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Structural characterisation of xyloglucan and xylans present in olive fruit (*Olea europaea* cv koroneiki)

E. Vierhuis, H.A. Schols, G. Beldman, A.G.J. Voragen*

Laboratory of Food Chemistry, Department of Food Technology and Nutritional Science, Wageningen University, Bomenweg 2, 6700 EV Wageningen, The Netherlands

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Abstract

Hemicellulose-rich fractions obtained from olive fruit were fractionated by anion-exchange chromatography, which resulted in a xyloglucan-rich pool and four xylan-rich pools. Sugar linkage analyses and degradation studies with specific enzymes were performed to obtain information about the structures. The results indicated a xyloglucan in olive fruit with a specific substitution pattern, which is not commonly found in plant cell walls: XXXG-type building units with both arabinosyl and galactosyl residues linked to it. The xylans present in olive fruit were all very low in substitution with mainly 4-*O*-methyl-glucuronic acid residues. Enzymatic degradation with endo-xylanases resulted in a mixture of neutral and acidic xylo-oligosaccharides. Striking were the identical degradation patterns on HPAEC for all xylan-rich pools while the elution of the pools on DEAE Sepharose differed markedly.

Keywords: Structural characterisation; Xyloglucan; Xylan; Olive fruit; Hemicellulose

1. Introduction

The cell wall material of olive fruit also contains besides pectic material considerable amounts of hemicelluloses (Coimbra, Waldron & Selvendran, 1994; Huisman, Schols & Voragen, 1996; Vierhuis, Schols, Beldman & Voragen, 2000). Hemicelluloses require relatively strong alkali, typically 1–4 M, for their extraction from the cell wall due to strong hydrogen bonding to cellulose microfibrils. The composition of the hemicelluloses of plant cell walls differs for each species (Brett & Waldron, 1990). The major hemicellulose components in olive fruit are xylans and xyloglucans (Coimbra et al., 1994). Xylans have a backbone of (1,4)-β-linked Xyl residues. Depending on their origin the backbone is substituted with GlcA or its 4-*O*-methylated derivative, Ara and acetyl groups (Wilkie, 1979). Xyloglucan consists of a backbone of (1,4)-β-linked Glc residues branched on C-6 with Xyl residues. Two general types of xyloglucan poly-XXXG and poly-XXGG can be distinguished which differ in the degree of backbone substitution with Xyl residues. Some of the Xyl residues are substituted with Gal or Ara residues or a disaccharide of Fuc 1,2-linked to Gal. In addition, xyloglucans are often *O*-acetylated as reviewed by Vincken, York, Beldman and Voragen (1997b).

From cell wall material of olive fruit, an acidic xylan and a xyloglucan were isolated and partially characterised by Gil-Serrano, Mateos-Matos and Tejero-Mateo (1986) and Gil-Serrano and Tejero-Mateo (1988). Coimbra and coworkers (1994) have continued the research on hemicellulose in olive fruit and reported the composition and structural features of hemicellulose-rich fractions. They have described the presence of xylan–xyloglucan complexes in the cell wall of olive pulp in which the xylan and xyloglucan moieties are strongly attached (Coimbra, Rigby, Selvendran & Waldron, 1995). In an earlier paper, we have described the isolation and analysis of hemicellulose-rich fractions from olive fruit (Vierhuis et al., 2000). These 1 and 4 M KOH soluble polymers were fractionated by anion-exchange chromatography, which resulted in a xyloglucan-rich pool and four xylan-rich pools. The present study investigates in more detail the polymers present in the 1 and 4 M KOH fractions. Besides analysis of the glycosidic linkage composition well-characterised enzymes will also be used to obtain information about the structure of the xyloglucans and xylans present in olive fruit cell walls.

* Corresponding author. Tel.: + 31-317-482888; fax: + 31-317-484893.

E-mail address: fons.voragen@chem.fdsci.wau.nl (A.G.J. Voragen).

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2. Experimental

2.1. Materials

2.1.1. Substrates

The xyloglucan-rich pools and xylan-rich pools were obtained by anion-exchange chromatography from 1 and 4 M KOH fractions of purple olive fruit as described by Vierhuis et al. (2000). The xylan-rich pools were named according to their order of elution from the anion-exchange column as xylan 1, 2, 3 and 4.

2.1.2. Enzymes

Endo-(1,4)-β-D-xylanase I and III (XylI, XylIII) were purified from a culture filtrate from *Aspergillus awamori* as described by Kormelink, Searle-van Leeuwen, Wood and Voragen (1993b). Endo-(1,4)-β-D-glucanase IV and V (EndoIV, EndoV) were purified from a commercial enzyme preparation from *Trichoderma viride* as described by Beldman, Searle-van Leeuwen, Rombouts and Voragen (1985).

2.2. Enzyme incubations

The xyloglucan-rich pools and xylan-rich pools were dissolved in a 150 mM NaOAc buffer of pH 5.0 containing 0.01% (w/v) NaN_3 and incubated with purified enzyme. The incubations were performed at 40°C for 24 h with a substrate concentration of 1 mg/ml. The amount of enzyme used was 0.2 and 1.0 μg protein/ml for EndoIV and EndoV, respectively, and 0.5 μg protein/ml for XylI and XylIII. The resulting digests were heated for 15 min at 100°C to inactivate the enzymes. The change in molecular weight distribution and the release of oligomeric end-products were studied by HPSEC and HPAEC, respectively. For each enzyme, it was checked that the limit of digestion was reached after 24 h for the concentration of the enzyme used.

2.3. Isolation and characterisation of XylI-treated polymeric material

Xylan 3 and 4 were incubated with endo-xylanase I (XylI) as described above. After incubation the residual polymeric fraction was separated from the oligosaccharides present in the digest by ultrafiltration (Nominal Molecular Weight Cut-off 30 kDa; Pall Filtron). The polymeric fraction was freeze-dried and analysed for sugar composition using methanolysis combined with a trifluoroacetic acid hydrolysis as described by De Ruiter, Schols, Voragen and Rombouts (1992) followed by an enzymatic hydrolysis of the incomplete hydrolysed aldobiuronic acids (Vierhuis et al., 2000). The neutral sugar and uronic acid composition was determined by HPAEC using the gradient for uronic acids described before (Vierhuis et al., 2000) which was also used for the analysis of neutral sugars.

