Carbohydrate Polymers 86 (2011) 1236–1243

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Isolation and characterization of model homogalacturonans of tailored methylesterification patterns

Abrisham Tanhatan-Nasseri, Marie-Jeanne Crépeau, Jean-François Thibault, Marie-Christine Ralet*

INRA, UR1268 Biopolymères Interactions Assemblages, Rue de la Géraudière, B.P. 71627, F-44300 Nantes, France

ARTICLE INFO

Article history:
Received 20 April 2011
Received in revised form 7 June 2011
Accepted 7 June 2011
Available online 14 June 2011

Keywords:
Pectin
Pectin methyl-esterase
Homogalacturonan
Degree of blockiness

ABSTRACT

A very highly methylated homogalacturonan (HG) was produced and further used to generate model HG series. HG samples were characterized with respect to their macromolecular features, to their degree and pattern of methyl-esterification, and to their calcium-binding properties. All HG samples were of similar molar mass. Randomly demethylesterified HG samples (B-series) of DM ≥ 40, exhibiting low degree of blockiness (DB), absolute degree of blockiness (DB_{abs}) and calcium binding properties, were classically obtained by alkali deesterification of the very highly methylated “mother” HG. HGs generated by the action of plant pectin-methylesterase (*p*-PME) on the very highly methylated “mother” HG (P-series) exhibited high DB, DB_{abs} and calcium binding properties, whatever their DM, in consistency with the presence of few long deesterified blocks of GalA. HGs generated by the action of *p*-PME on “mother” HGs of DM ~ 80 arising from the B-series (BP-series) exhibited intermediate DB, DB_{abs} and calcium binding properties, in agreement with the presence of numerous but short deesterified blocks of GalA. Manipulating the initial DM of randomly methyl-esterified “mother” HGs prior to enzymatic blockwise demethylesterification allowed the recovery of various HG types with respect to the length and number of demethylesterified GalA stretches.

1. Introduction

Plant cells are encapsulated in a cell wall, whose most prominent components are polysaccharides: cellulose, hemicelluloses and pectins. Those polysaccharides, together with some structural proteins, collectively determine the shape and mechanical properties of plant cells. The architecture of plant cell walls is governed by the fine structure of their constitutive polymers, this fine structure having profound effects on polymers functional properties after extraction or *in planta*. Pectins are amongst the most abundant polysaccharides in dicotyledon primary cell walls. Due to their diverse interaction properties, pectins can be considered as key components for the architecture of dicotyledon primary cell walls and are indeed involved in various cell functions and plant processes (Mohnen, 2008; Ridley, O’Neil, & Mohnen, 2001; Willats, Knox, & Mikkelsen, 2006; Willats, McCartney, Mackie, & Knox, 2001).

Pectin molecules are composed of several structural domains (Caffall & Mohnen, 2009). Homogalacturonan (HG), the most abundant pectic structural domain, is a linear α-(1,4)-linked D-galacturonic acid*p* (GalA) homopolymer with a typical degree of polymerization of 100–300 (Round, Rigby, MacDougall, & Morris, 2010; Thibault, Renard, Axelos, Roger, & Crépeau, 1993). GalA units can be partly methyl-esterified at C-6 and, in some species, partly acetyl-esterified at *O*-2 and/or *O*-3 (Ralet et al., 2005; Ralet, Crépeau, & Bonnin, 2008; Voragen, Pilnik, Thibault, Axelos, & Renard, 1995). The HG backbone can be substituted by xylose, apiose or complex side-chains to constitute xylogalacturonan, apiogalacturonan, and type II rhamnogalacturonan, respectively. Type I rhamnogalacturonan (RG-I) is the only pectic structural domain that is not built on a HG backbone. RG-I is indeed composed of the repeating disaccharide unit [2)-α-L-rhamnose*p*-(1,4)-α-D-galacturonic acid*p*-(1] decorated, mainly at *O*-4 of rhamnosyl residues, with several types of arabinose- and galactose-containing neutral sugar side-chains (Voragen et al., 1995). It is noteworthy that even at this low level of description, complexity and heterogeneity abound (Williams, Cucheval, Ström, & Ralet, 2009). Amongst this heterogeneity, the amount and distribution of methylesters onto HG domains are key features for pectin molecular functionality. Many of the properties and biological functions of pectins are indeed mediated by ionic interactions between HG domains (Ridley et al., 2001; Willats, Orfila, et al., 2001) and cell wall enzymes consistently tailor methyl-esters amount and distribution (Vincent, Cucheval, Hemar, & Williams, 2009; Willats, Orfila, et al., 2001). Two main patterns of methyl-esters distribution are recognized, random or blockwise. Fungal pectin-methyl-esterases (PMEs) and base lead to a random distribution of methyl-esters (Daas, Meyer-Hansen, Schols, De Ruiter, & Voragen, 1999; Kohn, Markovic, & Machova, 1983; Limberg et al., 2000a; Thibault & Rinaudo, 1985). Plant PMEs by

