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Homogalacturonans from lime pectins exhibit homogeneous charge density and molar mass distributions

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Abstract

Two series of lime pectins with different degrees and patterns of methyl-esterification were successively fractionated by size exclusion followed by anion exchange chromatography. 'Homogeneous' populations with respect to molar mass and charge density were thereby obtained and their physico-chemical properties were investigated. Recovered fractions were characterised by a high galacturonic acid and a low neutral sugars content. The homogeneous population obtained for each pectin sample was treated with side-chain releasing enzymes (rhamnogalacturonan hydrolase, endo-β-(1→4)-D-galactanase, endo-α-(1→5)-L-arabinanase, α-L-arabinofuranosidase and β-D-galactosidase) and combinations of these enzymes. The reaction products were fractionated by anion exchange chromatography and their constitutive homogalacturonans were recovered. Each homogalacturonan was found homogeneous with respect to charge density and to molar mass (polydispersity index close to 1).

Keywords: Pectin; Rhamnogalacturonan hydrolase; Homogalacturonan

1. Introduction

Pectins are complex polysaccharides that constitute a large proportion of the cell wall of many higher plants and play a key role in growth, development and senescence (Ridley, O'Neill, & Mohnen, 2001; Willats, McCartney, Mackie, & Knox, 2001). Pectins are made of several structural elements among which homogalacturonans (HGs) ('smooth' regions) and type I rhamnogalacturonans (RGs-I) ('hairy' regions) are the most abundant. HG is composed of (1→4)-linked α-D-Gal*p*A residues that can be partly methyl-esterified at *C*-6 (Pilnik & Voragen, 1970) and possibly partly acetyl-esterified at *O*-2 and/or *O*-3. (Rombouts & Thibault, 1986). The degree of methylation (DM) and degree of acetylation (DAc) are defined as the percentage of GalA units esterified with methanol or acetic acid, respectively. RG-I is composed of a repeating disaccharide unit [→2)-α-L-Rha*p*-(1→4)-α-D-Gal*p*A-(1→]$_n$. Rhamnosyl residues often carry neutral sugars side-chains. Pectins are generally described as an alternation of smooth and hairy regions, although an alternative macromolecular structure of pectin in which HG could be a side-chain of RG-I was recently proposed (Vincken et al., 2003).

Pectins extracted from several plant by-products are widely used as gelling agents in the food industry (May, 1990; Voragen, Pilnik, Thibault, Axelos, & Renard, 1995). The methyl-esterification of HG has been the subject of several investigations since it determines to a large extent the industrial applicability of pectin and their interaction ability in muro. Furthermore, many of the properties and biological functions of HGs are believed to be determined by ionic interactions (Ridley et al., 2001; Willats et al., 2001). Not only the DM, but also the distribution of methyl groups on the HG has a deep impact on pectin gelation properties, a blockwise arrangement of free carboxyl groups leading to enhanced calcium-gelling properties compared to pectins with a random distribution of free carboxyl groups (Kohn, Markovic, & Machova, 1983; Ralet, Dronnet, Buchholt, & Thibault, 2001; Thibault & Rinaudo, 1985).

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Treatment of pectin with fungal-pectin methylesterases (f-PMEs) leads to pectins with a random-like distribution of carboxyl groups, whereas the action of plant-pectin methylesterases (p-PMEs) results in a blockwise arrangement of free carboxyl groups in the pectin molecule (Kohn et al., 1983; Limberg et al., 2000; Ralet & Thibault, 2002; Thibault & Rinaudo, 1985).

In addition to the complex structure of a single molecule, pectins are characterised by a high degree of inter-molecular heterogeneity in terms of composition, molar mass and degree of substitution (DM and DAc). Studies on 'homogeneous' pectins with respect to DM or molar mass contributed to establish clearer relationships between pectin fine structure and functional properties (Kravtchenko, Voragen, & Pilnik, 1992; Ralet & Thibault, 2002). However, intra-chain heterogeneity with respect to the DM and pattern of methyl-esterification has not been studied yet.

The length of HGs and the ratio between HGs and RGs-I may also influence the pectin properties. HGs were successfully isolated by acidic means (Thibault, Renard, Axelos, Roger, & Crepeau, 1993). Acid-resistant HGs with chain lengths of 72–100 GalA residues were thereby obtained. However, conditions used were likely to degrade the HG backbone, leading to underestimation of the molar mass values. Moreover, since pectins have to be de-esterified prior to the acidic treatment, the role of DM and distribution of methyl groups cannot be studied. Enzymatic degradation of pectic polysaccharides using pure enzymes was also successfully used to recover HGs from sugar beet, lime and apple pectins (Bonnin, Dolo, Le Goff, & Thibault, 2002). Pectins were degraded by a rhamnogalacturonan hydrolase associated with pectin methylesterases and side-chain degrading enzymes (endo-galactanase and -arabinanase). HGs with chain lengths of 84–247 GalA residues were obtained, according to plant origin. The use of pectin methylesterases precludes again the study of DM and pattern of methyl-esterification.

In this work, lime pectins with different degrees and patterns of methyl-esterification were purified by means of size exclusion and anion exchange chromatography (SEC and AEC, respectively) to recover homogeneous fractions with respect to molar mass and charge density. These homogeneous pectins were degraded by a rhamnogalacturonan hydrolase associated with side-chain degrading enzymes (endo-galactanase, endo-arabinanase, α-arabinofuranosidase and β-galactosidase). Methyl-esterified HGs were recovered and characterised.

2. Experimental

2.1. Materials

2.1.1. Pectins

Polygalacturonic acid was purchased from Sigma (L'Isle d'Abeau, France). A commercial pectin (L72) from Mexican lime peel (*Citrus aurantifolia*), with a DM of 72 was esterified in acid–methanol medium to give a pectin (E81) of a DM of 81. A series of pectin with defined DM were prepared by enzymatic treatment of E81 as fully described previously (Limberg et al., 2000). F-series were prepared using an f-PME from *Aspergillus niger* purified from Pektolase™ (Danisco, Brabrand, Denmark). P-series were prepared using a p-PME purified from orange peels as described by Christensen, Nielsen, Kreiberg, Rasmussen, and Mikkelsen (1998). Three f-PME pectin (F69, F58 and F43, DM 69, 58 and 43, respectively) and three p-PME (P70, P60 and P41, DM 70, 60 and 41, respectively) were fractionated by SEC on a column (92×5 cm) of Sephacryl S-500. A detailed description of chromatography conditions and physicochemical characterisation of recovered fractions has been published elsewhere (Ralet & Thibault, 2002).

