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Extraction, purification and chemical characterisation of xylogalacturonans from pea hulls

A. Le Goff[a], C.M.G.C. Renard[b], E. Bonnin[a,*], J.-F. Thibault[a]

[a]*Unité de Recherche sur les Polysaccharides, leurs Organisations et Interactions, INRA, BP 71627, 44316 Nantes Cedex 03, France*
[b]*Station de Recherches Cidricoles-Biotransformation des Fruits et Légumes, INRA, BP 35327, 35653 Le Rheu Cedex, France*

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Abstract

Pea hulls contained 925 mg/g sugar including 659 mg/g cellulosic glucose and 90 mg/g uronic acid. They were de-esterified by NaOH (pH > 13 at 4°C, 2 h) and treated with HCl (0.1 mol/l, 80°C, 24 h). The HCl-soluble fraction represented 95 mg/g initial pea hulls. It was rich in galacturonic acid (259 mg/g), xylose (93 mg/g) and rhamnose (91 mg/g), which co-eluted in anion-exchange chromatography. The HCl-soluble fraction was degraded by a rhamnogalacturonan-hydrolase and the reaction products were fractionated by size-exclusion chromatography. Two fractions, representing together 18 mg/g initial pea hulls, were composed almost exclusively of galacturonic acid and xylose and could be defined as xylogalacturonans. The first fraction exhibited a high molar mass, a molar ratio Xyl/GalA of 1 and contained almost 5% of rhamnose. The molar mass of the second fraction was much lower and the molar ratio Xyl/GalA was 0.6. Methylation analysis showed the presence in both fractions of a α(1 → 4) galacturonan backbone highly substituted on O-3 either by terminal xylosyl residues or by short side-chains of (1 → 2) linked xylosyl residues.

Keywords: Pea hulls; Pectins; Enzymic degradation; Xylogalacturonans

1. Introduction

Pectins are one of the major polysaccharides of plant cell wall in dicotyledons, and are characterised by a high content of galacturonic acid. Their structure is based on a backbone consisting of alternating homogalacturonans and rhamnogalacturonans. Rhamnogalacturonans are composed of a repeating sequence of [-2)-α-L-Rha (1-4)-α-D-GalA (1-]. They can be ramified with long side-chains of arabinose (arabinans) and/or galactose (galactans, arabinogalactans) carried by the O-4 of rhamnose (O'Neil, Albersheim & Darvill, 1990). Homogalacturonans are composed of α(1 → 4)-linked D-galacturonic acid generally methyl-esterified on some of the carboxylic groups (Pilnik & Voragen, 1970). The hydroxyl groups can be acetylated on O-2 or/and O-3 (Rombouts & Thibault, 1986a,b; Voragen, Schols & Pilnik, 1986). The degrees of methylation (DM) and acetylation (DAc) are defined as the number of moles of methanol or acetic acid per 100 mol of galacturonic acid. In some cases, short side-chains can be carried by the homogalacturonan backbone. A typical example is apiogalacturonan (Hart & Kindel, 1970). Xylogalacturonans with a backbone of α(1 → 4) galacturonic acid residues carrying β-D-xylopyranose on O-3 (molar ratio Xyl/GalA = 0.5) have been described for the first time in the pollen of mountain pine (Bouveng, 1965). They were also reported in kidney beans (Matsuura, 1984), in Japanese radish (Matsuura & Hatanaka, 1988), in cultured carrot cells (Kikuchi, Edashige, Ishii & Satoh, 1996), and in ripe watermelon (Yu & Mort, 1996). A similar fraction has also been identified in the "hairy" regions of apple pectins (De Vries, Rombouts, Voragen & Pilnik, 1983; Voragen, Schols & Gruppen, 1993). This fraction had a degree of methylation of 39 and contained rhamnose residues (Schols, Bakx, Schipper & Voragen, 1995).

Our previous results suggested the presence of xylogalacturonans in pea hulls (Weightman, Renard & Thibault, 1994; Weightman, Renard, Gallant & Thibault, 1995). Sequential extractions by chelating agent, acid and alkali released arabinans, xylose-rich polymers associated with pectic substances and (linear) xylans, respectively. The hot acid-soluble polysaccharides were found to be xylose-rich-pectins, in which xylose was present as single units or short side-chains (Renard, Weightman & Thibault, 1997). These results suggested that apart from homogalacturonans and rhamnogalacturonans, pectins from the primary cell

* Corresponding author. Tel.: +33-2-40-67-50-00; fax: +33-2-40-67-50-66.

E-mail address: bonnin@nantes.inra.fr (E. Bonnin).

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walls of pea hulls could also contain a xylogalacturonan fraction.

In the present study, a procedure was investigated to extract pectins using acidic conditions in order to purify xylogalacturonans from pea hulls. Their structural features are also described.

2. Experimental

2.1. Material

The pea hulls (particle size ≈375 μm) were provided by Sofalia (Ennezat, France). Rhamnogalacturonan-hydrolase (RG-hydrolase) was a gift from Novo Nordisk A/S (DK). Rhamnogalacturonans were isolated from sugar beet pulp (laboratory collection).