2.4. Analytical methods

2.4.1. Sugar linkage composition

Samples were methylated according to a modification of the Hakomori method (Sandford & Conrad, 1966) without a carboxyl reduction and subsequently dialysed against water and dried by evaporation (air stream, room temperature). The methylation step was repeated in order to improve the completeness of the reaction. The methylated polysaccharides were hydrolysed with 2 M trifluoroacetic acid (1 h, 121°C), which was removed by evaporation (air stream, at <10°C). The released (partially methylated) sugars were converted into their alditol acetates (Englyst & Cummings, 1984), which were quantified by GC-FID and identified by GC–MS. Sodium borodeuteride was used for reduction. Quantification was performed by GC-FID via on-column injections on a fused silica capillary column (30 m × 0.32 mm; wall coated with DB 1701; 0.25 μm film thickness; J and W Scientific) in a Carlo-Erba HRGC 5160 gas chromatograph equipped with a flame ionisation detector set at 280°C. The temperature program was 80 → 180°C at 20°C/min, 180 → 230°C at 2°C/min, and 230°C for 3 min. Derivatives were quantified according to their effective carbon response (Sweet, Shapiro & Albersheim, 1975). Identification of the compounds was confirmed by GC–MS using a CP Sil 19 CB capillary column (25 m × 0.25 mm, 0.2 μm; Chrompack) in an HP 6890 gas chromatograph coupled to a HP 5973 mass-selective detector and using a HP Chem Station (Hewlett Packard). The temperature program was 160 → 185°C at 0.5°C/min, 185 → 230°C at 10°C/min and 230°C for 5.5 min.

2.4.2. High-performance anion-exchange chromatography

High-performance anion-exchange chromatography (HPAEC) was performed as described previously (Vierhuis et al., 2000). Xyloglucan oligosaccharides were analysed with the following NaOAc gradient in 100 mM NaOH: 0 → 5 min, linear gradient of 0 → 50 mM NaOAc; 5 → 45 min, linear gradient of 50 → 80 mM NaOAc; 45 → 60 min, linear gradient of 80 → 260 mM NaOAc; Xylan oligosaccharides were analysed as described by Verbruggen et al. (1998b). After each run the column was washed for 5 min with 100 mM NaOH containing 1 M NaOAc, and subsequently equilibrated for 15 min with the starting eluent.

2.4.3. High-performance size-exclusion chromatography

High-performance size-exclusion chromatography (HPSEC) was performed as described before (Vierhuis et al., 2000).

2.4.4. Mass spectrometry

MALDI-TOF MS analysis in the linear mode was performed using a Voyager-DE RP Biospectrometry Workstation (PerSeptive Biosystems) equipped with a nitrogen laser operating at 337 nm (3-ns pulse duration), a single

Table 1
Neutral sugar linkage composition (mol%) of the xyloglucan- and xylan-rich pools of the 1 M KOH extract from olive fruit; within brackets the neutral sugar composition determined by alditol acetates and the uronic acid content determined by *m*-hydroxy-diphenyl assay is given (mole per 100 mol neutral sugars)

Sugar linkage	Xyloglucan		Xylan 1		Xylan 2		Xylan 3		Xylan 4	
Rhamnose										
T-Rha*p*	–		–		–		–		0.3	
Total	–	(1)	–	(2)	–	(2)	–	(2)	0.3	(5)
Fucose										
T-Fuc*p*	0.7		–		–					
Total	0.7	(1)	–	(–)	–	(–)	–	(–)	–	(–)
Arabinose										
T-Ara*f*	10.9		5.0		1.1				1.9	
1,5-Ara*f*	0.6		2.2		–				2.9	
1,3,5-Ara*f*	–		–		–				1.0	
1,2,3,5-Ara*f*	–		–		–				3.0	
Total	11.5	(11)	7.2	(9)	1.1	(1)	–	(1)	8.8	(10)
Xylose										
T-Xyl*p*	15.7		3.7		1.8				1.1	
1,4-Xyl*p*	–		77.1		90.6				65.4	
1,2-Xyl*p*	19.3		–		–				–	
1,2,4-Xyl*p*	–		4.8		3.6				1.6	
1,3,4-Xyl*p*	–		1.8		1.3				0.7	
1,2,3,4-Xyl*p*	–		–		–				13.7	
Total	35.0	(31)	87.4	(79)	97.3	(94)	–	(94)	82.5	(74)
Mannose										
1,4-Man*p*	1.4		–		–				–	
Total	1.4	(2)	–	(1)	–	(1)	–	(1)	–	(1)
Galactose										
T-Gal*p*	8.6		0.7		–				0.3	
1,4-Gal*p*	0.6		–		–				–	
1,3,6-Gal*p*	–		2.2		–				–	
1,2,3,4,6-Gal*p*	–		–		–				0.6	
Total	9.2	(11)	2.9	(7)	–	(1)	–	(1)	0.9	(3)
Glucose										
1,4-Glc*p*	13.7		1.3		0.9				4.9	
1,4,6-Glc*p*	28.6		1.1		–				–	
1,2,3,4,6-Glc*p*	–		–		0.7				2.7	
Total	42.3	(43)	2.4	(2)	1.6	(1)	–	(1)	7.6	(7)
Uronic acid										
Total	nd[a]	(1)	nd	(16)	nd	(15)	nd	(12)	nd	(14)
Ratio terminal/branching	1.25		0.96		0.43		–		0.08	

[a] Not determined.

stage reflector and delayed extraction. The accelerating voltage used was 12 kV and the delay time setting was 200 ns. Each spectrum was produced by accumulating data from 100–256 laser shots. Sample preparation: the matrix solution was prepared by dissolving 9 mg 2,5-dihydroxybenzoic acid and 3 mg 1-hydroxy isoquinoline in 700 μl distilled water and 300 μl acetonitril. A 1 μl volume of this solution was placed on the sample plate and mixed with 1 μl of the enzyme digest and allowed to dry at room temperature. Mass spectra were calibrated with an external standard containing cellodextrins (DP 3-9).

3. Results and discussion

3.1. Chemical characterisation

In the previous paper, we have described the fractionation by anion-exchange chromatography of hemicellulosic polysaccharides extracted from olive fruit with 1 and 4 M KOH (Vierhuis et al., 2000). The extracts had similar elution behaviours on DEAE Sepharose and all contained a xyloglucan-rich pool and four xylan-rich pools. The xyloglucan-rich pool represented 20 and 61% of the sugars present in

the 1 and 4 M KOH extract, respectively. Glc, Xyl, Ara and Gal were identified as the major neutral sugar residues of the xyloglucan-rich pools. The four xylan-rich pools all bound to the column and were eluted by a sodium acetate gradient or by alkali. The major fraction bound to the DEAE column was xylan 1, which eluted as soon as the acetate gradient was applied. It comprised 40% of the xylans in the extract. The other xylan-rich pools were designated according to their order of elution from the anion-exchange column as xylan 2, 3 and 4. The xylan-rich pools contained mainly Xyl residues (65–83 mol%) and small amounts of uronic acids (11–18 mol%). The uronic acids of xylan 1, 2 and 3 comprised mainly 4-*O*-MeGlcA, whereas the uronic acids in xylan 4 appeared to be almost exclusively GalA indicating the presence of pectins. The presence of acetyl groups in olive fruit xyloglucan and xylan could not be determined due to the use of alkali to extract the hemicelluloses, which saponified the ester-linkages.