2.1.2. Enzymes

Rhamnogalacturonan hydrolase (Rg-ase, Swiss-Prot entry Q00018) was provided by Novozymes (Copenhagen, Denmark). It was originally cloned from *Aspergillus aculeatus* and expressed in *Aspergillus oryzae*. α-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-arabinanase (endo-A, E.C. 3.2.1.99) and endo-galactanase (endo-G, E.C. 3.2.1.89) were purified from *A. niger* as described previously (Bonnin et al., 2002). The enzymes were dissolved in 0.05 M Na-succinate buffer, pH 4.5 at 60 mg/mL for Rg-ase and 10 mg/mL for others and dialysed against the same buffer before use.

2.2. Methods

2.2.1. Acid hydrolysis of pectins

The mother pectin (L72) was deesterified by 0.1 M NaOH at 4 °C as previously described (Thibault et al., 1993) and the deesterified pectin (0.1%, w/v) was hydrolysed in 0.1 M HCl at 80 °C for 72 h in sealed tubes. The acid-soluble and the acid-insoluble fractions were separated after cooling by centrifugation of the reaction mixture at 15,000*g* for 20 min. The insoluble fraction was washed with water, treated by solvent exchange and dried under vacuum at 50 °C. The insoluble fraction was re-suspended in water (2%, w/v) and the pH was brought to seven with 0.02 M NaOH. The solution was left overnight under mild stirring and freeze-dried.

2.2.2. Enzymatic hydrolysis of pectins

Four-milligram per millilitre pectins were dissolved in 0.05 M Na-succinate buffer, pH 4.5 and were degraded by an enzymatic mixture containing 0.5 nKat/mg of Rg-ase, α-Ara-ase, β-D-Gal-ase, endo-A and endo-G. The enzymatic mixture exhibited no activity towards polygalacturonic acid

(Nelson, 1944). The reaction mixture was incubated for 48 h at 40 °C and the end products were fractionated by AEC.

2.2.3. Chromatography

AEC was performed at room temperature on DEAE-Sepharose CL-6B column (32×2.6 cm) equilibrated with 0.05 M Na-succinate buffer, pH 4.5. The samples were loaded and eluted at flow rate of 90 mL/h. The bound material was eluted with a linear NaCl gradient (0–0.4 M). Fifteen-millilitre fractions were collected and GalA and total neutral sugars quantified colorimetrically (Thibault, 1979; Tollier & Robin, 1979). Appropriate fractions were pooled, extensively dialysed against water and freeze-dried for further analysis.

2.2.4. Physicochemical characterisation

To evaluate the polymer distribution and average molar masses, pectic samples were analysed by aqueous high-performance size-exclusion chromatography (HP-SEC). Molar mass (M_w, weight-average molar mass; M_n, number-average molar mass) determinations were performed using a multi-angle laser-light-scattering (MALLS) (Mini Dawn, Wyatt, Santa Barbara, CA) operating at three angles (41°, 90° and 138°), an on-line differential viscosimeter (T-50A, Viscotek) (universal calibration curve established with pullulans 5×10^3–1600×10^3 g/mol) and a differential refractometer (RI) (ERC 7517A). A refractive index increment $dn/dc = 0.146$ g/mL was employed and M_w and M_n were calculated either using an Astra software (MALLS) or a TriSec software (Viscotek).

Pectin samples were gently dispersed into 0.05 M $NaNO_3$ buffer containing 0.02% NaN_3 as preservative (2–4 mg/mL for Viscotek and RI detection or ~12 mg/mL for Viscotek, RI and MALLS detection), and left for dissolution overnight under magnetic stirring. The solutions were centrifuged in a benchtop centrifuge and filtered on 0.45 μm Minisart RC15 Sartorius membranes. HP–SEC was performed at room temperature on a system constituted of one Shodex OH SB-G pre-column followed by two columns in series (Shodex OH-Pack SB-804 HQ and OH-Pack SB-805 HQ) eluted with 0.05 M $NaNO_3$ buffer containing 0.02% NaN_3 as preservative at a constant flow rate of 42 mL/h.

2.2.5. Analytical

Uronic acid (as GalA) and neutral sugars (as Ara) contents were determined colorimetrically by the automated *m*-phenylphenol (Thibault, 1979) and orcinol (Tollier & Robin, 1979) methods, respectively. Except for chromatographic fractions, GalA was quantified after saponification of the pectin samples and neutralisation. Individual neutral sugars were quantified after acid hydrolysis (2 M trifluoroacetic acid, 2 h, 121 °C) of pectins as their alditol acetates derivatives (Blakeney, Harris, Henry, & Stone, 1983) by GLC. DM was calculated after HPLC determination of methanol released by alkaline de-esterification of pectins (Levigne, Thomas, Ralet, Quéméner, & Thibault, 2002), Isopropanol was added as internal standard and DM was calculated as the molar ratio of methanol to GalA.

3. Results

3.1. Acid hydrolysis of a lime pectin and analysis of the reaction products

Deesterified HGs were recovered after acid hydrolysis of the mother pectin L72 as previously described (Thibault et al., 1993). They were nearly exclusively built of GalA residues (>99 mol%) with some residual Rha residues. HGs (~12 mg/mL) were analysed by HP-SEC-MALLS-Viscotek to assess the validity of the Viscotek method for molar mass determination of HGs. Indeed, the Viscotek molar mass determination is based on universal calibration with pullulan standards, a method that can have several potential pitfalls among which the main ones are: (i) an inconsistency of SEC elution profiles with respect to the amount of injected sample; (ii) the presence of non-SEC phenomena such as weak adsorption. HGs gave single narrow RI and Viscotek peaks on HP-SEC (Fig. 1). The MALLS signal revealed, however, traces of aggregates eluting at V_0 (Fig. 1). An intrinsic viscosity of 75 (±2) mL/g and a Mark-Houwink coefficient (a) of 0.87 (±0.02) were determined by Viscotek, revealing a quite extended conformation for those HGs. The molar masses determined by HP-SEC-Viscotek (M_n = 15,400, M_w = 19,800; average of three measurements, standard deviation = 5.6%) were in good agreement with the molar masses determined by HP-SEC-MALLS (M_n = 14,700, M_w = 18,500; average of three measurements, standard deviation = 5.7%). When lower concentrations of sample (~3 mg/mL) were injected onto the HP-SEC system, the MALLS signal became too weak to