2.2. Extraction of NaOH-, HCl-, and pH7-soluble materials

The pea hulls (400 g) were suspended in 2.3 l cold deionised water. Sodium hydroxide (1 mol/l) was added to bring the pH up to 13 and the suspension was stirred at 4°C for 2 h. The pH was then adjusted to 5 by adding glacial acetic acid. The de-esterified pea hulls were recovered by filtration on a G3 sintered glass filter and dried by solvent exchange (ethanol and acetone). The filtrate corresponded to the NaOH-soluble material. The de-esterified pea hulls (50 g) were then hydrolysed in 333 ml of 0.1 mol/l HCl at 80°C for 24 h. The HCl-soluble material was recovered by centrifugation of the slurry (13,500*g* for 20 min). The insoluble fraction was suspended in 500 ml of deionised water and the pH was adjusted to 7 by adding 1 mol/l NaOH. The pH7-soluble material was recovered by centrifugation as above. The final residue was washed with water and dried by solvent exchange.

After neutralisation of the NaOH- and HCl-soluble materials, the NaOH-, HCl- and pH7-soluble materials were concentrated, dialysed (MWCO 6000–8000) against deionised water and freeze-dried.

2.3. Degradation by a RG-hydrolase

RG-hydrolase was solubilised in acetate buffer, 0.05 mol/l pH 4.8. The solution was extensively dialysed against acetate buffer, 0.02 mol/l pH 4.8 at 4°C. After dialysis, RG-hydrolase activity was monitored by the increase in reducing end groups (Nelson, 1944) using rhamnogalacturonan as a substrate.

The HCl-soluble material (100 mg) in 10 ml of acetate buffer, 0.05 mol/l pH 4.8, were incubated at 40°C with 5.5 nkat of RG-hydrolase. Aliquots (2 ml) were withdrawn at intervals and analysed by size-exclusion chromatography after thermal inactivation of the enzyme (15 min, 100°C).

2.4. Chromatography

Two millilitres of the sample (10 mg/ml) (filtered on a 2.7-μm Whatman GF/D glass fibre filter) were injected on a size-exclusion chromatography column (Sepharose CL-6B, 810×22 mm^2) and eluted at a flow rate of 25 ml/h by 0.05 mol/l acetate buffer of pH 4.8. Fractions (5 ml) were collected and analysed for neutral sugars and uronic acid contents. Pooled fractions were dialysed against deionised water and freeze-dried before analysis.

Anion-exchange chromatography was performed on a DEAE-Sepharose CL-6B column (250×16 mm^2). Five millilitres of the sample (10 mg/ml) were loaded on the column. The column was eluted at a flow rate of 45 ml/h by 1.3 column volumes of 0.05 mol/l ammonium or sodium acetate of pH 4.8, followed by 4 column volumes of a linear gradient from 0.05 up to 1 mol/l of ammonium or sodium acetate and finally by 1 column volume of 1 mol/l ammonium or sodium acetate. Fractions (4.5 ml) were collected and analysed for neutral sugar and uronic acid contents. Pooled fractions were dialysed against deionised water and freeze-dried before analysis.

High Performance Size Exclusion Chromatography (HPSEC) was performed using two columns in series (Shodex OH-pack SB 805 HQ and OH-pack SB 804 HQ, exclusion limit 1×10^6 and 4×10^6 Da for pullulans, respectively) and a pre-column (Shodex OH-pack SB-G). Twenty-microlitre samples were injected after filtration on a 0.45-μm Whatman filter. The elution was carried out at room temperature and at a flow rate of 0.6 ml/min by 0.05 mol/l $NaNO_3$ containing 0.02% NaN_3. The column effluent was monitored using a Multi Angle Laser Light Scattering detector (MALLS, MiniDawn Wyatt Corporation, USA) and a differential refractometer. Molar masses were determined with Astra 1.4 software using a refractive index increment ($dn/dc = 0.146$ g/ml) normalised on pullulan.

2.5. Analytical

Dry matter was determined by drying the samples at 120°C for 2 h. All data were expressed on a moisture-free basis.

Uronic acids and neutral sugars were quantified by the automated *m*-phenyl-phenol method (Thibault, 1979) and orcinol method (Tollier & Robin, 1979), using galacturonic acid and arabinose as standards, respectively.

The neutral sugar composition was determined after 3-h pre-hydrolysis in 72% sulphuric acid (Seaman, Moore, Mitchell & Millet, 1954) and 6-h hydrolysis in 1 mol/l sulphuric acid. The pre-hydrolysis step was omitted for soluble polysaccharides. Myo-inositol was added as internal standard. Alditol acetates of monosaccharides (Blakeney, Harris, Henry & Stone, 1983) were analysed by gas–liquid chromatography on a DI 200 chromatograph (Delsi Nermag Instruments, Argenteuil, France) equipped with a DB225 fused silica capillary column (Scientific Glass Engineering Sarl, Villeneuve-St-Georges, France) and a flame ionisation detector.

Methanol and acetate were released by alkaline

Table 1
Sugar compositions (mg/g) of pea hulls and extracted pectic material (ND: not detected)

	Pea hulls		Materials extracted by			Residue
	Initial	De-esterified	NaOH	HCl	pH7	
Yield	1000	978	2	95	73	640
Rha	17	16	60	91	13	ND
Fuc	2	1	4	1	ND	ND
Ara	43	40	205	11	ND	ND
Xyl	103	110	59	93	32	124
Gal	11	10	57	38	ND	ND
Glc	659	591	28	75	5	451
AUA[a]	90	85	200	260	281	25
DAc[b]	40	22	10	3	0	33
DM[c]	33	10	ND	ND	ND	ND

[a] AUA: anhydro uronic acid.
[b] DAc: degree of acetylation.
[c] DM: degree of methylation.

de-esterification (0.4 mol/l NaOH for 2 h at room temperature) and quantified by HPLC analysis on Aminex HPX-87H (300 × 7.8 mm^2, Bio-Rad S.A., Ivry sur Seine, France) eluted at 35°C with 0.005 mol/l H_2SO_4 at a flow rate of 0.6 ml/min (Voragen et al., 1986). Then, DM and DAc were calculated as the molar ratio of methanol or acetic acid to galacturonic acid.