The glycosidic linkage compositions of the pools obtained from the 1 M KOH extract are shown in Table 1. Unfortunately, no sugar linkage analysis could be performed of xylan 3 because the amount of material was too low. Of this pool only the neutral sugar composition determined by alditol acetates (within brackets) is given. The sugar linkage analysis confirmed the presence of a xyloglucan and xylans. The sugar compositions derived from the sugar linkage analyses were in good agreement with the results from the analyses of alditol acetates. Only the Rha and Gal contents determined by methylation analysis were rather low for all pools. Despite of the good correlation between the sugar composition found after per-*O*-methylation and the composition determined by alditol acetates, the ratio of terminal over branched residues deviated from 1 for a number of pools. This indicates undermethylation, which is generally observed for uronide containing polysaccharides.

The xyloglucan-rich pool contained 1,4- and 1,4,6-linked Glc residues typical for the cellulosic backbone of xyloglucans. About 70% of the Glc residues of the backbone were branched compared to the value of 55% reported previously for a xyloglucan isolated from olive fruit (Gil-Serrano & Tejero-Mateo, 1988). Terminal Xyl and 1,2-linked Xyl residues were present in almost similar amounts. No 1,4-linked Xyl residues typical for the presence of xylans were detected in this pool. Almost all Ara and Gal residues were present as terminal residues. Very small amounts of 1,5-linked Ara and 1,4-linked Gal indicated that the xyloglucan-rich pool was slightly contaminated with arabinan and galactan. The data in Table 1 further indicated contamination with a small amount of 1,4-linked Man residues. Terminal Fuc residues suggested that also small amounts of α-L-Fuc*p*- containing side chains (<1%) could be present in olive fruit. However, the specific Gal 1,2-linkage present in this side chain could not be detected in the sugar linkage analyses. No Glc residues with a substitution at C-2 could be detected. This indicated that substitution of the backbone with α-L-Ara*f*-(1,2), β-D-Xyl*p*-(1,2) or α-L-Ara*f*-(1,3)-β-D-Xyl*p*-(1,2) at C-2 of the Glc residue (Hisamatsu, York, Darvill & Albersheim, 1992; Kiefer, York, Albersheim & Darvill, 1990) did not occur in olive fruit xyloglucan.

Xylan 1, 2 and 4 were mainly composed of 1,4-linked Xyl residues. Less than 10% of the Xyl residues were monosubstituted with branch points at the C-2 or C-3 position. No double-branched Xyl residues could be detected in xylan 1 and 2. Xylan 4 consisted of about 14% of unmethylated Xyl residues. These residues could be present as disubstituted Xyl, but it is more likely that some undermethylation has occurred. Especially, since also part of the Ara, Gal and Glc residues appeared to be present as unmethylated alditol acetates. Previous results showed that the xylan-rich pools contain 4-*O*-MeGlcA and GlcA which are expected to be attached to the backbone (Vierhuis et al., 2000). Xylan 1 contains GlcA as well as 4-*O*-MeGlcA residues that are most certainly attached to C-2 of the Xyl residues (Gil-Serrano et al., 1986). Xylan 2 and 3 contain mainly 4-*O*-MeGlcA. The uronic acids of xylan 4 appear to be almost exclusively GalA indicating that pectins are also present in this pool. Only 16% of the uronic acids present in xylan 4 were GlcA and 4-*O*-MeGlcA. The substituents on the xylan backbone are besides 4-*O*-MeGlcA and GlcA residues probably also single unit Ara residues for xylan 1, 2 and 4. The exact amount of branch points was difficult to estimate from the sugar linkage analyses of the xylan-rich pools because the uronic acid residues were not reduced and thus not included in the methylation analysis. Probably, only a small part of the very acid resistant 4-*O*-MeGlcA-Xyl and GlcA-Xyl linkages are hydrolysed in the procedure followed since TFA hydrolysis after per-*O*-methylation is not able to completely hydrolyse the acid resistant uronic acid glycosyl linkages (Vierhuis et al., 2000). In general, carboxyl reduction is used to determine the glucuronic and galacturonic acid as deuterated Glc and Gal in the sugar linkage analysis. The reduction makes the glycosyl linkages more susceptible to acid hydrolysis but it is also known that carboxyl reduction is often not complete and gives a poor recovery of the uronic acids (Coimbra et al., 1995; Verbruggen, Beldman & Voragen, 1995). Despite of the fact that the precise percentages of branch points could not be determined by the method followed, it can be concluded that the xylans extracted from olive fruit were low in substitution using data from a previous study (Vierhuis et al., 2000) for an estimation of the amounts of uronic acids present.

Coimbra et al. (1995) have reported the occurrence of a complex containing glucuronoxylan and xyloglucan in the 1 M KOH fraction from olive fruit. The extraction procedure we performed was not exactly identical to their method but we expected that the material they extracted with 1 M KOH at 1°C would appear in our fraction of 1 M KOH at 20°C. However, we were not able to confirm the occurrence of the complex containing glucuronoxylan and xyloglucan. Anion-exchange chromatography of our material resulted in pools that contained either xyloglucan or xylans, but not a

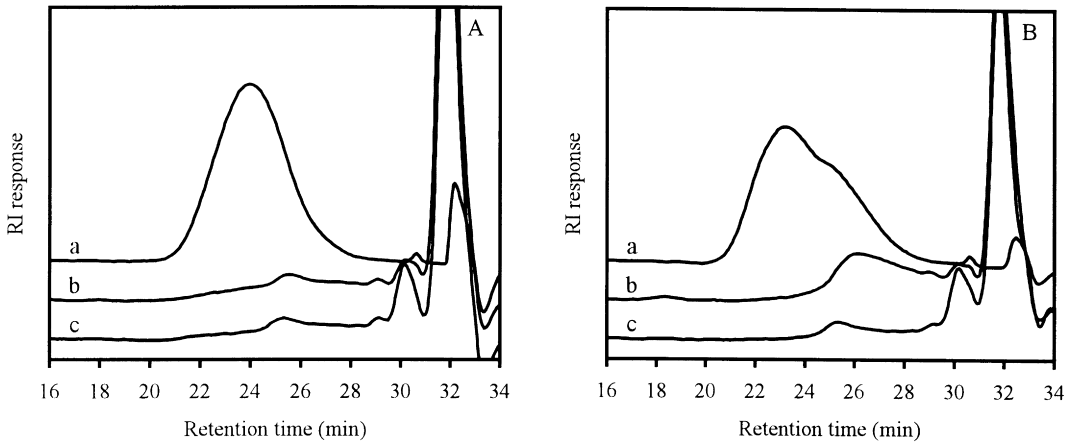

Fig. 1. HPSEC-patterns of the xyloglucan-rich pools of the 1 M KOH (A) and 4 M KOH (B) extract from olive fruit (a) before incubation and after incubation with (b) endo-glucanase IV (EndoIV) and (c) endo-glucanase V (EndoV).

mixture of both of them. The xylan-rich pools did contain some (1,4)- and (1,4,6)-linked Glc, but only in very small amounts compared to the amounts described by Coimbra et al. (1995).