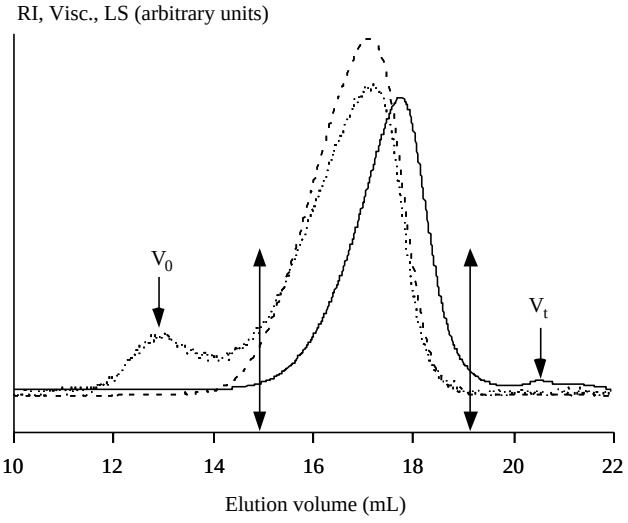

Fig. 1. HP-SEC-MALLS-Viscotek of the acid-insoluble HGs. (–) RI; (···) MALLS; (- - -) Viscotek.

allow any molar mass determination. On the contrary, the Viscotek signal could be analysed and the intrinsic viscosity, Mark-Houwink coefficient, M_n and M_w values obtained (73 mL/g, 0.85, 14,600, 21,300, respectively) were close to those determined for a higher sample concentration. The use of HP-SEC with RI and viscometry detectors and calculations based on universal calibration with pullulan standards appeared adequate for the measurement of macromolecular parameters of deesterified HGs.

3.2. Enzymatic hydrolysis of a lime pectin and analysis of the reaction products

The initial pectin P60 (DM 60) was chosen in order to optimise the enzymatic hydrolysis conditions for HGs isolation. This crude pectin was fully characterised in a previous work (Ralet et al., 2001). It exhibited a high content in GalA (865 mg/g), low contents in neutral sugars (36, 13 and 3 mg/g of Gal, Rha and Ara, respectively) and a M_w of 94,000.

Five specific and well-characterised enzymes (Rhamnogalacturonan hydrolase, Rg-ase; α-arabinofuranosidase, α-Ara-ase; β-D-galactosidase, β-Gal-ase; endo-arabinanase, endo-A; and endo-galactanase, endo-G) were tested for P60 hydrolysis and the reaction was followed by measuring M_w and polydispersity (Viscotek detection; injected sample concentration 4 mg/mL) as a function of time (Table 1). Endo-G, endo-A, and α-Ara-ase used alone only produced a minor decrease of the molar mass (data no shown). Rg-ase alone induced a significant decrease of M_w (94,000–59,800 mol/g) in the initial step (0–8 h) of the enzymatic reaction, suggesting a partial degradation of the RGI backbone. The combination of Rg-ase and endo-G resulted in a more significant decrease of the molar mass. The addition of either endo-A, α-Ara-ase or β-Gal-ase in the (Rg-ase + endo-G) enzymatic mixture did not significantly modify the degradation of the RG-I backbone. The degradation of P60 was, however, increased when Rg-ase was used in combination with endo-G, endo-A, β-Gal-ase and α-Ara-ase. M_w reached 24,800 mol/g after 24 h of incubation and decreased only slightly after fresh enzyme was added at $t = 24$ h (20,800 mol/g after 48 h of incubation). As already pointed out (Bonnin et al., 2002) no insoluble fraction was released from lime pectin, whatever the enzymes used.

The initial lime pectin P60 and the reaction products obtained after incubation with each enzyme or enzymatic combination were fractionated by AEC on DEAE-Sepharose CL-6B (Fig. 2). Chemical and physicochemical characteristics of major AEC-bound fractions are summarised in Table 2. GalA and neutral sugars recoveries were >95% for all samples. As previously shown (Ralet & Thibault, 2002), P60 eluted as a bound broad fraction (Fig. 2A), showing that P60 is heterogeneous in terms of charge density. No unbound fraction was evidenced. Hydrolysis by Rg-ase alone did not drastically modify the AEC profile (Fig. 2B). Small alterations of the profile were nevertheless observed, in particular, the appearance of a small unbound peak (fraction NR) (40 mg/g of recovered total sugars) rich in neutral sugars, and of a small fraction (fraction 1) eluting at the beginning of the NaCl gradient (0.04 M NaCl). The bulk of GalA was eluted as a broad unsymmetrical peak. Three GalA-rich fractions were recovered at 0.12, 0.23 and 0.31 M NaCl. These fractions exhibited increasing content in GalA to the detriment of neutral sugars for increasing ionic strength, similar M_w (Viscotek measurement; injected sample concentration 2–3 mg/mL), but differed widely in their DM (31–80). When endo-G was added to Rg-ase, the AEC profile was not significantly altered (Fig. 2C). Three GalA-rich fractions were recovered (fractions 2, 3 and 4 at 0.12, 0.22 and 0.30 M NaCl, respectively). Those fractions were very similar to the corresponding ones recovered after degradation with Rg-ase alone, except for M_w (Viscotek measurement; injected sample concentration 2–3 mg/mL) that were significantly lowered (around 45,000 instead of around 60,000 for Rg-ase alone). Similar profiles were also obtained when endo-A, α-Ara-ase or β-Gal-ase were added to Rg-ase (data no shown). When all these enzymes were used in combination (Fig. 2D), two peaks appeared in the not retained fraction, corresponding altogether to 133 mg/g of recovered total sugars. Those two peaks were previously

Table 1
Time changes in macromolecular characteristics of reaction mixtures containing P60 initial pectin incubated in the presence of various enzymes

	M_w (g/mol)	I
P60 initial pectin	94,000	2.1
Rg-ase		
8 h	59,800	2.0
24 h	55,600	1.9
48 h	45,000	1.8
Rg-ase + endo-G		
8 h	51,400	2.0
24 h	49,100	1.9
48 h	31,500	1.6
Rg-ase + endo-G + endo-A		
8 h	52,200	2.0
24 h	48,100	1.9
48 h	32,100	1.5
Rg-ase + endo-G + β-Gal-ase		
8 h	51,500	1.9
24 h	48,800	1.7
48 h	32,800	1.5
Rg-ase + endo-G + α-Ara-ase		
8 h	55,400	1.9
24 h	47,000	1.8
48 h	31,800	1.5
Rg-ase + endo-G + endo-Ara + α-Ara-ase + β-Gal-ase		
8 h	47,900	1.8
24 h	24,800	1.5
48 h	20,800	1.4

M_w, weight-average molar mass (Viscotek measurement; injected sample concentration 4 mg/mL); I, polydispersity index (M_w/M_n, number-average molar mass).

