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Pectins from citrus peel cell walls contain homogalacturonans homogenous with respect to molar mass, rhamnogalacturonan I and rhamnogalacturonan II

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Abstract

Pectins of different structures were extracted from citrus peels with water, oxalate, hot dilute hydrochloric acid and cold dilute sodium hydroxide. Homogalacturonans (HGs) were isolated from the four pectins by mild acid hydrolysis after deesterification. The study of their macromolecular properties revealed that they have similar number- and weight-average degrees of polymerisation, in the range of 81–117 galacturonic acid units, irrespective of the initial pectin. It was inferred that citrus peel pectins consist of HG strectches of similar lengths whatever the mode of extraction. Rhamnogalacturonan I (RG I) and rhamnogalacturonan II (RG II) were isolated from the oxalate- and the acid-extracted pectins using an endo-1,4-α-polygalacturonase followed by anion-exchange and size-exclusion chromatographies. RG I was composed mainly of arabinose, galactose, galacturonic acid and rhamnose, suggesting the presence of arabinan and/or (arabino)galactan side chains. Debranched RG I (dRG I) was obtained by treating the RG I arising from acid-extracted pectin with endo-1,5-α-L-arabinanase, endo-1,4-β-D-galactanase, α-L-arabinofuranosidase and β-D-galactosidase in admixture. The molar ratios of galacturonic acid to rhamnose of RGs I and dRG I were very close to 1/1, indicating a strict repeating $[GalA–Rha]_n$ pattern in the backbone. It is concluded that citrus pectins consist predominantly of HG, with a few RG I and a minor RG II fraction.

Keywords: Citrus peel; Sequential extraction; Pectin; Homogalacturonan; Rhamnogalacturonan I; Rhamnogalacturonan II

1. Introduction

Pectic substances are a family of complex heteropolysaccharides present in higher plant middle lamellae and primary cell walls. Their structure generally encompasses homogalacturonan (HG), type-one rhamnogalacturonan (RG I) and type-two rhamnogalacturonan (RG II). These three pectic polysaccharides are believed to be covalently linked to one another to form the pectin macromolecule although their structural arrangement is still an open question. Indeed, for a long time, a schematic representation of pectin macrostructure has shown HG, RG I (and RG II) in linear alternance (Schols & Voragen, 1996). In contrast, it has recently been suggested that HG and subsequently substituted galacturonans such as RG II could be connected as side chains to RG I (Vincken et al., 2003).

HG consists of 1,4-linked α-D-galacturonic acid (GalA) and may have a chain length of ~100 consecutive GalA residues (Thibault, Renard, Axelos, Roger, & Crépeau, 1993). RG I is a side-chain-containing polysaccharide whose backbone is believed to consist of [→4-α-D-Gal*p*A-(1 → 2)-α-L-Rha*p*-(1→] disaccharide repeating units. The side chain decorations, mainly linked to the *O-4* of some of the α-L-Rha units, could be arabinans, galactans,

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type-one and/or type-two arabinogalactans (Albersheim, Darvill, O'Neill, Schols, & Voragen, 1996). RG II is a low molecular weight (5–10 kDa) highly complex molecule whose backbone is composed of at least eight 1,4-linked α-D-Gal*p*A units to which four structurally conserved side chains, consisting of twelve different monosaccharides, including GalA and unusual sugars such as, apiose, 2-*O*-methyl-fucose, 2-*O*-methyl-xylose, 3-*C*-carboxy-5-deoxy-L-xylose (aceric acid), 2-keto-3-deoxy-D-*manno*-octulosonic acid (Kdo) and 3-deoxy-D-*lyxo*-heptulosaric acid (Dha), are attached (Darvill, McNeil, & Albersheim, 1978; Vidal et al., 2000).

Many of the functionalities of pectins are related to their contents in GalA and neutral sugars, the amounts and distribution of substituents (methoxyl and acetyl groups), and the molar mass and its distribution (Axelos & Thibault, 1991). The length of HG and the proportion of HG, RG I and RG II, in the molecule, may also influence the pectin properties (Bonnin, Dolo, Le Goff, & Thibault, 2002a). Thus, the determination of the macromolecular characteristics of HG, RG I and RG II could provide useful information, not only to improve the properties of extracted pectins, but also to envision possible biosynthesis routes of native pectins in the cell wall. In literature, various experimental procedures have been reported to isolate these pectic polysaccharides.

HGs have been isolated from various commercial acid-extracted pectins by mild acid or enzymatic hydrolysis (Bonnin et al., 2002a; Hellín, Ralet, Bonnin, & Thibault, 2005; Thibault et al., 1993). RG I polysaccharides have been isolated either directly from various purified plant cell walls or from extracted pectins using endopolygalacturonase (Endo-PG) in combination or not with pectin methyl esterase and side-chain degrading enzymes (Edashige & Ishii, 1997; McNeil, Darvill, & Albersheim, 1980; Øbro, Harholt, Scheller, & Orfila, 2004; Strasser & Amadò, 2001). RG II polysaccharides have been isolated with Endo-PG or other pectinases, either directly from purified plant cell walls or from buffer-, chelating-agent- and alkali-extracted pectins (Darvill et al., 1978; Hilz, Williams, Doco, Schols, & Voragen, 2006; Ishii, 1982; Pellerin et al., 1996; Strasser & Amadò, 2002; Thomas, McNeil, Darvill, & Albersheim, 1987; Vidal et al., 2000).

