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Investigation of the occurrence of xylan–xyloglucan complexes in the cell walls of olive pulp (*Olea europaea*)

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Previously we reported the possible occurrence of a complex containing glucuronoxylan and xyloglucan in the cell walls of olive pulp. In order to investigate the nature of this complex, the 1 M KOH (1°C)-soluble polysaccharides in which it was prevalent, were separated by graded ethanol precipitation followed by anion-exchange chromatography. A slightly acidic fraction was obtained and, by methylation analysis, glycosidic linkages typical of both xylan and xyloglucan were detected. Two distinct populations of the xylan–xyloglucan complexes were resolved by gel-filtration chromatography (2000 and 100 kDa) and the structural features were determined by methylation analysis. Cross-linking of the xylan–xyloglucan moieties was investigated by digestion of the xylan component with a purified, specific, endoxylanase. Although only the xylan element was digested, as verified by methylation analysis, the molecular weight of the xyloglucan moiety was also reduced. This confirmed that the xylan and xyloglucan moieties were strongly attached. The occurrence and structure of the xylan–xyloglucan complexes in the olive pulp cell walls is discussed.

INTRODUCTION

The plant cell wall consists of cellulose microfibrils within a matrix of non-cellulosic polysaccharides, glycoproteins and phenolics, the types and composition of which are related to the maturity and function of the cell and the plant type. Most non-cellulosic cell wall components are held into the microfibrillar array by a combination of cross-links, including those which are covalent and non-covalent (Fry, 1986). Some information may be elucidated by investigating the extraction properties of the component polymers (Selvendran, 1985; Redgwell & Selvendran, 1986). Many of these cross-links have been shown to change during physiological events such as stem extension (Waldron & Selvendran, 1992) and it is generally accepted that control of the location and metabolism of cell wall interpolymeric cross-links may constitute key mechanisms by which cell growth and extension are regulated (Fry, 1986). However, there is a paucity of information concerning the nature and extent of covalent cell wall cross-links and the polysaccharides involved.

During the isolation and characterisation of cell wall polymers from olive pulp (Coimbra *et al.*, 1994), we reported the possible occurrence of a complex containing mainly a glucuronoxylan and a xyloglucan in the 1 M KOH (1°C) extract. We have now carried out further fractionation studies on this polysaccharide and have partially characterised two of the fractions after digestion with a highly purified, specific xylanase. The results of these investigations are reported in this paper.

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MATERIALS AND METHODS

General

Neutral sugars were released by Saeman hydrolysis and analysed (Selvendran *et al.*, 1979) as their alditol acetates by gas–liquid chromatography (GLC). Uronic acids were determined colorimetrically by a modification (Selvendran *et al.*, 1989) of the method of Blumenkrantz and Asboe-Hansen (1973).

Preparation and sequential extraction of cell wall material (CWM)

CWM of olive pulp (var. Douro) and the 1 M KOH 1 C (hemicellulose B) extract were from the batch previously prepared (Coimbra *et al.*, 1994).

Graded precipitation with ethanol

The frozen, stored material of the 1 M KOH 1 C (hemicellulose B) extract (390 mg), was thawed, dispersed in water (40 ml) and stirred overnight at 1°C. The insoluble material was separated by centrifugation and dispersed in 1 M NaOH for 2 h at 1°C. The materials solubilised in water and in 1 M NaOH (10 mg/ml) were submitted separately to graded precipitation with ethanol. The alcohol concentration was increased in steps of between 10 and 20% (Selvendran & King, 1989). Each mixture was placed at 4°C for 1 h and the precipitate was collected by centrifugation. In order to remove the ethanol completely, each precipitate was dissolved in water and rota-evaporated (Coimbra *et al.*, 1994). The alkali fractions were neutralised with acetic acid and dialysed. All fractions were freeze-dried.