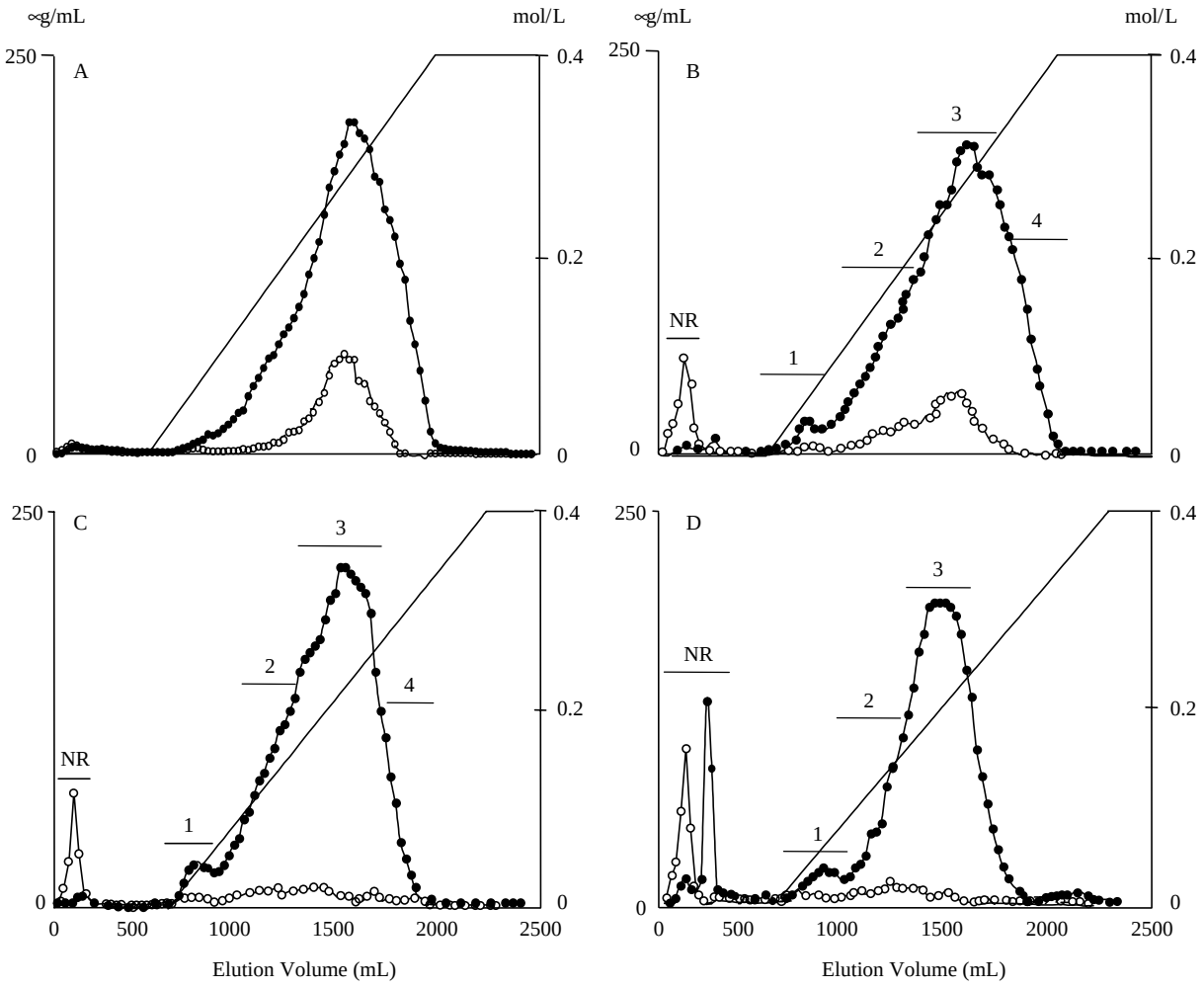

Fig. 2. Anion exchange chromatography (DEAE Sepharose CL-6B) elution patterns of degradation media containing initial P60 lime pectin incubated with various enzymes. (A) P60 (initial lime pectin, DM 60); (B) P60 + Rg-ase; (C) P60 + Rg-ase + endo-G; (D) P60 + Rg-ase + endo-G + endo-A + α-Ara-ase + β-Gal-ase. (●) Galacturonic acid (μg/mL); (○) neutral sugars (μg/mL); (–) NaCl (mol/L).

attributed to neutral sugars from side-chains and short RG-I oligomers, respectively (Bonnin et al., 2002). A small peak (fraction 1) was again eluted at the beginning of the NaCl gradient (0.05 M NaCl). The bulk of GalA was eluted as a fairly narrow and symmetrical peak, contrary to what was observed when Rg-ase was used alone or in combination with endo-G. Two GalA-rich fractions were recovered (fractions 2 and 3 at 0.12 and 0.19 M NaCl, respectively). These two fractions exhibited very low neutral sugars content and are assumed to correspond to HGs. When analysed by HP-SEC-Viscotek (injected sample concentration ~3 mg/mL), both fractions exhibited an average intrinsic viscosity of 84 mL/g. Mark-Houwink coefficient (a) values of 1.25 and 1.22 were determined for fractions 2 and 3, respectively, indicating an extended conformation for those partly methylated HGs. M_w (Viscotek measurement) of 17,900 and 21,300 g/mol were calculated for fractions 2 and 3, values in good agreement with the average value obtained on the un-fractionated hydrolysate (Table 1). A polydispersity of 1.4 was observed for both fractions (Table 2). Fraction 3 was also analysed by HP-SEC-MALLS (injected sample concentration ~12 mg/mL). A M_w of 22,400 was determined, value in close agreement with that estimated by Viscotek for a lower sample concentration. The use of HP-SEC with RI and viscometry detectors and calculations based on universal calibration with pullulan standards appeared adequate for the measurement of macromolecular parameters of partly methyl-esterified HGs.