Pectins, isolated from various plant cell walls by the same extracting agent or from a given plant cell wall by various extractants, may differ in their HG and RG I regions, which may result in a structural variability and different chemical and physicochemical properties. As a first study of this structural variability, one plant material (citrus peel) was chosen and pectins were extracted using four chemical agents of different strengths. HGs were isolated from the sequentially extracted pectins by a mild acid procedure. RG I and RG II were isolated from the oxalate- and acid-extracted pectins using Endo-PG followed by anion-exchange and size-exclusion chromatographies. The macromolecular properties of the extracted pectins, HGs and one of the RGs I thereof obtained were examined using high-performance size-exclusion chromatography in combination with multi-angle laser light scattering and viscometry.

2. Materials and methods

2.1. Material

2.1.1. Citrus peels

Dried industrial citrus peels from Degussa Texturant Systems (Carentan, France) were stored at room temperature until used.

2.1.2. Enzymes

Endo-poly-1,4-α-D-galacturonide glycanohydrolase II (Endo-PG II, E.C. 3.2.1.15) was purified from a crude preparation of a cloned *Aspergillus niger* from Novozymes (Bagsvaerd, Denmark) as previously described (Bonnin et al., 2002b). α-L-Arabinofuranosidase (α-Ara-ase, E.C. 3.2.1.55) from *A. niger* was purchased from Megazyme (Bray, Ireland). β-D-Galactosidase (β-Gal-ase, E.C. 3.2.1.23), from *A. oryzae*, was purchased from Sigma (L'Isle d'Abeau, France). Endo-1,5-α-L-arabinanase (Endo-A, E.C. 3.2.1.99) and endo-1,4-β-D-galactanase (Endo-G, E.C. 3.2.1.89) were purified from *A. niger* as previously described (Bonnin et al., 2002b). Each of the enzymatic preparations had no measurable activity other than its main activity.

2.2. Methods

2.2.1. Preparation of citrus peel cell wall material (CWM)

Dried citrus peels (1 kg) were heated in 3 L of boiling 80% v/v ethanol for 20 min and filtered on a G3 sintered glass. The insoluble residue was washed with 70% v/v ethanol until the filtrate gave a negative reaction with the phenol-sulfuric acid test (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). The solid residue was dried by solvent exchange (95% ethanol, acetone), and finally dried at 40 °C in a ventilated oven for 72 h. The CWM preparation was carried out in duplicate. Dried CWM was stored at room temperature until used.

2.2.2. Sequential extraction of pectins from citrus peel CWM

Five grams of CWM were suspended in 150 mL of deionised water and adjusted to pH 4.5 with 0.1 M hydrochloric acid. The suspension was shaken at 120 rpm, for 30 min, at 25 °C. The resulting slurries were filtered through a G3 sintered glass and the pH of the filtrate was adjusted to 4.5. The residue was extracted twice again under the same conditions. The filtrates were combined and concentrated at 40 °C to approximately 200 mL and extensively dialysed (6000–8000 MWCO tubing) against deionised water. The material remaining in the dialysis tubing was freeze-dried, vacuum-dried at 40 °C overnight and weighed. This product was referred to as water-extracted pectin (WEP). The solid residues were then extracted with

1% w/v potassium oxalate, at pH 4.5 and at 25 °C (3× 150 mL, 3× 30 min), and treated as before to yield an oxalate-extracted pectin (OEP). The residue was rinsed twice with water to remove oxalate and extracted with 0.05 M hydrochloric acid at 85 °C (3× 150 mL, 3× 30 min), thereby leading to an acid-extracted pectin (HEP). The residue, after being neutralized to pH 5 with 0.1 M sodium hydroxide and cooled to 4 °C, was extracted with 0.05 M sodium hydroxide at 4 °C (3× 150 mL, 3× 30 min), and treated as before to yield a sodium hydroxide-extracted pectin (OHEP). The remaining residue was brought to pH 5 with 0.1 M hydrochloric acid and washed three times with 70% ethanol, dried by solvent exchange (95% ethanol, acetone), air-dried in a ventilated oven at 40 °C for 72 h and weighed. For each crude extract, the pH was brought to 4.5 using either 0.1 M hydrochloric acid or 0.1 M sodium hydroxide. Sequential extraction was performed in duplicate.

2.2.3. Isolation of the HG domains

The pectins were saponified with 0.1 M sodium hydroxide at 4 °C to remove the methyl and acetyl ester groups. Deesterified pectins (0.1%, w/v) were hydrolysed with 0.1 M hydrochloric acid, at 80 °C, for 72 h in sealed tubes (Thibault et al., 1993). The acid-soluble and acid-insoluble fractions were separated, after cooling, by centrifugation of the reaction mixture at 15,000*g* for 20 min in a tabletop centrifuge. The insoluble material containing the HGs was suspended in water and the HGs were solubilised by gradually increasing the pH to about 7 with 0.2 M sodium hydroxide. The solution was kept under gentle stirring at 4 °C overnight, exhaustively dialysed (6000–8000 MWCO tubing) against deionised water, freeze-dried, and weighed.

2.2.4. Isolation of the RG domains

Six milligrams per millilitre of deesterified OEP or HEP were dissolved in 50 mM sodium acetate buffer, pH 4.5 and an Endo-PG II from *A. niger* (3× 5 μL, 7000 nkat/mL) was added. The mixture was incubated at 35 °C for 48 h. The end products were extensively dialysed (1 kDa MWCO tubing) against deionised water and freeze-dried, and then fractionated by anion-exchange and size-exclusion chromatographies, as described below, thereby leading to the isolation of RG I and RG II. Subsequently, RG I from HEP was treated with Endo-A (2× 30 μL, 21 nkat/mL), Endo-G (2× 50 μL, 8 nkat/mL), α-Ara-ase (2× 20 μL, 4170 nkat/mL) and β-Gal-ase (2× 20 μL, 500 nkat/mL) in admixture at 35 °C, for 48 h and the reaction products were desalted using a 1 kDa MWCO dialysis tubing. The material remaining within the membrane was freeze-dried and referred to as debranched RG I (dRG I).