3.2. Enzymatic characterisation

More information about the structure of the xyloglucan present in the 1 and 4 M KOH extract was obtained by incubating them with two endo-glucanases from *Trichoderma viride* having a high xyloglucanase activity, EndoIV and EndoV. Both enzymes cleave the glucan backbone next to an unbranched Glc residue but have different subsites (Vincken, Beldman & Voragen, 1997a). The HPSEC elution profiles of the incubations are shown in Fig. 1. The elution pattern of the xyloglucan-rich pool isolated from the 1 M KOH extract showed a major population with a molecular mass of about 150 kDa as based on calibration with dextrans. Incubation with EndoIV or EndoV degraded the xyloglucan entirely into oligosaccharides. The elution pattern of the xyloglucan-rich pool isolated from the 4 M KOH extract showed a major population with a molecular mass of about 150 kDa and in addition a smaller population of about 50 kDa. HPSEC analysis of this xyloglucan-rich pool treated with EndoIV showed a residual population, which probably consisted of a Man-containing polysaccharide. The same phenomenon has been described for a xyloglucan-rich fraction isolated from potato with a mannan contamination (Vincken, Wijsman, Beldman, Niessen & Voragen, 1996a). Incubation with EndoV degraded both populations present in this pool entirely into oligosaccharides. Apparently, EndoV was able to degrade the Man-containing polysaccharide of the xyloglucan-rich pool. It is not sure whether EndoV is able to cleave this polysaccharide or it contains a residual endo-mannanase activity (Vincken et al., 1996a).

The elution patterns on HPAEC of the digests of the xyloglucan-rich pools showed a rather complicated mixture of oligosaccharides with two major peaks at about 52 and 54 min (Fig. 2). Products eluting between 7 and 15 min

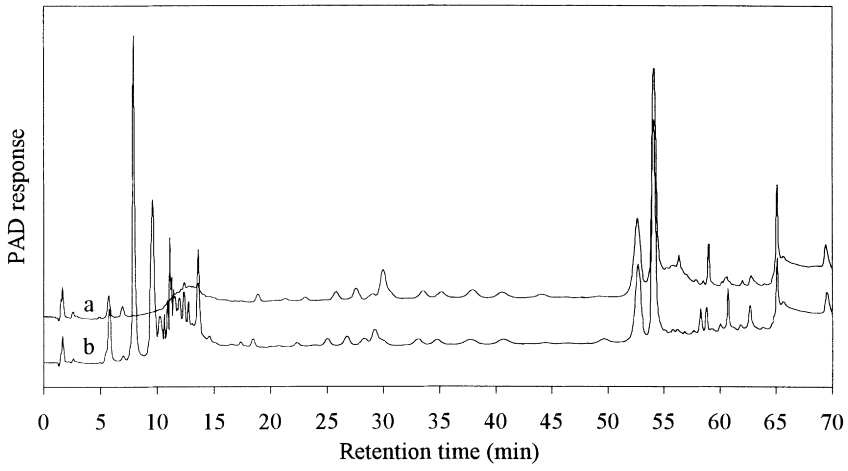

Fig. 2. HPAEC-patterns of the xyloglucan-rich pool of the 4 M KOH extract from olive fruit digested with (a) endo-glucanase IV (EndoIV) and (b) endo-glucanase V (EndoV).