3.3. Recovery of homogeneous pectins

The isolation procedure of homogeneous pectins followed by HGs recovery is summarised on Fig. 3. Three f-PME de-methoxylated samples (F69, F58 and F43) and three p-PME de-methoxylated samples (P70, P60 and P41) were fractionated by preparative SEC on Sephacryl S-500 in a previous work (Ralet & Thibault, 2002). Intermediate size fractions (P70a, P60a and P41a; F69a, F58a and F43a), representing the bulk of the samples, were further

Table 2
Composition and physico-chemical properties of initial P60 and DEAE Sepharose CL-6B GalA-rich fractions of enzymatically degraded P60

DEAE-fractions	Yield (mg/g)	NaCl (M)	Sugar content (mol%)				DM	M_w (g/mol)	I
			GalA	Rha	Ara	Gal			
A			93.7	1.7	0.4	4.2	60	94,000	2.1
B									
2	253	0.12	95.5	1.3	1.0	2.2	80	57,400	1.8
3	587	0.23	97.9	0.8	0.2		63	63,300	1.8
4	81	0.31	Nd	Nd	Nd	Nd	31	61,300	1.9
C									
2	329	0.12	96.6	1.6	0.4	1.4	78	47,000	1.7
3	502	0.22	97.1	1.3	0.3	1.3	63	43,100	1.8
4	82	0.30	97.9	1.0	0.3	0.8	29	46,500	1.7
D									
2	250	0.12	95.9	1.0	0.5	2.6	63	17,900	1.4
3	548	0.19	97.5	0.6	0.2	1.6	57	21,300	1.4

A, P60; B, P60 + Rg-ase; C, P60 + Rg-ase + endo-G; D, P60 + Rg-ase + endo-G + endo-A + α-Ara-ase + β-Gal-ase; DM, degree of methylation; M_w, weight-average molar mass (Viscotek measurement; injected sample concentration ~3 mg/mL); I, polydispersity index (M_w/M_n, number-average molar mass); Nd, not determined.

chromatographed by AEC on DEAE-Sepharose CL-6B, in order to fractionate those samples primarily according to the charge density of their constituent molecules (Fig. 4). GalA and neutral sugars recoveries were >98% for all samples. Elution patterns very similar to those obtained by Ralet and Thibault (2002) on initial pectins were observed. F-PME de-methoxylated samples eluted as a single homogeneous peak, progressively more thin and symmetrical, and eluting at increasing ionic strength when the average DM of the pectin sample lowered. P-PME de-methoxylated samples with DM ≥ 60 (P70a and P60a) exhibited a broad elution pattern, showing that both were quite heterogeneous in terms of charge density while P41a was eluted as a fairly thin peak.

The chemical and physicochemical characteristics of the main fractions (Fig. 4) which represented 48–63% of the recovered material are summarised in Table 3. No significant difference was evidenced between f- and p-PME samples with respect to their chemical composition. These fractions were characterised by a high GalA content and a low content in neutral sugars. GalA and neutral sugars represented more than 950 mg/g of sample for all pectins. The high GalA/Rha molar ratios values (64–88) suggest that the number and/or size of RGIs are limited in these populations, in agreement with Bonnin et al. (2002) who reported short RG-I regions for lime pectins. All homogeneous fractions exhibited a DM similar to that of the corresponding initial pectin. Those fractions were eluted as a single peak on HP-SEC, in agreement with Ralet and Thibault (2002) findings. It is noteworthy that M_w (Viscotek measurement; injected sample concentration 2–3 mg/mL) were significantly reduced compared to initial pectins. Very high M_w constituents (~300,000 g/mol) were shown to be present in initial pectic samples together with intermediate M_w constituents (~50,000–100,000 g/mol) (Ralet & Thibault, 2002). The removal of high M_w constituents by SEC and AEC led to a decrease in the overall M_w.

After consecutive fractionations by preparative SEC and AEC, the molar mass and charge density inter-chain heterogeneity of recovered intermediate fractions were significantly reduced compared to that of initial pectins.

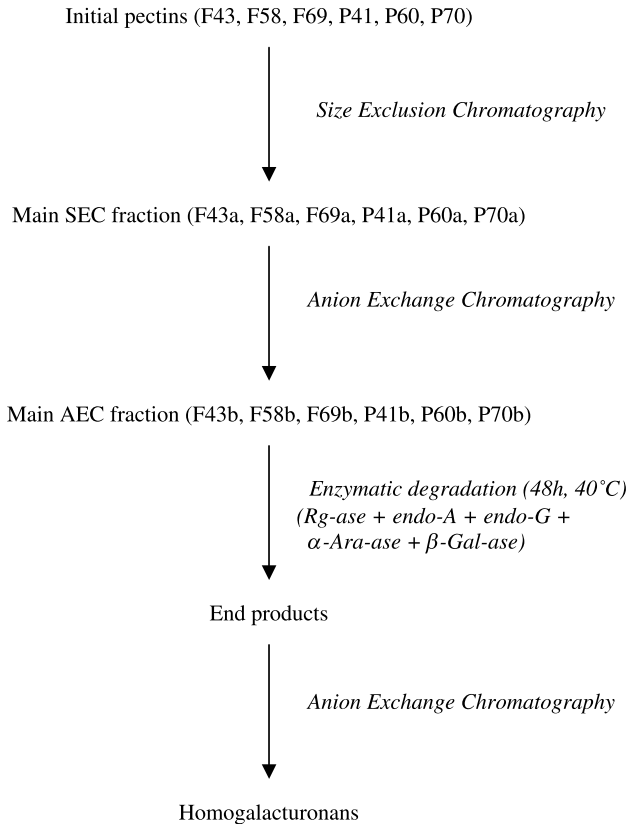

Fig. 3. 'Homogeneous' pectins and HGs isolation procedure.