Isolation of xylan–xyloglucan complexes

(i) The material precipitated with 30% ethanol (100 mg) was suspended in water (50 ml) and stirred at 20°C. A small insoluble residue (5 mg) was removed by centrifugation and potassium phosphate buffer (pH 6.5) was added to the supernatant to a final concentration of 50 mM and 1 mg/ml of material. The solution was eluted from a column of DEAE–Trisacryl M (6 × 1 cm) at 10 ml/h (Waldron & Selvendran, 1992). The fractions were eluted sequentially with the same volume of buffer and buffer containing 0.125, 0.25, and 0.5 M NaCl. Fractions (2 ml) were collected, the UV absorption at 280 nm was measured and aliquots (20 μl) were assayed for carbohydrate by the phenol–sulphuric acid method (Dubois *et al.*, 1956). The appropriate fractions were combined, dialysed, concentrated and freeze-dried.

(ii) The material not retained in the DEAE-Trisacryl column (fraction X-XG) (65 mg) was suspended in water (15 ml) and stirred at 20°C. A small insoluble residue (4 mg) was removed by centrifugation and to the supernatant, sodium borate (pH 8.0) was added to a final concentration of 30 mM (5 mg/ml). The solution was eluted from a column (30 × 1 cm) of DEAE–Sephacel (borate form), initially with sodium borate buffer (45 ml), then with a linear gradient from 30 mM to 1 M (200 ml) and then with 1.2 M sodium borate. Fractions (3 ml) were collected, monitored and processed as in (i).

Gel-filtration chromatography

Gel-filtration chromatography was performed on a column (100 × 1 cm) of Sephacryl S-400 at a flow rate of 20 ml/h. Samples were dissolved in 0.5–1 ml 50 mM potassium phosphate buffer, pH 6.5, with 0.2 M NaCl. Fractions (2 ml) were collected, monitored, and processed as in (i). To calibrate the column, standard dextrans of 2000, 487, 266, and 72 kDa were used. The internal volume of the column was determined by elution of glucose.

Xylanase purification

Xylanase 5 g (Bioxylanase p78, Biocon (UK) Ltd, Worcester, UK) was dissolved in 20 mM sodium acetate buffer pH 4 and centrifuged at 2000*g* for 30 min to remove insoluble material. The first stage of the purification was similar to that used by Gibson and McLeary (1987) and the solution was applied to a column of CM Sepharose CL6B (35 cm × 2.8 cm) at a flow rate of 4.5 ml/h. The column was washed with buffer (sodium acetate 20 mM, pH 4, 700 ml) then with a gradient of 0.0–0.5 M NaCl in the same buffer. The peak of enzyme activity corresponding to Xylanase 1 (Gibson & McLeary, 1987) which eluted at 0.1–0.15 M NaCl was collected and used in the next stage of the purification. The partially purified enzyme was applied in 5 ml portions to a gel filtration column (Sephacryl S200 superfine, Pharmacia UK, Milton Keynes, UK0 2.2 cm × 145 cm equilibrated in sodium acetate pH 5.0, 50 mM. The column was eluted in this buffer at 4 cm/h and 2 ml fractions monitored for protein and enzyme activity. Most (83%) of the activity applied to the column was recovered in a single peak of activity coinciding with the main protein peak eluted from the column. This was the source of the enzyme used for digestion of xylan-containing polysaccharide complexes.

Enzyme activity of column fractions was determined by incubating an aliquot of the fraction (10 μl) with a solution of xylan from larch (1.0 ml, 0.5% in 0.1 M sodium acetate, pH 5.0) for up to 10 min (depending on the expected enzyme concentration) and then assaying the incubation mixture for reducing sugar using the Nelson Somogyi assay (Somogyi, 1966).