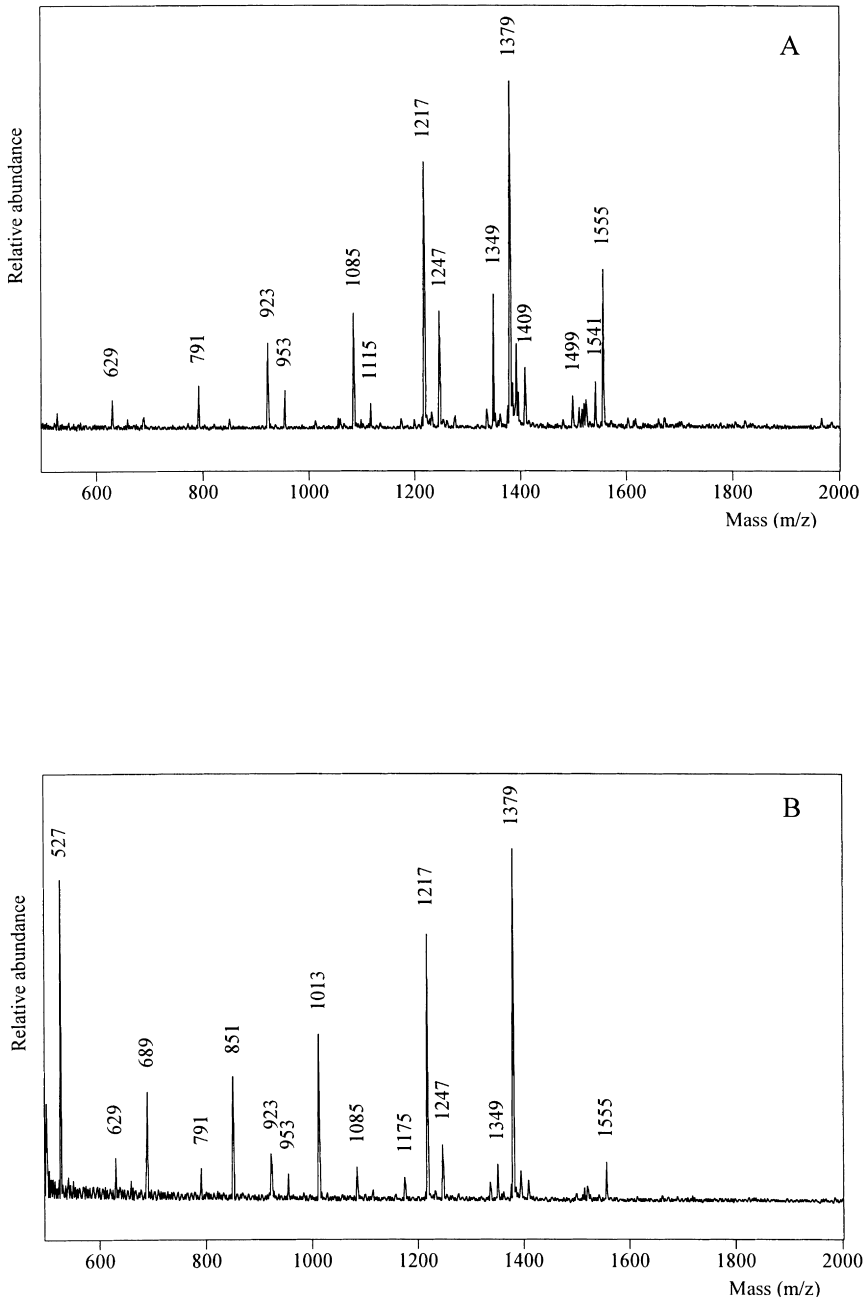

Fig. 3. MALDI-TOF mass spectra of the xyloglucan-rich pool of the 4 M KOH extract from olive fruit digested with (A) endo-glucanase IV (EndoIV) and (B) endo-glucanase V (EndoV).

probably originated from the mannan contamination because they mainly appeared in the xyloglucan-rich pool from the 4 M KOH extract incubated with EndoV. Analysis on HPAEC of a galactomannan digest confirmed that Man-containing oligosaccharides eluted in this region. The degradation patterns of the xyloglucans isolated from the 1 and 4 M KOH extract were identical between 15 and 55 min. Only a small amount of free Glc was produced on enzymic hydrolysis (ca 1–2% of Glc present). Degradation of the xyloglucan-rich pools by EndoIV and EndoV gave identical products although small differences in the relative amounts of products were noticed. Comparison of the degradation patterns of olive fruit xyloglucan with degradation patterns of xyloglucans with known structural features (apple: XXXG core with Gal residues; potato: XXGG core with Gal and Ara residues in a ratio of 1/1) incubated with the same endo-glucanases (Vincken et al., 1996a; Vincken, Beldman, Niessen & Voragen, 1996b) indicated that xyloglucan in olive fruit had a different substitution pattern.

The MALDI-TOF mass spectra of the digests of the xyloglucan-rich pool from the 4 M KOH extract are given in Fig. 3. Both spectra showed a diversity of masses. Tentative

Table 2
Data on MALDI-TOF mass spectra analysis of the xyloglucaan-rich pools of the 1 and 4 M KOH extracts from olive fruit incubated with endo-glucanase IV (EndoIV) and endo-glucanase V (EndoV)

Mass		1 M KOH		4 M KOH		Tentative structures[a]
$(M+Na^+)$		EndoIV	EndoV	EndoIV	EndoV	
527	$(Hex)_3$	nd[b]	+	nd	+++	Mannan oligosaccharide
851	$(Hex)_5$	nd	w	w	++	Mannan oligosaccharide
1013	$(Hex)_6$	nd	w	w	++	Mannan oligosaccharide
1085	$(Hex)_4(Pent)_3$	+	w	+	w	XXXG/G[SX]G/[SX]GG
1217	$(Hex)_4(Pent)_4$	+++	+++	+++	+++	X[SX]G/GSSG/SSGG
1247	$(Hex)_5(Pent)_3$	+	+	++	+	X[LX]G/G[SL]G/[SL]GG
1349	$(Hex)_4(Pent)_5$	+	w	++	w	XSSG
1379	$(Hex)_5(Pent)_4$	+++	+++	+++	+++	X[SL]G/GSSGG
1555	$(Hex)_6(Pent)_3(Deoxyhex)_1$	+	w	++	w	X[LF]G

[a] Nomenclature according to Fry et al. (1993) with specific code letters for each segment (G: β-D-Glc*p*-; X: α-D-Xyl*p*-(1, 6)-β-D-Glc*p*-; L: β-D-Gal*p*-(1, 2)-α-Xyl*p*-(1, 6)-β-D-Glc*p*-; S: α-L-Ara*f*-(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*-).
[b] +++, ++, +, w and nd denote high, medium, minor, weak and not detectable peaks in the spectra.

structures were proposed for the different oligosaccharides considering the glycosidic linkage composition of the xyloglucan-rich pool, the mode of action of the endo-glucanases and the molecular masses of the oligosaccharides in the digests (Table 2). The tentative structures of the masses with an accumulated intensity of at least one third of the main peak (*m*/*z* 1379) in the mass spectra are shown to emphasise the main characteristics of the digests. It should be kept in mind that these intensities do not necessarily have to correlate completely with the amount of material present in the digests although it is thought that peaks with the highest intensities are the most abundant. Also it is not possible to compare the intensities of different spectra because the intensity may be influenced by many factors like concentration on a specific spot, laser intensities, average scans, etc.

The mass spectra of the digests showed two major components with a mass that corresponded to a sodium adduct of oligosaccharides from xyloglucans. Oligosaccharide structures originating from xyloglucans composed of either XXXG-type or XXGG-type building units could be proposed. In principle, there were three possible structures for $(Hexose)_4(Pentose)_4$: X[SX]G, GSSG and SSGG. For $(Hexose)_5(Pentose)_4$ two structures could be proposed: X[SL]G and GSSGG. The structural elements containing Ara (S) and Gal (L) were indicated within brackets to indicate that the exact position of these residues was not known.

Based on the sugar linkage composition, the gross formula of $(Hexose)_5(Pentose)_4$ could represent two possible structures, X[SL]G and GSSGG, as mentioned before. However, from literature it is known that EndoV is able to release Glc from the reducing end of oligosaccharides with two unbranched Glc residues (Vincken et al., 1996a). Consequently, the major peak with *m/z* 1379 was not likely to correspond to GSSGG in the EndoV digest. Re-incubation of the EndoV digest showed that the profile of the mass spectrum did not change which further substantiated that X[SL]G and not GSSGG was present as a major compound in the digest. The fact that very low amounts of free Glc were detected in the fractions treated with endo-glucanases was also in agreement with the above results.

The other major component in the mass spectrum (*m*/*z* 1217) could represent structures of oligosaccharides from xyloglucan of the XXGG-type as well as the XXXG-type. In case of a structure with XXGG-type building units, SSGG and GSSG could be present as a major component in the digests of EndoIV and EndoV, respectively. In case of XXXG-type building units both enzymes would have released X[SX]G. So, evidence about the branching pattern of the xyloglucans present in olive fruit could not be obtained from this *m*/*z* value.