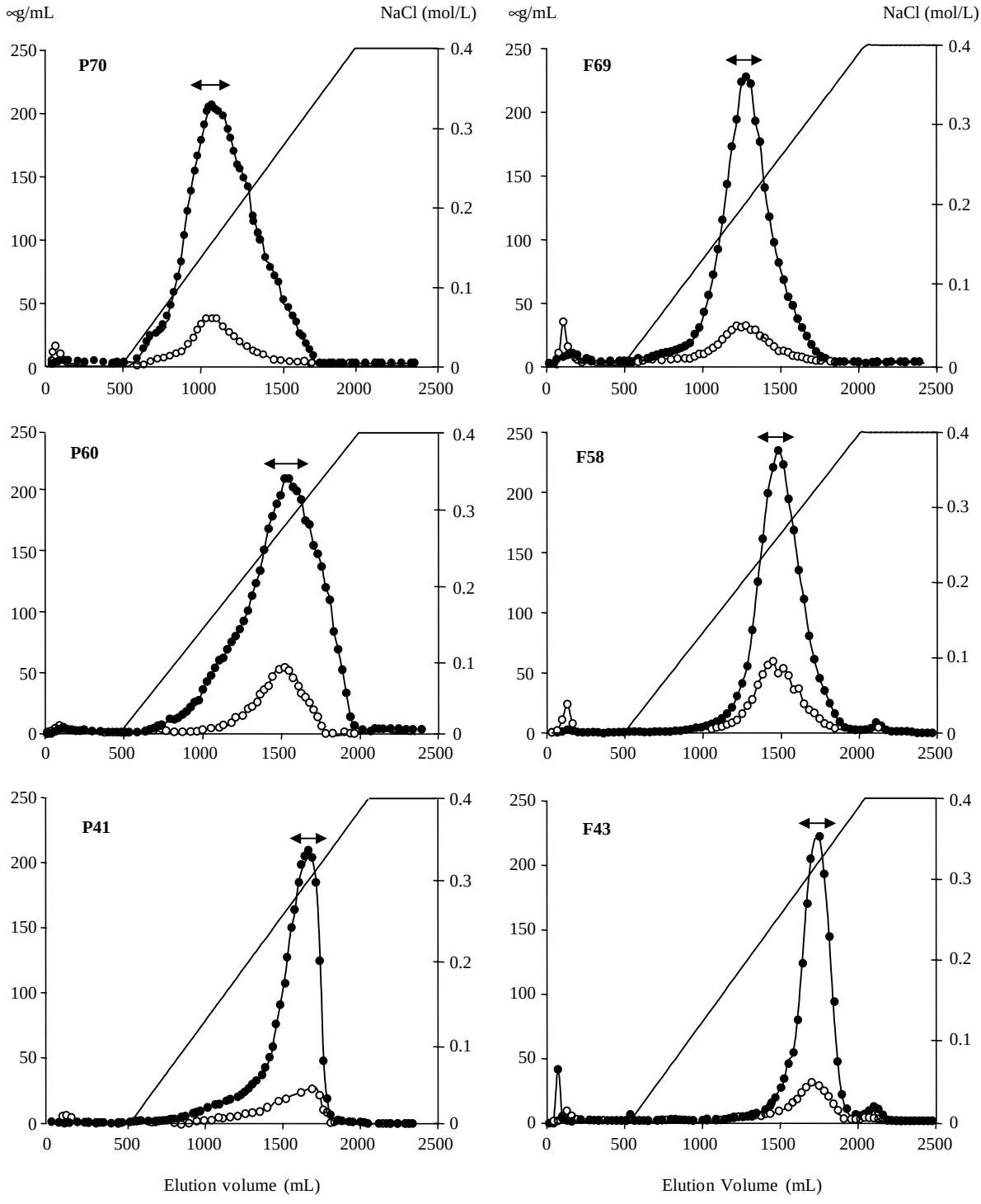

Fig. 4. Anion exchange chromatography (DEAE Sepharose CL-6B) elution patterns of P-(P70, P60, P41) and F-(F69, F58, F43) series Sephacryl S-500 main fraction. (●) Galacturonic acid (μg/mL); (○) neutral sugars (μg/mL); (–) NaCl (mol/L).

3.4. Isolation of partly methylated homogalacturonans from homogeneous pectins

The enzyme combination and reaction conditions selected to degrade initial P60 were used towards homogeneous populations obtained after sequential fractionations by preparative SEC and AEC. P70b, P60b, P41b, F69b, F58b and F43b were hydrolysed using the combined action of Rg-ase, endo-A, endo-G, α-Ara-ase and β-Gal-ase. The reaction products were fractionated by AEC and the elution patterns are showed on Fig. 5. Chemical and physico-chemical characteristics

Table 3
Composition and physico-chemical properties of 'homogeneous' pectin fractions from the P- and the F-series recovered after SEC and AEC

	P41b	P60b	P70b	F43b	F58b	F69b
Yield (wt%)	59	48	52	63	59	56
Sugar content (mol%)						
GalA	96.0	96.7	96.7	95.4	96.6	96.6
Rha	1.4	1.1	1.2	1.5	1.2	1.1
Ara	0.7	0.6	0.4	0.7	0.6	0.4
Gal	2.0	1.7	1.7	2.3	1.6	1.8
DM	40	58	68	45	59	70
M_w (g/mol)	57,200	46,600	59,300	46,700	44,200	72,200
I	1.7	1.5	1.7	1.4	1.8	1.9

Traces (<0.2% weight) of Fuc, Xyl and Glc were detected; DM, degree of methylation; M_w, weight-average molar mass (Viscotek measurement; injected sample concentration ~3 mg/mL); I, polydispersity index (M_w/M_n, number-average molar mass).

of the major AEC fractions are summarised in Table 4. The chromatographic recoveries in GalA and neutral sugars were close to 98% for all samples.