The xylanase was assayed for specific activity and contaminating activities. The partially purified xylanase stock solution was diluted with 0.1 M sodium acetate pH 5 by a factor of 100. This diluted enzyme solution (100 μl) was incubated with a series of carbohydrate substrates (10 ml) at 40°C. Aliquots (1.0 ml) were removed at 5 min intervals for up to 40 min and assayed for reducing sugars (Somogyi, 1966). The substrates were 0.5% solutions in 0.1 M sodium acetate pH 5 of each of the following carbohydrates: xylan ex larch (Aldrich Chemical Co Ltd, UK); galactan ex lupin (Megazyme, Australia); debranched arabinan (Megazyme, Australia);

galactomannan ex carob (Megazyme, Australia); polygalacturonic acid (Fluka Chemicals, UK); carboxymethyl cellulose Na salt (Sigma Chemical Co., UK); ball milled filter paper (Whatman No. 1 filter paper, Whatman International Ltd, UK) and cauliflower stem xyloglucan (Femenia, Selvendran & Waldron, unpublished). The only detectable enzyme activity other than endoxylanase was a carboxymethyl cellulase with an activity of 0.08% of that of the xylanase.

Protein measurement

Protein concentrations were measured using the Lowry protein assay (Layne, 1985) with BSA (Sigma Chemical Co. Ltd, UK) as standard. Protein content of column fractions was monitored by measuring the UV absorption at 280 nm in 1 cm quartz cuvettes using a Uvikon 860 UV spectrophotometer (Kontron Instruments Ltd, UK).

Xylanase digestion

Fractions A1 and C3 (2 mg), obtained from gel-filtration of fraction X–XG2, were digested with xylanase (40 units) in 1 ml 25 mM sodium acetate buffer, pH 5.0. The mixture was allowed to digest at 20°C for 20 h. The digested mixture was heated at 90°C for 10 min, cooled, and centrifuged at 12 000 r.p.m. for 10 min. The supernatant was directly introduced in the Sephacryl S-400 column and eluted as described previously.

Methylation analysis

Polysaccharides were methylated by a modification of the method of Ciucanu and Kerek (1984) and Isogai *et al.* (1985) as described by Coimbra *et al.* (1994).

RESULTS AND DISCUSSION

Fractionation of the 1 M KOH 1°C extract (hemicellulose B)

Purified CWM from olive pulp was sequentially extracted with CDTA, Na_2CO_3, 1 M KOH and 4 M KOH as previously reported (Coimbra *et al.*, 1994). Extraction of the depectinated cell walls with 1 M KOH at 1°C solubilised 8.6% of CWM which, on dialysis and concentration, gave insoluble material (hemicellulose A) and soluble material (hemicellulose B) in the ratio 3:2.

Thawing the frozen, stored hemicellulose B (soluble) gave rise to an additional white precipitate (WPPT) which was recovered by centrifugation and then dissolved in 1 M NaOH. The NaOH-soluble polymers, and those which had remained water soluble (WS), were subjected to graded ethanol (EtOH) precipitation (Table 1). Both the 75% EtOH-precipitated fraction of WS (WS75) and the 30% EtOH-precipitated fraction of the NaOH-soluble WPPT (WN30) were rich in xylose, with significant quantities of glucose, arabinose and uronic acid consistent with the presence of a complex or complexes containing acidic xylans and xyloglucans associated with small amounts of pectic polysaccharides. Since WN30 contained much less contaminating pectic polysaccharides, this fraction was investigated further.

To obtain supporting evidence for the occurrence of xylan–xyloglucan complexes in WN30, the dialysed, buffer-soluble material was first separated on DEAE-Trisacryl M, giving rise to four fractions. The major fraction (X–XG), which was eluted with buffer, accounted for 69% of the applied material. The carbohydrate composition of the eluted fractions are shown

Table 1. Sugar composition of fractions from solubilised 1 M KOH at 1°C (soluble fraction)