2.6. Methylation analysis

The samples were initially converted to their H^+ form using a Dowex 50W resin, and freeze dried.

Two procedures for the reduction of the uronic acids were used. In the first one (York, Darvill, Mc Neil, Stevenson & Albersheim, 1985), 10 mg of the H^+ sample were dissolved in 2 ml of 8 mol/l urea and pH was adjusted to 4.75 by using 0.025 mol/l HCl. *N*-Cyclohexyl-*N′*-2-morpholinoethyl-carbodiimide-methyl-*p*-toluene sulfonate (CMC, Fluka) was slowly added to obtain a CMC/polymer weight ratio of 10:1. After 1 h, the pH was adjusted to 7 by using 2 mol/l HCl. The reduction was carried out by $NaBH_4$ (250 mg in 3 ml H_2O) during 1 h. $NaBH_4$ was added using a peristaltic pump at 0.1 ml/min (the pH was maintained at 7 with 1 mol/l HCl). 1-Octanol was used as an anti-foaming agent. After reduction, the pH was decreased to 5–6 with 2 mol/l HCl. The carboxy-reduced samples were extensively dialysed (Spectra-Por Membrane MWCO 1000) against deionised water and freeze-dried. Traces of boric acid were removed by solubilisation of the freeze-dried samples in 0.5 ml water and addition of 0.5 ml acetic acid–methanol mixture (1:9). Then, the methyl borate esters were eliminated by evaporation. After repeating three times the extraction of boric acid, the residue was extracted by 1 ml methanol. The reduced polymers were methylated according to the method of Hakamori (1964) using buthyl lithium carbanion and methyl iodure. After dialysis and freeze-drying, 4–5 mg of methylated samples were hydrolysed by 1 mol/l H_2SO_4 for 2 h at 100°C and converted to alditol acetates (Blakeney et al., 1983) using $NaBD_4$ for reduction.

In the second procedure, the samples were methylated using buthyl lithium carbanion, then the carboxylic groups were deuterio-reduced using superdeuteride (lithium triethyl-borodeuteride) for 2 h at room temperature (Pellerin, Doco, Vidal, Williams, Brillouet & O'Neill, 1996). They were hydrolysed using 2 mol/l TFA for 75 min at 120°C, reduced by $NaBH_4$ and acetylated. In both procedures, myo-inositol was used as the internal standard.

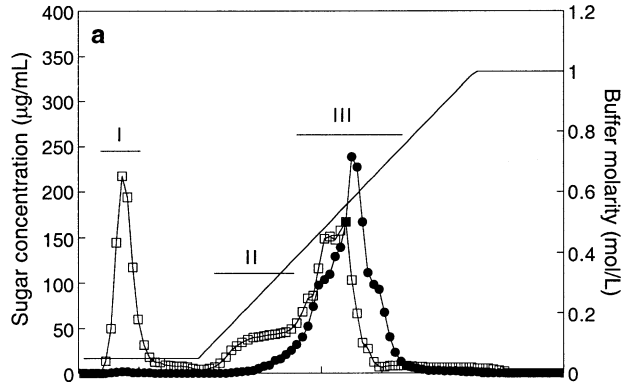
Identification of the linkages among sugars was based on the separation of the partially methylated sugars by gas–liquid chromatography on a DI 200 chromatograph (Delsi Nermag Instruments, Argenteuil, France) equipped with a OV225 fused-silica capillary column (Scientific Glass Engineering Sarl, Villeneuve-St-Georges, France) and a flame ionisation detector. The temperature program was as follows: 165°C for 10 min, +5°C/min to 205°C, 205°C for 10 min; injector: 220°C; detector: 220°C. Elution was carried out by hydrogen at 80 kPa. The mass fragmentation patterns were obtained by GC–MS (DI 700-Delsi Nermag Instruments R-10.10-C, Argenteuil, France) equipped with a OV225 column (180°C for 15 min; +5°C/min to 210°C; 210°C for 20 min) and eluted with helium.

3. Results

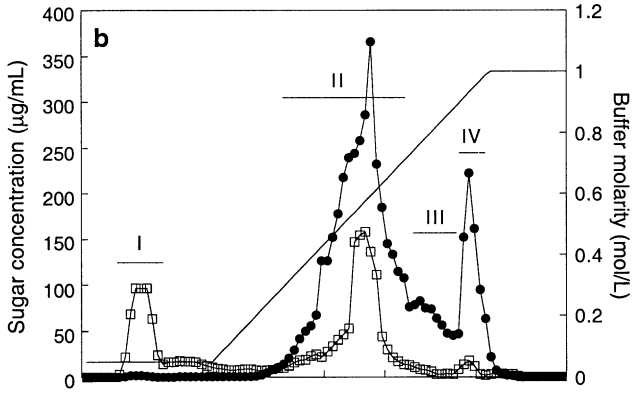
3.1. Extraction of NaOH-, HCl- and pH7-soluble materials from pea hulls