For several components in the mass spectra, it was not possible to compose a structure of XXXG-type as well as XXGG-type building units. For example, the peak with *m*/*z* 1349 with a gross formula of $(Hexose)_4(Pentose)_5$ suggested almost certainly an XXXG-type building unit with two Xyl residues substituted with Ara residues. An XXGG-type building unit with a short-side chain of Ara residues might be a possibility but has not been published yet (Vincken et al., 1997b). Also for the oligosaccharide with *m*/*z* 1555 the structure X[LF]G with a XXXG-type building unit is more likely than a structure with an XXGG-type building unit. Oligosaccharides with Fuc residues like XLFG have been reported before in literature. Besides XLFG also XXFG and XFFG have been described (Hisamatsu, Impallomeni, York, Albersheim & Darvill, 1991; York, Halbeek, van Darvill & Albersheim, 1990). The spectrum of the EndoV digest of the xyloglucan-rich fraction from the 4 M KOH extract contained besides the masses characteristic for xyloglucan oligosaccharides also peaks with a high intensity consisting of only hexose residues. These peaks most probably originated from glucomannan or galactomannan oligosaccharides formed by EndoV. The absence of these peaks in the EndoV digest of the 1 M KOH

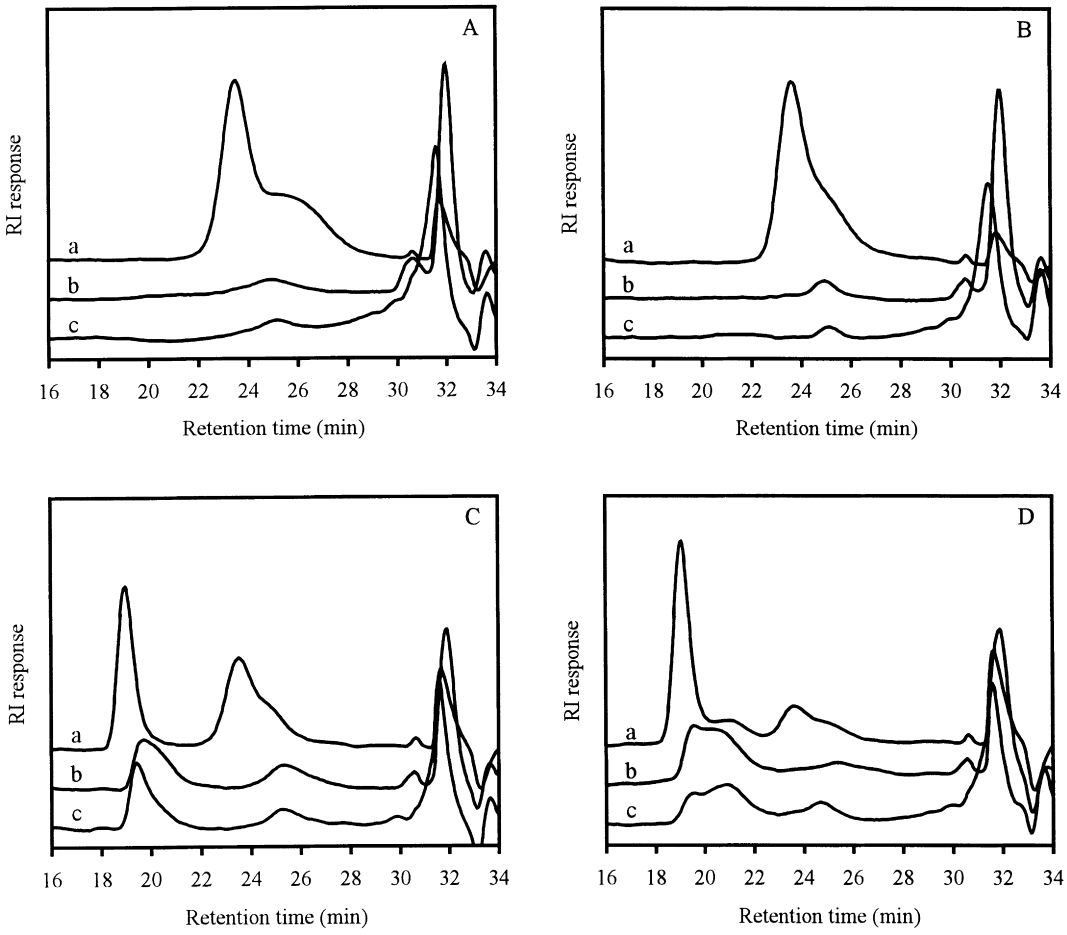

Fig. 4. HPSEC-patterns of the xylan-rich pools of the 1 M KOH extract from olive fruit (a) before incubation and after incubation with (b) endo-xylanase I (XylI) and (c) endo-xylanase III (XylIII): (A) xylan 1; (B) xylan 2; (C) xylan 3; and (D) xylan 4.

extract and the EndoIV digests confirmed this observation.

The data of the MALDI-TOF mass spectra combined with the sugar linkage composition and the knowledge of the mode of action of both endo-glucanases indicated the presence of a xyloglucan in olive fruit with a different structure compared to xyloglucan from other plant sources. Based on the results, it can be concluded that olive fruit contained a xyloglucan with an XXXG core with Gal as well as Ara residues linked to the Xyl residues. The presence of terminal Ara as well as terminal Gal residues are usually described for xyloglucans with an XXGG core which are present in various solanaceous plants (Vincken et al., 1997b). Xyloglucans isolated from other dicotyledonous plants in general have XXXG-type building units and terminal Ara residues attached to Xyl residues have not been described very often. Side chains of α-L-Ara*f*-(1,2)-α-D-Xyl*p*-(1,6)- have been suggested to be present in xyloglucan from non-solanaceous plants as runner bean (O'Neill & Selvendran, 1983) and tamarind (Niemann, Carpita & Whistler, 1997) but no clear evidence has been reported (Vincken et al., 1997b). However, the presence of especially the peak with *m/z* 1379 as a major component in the digest and the other peaks with masses that could only correspond to an oligosaccharide with a structure of the XXXG-type, supported the finding that olive fruit xyloglucan consisted of XXXG-type building units with terminal Ara as well as terminal Gal residues linked to it.