Fraction total	Recovery (%) (of 1 M KOH 1°C)	Cell wall sugars (mol%)								Total sugar[a] (μg/mg)
		Rha	Fuc	Ara	Xyl	Man	Gal	Glc	UA	
1 M KOH 1°C (hemicellulose B) WS										
EtOH 50% (WS50)	11.6	1	1	15	32	1	9	34	7	904
EtOH 75% (WS75)	22.8	1	t	13	50	2	4	13	17	929
EtOH 75% soluble (WS75S)	2.6	5	t	34	29	2	6	5	19	185
WPPT NaOH soluble										
EtOH 30% (WN30)	24.2	1	t	16	56	1	4	15	7	907
Insoluble residue 2	5.0	1	1	4	75	t	1	3	15	858
Buffer (X–XG)	69.0	1	1	13	58	t	4	14	9	920
0.125 M NaCl	9.0	2	1	48	26	t	4	4	15	549
0.25 M NaCl	5.0	3	1	60	11	1	5	5	14	593
0.5 M NaCl	5.0	6	—	34	11	2	3	9	35	139
EtOH 50% (WN50)	2.6	1	t	17	36	9	9	24	4	913
EtOH 50% soluble (WN50S)	3.7	3	1	38	17	3	15	9	14	212
Insoluble residue 1	32.1	3	t	69	2	t	5	3	18	849

[a]Values are expressed as μg anhydrosugar per mg dry polymers. t, trace; WS, water-soluble; EtOH, ethanol-soluble; WPPT, white precipitate; UA, uronic acid.

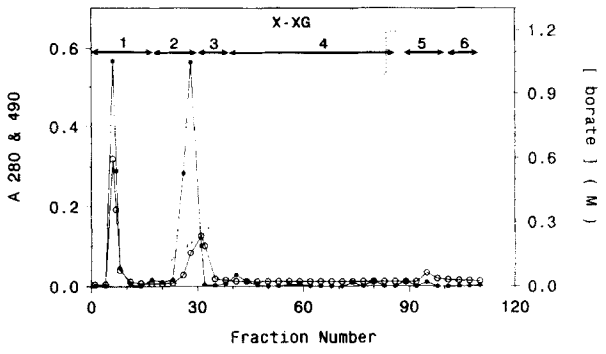

Fig. 1. Chromatography on DEAE-Sephacel of fraction X-XG. ●–●, total carbohydrate (A_{490}); ○–○, total phenolic (A_{280}); ---, sodium borate gradient.

in Table 1. All the fractions contained various amounts of xylose, arabinose, glucose and uronic acid. However, it is clear that the small amounts of pectic polysaccharides relatively rich in arabinose and uronic acid were retained on the column, and were only eluted with buffer containing increasing amounts of NaCl. Whilst the predominant neutral sugar in the acidic polymers was arabinose, the presence of a significant amount of xylose co-eluting with such pectic polysaccharides is consistent with the presence of pectic–xylan complexes. The presence of such complexes has been postulated in other plant tissues (Waldron & Selvendran, 1992). The major fraction (X-XG), which was relatively low in uronic acid, was further fractionated on DEAE-Sephacel, borate form, pH 8.0, which gave rise to six fractions (X-XG 1–6; Fig. 1). All these fractions contained various amounts of xylose, glucose, arabinose and uronic acid, indicative of xyloglucans, xylans and some pectic polysaccharides (Table 2). The major, weakly acidic, fraction X-XG2, which accounted for 39% of the material eluted from the column, was virtually free of contaminating pectic polysaccharides. Methylation analysis in conjunction with carboxyl reduction confirmed that the fraction was composed mainly of glucuronoxylans and xyloglucans (Table 4, columns 1 and 2).

Table 2. Sugar composition of fractions obtained from X-XG after DEAE-Sephacel chromatography

Fraction total	Recovery (%)	Cell wall sugars (mol%)								Total sugar[a] (μg/mg)
		Rha	Fuc	Ara	Xyl	Man	Gal	Glc	UA	
X-XG										
Insoluble residue	6.6	2	1	21	49	t	3	10	14	752
X-XG1	12.6	1	1	24	55	t	3	2	14	687
X-XG2	38.9	1	1	8	51	1	6	26	6	858
X-XG3	4.0	1	t	6	54	8	5	19	7	553
X-XG4	4.2	2		6	63	2	4	10	13	436
X-XG5	11.2	3		32	45	t	2	3	15	719
X-XG6	4.0	3		26	39	1	3	7	21	347

[a]Values are expressed as μg anhydrosugar per mg dry polymers. t, trace; UA, uronic acid.