2.2.5. Chromatographies

Low-pressure anion-exchange chromatography (AEC) was performed at room temperature on a DEAE-Sepharose Fast Flow (Pharmacia, Uppsala, Sweden) column (25 × 2.6 cm) equilibrated with a degassed 50 mM sodium succinate buffer, pH 4.5 as previously described (Ralet et al., 2005). Fractions were analysed for their content in GalA and neutral sugars (Thibault, 1979; Tollier & Robin, 1979). The fractions of interest were pooled, concentrated at 40 °C, extensively dialysed (1 kDa MWCO tubing) and freeze-dried.

Low-pressure size-exclusion chromatography (SEC) was performed at room temperature on a (80 × 1.6 cm) column of Sephacryl S-200 (Pharmacia, Uppsala, Sweden) as described previously (Ralet et al., 2005). Fractions were analysed for their content in GalA and neutral sugars (Thibault, 1979; Tollier & Robin, 1979). The fractions of interest were pooled, concentrated, extensively dialysed (1 kDa MWCO tubing) and freeze-dried.

High-pressure size-exclusion chromatography (HPSEC) was carried out on two columns (Shodex OH-Pack SB-804 HQ and OH-Pack SB-805 HQ) equipped with one Shodex OH SB-G pre-column as described previously (Hellín et al., 2005). Solutions (~5 mg/mL of pectins, RG I or dRG I and 10 mg/mL of HGs) prepared in 50 mM sodium nitrate containing 0.02% sodium azide as a preservative were analysed. The refractive index increment (dn/dc) value was taken as 0.146 mL/g (Chapman, Morris, Selvendran, & O'Neill, 1987). The M_w and M_n were calculated by the Astra software (Wyatt, Santa Barbara, CA). The intrinsic viscosity ([η]) was calculated using the TriSEC software (Version 3.0, Viscotek, Houston, TX).

2.2.6. Analytical

Analyses were performed in duplicate. The GalA content was quantified according to the *meta*-hydroxybiphenyl method (Ahmed & Labavitch, 1977; Thibault, 1979) for insoluble and soluble fractions, respectively. Total neutral sugars (Gal, Ara or Rha standards) were determined by the automated orcinol method (Tollier & Robin, 1979). Total neutral sugars were corrected for interfering GalA. Individual neutral sugars were analysed as their alditol acetates (Blakeney, Harris, Henry, & Stone, 1983) by gas-liquid chromatography (GLC) on a DB-225 fused-silica capillary column (30 mL × 0.32 mm i.d., J&W Scientific, Courtaboeuf, France). Acid hydrolysis of the samples was optimised for each set of samples as described previously (Levigne, Ralet, & Thibault, 2002a). *Myo*-inositol was used as an internal standard.

To determine the glycosyl residue composition of RG II, samples were submitted to methanolysis (2 M MeOH/HCl, 18 h, 105 °C) and silylation (hexamethyldisilizane: trimethylchlorosilane:pyridine, 3:1:9 v/v at 80 °C for 20 min). The resulting 1-*O*-methyl persilyl methyl ester derivatives of sugars were quantified by GLC. Presence of glycosyl residues specific of RG II was confirmed by GLC coupled to electronic impact mass spectrometry (GLC–EIMS) as described by Doco, O'Neill, & Pellerin (2001).

Methyl and acetyl contents were determined by high-performance liquid chromatography (HPLC) on a (250 × 4.6 mm) superspher-100 RP18e C-18 reverse-phase column (Merck, Germany) as previously published

(Levigne, Thomas, Ralet, Quemener, & Thibault, 2002b) using isopropanol as internal standard. Degrees of methylesterification (DM) and acetylesterification (DA) were calculated as the percent molar ratios of methanol and acetic acid to GalA, respectively.

3. Results

3.1. Yields and chemical features of pectins

The CWM accounted for 72% of the citrus peel dry weight. Pectins were sequentially extracted from the CWM with water (WEP), potassium oxalate (OEP), hot dilute hydrochloric acid (HEP) and cold dilute sodium hydroxide (OHEP). The yields of WEP, OEP, HEP and OHEP were 5.8%, 14.7%, 27.3% and 4.8%, respectively (Table 1). The insoluble residue represented 33.2%, indicating a total recovery of 85.8%. The WEP yield was close to a value of 4% previously reported (Ralet & Thibault, 1994) and the HEP yield was in the range of the values of 24.1–34.1% (Ralet & Thibault, 1994; Thibault, De Dreu, Geraeds, & Rombouts, 1988). The OHEP yield was lower than the 13.9% reported by Ros, Schols, & Voragen (1996), presumably because of the removal after centrifugation of the water insoluble fraction formed during dialysis. A similar water insoluble fraction has been separated from the water soluble one in Ros, Schols, & Voragen (1998) but not removed in their first study.

The chemical composition of the CWM, pectin fractions, and residue of extraction is shown in Table 1. The CWM consists of 81.7% of carbohydrates, mainly GalA (32.9%), Glc (25.8%), Ara (8.4%) and Gal (6.4%). This amount was higher than the amounts of 67.4% (Ralet & Thibault, 1994) in commercial lemon dietary fibres, 59.4% (Ros et al., 1996, 1998) in lemon albedo-alcohol insoluble solids and 69.9% (Brillouet et al., 1988) in lemon pulps, indicating the importance of the method of preparation of cell wall material and/or the origin of the plant material. The GalA content of CWM was 32.9%, a value rather consistent with those previously found (20.7–28.3%) for lemon alcohol insoluble solids (De Vries, Rombouts, Voragen, & Pilnik, 1984; Ros et al., 1996, 1998). It could be calculated that the citrus peels consist of 23.7% of pectic substances (on a GalA basis), assuming that no GalA residue was removed during CWM preparation, a content in good agreement with those found (22.3–26.0%) in lemon pulps and lemon dietary fibres (Brillouet et al., 1988; Ralet & Thibault, 1994). The DM (69) and DA (13) of the CWM are consistent with those previously reported (De Vries et al., 1984; Ros et al., 1996, 1998).