All elution patterns look similar to that obtained from initial P60. An unbound population (NR) consisting of two peaks, one rich in neutral sugars and the other rich in GalA, and a bound population rich in GalA were observed (Fig. 5). The unbound population represented 110–170 mg/g of recovered total sugars, values lower than those previously reported by Bonnin et al. (2002). Fraction 1, eluting at the beginning of the gradient, represented 40–120 mg/g of recovered total sugars. The GalA peak top was observed at increasing ionic strength when the average DM of the pectin sample decreased. Furthermore, pectins of similar DM but differing in their pattern of methyl-esterification eluted for similar ionic strength (0.18 M NaCl for F69 and P70; 0.22 M NaCl for F58 and P60; and 0.30 M for F69 and P70). This is in agreement with previously reported data (Ralet & Thibault, 2002) and suggests that the elution on DEAE-Sepharose CL-6B is mainly governed by charge density, independently of charge distribution patterns.

The bulk of GalA was fractionated into three populations (fractions 2, 3 and 4) that are assumed to correspond to HG regions. Fractions 2, 3 and 4 represented 160–240, 400–570 and 60–200 mg/g of recovered total sugars, respectively. Whatever the pectin sample, fractions 2–4 were very rich in GalA (95.3–98.8 mol%). The amount of Rha decreased along the fractionation and molar ratios GalA/Rha of 50–109, 97–163 and 89–328 were observed for fractions 2, 3 and 4, respectively. DM decreased also slightly along the fractionation, although an overall narrow distribution of DM was observed whatever the sample. Average DM values recalculated from the contribution of each of their constituting fractions were in good agreement with average DM measured on the whole samples. M_w (Viscotek measurement; injected sample concentration 2–3 mg/mL) of 20,400–27,500 g/mol were observed for fractions 2–4 of P-and F-series. Fractions 3, representing the bulk of the bound material whatever the pectin sample, exhibited M_w of 20,400–23,400, corresponding to degrees of polymerisation of 113–126. These fractions were particularly homogeneous with respect to M_w, as revealed by their polydispersity index very close to 1.

4. Discussion

Pectins are complex polysaccharides characterised by a high degree of heterogeneity with respect to composition, molar mass, and degree of substitution. They were defined as 'chemically homogeneous polydisperse systems consisting of structurally related molecular species with continuously variable proportions of neutral sugar residues' (Aspinall, 1970). More recently, pectin was depicted as a block co-polymer consisting of different proportions of HGs, RGs-I with associated-neutral sugars side-chains and substituted galacturonans (mainly RGs-II) (O'Neill & York, 2003; Zhan, Janssen, & Mort, 1998). Current models of the organisation of these pectic subunits envision a macromolecular complex in which HGs, RGs-I and RGs-II are covalently linked to one another (O'Neill & York, 2003). The way in which these structural elements are combined into the macromolecular structure is, however, still a subject of debate (Vincken et al., 2003).

In the present work, lime pectins differing in their degrees of methyl-esterification and in the distribution of methyl groups were purified by chromatographic means (SEC + AEC) in order to reduce their inter-chain heterogeneity. Rhamnogalacturonan hydrolase in combination with various side-chain-degrading enzymes were applied to these homogeneous pectins with the aim of isolating HG regions. Prior to that, the level of degradation of the RG-I regions using different combinations of enzymes was appraised on a raw lime pectin (DM 60). As previously reported (Bonnin et al., 2002), the use of Rg-ase alone induced only a limited hydrolysis of the RG-I regions. The hydrolysis was more efficient when Rg-ase was used in combination with endo-G and endo-A. The degradation of RG-I was even increased by addition of α-Ara-ase and β-Gal-ase to the [Rg-ase + endo-G + endo-A]-mixture. [Rg-ase + endo-G + endo-A + α-Ara-ase + β-Gal-ase]-released HGs consisted of almost pure galacturonans (GalA 95.9–97.5 mol%). They exhibited M_w of 17,900–21,300 (weight-average degrees of