The initial pea hulls were almost completely composed of sugars (925 mg/g, Table 1), mainly glucose (659 mg/g), xylose (103 mg/g), uronic acid (90 mg/g) and arabinose (43 mg/g). According to previously published data, glucose was mostly of cellulosic origin (Weightman et al., 1994), arabinose from arabinans and xylose from xylans (Ralet, Della Valle & Thibault, 1993a; Ralet, Saulnier & Thibault, 1993b). Uronic acids were essentially composed of galacturonic acid with a reported GalA/glucuronic acid ratio of 97:3 (Weightman et al., 1994). Concomitant presence of galacturonic acid, rhamnose, arabinose, and galactose indicated the presence of a pectic fraction which was slightly methyl esterified and highly acetylated. The composition of the initial pea hulls was similar to previous results (Ralet et al., 1993a; Weightman et al., 1994).

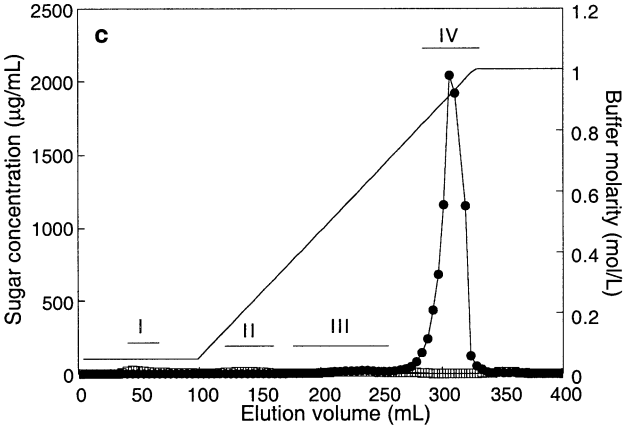
The procedure used to obtain xylogalacturonan was previously set up by Thibault, Renard, Axelos, Roger and Crépeau (1993) for the preparation of homogalacturonan from various pectins. However, the time of hydrolysis was reduced from 72 to 24 h in order to preserve the linkages between galacturonic acid and xylose. The pea hulls were de-esterified in order to limit differences of susceptibilities of glycosidic bonds to hydrolysis because of ester groups. The de-esterification was carried out in alkaline conditions at low temperature in order to minimize β-elimination of



	I	II	III
yield *	17	7	72
Sugar (mol %)			
Rha	4	3	17
Ara	69	39	19
Xyl	1	24	10
Gal	3	16	9
Glc	24	3	1
AUA	2	12	43



	I	II	III	IV
yield *	7	60	9	13
Sugar (mol %)				
Rha	0	20	7	1
Ara	1	0	1	1
Xyl	7	12	6	0
Gal	3	6	1	0
Glc	85	1	1	0
AUA	2	61	83	97



	I	II	III	IV
yield *	2	1	4	92
Sugar (mol%)				
Rha	0	0	16	1
Ara	1	5	5	0
Xyl	63	80	15	1
Gal	1	1	6	0
Glc	24	5	2	0
AUA	19	18	53	97

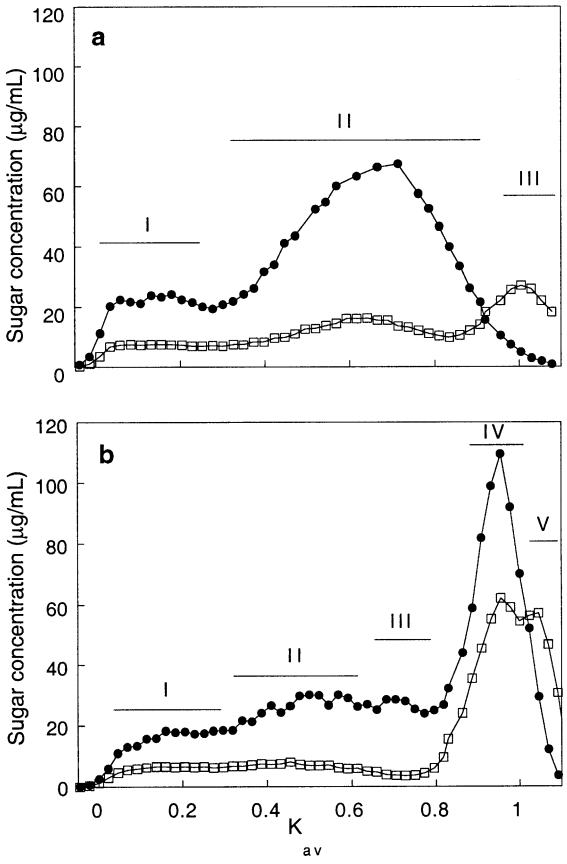
* : yield expressed as a percentage of sugars loaded on the column

Fig. 1. Anion-exchange chromatography on DEAE-Sepharose CL-6B column of NaOH-soluble (a), HCl-soluble (b) and pH 7 soluble (c) materials. Elution was carried out by acetate buffer, 0.05 mol/l pH 4.8, with a linear gradient 0.05 up to 1 mol/l of acetate. ●: anhydro uronic acid (AUA); □: neutral sugars; —: sodium acetate (a and b) or ammonium acetate (c).

pectins and the NaOH-soluble fraction was recovered. Then, a HCl-soluble material was removed by acidic treatment. Finally, a pH7-soluble fraction was recovered by neutralisation of the acidic insoluble material.