Table 3. Sugar composition of fractions obtained from X-XG2 after gel filtration chromatography followed by refractionation of selected fractions

Fraction total	Recovery (%)	Cell wall sugars (mol%)								Total sugar[a] (μg/mg)
		Rha	Fuc	Ara	Xyl	Man	Gal	Glc	UA	
X-XG2										
X-XG2 A	21.1									
X-XG2 A1 (A1)	55.0	t		2	53	t	1	24	20	576
X-XG2 A2 (A2)	47.5	1		4	48	1	3	19	24	301
X-XG2 B	27.5	t	1	9	32	t	8	41	9	615
X-XG2 C	46.0									
X-XG2 C1 (C1)	19.5	1		2	37	1	1	20	38	162
X-XG2 C2 (C2)	35.6		1	9	33	t	7	36	14	481
X-XG2 C3 (C3)	51.7	t	1	9	37	t	8	38	7	827
X-XG2 C4 (C4)	9.2	t	t	6	44	2	3	26	19	435
X-XG2 D	16.9	1	1	6	48	1	4	23	16	577
X-XG2 E	2.6									

[a]Values are expressed as μg anhydrosugar per mg dry polymers. t, trace; UA, uronic acid.

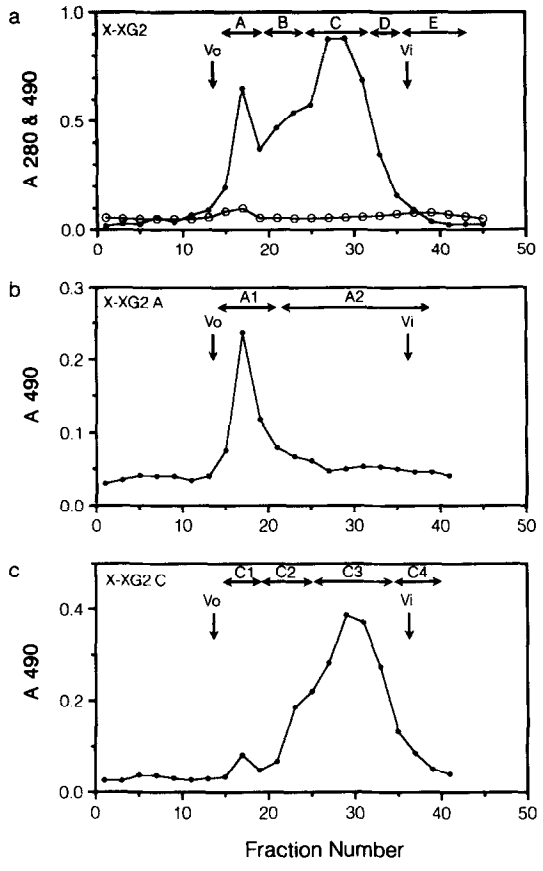

Fig. 2. Chromatography on Sephacryl S-400 of fractions: (a) X–XG2; (b) X–XG2A; (c) X–XG2C. ●–●, total carbohydrate (A_{490}); ○–○, total phenolic (A_{280}). Vo, void volume; Vi, included volume.