More information about the xylan-rich pools isolated from the 1 and 4 M KOH extract was obtained by incubation with two different endo-xylanases from *Aspergillus awamori*, XylI and XylIII. Kormelink, Gruppen, Vietor and Voragen (1993a) have shown clear differences in the mode of action of these two endo-xylanases. The enzymes are in a different way restricted in the hydrolysis of xylosidic linkages in the vicinity of branch points, which is reflected in many types of heterogenous oligosaccharides released. In general, it can be concluded that XylIII is more hindered by substitution of the xylan backbone than XylI. The digests of xylan-rich pools were subjected to analysis by HPSEC, HPAEC and MALDI-TOF MS. It appeared that the results of the analyses were identical for the xylans isolated from the 1 and the 4 M KOH extract, therefore only the results of the xylan-rich pools isolated from the 1 M KOH extract will be discussed in the next paragraphs.

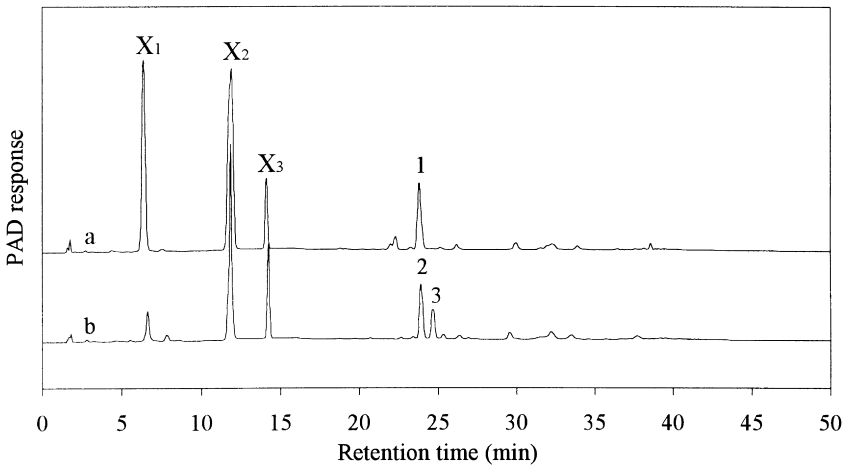

Fig. 5. HPAEC-patterns of xylan 1 of the 1 M KOH extract from olive fruit digested with (a) endo-xylanase I (XylI) and (b) endo-xylanase III (XylIII). Xylan oligosaccharides (X1, X2 and X3) are denoted above their corresponding peaks; the peak numbers 1, 2, and 3 correspond to tentative structures discussed in the text.

The HPSEC elution pattern of xylan 1 showed a major population with a molecular mass of about 150 kDa which corresponded to a DP of ca 1100 as based on calibration with dextrans (Fig. 4). The shoulder of the main peak in the elution pattern indicated the presence of another population in this pool with a molecular mass of about 25 kDa. Incubation with XylI as well as XylIII resulted in a shift to lower molecular mass ranges of the material in this pool. Only 5–10% of the polymers remained as material with a high-molecular mass indicating that the substitution pattern of the xylans hardly hindered the action of the endo-xylanases. Xylan 2 had an elution pattern quite similar to xylan 1 and XylI and XylIII were also able to degrade this xylan-rich pool almost entirely. Xylan 3 and 4 both contained a population which eluted in the void volume of the column set used so that no exact molecular mass could be determined. The molecular mass of these populations was at least 500 kDa. Unlike the first two xylan-rich pools, xylan 3 and 4 were less degradable to fractions with a low-molecular mass. Although XylI is less hindered by substitution than XylIII both enzymes were not able to degrade these pools completely. This might indicate that these pools contained besides low-substituted xylans also highly substituted xylans. The xylans with highly branched regions could be interlinked with more linear degradable regions but the existence of different populations of xylans in the partly degradable pools was also possible.

Xylan 3 and 4 were incubated with XylI on a larger scale to investigate the composition of the undegradable polymers. The residual polymeric material was isolated to yield a XylI-treated xylan 3 and a XylI-treated xylan 4 fraction. Characterisation of the XylI-treated xylan 3 showed that the sugar composition was identical to that of the original xylan 3 (no further data shown). The sugar composition of the XylI-treated xylan 4 contained relatively more pectic material than the original xylan 4 (factor 1.5), but low-substituted xylans were still the main part of the polysaccharides in this pool (no further data shown). These data indicated that the structure of the xylans could not explain the restriction of both xylanases towards these substrates but that another factor was involved. Measurement of the A_{280} signal during the elution on the DEAE Sepharose column showed that all xylan-rich pools exhibited UV absorption. Especially, xylan 3 and 4 contained a significant amount of UV absorbing material. Therefore, the presence of lignin-like material or proteins (A_{280}) in these pools might explain the incomplete degradability of the residual xylans.

XylI degradation resulted in very similar elution patterns on HPAEC for all xylan-rich pools. The degradation products as well as the relative amounts were identical, showing that the same oligomers were formed in the same amounts. The degree of degradation of the pools by XylI differed: XylI gave a two times higher degree of degradation of xylan 1 and 2 compared to xylan 3 and 4. Analysis of the xylan-rich pools incubated with XylIII on HPAEC showed that also in this case the degradation products as well as the relative amounts were identical for all pools. In Fig. 5, it can be seen that XylIII released mainly xylobiose and xylotriose and smaller amounts of Xyl monomer, while XylI degraded the xylan mainly into Xyl monomer and xylobiose. This was in agreement with the results of Kormelink et al. (1993a) and confirmed the difference in the mode of action and substrate specificity of XylI and XylIII. Besides the main end-products Xyl monomer, xylobiose and xylotriose, XylI released another major component which eluted around 24 min (peak 1) whilst XylIII released oligomers eluting at 24–25 min (peaks 2 and 3).

MALDI-TOF MS was employed to determine the molecular masses of the unknown oligosaccharides. The spectra of xylan 1 incubated with XylI and XylIII are depicted in Fig. 6. The spectrum of xylan 1 incubated with XylI showed a main peak at *m/z* 627 that corresponded to a 4-*O*-MeGlcA linked to three pentose residues. Although mass analysis