The compositions of the soluble extracts and the residue are in Table 1. Saponification did not significantly change the sugar composition of the pea hulls. The loss of polymeric material (NaOH-soluble) was only 2 mg/g, mainly composed of arabinose and galacturonic acid. The DM and the DAc of the pea hulls markedly decreased (from 33 to 10 and from 40 to 22, respectively).

The initial pea hulls (95 mg/g) were solubilised by the HCl treatment, with high levels of galacturonic acid and rhamnose indicating that pectic polymers were solubilised. The extract was also rich in xylose. Very low amount of arabinose was recovered indicating that arabinans were



	I	II	III
yield *	16	65	20
Sugar (mol %)			
Rha	10	23	9
Ara	0	0	1
Xyl	26	10	16
Gal	4	5	5
Glc	2	5	43
AUA	57	57	25

	I	II	III	IV	V
yield *	14	21	10	44	13
Sugar (mol %)					
Rha	5	2	10	49	14
Ara	2	1	1	0	3
Xyl	41	36	15	5	23
Gal	4	3	6	6	5
Glc	2	1	8	1	32
AUA	43	55	57	37	22
Rha/AUA	0.1	0.0	0.2	1.3	0.6
Xyl/AUA	1.0	0.6	0.3	0.1	1.0

* : yield expressed as a percentage of sugars loaded on the column

Fig. 2. Size-exclusion chromatography of HCl-soluble materials before (a) and after 24 h of RG-hydrolase degradation (b). Elution was carried out by 0.05 mol/l acetate buffer pH 4.8. ●: anhydro uronic acid (AUA); □: neutral sugars.

degraded by acidic hydolysis and eliminated during dialysis. The HCl-soluble material was not methylated and almost devoid of acetic acid. Presence of glucose indicated that starch (known to represent 5 mg/g of pea hulls and probably originating from the cotyledon, Weightman et al., 1994) was also extracted by hot acid.

The pH7-soluble material (73 mg/g of initial pea hulls) was mainly rich in galacturonic acid (81.4%) but still contained rhamnose and a small amount of xylose. Thus, the NaOH-, HCl- and pH7-soluble materials contained significant amounts of xylose and galacturonic acid.

The final residue was almost exclusively composed of neutral sugars, namely glucose and xylose. They corresponded to cellulose and xylan, which could only be extracted using concentrated alkali (Weightman et al., 1994). Minor quantities of uronic acid were also detected, probably glucuronic acid from acidic xylans (Weightman et al., 1994, 1995).

3.2. Anion-exchange chromatography of soluble material

The NaOH-, HCl- and pH7-soluble materials were fractionated by anion-exchange chromatography on DEAE Sepharose CL-6B (Fig. 1).

The non-retained fraction (I) of the NaOH-soluble material (Fig. 1a) contained mainly arabinans. It contained also glucose, probably coming from starch. In fraction II, the presence of arabinose, xylose and low concentration of uronic acid suggested the presence of an acidic heteroxylan (Ralet et al., 1993a,b). The most retained fraction (III) was rich in galacturonic acid, arabinose, rhamnose and galactose suggesting the presence of rhamnogalacturonan with arabinan and galactan side-chains. It also contained a lot of xylose.

The elution profile of the HCl-soluble material (Fig. 1b) showed that the non-retained fraction (I) was almost completely composed of glucose and probably corresponded to starch co-extracted with pectins (Weightman et al., 1994). Fraction II corresponded to rhamnogalacturonans which were almost completely devoid of arabinan side-chains, as expected from sensitivity of the linkages to acidic hydrolysis. Xylose was also present in this fraction. Fractions III and IV, eluted by a high ionic strength, were constituted almost completely of galacturonic acid.

Very low column yield of about 9% was obtained when sodium acetate was used for the elution of the pH7-soluble material. In contrast, good recoveries (≈100%) were obtained with ammonium acetate (Cheng & Kindel, 1995), which was therefore chosen as eluent for the fractionation of the pH7-soluble material. The elution profile (Fig.

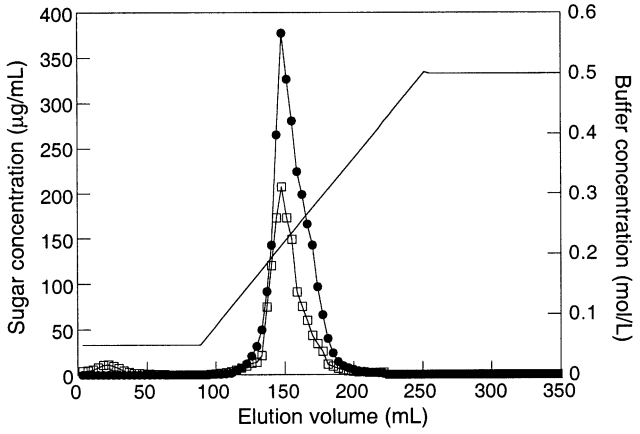


Fig. 3. Anion-exchange chromatography of xylogalacturonans on DEAE-Sepharose CL-6B. Elution was carried out by acetate buffer, 0.05 mol/l pH 4.8, with a linear gradient 0.05 up to 1 mol/l acetate. ●: anhydro uronic acid (AUA); □: neutral sugars; —: sodium acetate.