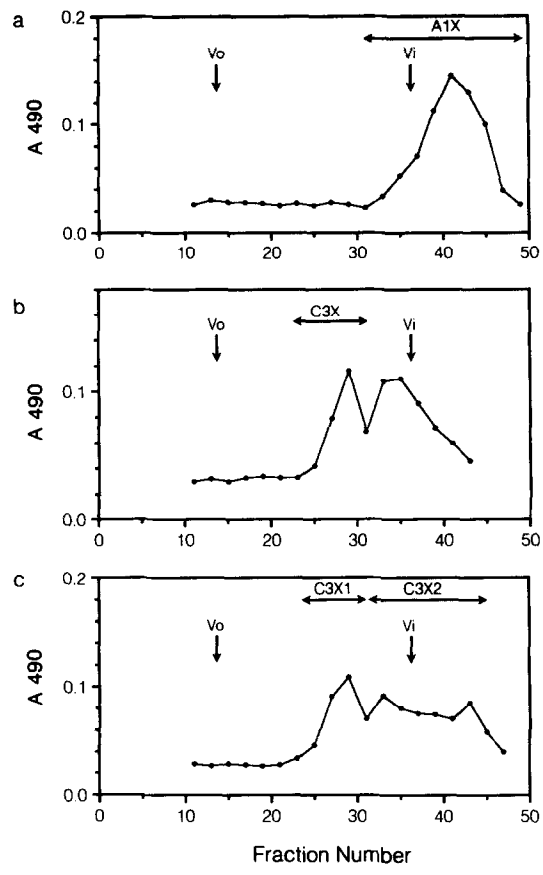

Fig. 3. Chromatography on Sephacryl S-400 of fractions: (a) A1 (after xylanase digestion); (b) C3 (after xylanase digestion); (c) C3X. ●–●, total carbohydrate (A_{490}); Vo, void volume; Vi, included volume.

Gel-filtration chromatography

Fraction X–XG2 was submitted to gel-filtration chromatography on Sephacryl S-400, giving rise to five poorly defined fractions, A–E (Fig. 2a). In order to obtain better resolution, fractions A and C were refractionated on Sephacryl S-400 (Fig. 2b and c, respectively). Much of fraction A was resolved into a well defined peak, X-XG2A1 (A1) (Fig. 2b), and the bulk of fraction C was resolved into four ill-defined fractions, X–XG2 C1–4 (C1–C4) (Fig. 2c). The bulk of fraction C was present in X–XG2 C3 (C3). The carbohydrate composition of the various fractions resolved on Sephacryl S-400 are shown in Table 3.

It is clear that despite the fact that all the fractions were (initially) not well resolved, their compositions are comparable. They all appear to contain various amounts of acidic xylans associated with xyloglucans. Small quantities of pectic polysaccharides were detected in some of the minor fractions, as indicated by the levels of uronic acid and the presence of rhamnose. The compositions of A1 and A2 are highly comparable, despite the fact that fraction A2 had an indefinite maximum. A1 was found to have a molecular weight of 2000 kDa, using molecular weight dextran standards. Fractions C2 and C3 were poorly resolved and had highly comparable compositions. Fraction C3 was found to have a molecular weight of 100 kDa.

Methylation analysis of A1 (Table 4) showed that it had structural features of a slightly branched acidic xylan, and a xyloglucan with a very low degree of polymerisation (DP), with the former being the predominant one. Methylation analysis of C3 (Table 4) showed that it had structural features of a highly branched acidic xylan, and a very highly branched xyloglucan with a high DP, present in comparable amounts. Carboxyl reduction of methylated C3 confirmed the presence of T-Glc*p*A in the highly branched xylan moiety ($C3^a$, Table 4). Generally, methylation analysis was complete, as indicated by the comparability between terminal and branched residues.

Purified xylanase digestion studies

The composition of fractions A1 and C3, obtained after a number of purification steps, gave (fairly) firm evidence that they contained acidic xylans and xylo-

glucans in close, possibly covalent, association. To obtain unambiguous evidence for the occurrence of complexed acidic xylans and xyloglucans, fractions A1 and C3 were treated with highly purified xylanase in which xyloglucanase (Femenia, Selvendran and Waldron, unpublished) and cellulase activity was not detectable. For details of the preparation and specificity of the purified xylanase, see Materials and Methods. The products of digestion were fractionated by Sephacryl S-400 (Fig. 3a–c).