The sugar compositions (Table 1) of the extracted pectins were rather similar to those reported in previous studies (Ralet & Thibault, 1994; Ros et al., 1996, 1998). The HEP or OHEP were richer in neutral sugars than the WEP and OEP as reported from various plant sources (Fügel, Carle, & Schieber, 2004; Yapo & Koffi, 2006). In addition, the Rha contents of the HEP and OHEP were higher than those of the WEP and OEP, suggesting that HEP and OHEP are richer in "hairy" regions than the WEP and OEP. All of the extracted pectins possessed high DM and low DA, except the DM of the OHEP which was deesterified during extraction. The high DM of the OEP showed that a chelating agent such as oxalate could solubilise pectins of high methoxyl content as also reported previously (Ros et al., 1996, 1998). The mechanism of extraction, however, remains unclear. The OHEP still contains methyl esters groups, consistent with literature reports (Ros et al., 1996, 1998). The GalA residues of the WEP, OEP, HEP and OHEP represented approximately 11%, 32%, 45% and 7% of the GalA initially present in the CWM, which accounted for a total amount of 95%, thus indicating that the bulk of the pectic substances

Table 1
Yield (g/100 g of the CWM dry weight) and some chemical features of the initial pectin fractions[a]

	CWM	WEP	OEP	HEP	OHEP	Residue
Yield	100	5.8	14.7	27.3	4.8	33.2
Sugar composition (%w/w)[b]						
GalA	32.9 (36.9)	61.5 (80.1)	70.4 (86.5)	54.5 (66.3)	46.3 (71.3)	4.6 (4.8)
Rha	0.9 (1.2)	0.9 (1.4)	0.9 (1.3)	1.6 (2.3)	3.2 (5.9)	2.4 (3.0)
Fuc	0.6 (0.8)	0.1 (0.2)	nd	0.2 (0.3)	nd	1.2 (1.5)
Ara	8.4 (12.6)	4.7 (8.2)	3.8 (6.2)	13.5 (21.9)	5.9 (12.1)	1.0 (1.4)
Xyl	3.7 (5.5)	0.8 (1.4)	0.4 (0.7)	0.1 (0.2)	1.2 (2.5)	7.9 (11.1)
Man	3.0 (3.7)	nd	nd	0.6 (0.8)	0.2 (0.3)	7.0 (8.0)
Gal	6.4 (7.8)	3.3 (4.7)	2.5 (3.3)	3.5 (4.6)	4.2 (7.0)	5.3 (6.0)
Glc	25.8 (31.5)	2.9 (4.1)	1.5 (2.0)	2.7 (3.6)	0.5 (0.8)	56.2 (64.1)
Degrees of esterification (mol%)						
DM	69.4	76.5	73.7	65.1	10.0	ND
DA	12.8	5.5	2.3	3.0	nd	ND

nd, not detected.
ND, not determined.
[a] Values are the average of two replicates.
[b] Values in brackets represent the sugar composition in mol percent.

initially present in the CWM were extracted. The greatest part (77%) was extracted during the oxalate and hot dilute acid extraction steps. The molar ratios of GalA to Rha were calculated and values of 57/1, 65/1, 29/1 and 12/1 were found for WEP, OEP, HEP and OHEP, respectively. The HEP and OHEP appeared to be richer in RG regions than the WEP and OEP. The length of each HG or RG stretch as well as the proportion HGs and RG Is could explain these differences. These pectic polysaccharides were therefore isolated and characterized.

3.2. Chemical and macromolecular features of the deesterified pectins

Pectins were first deesterified before isolating HG and RG regions in order to avoid differences in susceptibilities to acid hydrolysis of the glycosidic bonds because of the ester groups, and to allow the Endo-PG to be active. The deesterification was performed under cold alkaline conditions to minimize β-elimination of the GalA chains. The chemical and physicochemical features of the deesterified pectins are given in Table 2. The deesterified WEP, OEP and HEP consist of approximately 90% of sugars whereas the sugar content in deesterified OHEP, that was richer in proteins (Table 2), represented only ~75% of the extract. Furthermore, the molar ratios of GalA to Rha of the deesterified WEP, OEP, HEP and OHEP were 55/1, 68/1, 29/1 and 10/1, respectively, in agreement with the values obtained before being chemically deesterified, indicating that the pectin backbones were not affected. The number ($\langle M_n \rangle$)- and weight ($\langle M_w \rangle$)-average molar masses of the deesterified pectins were high, ranging from 83,000 to 251,000 g/mol and from 111,000 to 380,000 g/mol, respectively. The values of radius of gyration of the pectins were relatively low, ranging from 16 to 25 nm, suggesting rather flexible and/or branched macromolecules.