1c) showed a very important retained fraction (IV) eluted at a very high ionic strength (0.9 mol/l ammonium acetate). It represented ≈92% of the total sugars loaded on the column and was composed of ≈97 mol% of galacturonic acid. This fraction could therefore be considered as consisting of homogalacturonans.

Finally, xylose co-eluted with galacturonic acid during the ion-exchange chromatography in the NaOH- and HCl-soluble materials, suggesting the presence of xylogalacturonans. The yield of the NaOH-soluble material was low (2 mg/g) compared to the yield of the HCl-soluble material (95 mg/g). The HCl-soluble fraction was further investigated as a possible source of xylogalacturonans. However, the xylose- and galacturonic acid-rich fraction was also rich in rhamnose indicating that an extra step was needed for the purification of xylogalacturonans.

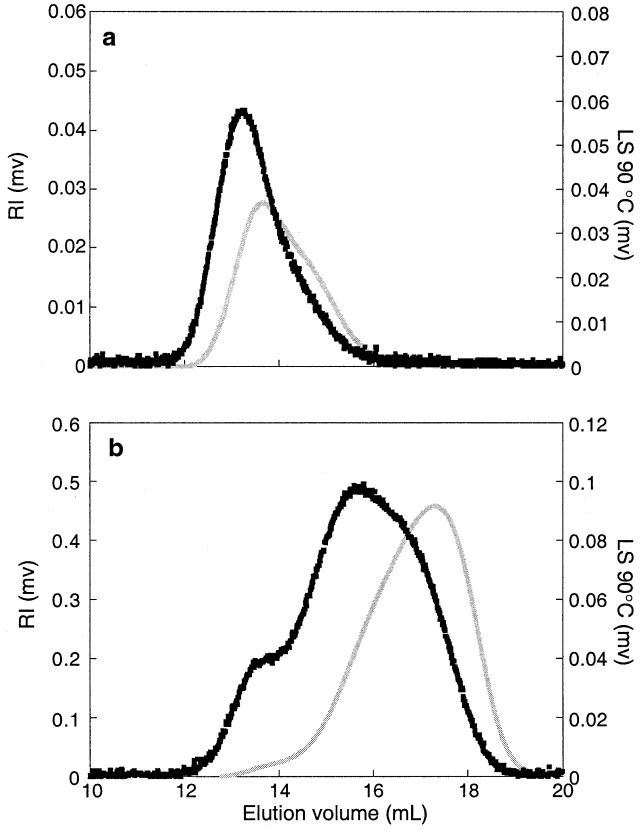


Fig. 4. HPSEC-MALLS of FI (a) and FII (b). ■: light scattering at 90° (LS). ▬: Light scattering at 90° (LS): refractive index (RI).

3.3. Size-exclusion chromatography of the HCl-soluble material

The HCl-soluble material was fractionated by size-exclusion chromatography on a Sepharose CL-6B column (Fig. 2a), eluted with sodium acetate. The column yield was approximately 100%.

Three different peaks eluted from $K_{av} = 0–1$, showing that polysaccharides from the HCl-soluble material had a broad range of molar masses. Fraction I eluted close to the void volume of the column (K_{av}: 0–0.2). It was almost completely composed of galacturonic acid and xylose (≈84 mol% of the total sugars) but still contained 10% of rhamnose and 4% of galactose. Fraction II was the major one and contained galacturonic acid, rhamnose and xylose. It could correspond to the xylose-rich rhamnogalacturonan fraction previously identified by anion-exchange chromatography (Fig. 1b, fraction II). Fraction III was mainly composed of glucose and xylose and could correspond to the non-retained fraction of anion-exchange chromatography (Fig. 1b, fraction I).

3.4. RG-hydrolase degradation

In order to decrease the rhamnose content in the fractions eluting with the lowest K_{av}, the HCl-soluble material was degraded by a RG-hydrolase. The reaction products after 10 min, 60 min (data not shown) or 24 h of degradation were fractionated by size-exclusion chromatography (Fig. 2b).

After 24-h treatment, the products were separated into five fractions (Fig. 2b) and the main peak shifted from $K_{av} = 0.6$ before degradation to $K_{av} = 0.9$ after RG-hydrolase treatment.

In fraction I (FI, K_{av}: 0.05–0.3), xylose and galacturonic acid represented ≈84 mol% of total sugars and the molar ratio Xyl/GalA was 1. It also contained galactose and rhamnose and exhibited a molar ratio Gal/Rha of about 1. These results suggested that FI could contain "hairy" regions composed of rhamnogalacturonans and xylogalacturonans and indicated resistance of this galactose-rich rhamnogalacturonan fraction to the degradation by RG-hydrolase. FI also contained a low amount of arabinose that could hinder accessibility to RG-hydrolase.