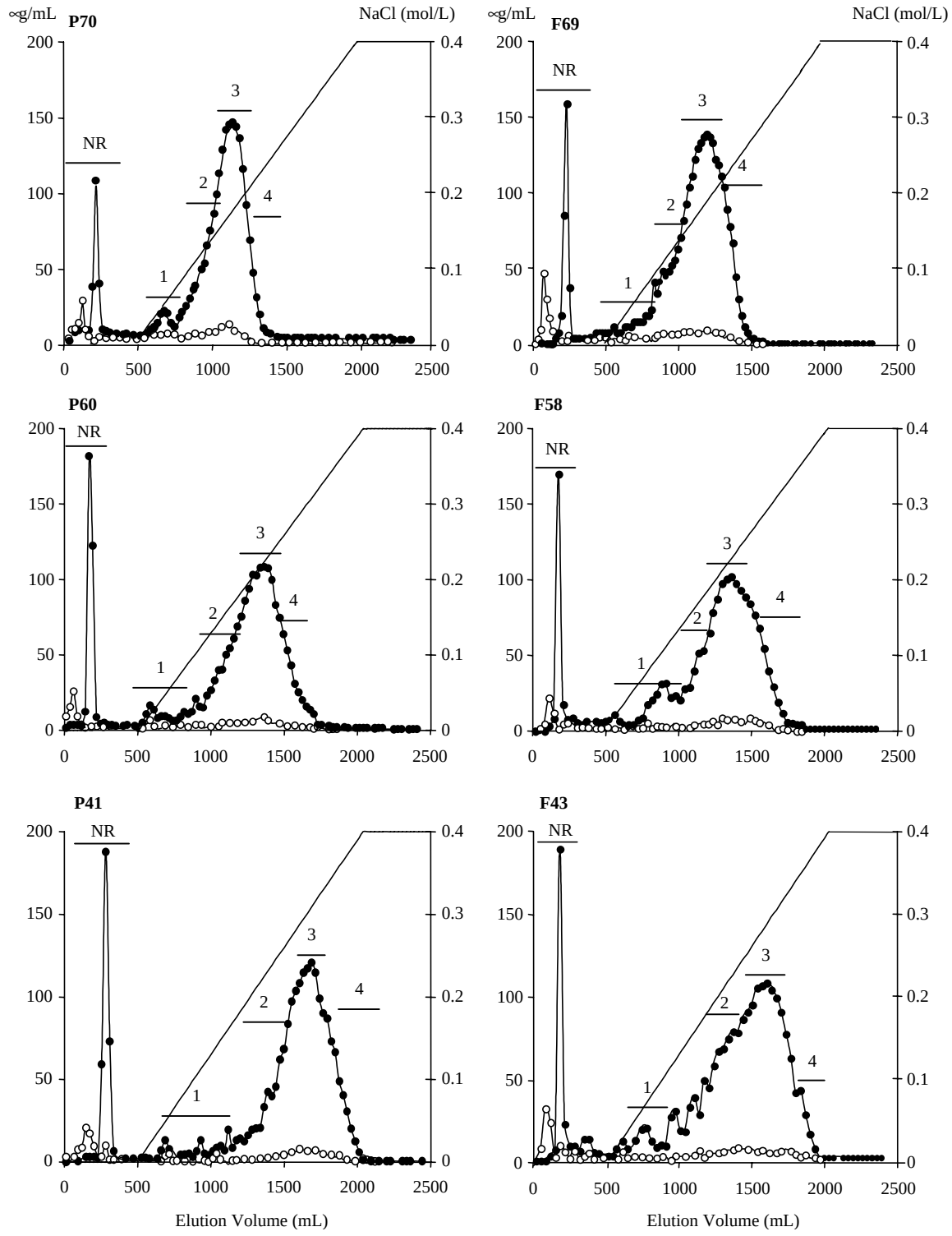

Fig. 5. Anion exchange chromatography (DEAE Sepharose CL-6B) elution patterns of 'homogeneous' P-(P70b, P60b, P41b) and F-(F69b, F58b, F43b) series after degradation with Rg-ase + endo-G + endo-A + α-Ara-ase + β-Gal-ase. (●) Galacturonic acid (μg/mL); (○) neutral sugars (μg/mL); (–) NaCl (mol/L).

polymerisation of 97–116), in good agreement with values observed on HGs isolated after mild acid hydrolysis of lime pectins (Thibault et al., 1993). These results suggest that an intense degradation of neutral sugars side-chains is necessary to provide a good accessibility to RG-I for the Rg-ase. The use of the sole [Rg-ase + endo-G + endo-A] seems insufficient to individualise all HG regions. This could explain the high weight-average degree of polymerisation (193) observed by Bonnin et al. (2002) on HGs isolated from lime pectins using this combination of enzymes.

Table 4
Composition and physico-chemical properties of GalA-rich fractions recovered by DEAE-Sepharose CL-6B after enzymatic degradation of F43b, F58b, F69b, P41b, P60b and P70b

DEAE-fractions	Yield (mg/g)	Sugar content (mol%)				DM	M_w (g/mol) (dp_w)	I
		GalA	Rha	Ara	Gal			
F43b								
2	240	95.3	1.5	0.7	2.4	Nd	27,500 (nd)	1.09
3	460	97.6	1.0	0.4	1.0	44	22,800 (125)	1.02
4	100	97.5	1.1	0.3	1.1	41	24,400 (134)	1.09
F58b								
2	160	97.8	0.9	0.3	1.0	61	26,900 (146)	1.09
3	400	97.7	0.9	0.3	1.1	59	22,500 (122)	1.02
4	200	98.8	0.4	0.2	0.6	53	22,800 (124)	1.12
F69b								
2	210	96.7	1.3	0.7	1.4	71	26,300 (141)	1.12
3	570	98.0	0.6	0.3	1.1	68	23,400 (126)	1.02
4	60	98.5	0.3	0.3	1.0	63	21,200 (115)	1.10
P41b								
2	170	95.9	1.9	0.3	1.5	45	25,100 (138)	1.18
3	430	96.7	1.0	0.3	1.7	38	20,400 (113)	1.01
4	130	98.1	0.5	0.3	0.8	35	24,200 (134)	1.10
P60b								
2	170	96.8	1.2	0.5	1.2	63	25,800 (140)	1.19
3	480	97.7	0.7	0.3	1.1	59	22,200 (120)	1.02
4	150	98.6	0.4	0.3	0.6	53	25,200 (137)	1.11
P70b								
2	210	96.4	1.4	0.6	1.7	72	24,300 (131)	1.05
3	540	97.7	0.7	0.3	1.3	69	22,300 (120)	1.00
4	90	Nd	Nd	Nd	Nd	Nd	21,200 (nd)	1.17

DM, degree of methylation; M_w, weight-average molar mass (Viscotek measurement; injected sample concentration 2–3 mg/mL); dp_w, weight-average degree of polymerisation; I, polydispersity index (M_w/M_n, number-average molar mass), Nd, not determined.

The presence of methyl groups on the pectin sample (DM 60) did not seem to preclude the degradation of RGs-I. Indeed, Schols, Geraeds, Searle-van Leeuwen, Kormelink, and Voragen (1990), suggested that methyl groups do not hinder the Rg-ase. Moreover, there is no strict evidence that the GalA residues are methyl-esterified in the RG-I region (O'Neill & York, 2003). The optimal enzyme combination [Rg-ase + endo-G + endo-A + α-Ara-ase + β-Gal-ase] was applied to homogeneous pectins differing in their degrees and patterns of methyl-esterification. Released-HGs were isolated by AEC. HG populations represented around 800 mg/g of recovered material for all pectic samples, value in good agreement with previous findings (Thibault et al., 1993; Zhan et al., 1998). An overall narrow distribution of DM in HGs was observed whatever the pectin sample, showing that the intra-chain heterogeneity with respect to charge density is rather limited, whatever the deesterification mode. The main HG fraction recovered for each pectin (fraction 3) consisted of almost pure galacturonans (GalA 96.7–98 mol%) exhibiting DM similar to that of the corresponding initial raw pectins. These HGs display similar weight-average degrees of polymerisation (121 ± 5), showing that RG-I regions were efficiently degraded without degradation of HG regions whatever the DM and methyl groups distribution of lime pectins. These weight-average degrees of polymerisation were in good agreement with values observed on HGs isolated after mild acid hydrolysis of lime pectins (Thibault et al., 1993) indicating that this chemical procedure does not lead to an underestimation of the HGs length as previously assumed. HGs isolated in the present work were all characterised by a very low polydispersity index (1.00–1.02), revealing that they all were very homogeneous with respect to molar mass. The length periodicity of HGs isolated by acidic means is here confirmed. The methyl-esterification (amount and distribution) of HGs determines many properties and biological functions of pectins. The isolation of HGs by enzymatic means preserving their substitution by methyl groups is indubitably of interest for further studies on the impact of the distribution of those substituents on pectins ionic interaction properties.

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