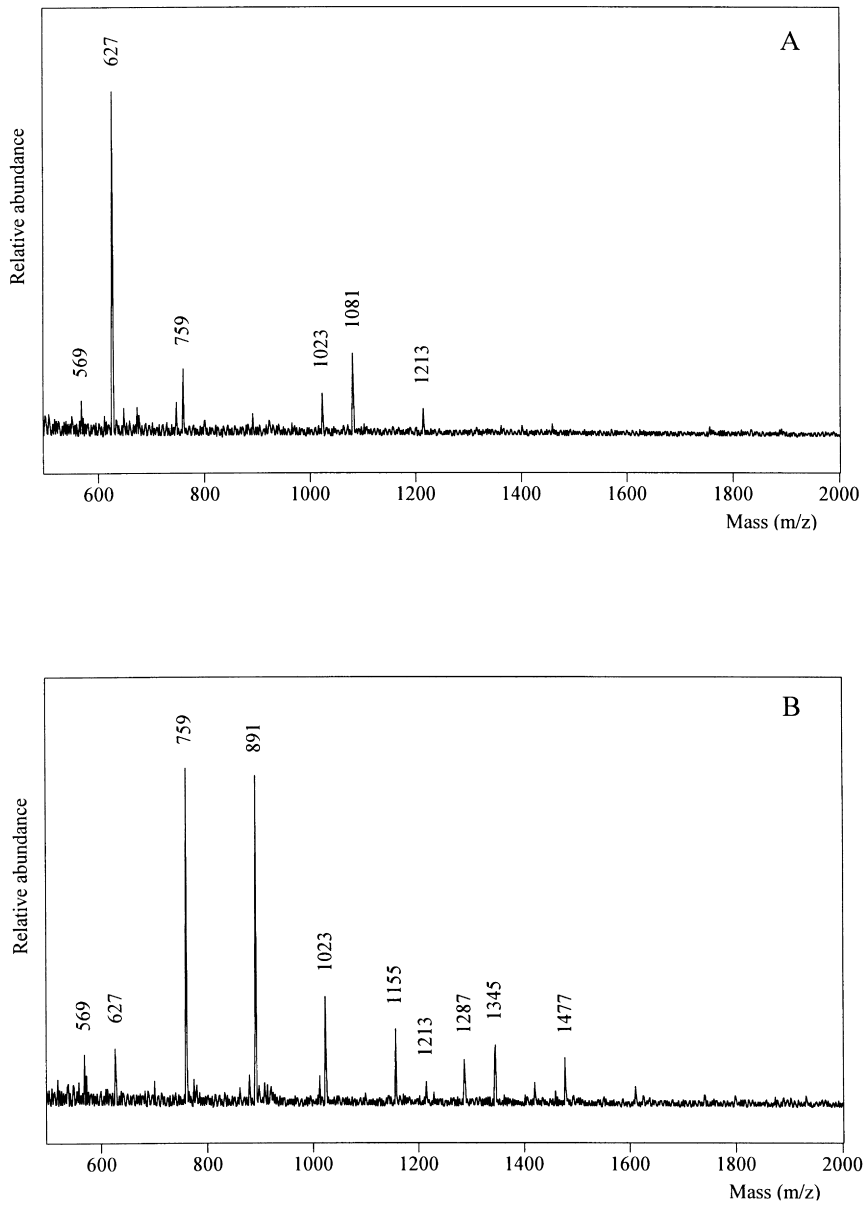

Fig. 6. MALDI-TOF mass spectra of xylan 1 of the 1 M KOH extract from olive fruit digested with (A) endo-xylanase I (XylI) and (B) endo-xylanase III (XylIII).

could not differentiate between the pentoses Ara or Xyl, the mode of action of XylI pointed to a xylotriose substituted with 4-*O*-MeGlcA at the non-reducing terminus. XylI is not able to remove at least two unsubstituted Xyl residues towards the reducing end adjacent to the Xyl residue substituted with GlcA (Kormelink et al., 1993a; Verbruggen et al., 1998b). Although the mode of action of the XylI towards a 4-*O*-MeGlcA linked to the xylan backbone has not been revealed yet, we expected that this enzyme would act similarly on a backbone substituted with a 4-*O*-MeglcA residue instead of a GlcA residue. The main peak at *m*/*z* 627 in the mass spectrum corresponded almost certainly to peak 1 on HPAEC, which was the only major compound detected besides Xyl monomer, xylobiose and xylotriose. Besides the main peaks, the HPAEC patterns as well as the MALDI-TOF MS spectra also contained smaller peaks. Probably of xylo-oligomers containing besides 4-*O*-MeGlcA side groups also Ara side groups. The glycosidic linkage composition of the xylan-rich pools especially xylan 1 gave indications for the presence of Ara residues linked to the xylan backbone. However, no major peaks corresponding to Ara-rich oligomers were detected on HPAEC or in the MALDI-TOF MS spectra. Probably, several xylo-oligomers with Ara substituents were formed but all in very small amounts. Also part of the undegradable material of xylan 1 might have consisted of an Ara-rich polymer.

The MALDI-TOF MS spectrum of olive fruit xylan incubated with XylIII showed two main peaks, which probably

corresponded to peaks 2 and 3 on HPAEC. The masses were *m*/*z* 759 and 891 equal to an oligomer with a 4-*O*-MeGlcA linked to four and five pentoses, respectively. XylIII is not able to remove at least two unsubstituted Xyl residues adjacent to a substituted Xyl residue towards the reducing end (Kormelink et al., 1993a). Thus, it could be concluded that these oligomers consisted most certainly of 4-*O*-MeGlcA residues linked to the third Xyl residue counting from the reducing end of a xylotetraose and xylopentaose.

Although clear differences in the mode of action of XylI and XylIII were observed towards wheat and barley arabinoxylans (Kormelink et al., 1993a) and sorghum glucuronoarabinoxylan (Verbruggen et al., 1998a), both enzymes were able to degrade olive fruit glucuronoxylan to the same extent. The substitution of the backbone with 4-*O*-MeGlcA residues and perhaps Ara residues did not hinder both endoxylanases which suggested that the substituents were distributed very evenly over the xylan backbone. The results also confirmed earlier observations that the xylans extracted from olive fruit are low in substitution.

4. Conclusions

The glycosidic linkage composition of the xyloglucan-rich pools and the enzymatic degradation with endo-glucanases indicated the presence of an arabinogalactoxyloglucan with an XXXG core in olive fruit. The substitution of Xyl residues with Ara as well as Gal residues is commonly described for xyloglucans belonging to the poly-XXGG group but has only been suggested before in literature for xyloglucans belonging to the poly-XXXG group. No differences in the branching patterns of the xyloglucans extracted with a 1 or a 4 M KOH solution could be noticed.

Based on the sugar linkage composition and the incubation with two endo-xylanases, it can be concluded that the xylans in olive fruit were low in substitution. Although anion-exchange chromatography of the 1 and 4 M KOH fractions resulted in four xylan-rich pools, the structures of these xylans were almost identical as concluded from the results of the degradation studies with endo-xylanases. The 4-*O*-MeGlcA substituents of the xylans in olive fruit were probably rather regular distributed which resulted in identical patterns on HPAEC for all endo-xylanase digests of the xylan-rich pools. The differences in elution on DEAE Sepharose were most likely explained by the presence of proteins or lignin-like material (A_{280}), which not only affected the elution on DEAE Sepharose but also the degradability of two of the xylan-rich pools.

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