The fraction II (FII, K_{av}: 0.3–0.65) was rich in xylose and galacturonic acid (≈91 mol% of total sugars) and had a molar ratio Xyl/GalA of 0.6. It contained very low amounts

Table 2
Glycosyl linkages in FI, FII and FI + FII expressed in mol% of their sugar composition (ND: not detected; Tr: traces)

Residue	Reduction of GalA before methylation					Methylation of GalA before reduction		
	Methyl ether	FI (mol%)	FII (mol%)	FI + FII (mol%)	Deduced linkages	Methyl ether	FI + FII (mol%)	Deduced linkages
Rhamnosyl	2,3,4 Rha	0.3	0.4	0.4	Terminal	2,3,4 Rha	1	Terminal
	3,4 Rha	4	1	2	2-Linked	3,4 Rha	2	2-Linked
	3 Rha	Tr	Tr	Tr	2,4-Linked	3 Rha	0.1	2,4-Linked
Arabinosyl	2,3,5 Ara				Terminal	2,3,5 Ara	1	Terminal
	2,3 Ara				5-Linked	2,3 Ara	1	5-Linked
Xylosyl	2,3,4 Xyl	34	30	27	Terminal	2,3,4 Xyl	28	Terminal
	3,4 Xyl	5	4	8	2-Linked	3,4 Xyl	8	2-Linked
	2,4 Xyl	1	1	2	3-Linked	2,4 Xyl	1	3-Linked
Galactosyl	2,3,4,6 Gal	1	1	4	Terminal	2,3,4,6 Gal	8	Terminal Gal
	2,3,6 Gal	11	10	7	4-Linked	2,3,4 Gal	3	Terminal GalA
	2,6 Gal	5	6	12	3,4-Linked	2,3 Gal	10	4-Linked GalA
	2,3 Gal	14	21	ND	4-Linked	2 Gal	33	3,4-Linked GalA
	2 Gal	3	4	3				
Yield		69[a]	60[a]	59[a]			49[b]	

[a] From reduced sample.
[b] From initial sample.

of rhamnose and galactose. Thus, FII could mainly correspond to xylogalacturonans and portions of rhamnogalacturonans.

Fraction III (K_{av}: 0.65–0.8) was rich in galacturonic acid and xylose, but its content in rhamnose was higher than in the two first fractions. It could be composed of stubs of xylogalacturonans associated with rhamnose residues or short rhamnogalacturonans.

Fraction IV contained almost pure rhamnogalacturonans with molar ratio Rha/GalA of 1.3, and some short galactan side-chains (ratio Gal/Rha lower than 0.2). It eluted at $K_{av} = 0.9$ and probably corresponded to rhamnogalacturonan oligomers.

Fraction V mainly contained neutral sugars and had basically the same composition and elution volume as fraction III before RG-hydrolase degradation.

FI and FII, the fractions richest in xylose, were pooled and further fractionated by anion-exchange chromatography on DEAE-Sepharose CL-6B (Fig. 3). Almost all the carbohydrates (≈96%) were bound to the column and eluted in a single peak. Moreover, the ratio neutral sugars/GalA was constant (about 0.65) along the peak confirming that xylose and galacturonic acid belonged to the same polymer.

3.5. Characterisation of xylogalacturonans

FI and FII had a number-average molar mass of about 287 and 31.9 kDa, respectively, as determined by HPSEC-MALLS (Fig. 4). FI was more homogeneous than FII as polydispersities were 1.22 and 1.45, respectively. The degrees of polymerisation, calculated taking into account the xylose content, were 880 and 119 for FI and FII, respectively. FI was characterised by a higher mass than FII, as expected from the Sepharose CL-6B elution profile (Fig. 2).

To determine the structure of FI and FII, their glycosyl-linkage composition was determined by GC–MS of the derivatives permethylated before and after reduction of the galacturonic acid residues to galactose.

In the procedure including reduction before methylation, 79.5% of the initial galacturonic acid were reduced in galactose producing 92% of the final galactose. After dialysis, about 65% of the neutral sugars were lost, demonstrating degradation during reduction. The content of residual galacturonic acid (not reduced) was not decreased by dialysis, showing that galacturonans were still present. Afterwards, reduced samples were methylated, hydrolysed, deuterio-reduced and converted to alditol acetates and analysed by GC.

The results of GC (Table 2) showed two major products, identified as terminal xylosyl and 1,2- or 1,4-linked xylosyl residues. The fragmentation profile obtained by mass spectroscopy confirmed that xylaryl residues were 1,2-linked. A low proportion of 1,3-linked xylosyl residue was also identified. Some 1,2-linked rhamnosyl residues and terminal rhamnosyl residues were identified, whereas the 1,2,4-linked rhamnosyl residue was not found. 1,4-Linked and 1,3,4-linked galactosyl residues were also identified and corresponded to the reduced galacturonic acid residues. Some terminal galactosyl residues were also detected. The proportion expected of about 1:1 for the 1,3,4-linked galacturonosyl residue (from galacturonic acid) and the terminal xylosyl was not found. This could be explained by a loss of reducing sugars, especially from galacturonic acid oligomers, during the dialysis step.

A second procedure was carried out in order to confirm the identity and origin of the galactosyl residues. Samples

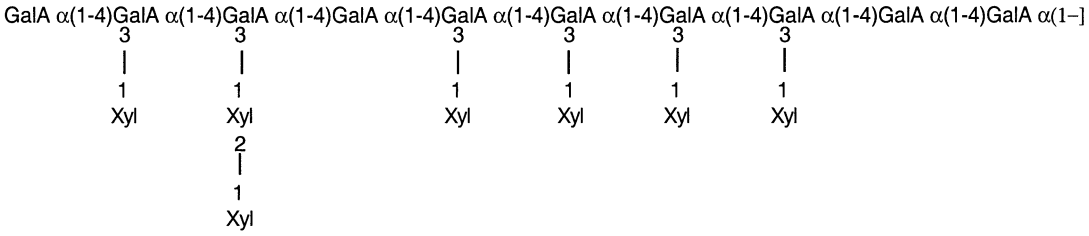


Fig. 5. Proposed structure of xylogalacturonans from pea hulls. GalA: galacturonic acid; Xyl: xylose.