As a result of xylanase treatment, the polysaccharides in A1 were reduced to fragments which were less than 10 kDa (the resolving limit of the gel; Fig. 3a). This material (A1X) was pooled and dialysed exhaustively prior to being freeze-dried. Because of the small quantities of material involved (1–2 mg), carbohydrate analysis was not performed. Instead, samples were subjected to methylation analysis with carboxyl reduction if quantities permitted.

Methylation analysis of A1X demonstrated that the linkages associated with the xylan component had decreased considerably during digestion; the ratio of (1 4)-linked xylose to terminal xylose (of xyloglucan) decreased to 7% of its former value, which was consistent with the depolymerisation of the xylan moiety. However, the ratios of the xyloglucan linkages to that of terminal xylose remained similar (A1X[a], Table 5) indicating that there had been little significant change in the xyloglucan. This, and the xylanase-catalysed decrease in molecular weight of A1 provide unequivocal evidence for cross-linking of the xyloglucan and xylan moieties within this fraction. The decrease in (1–2,4)-linked xylose (relative to xyloglucan linkages) was much less than that of the (1–4)-linked moiety. It is possible that some of these linkages contributed to the cross-linking between the xylan and xyloglucan; the majority will have served as the points of attachment for terminal Glc*p*A (A1X[a], Table 5). There was good equivalence between the branched xylan residues and terminal Glc*p*A.

In contrast to A1, the elution profile of fraction C3 after xylanase treatment demonstrated a much larger molecular weight distribution. The eluted carbohydrate was divided into two fractions; the first contained material of low molecular weight which was not analysed further and the second consisted of material the molecular weight of which had only slightly shifted, remaining in the region of 50 kDa (C3X) (Fig. 3b). The

Table 4. Glycosyl linkage composition of polysaccharides from X–XG2 after gel-filtration chromatography

Residue	Methylated positions	Deduced linkage	X XG2	X-XG2[a]	X-XG2		
					A1	C3	C3[a]
Fuc	2,3,4	T-Fuc*p*	0.1			0.8	0.1
Ara	2,3,5	T-Ara*f*	6.5	6.8	1.7	8.3	8.1
Ara	2,5	3-Ara*f*	0.3				0.2
Ara	2,3	5-Ara*f*	0.5		0.4	0.7	1.0
Ara	3	2,5-Ara*f*			0.5		
Ara	2	3,5-Ara*f*				0.3	
Arabinitol			0.1	0.1			
Xyl	2,3,4	T-Xyl*p*	13.4	12.8	2.8	11.5	12.3
Xyl	3,4	2-Xyl*p*	18.1	16.1		10.1	12.2
Xyl	2,3	4-Xyl*p*	36.1	32.3	73.2	13.4	8.1
Xyl	3	2,4-Xyl*p*	2.5	4.2	5.5	1.3	2.2
Xylitol			0.1	0.1	0.5	0.4	0.3
Man	2,3,6	4-Man*p*	0.5			0.2	
Gal	2,3,4,6	T-Gal*p*	0.3	0.1		10	11.5
Gal	3,4,6	2-Gal*p*				0.5	0.4
Gal	2,3,6	4-Gal*p*	0.5	0.5		0.1	0.3
Gal	2,3,4	6-Gal*p*	0.2			0.3	
Gal	2,3	4,6-Gal*p*					0.4
Glc	2,3,4,6	T-Glc*p*	0.5	0.4	0.7		0.2
Glc	2,3,6	4-Glc*p*	4.6	5.4	6.1	12.4	11.8
Glc	2,3	4,6-Glc*p*	13.2	16.8	3.9	27.1	28.0
Glc	6	2,3,4-Glc*p*		—	—	—	0.5
Glc	3	2,4,6-Glc*p*	0.1				0.2
Glc	2	3,4,6-Glc*p*	1.0				
Glucitol			1.4	0.2	4.7	2.6	0.5
Glc[b]	2,3,4	T-Glc*p*A		4.2			2.2

[a] Sample reduced with $LiAlD_4$.
[b] Deuterium-labelled.