Table 2
Some chemical and physicochemical features of the deesterified pectins[a]

	WEP	OEP	HEP	OHEP
Sugar composition (%w/w)[b]				
GalA	79.1 (90.4)	80.7 (88.7)	77.7 (87.8)	55.1 (67.9)
Rha	1.2 (1.6)	1.0 (1.3)	2.2 (3.0)	4.8 (7.1)
Fuc	0.1 (0.1)	nd	nd	nd
Ara	2.6 (4.0)	3.9 (5.7)	3.4 (5.1)	10.5 (17.3)
Xyl	0.5 (0.8)	0.6 (0.9)	0.6 (0.9)	0.3 (0.5)
Man	nd	nd	nd	0.2 (0.3)
Gal	2.2 (2.7)	2.1 (2.5)	2.3 (2.8)	3.6 (4.8)
Glc	0.3 (0.4)	0.7 (0.8)	0.3 (0.4)	1.6 (2.1)
Protein	2.7	3.6	4.1	10.4
DM (mol%)	2.0	3.0	3.0	0.0
DA (mol%)	0.0	0.0	0.0	0.0
$[\eta]$ (mL/g)	404	489	490	302
$\langle M_n \rangle$ (g/mol)	83,300	100,800	251,000	99,250
$\langle M_w \rangle$ (g/mol)	111,000	142,700	379,700	134,100
I_p	1.3	1.4	1.5	1.4
R_g (nm)	20.0	21.9	25.0	16.3

nd, not detected.
$[\eta]$, intrinsic viscosity.
$\langle M_n \rangle$, number-average molar mass.
$\langle M_w \rangle$, weight-average molar mass.
I_p, polydispersity index.
R_g, radius of gyration.
[a] Values are the average of two replicates.
[b] Values in brackets represent the sugar composition in mol percent.

3.3. Chemical and macromolecular features of the HGs

The yield, chemical and physicochemical properties of the isolated HGs are shown in Table 3. The HGs from the WEP, OEP or HEP accounted for ~90% by weight of the deesterified pectins and that from the OHEP accounted for ~60%. Each of them contained a very high amount of GalA (80–90%) and a minor amount of neutral sugars (0.7–1%). The GalA content was >98 mol% of all of the sugars identified and quantified, and Rha content was <0.2 mol%, indicating that the HGs in citrus pectin consist almost exclusively of GalA, in agreement with previous findings (Hellín et al., 2005; Thibault et al., 1993). As it can be calculated from Table 3, the GalA residues of each HG accounted for 95% of the GalA present in the starting pectin for WEP, OEP and HEP and for 85% of the GalA present in the starting pectin for OHEP, showing that HGs are predominant in citrus pectins.

Fig. 1 illustrates the HPSEC elution patterns of the HGs. All of them exhibited fairly narrow and very homogenous molar mass distributions with the same elution vol-

Table 3
Some chemical and physicochemical features of the HGs isolated from the deesterified pectins[a]

	HGs			
	WEP	OEP	HEP	OHEP
Yield%[b]	90.2	90.5	88.3	56.5
Sugar composition (%w/w)[c]				
GalA	83.7 (98.9)	85.2 (98.8)	83.3 (98.5)	83.1 (98.9)
Rha	tr	tr	0.1 (0.1)	0.1 (0.1)
Fuc	nd	nd	nd	nd
Ara	nd	nd	nd	nd
Xyl	0.5 (0.7)	0.4 (0.5)	0.6 (0.8)	0.2 (0.3)
Man	nd	nd	nd	nd
Gal	nd	0.2 (0.3)	0.2 (0.3)	0.3 (0.4)
Glc	0.2 (0.3)	0.2 (0.3)	0.1 (0.1)	0.2 (0.3)
$[\eta]$ (mL/g)	90.3	86.8	85.1	75.2
$\langle M_n \rangle$ (g/mol)	16,700	16,900	15,300	14,200
$\langle M_w \rangle$ (g/mol)	20,600	19,700	18,800	17,000
I_p	1.2	1.1	1.1	1.2
R_g (nm)	9.6	9.1	9.5	8.8

$[\eta]$, intrinsic viscosity.
$\langle M_n \rangle$, number-average molar mass.
$\langle M_w \rangle$, weight-average molar mass.
I_p, polydispersity index.
R_g, radius of gyration.
nd, not detected.
tr, trace: <0.05%.
[a] Values are the average of two replicates.
[b] Yield%, g of HG/100 g of deesterified pectin.
[c] Values in brackets represent the sugar composition in mol percent.

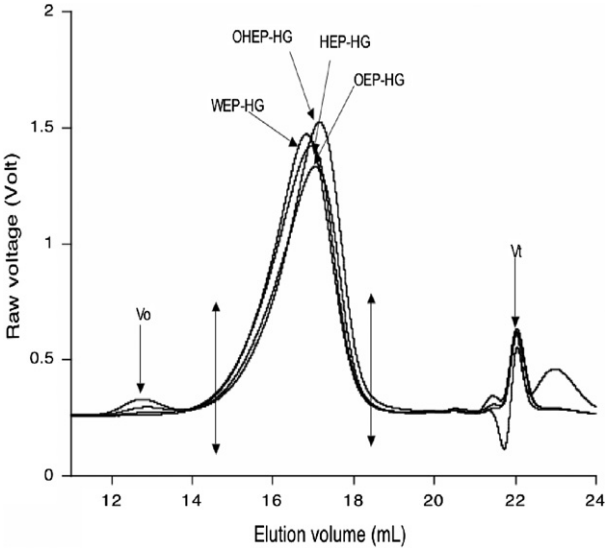

Fig. 1. HPSEC elution profiles of acid-isolated HGs from deesterified pectins.

ume (16.8 mL). The intrinsic viscosity of the HGs ranged from 75 to 90 mL/g, comparable to a previous value of 86 mL/g (in 0.155 M NaCl) reported for an HG from a commercial citrus pectin (Thibault et al., 1993). The values of the $\langle M_n \rangle$ and $\langle M_w \rangle$ were very similar, leading to an I_p value very close to 1, thus showing very homogenous polysaccharide fractions with respect to polymer size. The radius of gyration ranged narrowly from 8.8 to 9.6 nm, showing that HGs are macromolecules of rather rigid and extended conformation (Boutherin, Mazeau, & Tvaroska, 1997; Hellín et al., 2005). Number- and weight-average degrees of polymerisation of 95–117, 96–112, 87–107 and 81–97 consecutive GalA residues could therefore be estimated for the HGs from WEP, OEP, HEP and OHEP, respectively, a range value in good agreement with that found (114–138) for HGs from a commercial citrus pectin (Thibault et al., 1993). From these results, it could be inferred that all of the HGs exhibit similar macromolecular properties irrespective of the initial pectin and therefore independently of the extracting agent used.

