, possibly, the evolution of the xyloglucan as the cell wall matures.

EXPERIMENTAL

Preparation of xyloglucan oligosaccharides.—(a) Cell wall preparations. Partially depectinated apple cell walls were prepared by treatment of apple alcohol-insoluble solids with CDTA (cyclohexanediaminetetra-acetic acid) [18]. The xyloglucans were prepared from M and 4 M NaOH extracts of apple cell walls as described [21].

(b) Enzyme digestion. Partially depectinated apple cell walls were treated with endo-glucanase [(1 → 4)-β-D-glucan glucanohydrolase; EC 3.2.1.4] (endo IV purified from *Trichoderma viride* [26]) as described [22]. The xyloglucans prepared from the alkali extracts were incubated with 0.25 nkat of endo-glucanase/mg of xyloglucan for 24 h at 37° in 0.1 M succinate buffer (pH 4.5).

Chromatography on Bio-Gel P-4.—The endoglucanase digests (5 mL) were applied to a column (90 × 2.6 cm) of Bio-Gel P-4 (at 50°) and eluted (ascending)

with water. Fractions (4 mL) were assayed for neutral sugars and uronic acids. Appropriate fractions were combined and concentrated, and the residue was rechromatographed on Bio-Gel P-4 with recycling (3 or 4 cycles) before the fractions were collected and assayed.

Analytical methods.—Glycosyl composition was determined by GLC of alditol acetates [18]. Neutral sugars and uronic acid were determined by the automated orcinol and metahydroxybiphenyl assays [18]. The activity of the endo-glucanase was measured by the increase in reducing-end groups detected by the Nelson–Somogyi test [33] on carboxymethylcellulose (CM cellulose type Akucell AF 0305, from Akzo, Arnhem, The Netherlands) at 37° in succinate buffer (pH 4.5). Each sample was desalted, before further analysis, using a column (5 × 0.5 cm) of Dowex 50W-X4 (H^+) resin.

Methylation analysis.—Methylation was carried out by the method of Hakomori [34]. Each oligosaccharide (~ 1 mg) was reduced with an excess of $NaBD_4$ for 3 h at room temperature. The excess of $NaBD_4$ was then destroyed with a few drops of glacial acetic acid, and the borate was removed by repeated evaporations with acidified MeOH and MeOH. The oligosaccharide-alditols were desalted on Dowex 50W-X4 (H^+) resin and dried overnight over P_2O_5. Fresh potassium dimsyl was added to a solution of the oligosaccharide-alditol in dry Me_2SO. After 2 h, the mixture was cooled, and MeI was added in portions during 30 min. The mixture was stored at room temperature for 2 h, satd aq KCl was added, and the methylated oligosaccharide-alditol was extracted with CH_2Cl_2. A portion (~ 20%) of each product was kept for DCI-MS and high-temperature GLC. The rest was hydrolysed with 2 M trifluoroacetic acid for 1 h at 120°. The partially methylated sugars were reduced, and the excess of borate was eliminated by evaporation with acidified MeOH and MeOH. Acetic anhydride (1 mL) was added, and the mixture was kept for 5 h at 100°. Water was added, and the partially methylated alditol acetates were extracted with CH_2Cl_2. The extract was concentrated under a stream of N_2 at room temperature.

GLC.—The partially methylated alditol acetates were analysed on a Packard 438S gas chromatograph, using CPWAX85CB and CPSIL5CB wall-coated silica columns (50 m × 0.32 mm, Chrompak). On-column injection was performed at 50°; the temperature of the oven was raised rapidly to 220° and maintained there for 55 min. Peaks were identified by their relative retention times and by GLC–MS, using the CPSIL5CB column coupled to a JEOL JMS-DX 303 mass spectrometer.

High-temperature GLC.—The purity of the oligosaccharides was checked by high-temperature GLC. The methylated oligosaccharide-alditols were separated on a Chrompak Sim-Dist capillary column (0.33 mm × 10 m) by on-column injection using a Carlo Erba SFC 3000 high-temperature gas chromatograph equipped with a flame-ionisation detector. The carrier gas was He at 2 mL/min, except during injection when constant pressure was used. Injections were carried out at 50°, the oven was heated rapidly to 250° then programmed to 370° at 3°/min, and kept there for 10 min.

Mass spectrometry.—DCI-MS was performed with a Jeol DX-303 double-focussing mass spectrometer. Each sample was dissolved in CH_2Cl_2, an aliquot (~ 2 μL) was applied to the tip of the Pt/Rh wire of an insertion probe, and the solvent was evaporated *in vacuo* prior to analysis. Analysis conditions: acceleration voltage, 2.2 kV; mass range, 60–2000 amu; ammonia as the reagent gas at ~ 10 Pa in the source; heating rate of the wire, 1 A/min to a final temperature of 200°, source temperature, 180°.

NMR spectroscopy.—The exchangeable protons of the oligosaccharides were replaced by deuterons and the samples were dissolved in D_2O (99.96% D, Aldrich). ^{1}H-NMR spectra (500 MHz) were recorded at 20° with a Bruker AM500 spectrometer.

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