Table 5. Glycosyl linkage composition of polysaccharides from X–XG2 after gel filtration chromatography of xylanase-digested samples (values expressed as relative mol%)

Residue	Methylated positions	Deduced linkage	Fraction	A1X[a]	C3X1	C3X2
Fuc	2,3,4	T-Fuc*p*		—	0.6	0.7
Ara	2,3,5	T-Ara*f*		2.3	6.7	7.9
Ara	2,5	3-Ara*f*		—	-	0.5
Ara	2,3	5-Ara*f*		—	1.2	1.3
Ara	2	3,5 Ara*f*		1.4	0.6	—
Arabinitol					0.7	0.3
Xyl	2,3,4	T-Xyl*p*		11.9	8.6	10.0
Xyl	2,4	2-Xyl*p*		7.8	11.3	10.3
Xyl	2,3	4-Xyl*p*		19.7	2.3	1.7
Xyl	3	2,4-Xyl*p*		5.7	1.1	0.8
Xylitol				2.3	1.3	0.7
Man	2,3,6	4-Man*p*		2.4	1.0	1.1
Gal	2,3,4,6	T-Gal*p*		-	8.6	11.3
Gal	2,3,4	6-Gal*p*		—	0.2	0.3
Gal	2,3	4,6-Gal*p*		—	0.4	0.3
Glc	2,3,4,6	T-Glc*p*		2.1	0.6	0.9
Glc	2,3,6	4-Glc*p*		22.1	21.1	19.7
Glc	2,3,4	6-Glc*p*		—	0.7	0.6
Glc	3,6	2,3-Glc*p*		0.3	0.2	0.4
Glc	2,3	4,6-Glc*p*		9.0	23.7	26.6
Glc	3	2,4,6-Glc*p*		—	0.4	0.3
Glc	2	3,4,6-Glc*p*		0.3	0.6	0.4
Glucitol				6.0	8.3	3.9
Glc[b]	2,3,4	T-Glc*p*A		6.7	—	—

[a] Sample reduced with $LiAlD_4$.
[b] Deuterium-labelled.

material of fraction C3X was dialysed, freeze-dried and re-applied to the column to give two fractions (Fig. 3c), one with a molecular weight of approximately 50 kDa (C3X1) and a second one (C3X2) with a molecular weight of less than 10 kDa.

As in the case of AX1, methylation analysis of fractions C3X1 and C3X2 (Table 5) showed that the xylanase activity had considerably reduced the xylan moiety whilst having little effect on the linkages associated with the xyloglucan (Table 4). The relative, but small increase in (1–4,6)-linked glucose was probably due to partitioning of heterogeneous xylan–xyloglucan fragments by the gel filtration step immediately following xylanase digestion (Fig. 3b). Such partitioning would not have affected the results for A1X because all of the digested carbohydrate was pooled after gel filtration. The linkage profiles of C3X1 and 2 were similar, although C3X2 generally contained more terminal groups which was consistent with its lower molecular weight.

CONCLUSIONS

The results of this investigation from the 1 M KOH (1°C) extract of olive-pulp CWM, containing Xyl*p*, Glc*p*, Ara*f* and uronic acid on two anion exchange columns provided good evidence for the occurrence of a range of closely associated acidic xylans and xyloglucans, possibly in covalent association. Two of the fractions, on further chromatography by gel filtration, and subsequent methylation analysis, confirmed the heterogeneity of the xylan–xyloglucan complexes, and suggested that two of the major complexes from the above fractions had apparent molecular weights of 2000 and 100 kDa. Digestion of the above fractions with a highly purified xylanase, followed by chromatography on Sephacryl S-400, and methylation analysis of the degraded polymeric material, provided unambiguous evidence that their parent compounds are indeed complexes of glucuronoxylans and highly branched xyloglucans in covalent association. A similar approach, using polysaccharide degrading enzymes, has been used in the analysis of extruded fibre from pea hulls (Ralet *et al.*, 1993). The present paper is one of the few reports that have provided such evidence for the occurrence of such interpolymeric cross-linking.

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