3.4. Endo-PG treatment of pectin and analysis of the reactions products

An Endo-PG was used to degrade the HG regions of deesterified HEP, thus releasing the RG regions as intact as possible. After extensive dialysis, the Endo-PG-treated HEP was fractionated by AEC on a DEAE Sepharose Fast Flow column (Fig. 2a). The unbound fractions, containing mono-, di- and tri-mers of GalA (Ralet et al., 2005), were eluted with 0.05 M sodium succinate buffer, and the bound population was released from the column with a linear gradient of NaCl. The bound population was pooled as indicated in Fig. 2a and further fractionated by SEC on a Sephacryl S-200 column. Three peaks referred to as pools

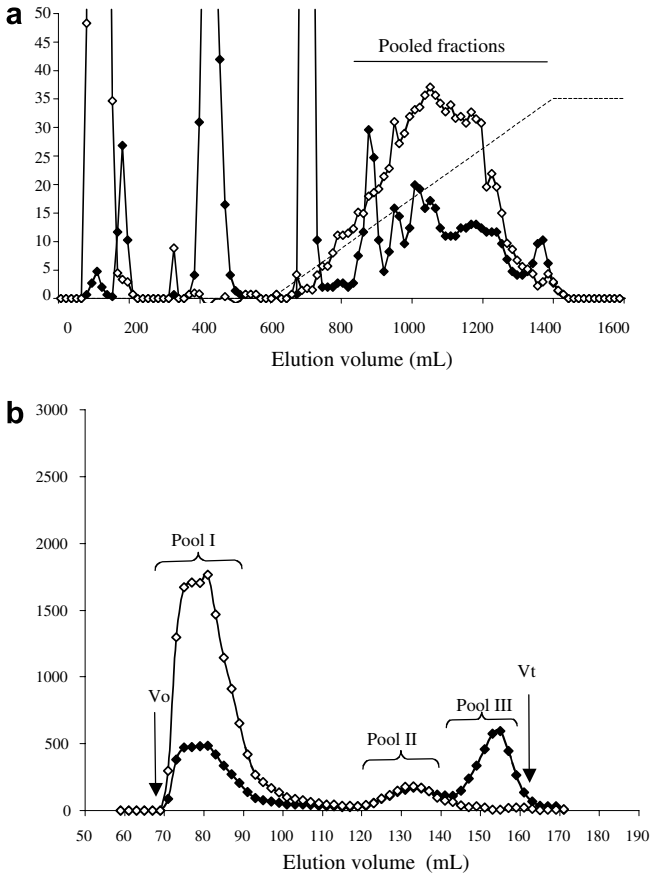

Fig. 2. (a) Anion-exchange chromatography of Endo-PG II-treated deesterified HEP fraction, GalA (♦); neutral sugars (◇); (—) NaCl gradient. (b) Size-exclusion chromatography of the AEC-pooled fractions, GalA (♦); neutral sugars (◇).

I, II and III (Fig. 2b) were recovered. Pool III, eluting near the total volume of the column, corresponded to oligogalacturonides and was not further analysed. Pools I and II were further studied. As shown in Table 4, pool II, accounting for <1% by weight of deesterified HEP, contained diagnostic sugars of RG II such as 2-*O*-methyl Fuc, aceric acid, 2-*O*-methyl Xyl, Kdo and apiose, in addition to the most common sugars (GalA, Rha, Ara and Gal) of pectic polysaccharides, thus confirming the presence of RG II. The glycosyl-residue composition (mol%) of the isolated RG II from citrus pectin is similar to that reported for

Table 4
Sugar composition (mol%) of RG II isolated from the HEP

Sugars			
GalA	43.3	Fuc	4.2
GlcA	1.5	2-*O*-methyl Fuc[a]	0.7
Rha	16.9	2-*O*-methyl Xyl[a]	2.4
Ara	12.1	Kdo[a]	0.4
Xyl	2.6	Dha[a]	ND[b]
Gal	10.5	Apiose[a]	1.6
Glc	1.3	Aceric acid[a]	0.6

[a] Confirmed by GLC–EIMS.
[b] Not determined.

RG II from other plant sources as summarised by Strasser & Amadò (2002), except that the amount of Api was relatively low in our case, presumably due to partial degradation during pectin extraction by acid.

Pool I, accounting for ~92% of the Endo-PG resistant material and >10% of deesterified HEP (Table 5), contained mainly Ara next to Gal, GalA and Rha (Table 4) and minor amounts of Xyl. The molar ratio of GalA/Rha was ~1.06/1, indicating that the polysaccharides in pool I corresponded to RG I containing nearly equimolar amounts of GalA and Rha. The GalA amount in the RG I accounted for 3.3% of the GalA initially present in deesterified HEP. Likewise, the Rha amount in the RG I accounted for 86.9% of the Rha present in the deesterified HEP, showing that the bulk of GalA in citrus pectin is located in the HG regions and the bulk of Rha in RG I regions as also reported by Zhan, Janssen, & Mort (1998).

RG I regions were also recovered from deesterified OEP using the same experimental procedure. Results similar to those fully presented for deesterified HEP were obtained. The SEC fractionation yielded three main populations that could be assigned to RG I, RG II and remaining oligogalacturonides. The OEP-RG I fraction was rich in Gal, Ara, GalA and Rha, the two latter being again present in equimolar amounts (Table 5). However, due to limited quantities, OEP-RG I could not be further investigated.