were methylated before deuterio-reduction of the carboxylic group, which allowed positive identification of the derivatives coming from galacturonic acids. The main derivatives were 1,3,4-linked and 1,4-linked galactosyl residues. Terminal xylosyl, 1,2-linked xylosyl, terminal rhamnosyl and 1,2-linked rhamnosyl residues were also identified (Table 2). These results were consistent with a polymer having an $\alpha(1 \rightarrow 4)$-linked galacturonic acid backbone with some insertions of rhamnose. Taking into account the results from the second procedure, the degree of substitution of galacturonic acid by xylose residues was ≈72% (including 73% of terminal xylosyl, 22% of 2-linked xylosyl and 4% of 3-linked xylosyl residues). Hence, galacturonic acid residues carried mainly single terminal xylosyl residues attached on O-3 and few short side-chains of xylosyl residues linked on O-2 or sometimes on O-3 (Fig. 5).

4. Discussion

Large amounts of xylose were present in pea hulls. It originated mainly from xylans (Ralet et al., 1993a,b; Weightman et al., 1994). However, xylose-rich pectins were also identified in the copper precipitate of the hot acid extract, suggesting that xylogalacturonans could be present (Renard et al., 1997). Our results confirmed that pea hulls contained xylogalacturonans.

They were extracted from de-esterified pea hulls by acidic hydrolysis. In this way, rhamnogalacturonans were also solubilised whereas homogalacturonans were insoluble. The difference in solubility of homogalacturonans and xylogalacturonans, which have the same backbone, was apparently due to the substitution by xylose which prevented association between molecules.

Xylose and galacturonic acid residues co-eluted during ion-exchange as well as size-exclusion chromatography. Xylose and galacturonic acid still co-eluted after enzymic treatments either by RG-hydrolase (this paper), endopolygalacturonase or endoxylanase (Renard et al., 1997). These results strongly indicated that xylose and galacturonic acid belonged to the same polymer.

Size exclusion chromatography of the RG-hydrolase digest of the HCl-soluble material provided two xylogalacturonans fractions (FI and FII). FI contained rhamnose residues, but was not further degraded by RG-hydrolase suggesting that: (i) rhamnogalacturonans regions were shorter than required for the recognition by the RG-hydrolase, since it needed four successive alternating GalA–Rha residues to act on the rhamnogalacturonic backbone (Mutter, Renard, Beldman, Schols & Voragen, 1998); (ii) rhamnose and galacturonic acid residues did not strictly alternate. The presence of resistant rhamnogalacturonan sequences could explain the high molar mass of FI. In contrast, rhamnogalacturonans were degraded by RG-hydrolase allowing the release of FII, suggesting that xylogalacturonans from FII could be rather included in the rhamnogalacturonan region.

Methylation analysis of FI and FII allowed three main derivatives. 1,4- and 1,3,4-Linked galacturonic acid residues and terminal xylosyl residues to be identified, suggesting substitution of the galacturonan backbone by xylose units on O-3.

Some xylogalacturonans have been previously described (Bouveng, 1965; De Vries et al., 1983; Kikuchi et al., 1996; Matsuura, 1984; Matsuura & Hatanaka, 1988; Schols et al., 1995; Voragen et al., 1993; Yu & Mort, 1996). Our xylogalacturonans were extracted after the saponification of pea hulls which explains why they had low DM compared to xylogalacturonans from apple (Schols et al., 1995). The main difference compared with other xylogalacturonans concerned the pattern of substitution of xylogalacturonans. They were more substituted than xylogalacturonans from mountain pine pollen (Xyl/GalA = 0.5), kidney bean (Xyl/GalA = 0.5) or soy sauce (Xyl/GalA = 0.2). Xylose was mainly present as single units on O-3. Moreover, some oligomeric side-chains of 2-linked xylose were identified in pea hulls. They were shorter than the side-chains previously described by Renard et al. (1997), probably because extraction was carried out under acidic conditions. Glycosidic bonds are known to have different susceptibilities to acid hydrolysis and glycosidic bonds between neutral sugars were first cleaved (Thibault et al., 1993). The side chains of xylose could thus be partially hydrolysed during extraction, even when linkages between galacturonic acid and xylose are preserved.

5. Conclusions

Xylogalacturonans were extracted from pea hulls by

acidic hydrolysis after saponification, purification by RG-hydrolase treatment, and size-exclusion chromatography with a yield 18 mg/g of the initial pea hulls. They were characterised by a molar ratio Xyl/GalA of 0.76. The behaviour of the xylogalacturonans during the purification led to consider them as a sub-fraction of "hairy" regions.

As the xylose substitution in xylogalacturonans inhibited endopolygalacturonase degradation (Yu & Mort, 1996), they could protect the galacturonan backbone from microbial contamination during seed dormancy. However, Bouveng (1965) observed an enzyme able to partially degrade xylogalacturonans from pollen mountain pine. More recently, Beldman, Van den Broek, Schols, Searle-van Leeuwen, van Laere and Voragen (1996) had identified an exogalacturonase able to degrade xylogalacturonans from soy. Kester, Benen and Visser (1999) had also found an exogalacturonase active on saponified xylogalacturonans from apple. It could be of interest to look for enzymes specific for xylogalacturonans degradation, either to split linkages in the backbone or to lower the level of xylose.

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