The HEP-RG I was further analysed by HPSEC to determine its molar mass distribution and physicochemical parameters values. The RG I eluted in a wider range of molar masses (Fig. 3a) compared to the corresponding HG (see Fig. 1), including one shouldered peak. To better determine its average molar mass, the two peaks were individually integrated. Peak I accounted for 28% of the total amount. The average molar mass of this material could not be correctly measured due to a low response from the MALLS detector. In contrast, peak II, accounting for 72% of the material, was well detected by the MALLS. The $\langle M_w \rangle$ and I_p values of this peak material were 56,500 g/mol and 1.1, respectively (Table 5).

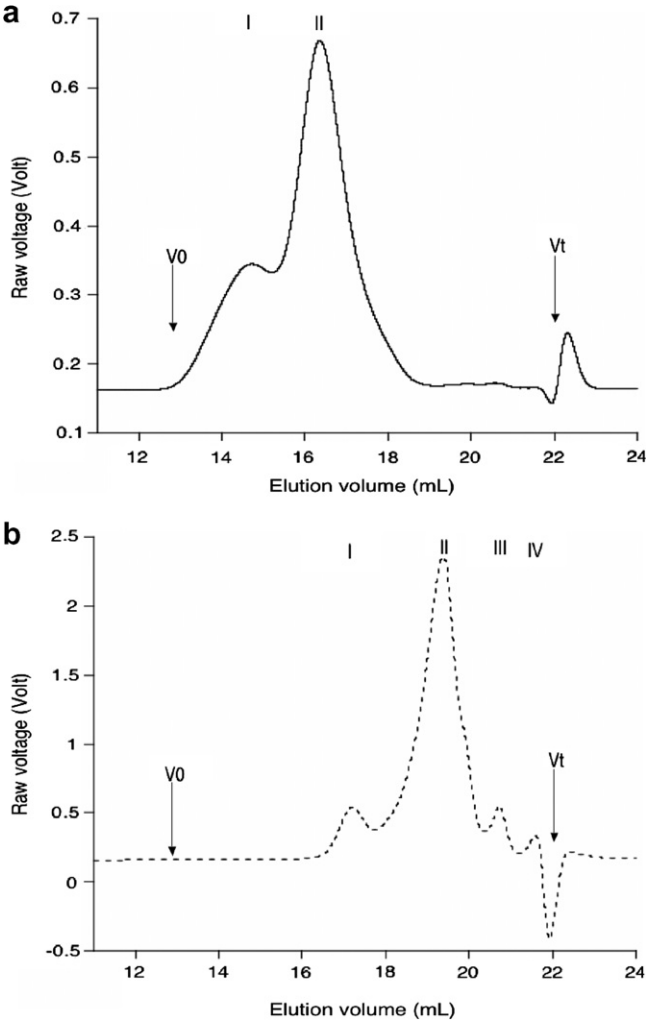

Fig. 3. (a) HPSEC elution profile of the highest molar mass material (RG I) obtained after SEC fractionation of the Endo-PG II-treated deesterified HEP. (b)HPSEC elution profile of the side-chains degrading enzymes-treated RG I (dRG I).

Table 5
Sugar composition (mol%) and some physicochemical features of RGs I and debranched RG I (dRG I)

Extraction mean	OEP	HEP	HEP
	RG I	RG I	dRG I
Yield%[b]	3.5	10.8	4.9
GalA[a]	11.0	20.1	45.9
Rha[a]	10.9	19.0	42.5
Ara[a]	32.3	36.2	2.3
Xyl[a]	nd	0.9	1.5
Gal[a]	45.9	23.8	7.8
$[\eta]$ (mL/g)	ND	54.7	5.3
$\langle M_n \rangle$ (g/mol)	ND	52,600	12,500
$\langle M_w \rangle$ (g/mol)	ND	56,500	15,000
I_p	ND	1.1	1.2
R_g (nm)	ND	10.7	3.1

nd, not detected.
ND, not determined.
$[\eta]$, intrinsic viscosity.
$\langle M_n \rangle$, number-average molar mass.
$\langle M_w \rangle$, weight-average molar mass.
I_p, polydispersity index.
R_g, radius of gyration.
[a] Values are the average of two replicates.
[b] Yield%, g of RG I or dRG I/100 g of deesterified pectin.

In order to investigate the backbone of RG I, Endo-A, Endo-G, α-Ara-ase and β-D-Gal-ase were used to degrade the neutral sugar side-chains, thereby releasing debranched RG I (dRG I). The material obtained after dialysis of the side-chain degrading enzymes-treated RG I, represented 45.4% of initial RG I and 4.9% of deesterified HEP (Table 5). The main glycosyl residues in this material were GalA and Rha as expected (Table 5), indicating that the neutral sugar side-chains were effectively degraded. It also contained relatively low amounts of others sugars. The presence of Ara and Gal indicated that arabinan and/or (arabino)galactan side-chains attached to the backbone of RG I were not completely degraded by the enzymatic

mixture. The presence of Xyl suggested a direct link of this glycosyl residue to GalA. The molar ratio of GalA/Rha was 1.08/1, consistent with that found before treating RG I with side-chain degrading enzymes. This suggested that no GalA and Rha were present as side-chain residues. Furthermore, this result confirmed that GalA strictly alternates with Rha in the backbone of RG I. The GalA amount in dRG I accounted for 3.3% of the GalA present in deesterified HEP and the Rha amount in dRG I accounted for 82.7% of the Rha present in deesterified HEP, consistent with the amounts in the RG I carrying neutral sugar side-chains, thus confirming no loss of GalA or Rha after the side-chain degrading enzymes treatment.

Fig. 3b illustrates the HPSEC elution profile of dRG I. The molar mass distribution was polydisperse and multimodal. Peak I, the highest size material accounting for ~10% of the total amount, could correspond to long RG I segments and/or to RG I segments bearing relatively short neutral sugar side-chains (not removed by the enzymes). Peak II, accounting for 89% of the total, could correspond to the portion of dRG I devoid of side-chain residues. Peaks III and IV eluting closer to the total volume of the columns may correspond to relatively long neutral sugar oligomers that were not eliminated from the dRG I fraction after dialysis. Only, the material in peak II may be analysed by the MALLS and its estimated $\langle M_n \rangle$ and $\langle M_w \rangle$ were 12,500 and 15,000 g/mol, respectively (Table 5), leading to number- and weight-average degrees of polymerisation of 34–40 [GalA–Rha] disaccharide repeating units, in good agreement with previous reports on citrus pectin (Prade, Zhan, Ayoubi, & Mort, 1999; Zhan et al., 1998).

4. Discussion

With the hypothesis that a sequential extraction scheme using various chemical extracting agents may solubilise, pectins of different structural features, water, potassium oxalate, hydrochloric acid and sodium hydroxide were used to extract pectins from citrus peel CWM. The sugar composition analysis indicated that pectins of different backbone compositions and degree of branching were extracted. Indeed, the molar ratio of GalA to Rha ranged as OEP > WEP > HEP > OHEP. Hence, the backbone of free and loosely bound pectic substances in citrus peel CWM, generally extractable with weak agents such as water and oxalate are composed predominantly of GalA and a few Rha. On the contrary, the more-"anchored" ones, extractable with acid and alkali consist mainly of GalA and relatively high amounts of Rha. These structural differences may be due to various lengths of the HGs and/or to various sizes of the RG I regions. HGs were therefore isolated from these pectins. It was observed that all of the isolated HGs had very similar macromolecular characteristics irrespective of the initial extracted pectins, indicating that the HG stretches in pectin are well conserved whatever the mode of extraction of pectins and, presumably whatever their location in the cell wall. The degrees of polymerisation of the isolated HGs are consistent with those from acid-extracted, citrus, beet and apple pectins (Bonnin et al., 2002a, 2002b; Hellín et al., 2005; Thibault et al., 1993). It could then be postulated that HGs are of the same length magnitude whatever the plant origin. Studies of HGs from pectins from various cell walls of plants phylogenically selected are underway to elucidate this hypothesis.

Therefore, the structural differences may be ascribed to the RG I domains. The RG I regions of all of the four extracted pectins could not be studied, because of insufficient materials, however, results obtained on HEP show that RG I is not as homogenous as HG is. Indeed, RG I eluted in a broad and wide molar mass range whereas HG eluted in a single narrow and symmetrical peak as analysed by HPSEC. Moreover, after degradation of the neutral sugar side-chains of RG I, the HPSEC elution profile of dRG I was polydisperse, indicating that polysaccharidic populations of various lengths were present in RG I.

Using an Endo-PG followed by ultrafiltration, Zhan et al. (1998) isolated, from a citrus pectin, a high-molecular weight material shown to correspond to a rhamnogalacturonan. Although these workers did not use the terminology RG I, the presence of GalA–Rha disaccharide repeats, in oligomers generated by a partial acid hydrolysis indicated that the polysaccharide was a RG I. However, the molar ratio of GalA to Rha of 1.7/1 is not consistent with a strictly repeating GalA–Rha disaccharide units in a typical RG I. It is possible that their conditions of enzymatic hydrolysis is not complete, thus leaving a HG fragment attached to RG I, thereby yielding a molar ratio of GalA to Rha by far >1/1. We showed, in this study, that the backbone of RG I from citrus pectin, indeed, contained an equal amount of GalA and Rha, thus confirming the hypothesis of strict alternance of GalA with Rha. Furthermore, using Endo-PG, we also isolated RG II from the HEP after SEC fractionation. To our knowledge, this is the first time that RG II is reported, not only for citrus pectin, but also for an acid-extracted pectin. Indeed, since acid is believed to cause degradation, RG II hitherto reported in literature have been isolated using Endo-PG or other pectinases either directly from purified plant primary cell walls or from buffer-, chelating agent- or alkali-extracted plant materials (Darvill et al., 1978; Ishii, 1982; Pellerin et al., 1996; Strasser & Amadò, 2002; Thomas et al., 1987; Vidal et al., 2000; Zablackis, Huang, Müller, Darvill, & Albersheim, 1995). This result shows that RG II could be isolated from acid-solubilised material from plant cell walls.

5. Conclusion

This study shows that pectins of different structures are obtained from citrus peel using various chemical extracting agents. The structural variability appeared not to be related to the homogalacturonic regions but likely to the rhamnogalacturonic regions. Indeed, HG was shown to be a homogenous polysaccharide with a rather constant

chain length. In contrast, RG I seems to consist of various polysaccharide portions. HGs are predominant in all extracted citrus pectins, portending a structural model consisting of much more HG stretches than RG I ones, in disagreement with that proposed by Schols & Voragen (1996). RG II was shown to be also present in the acid-extracted pectin. For deesterified acid-extracted pectin, a mass repartition HG/RG I/RG II of ~88/11/1 was evidenced. Calculations show that water- and oxalate-extracted pectins are probably slightly richer in HG regions (~90%) and alkali-extracted pectin much poorer in HG regions (~60%). Further studies, however, are necessary to determine the most adequate structural arrangement of HG, RG I and substituted galacturonan (RG II for e.g.) to form a pectin macromolecule and to envision possible biosynthesis routes.

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