---

* Corresponding author. Tel.: +33 0240675196; fax: +33 0240675084.
E-mail address: marie-christine.ralet@nantes.inra.fr (M.-C. Ralet).

0144-8617/$ – see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carbpol.2011.06.019

contrast demethylate pectin in a processive manner, leading to the appearance of demethylated stretches (Cameron, Luzio, Goodner, & Williams, 2008; Daas et al., 1999; Denès, Baron, Renard, Péan, & Drilleau, 2000; Kohn et al., 1983; Limberg et al., 2000a; Ralet, Dronnet, Buchholt, & Thibault, 2001; Savary, Hotchkiss, & Cameron, 2002; Thibault & Rinaudo, 1985).

Since the degree of methyl-esterification (DM) and the distribution of methyl esters upon HG domains have a great impact on pectin functionality, much work has been carried out to elucidate pectins' patterns of methyl-esterification and to correlate those patterns with calcium binding properties or with the storage modulus of calcium–pectin gels. Two main approaches have been investigated in the elucidation of methyl-esterification patterns: (i) either using NMR to directly measure the frequency of the possible triad sequences of methylated and non-methylated GalA units within the chain (Grasdalen, Barkoy, & Larsen, 1988), or (ii) hydrolysing pectins by relevant enzymes, examining the liberated oligomers and making inferences about the initial structure prior to enzymatic digestion (Coenen, Kabel, Schols, & Voragen, 2008; Daas, Arisz, Schols, De Ruiter, & Voragen, 1998; Daas et al., 1999; Daas, Voragen, & Schols, 2000, 2001; Guillotin et al., 2005; Körner, Limberg, Christensen, Mikkelsen, & Roepstorff, 1999; Limberg et al., 2000a, 2000b). The enzyme specificity indeed renders the oligomers patterns methyl-ester-sequence-dependent. Several parameters have been introduced to characterize the presence or absence of demethylated blocks amongst which the degree of blockiness (DB, i.e. amount of non-methylated monomers, dimers and trimers released by endo-polygalacturonase (endo-PG) divided by the amount of non-methylated GalA present in the pectin sample) (Daas et al., 1999) and absolute degree of blockiness (DB_{abs}, i.e. amount of non-methylated monomers, dimers and trimers released by endopolygalacturonase divided by the amount of total GalA-methylated GalA included-present in the pectin sample) (Guillotin et al., 2005) are the most widely used. Rheological properties of calcium–pectin gels have been related to measurements of DB and DB_{abs} and high DB_{abs} values were shown to correlate with high strength of calcium–pectin gels (Fraeye et al., 2009; Löfgren, Guillotin, Evenbratt, Schols, & Hermansson, 2005; Ström et al., 2007).

Although extensive analyses for the presence of demethylated stretches and how this presence correlated with calcium binding properties have been published, a consistent pitfall is the variety of initial pectin samples used to generate plant-PME-deesterified samples and variability in deesterification conditions applied. Although pectins with random distribution of non-methylated GalA units can easily be differentiated from pectins with an extreme blockwise distribution of non-methylated GalA units, subtle differences regarding demethylated blocks length and distribution for a similar overall DM still are difficult to define. Designing HG models could simplify the complexity of the molecule and illustrate in more detail methyl ester distributions over the HG backbone. In the present work, we report on the preparation and characterization of model HGs series with tailored patterns of methyl-esterification.

2. Materials and methods

2.1. Preparation of model HGs

2.1.1. Preparation of unmethylated HG

Unmethylated HG has been prepared according to Thibault et al. (1993). Citrus pectin (Danisco, Lot 0080677, DM% 70.7) was first deesterified. Pectin (~25 g) was solubilised in 3.6 L of water under gentle magnetic stirring overnight at 4 °C. 500 mL of cold 0.2 N NaOH were added drop by drop to raise pH 11.8. After 20 min at 4 °C, 0.2 N NaOH was added to maintain pH at around 12. After 17 h at 4 °C, 1 N HCl was added drop by drop to reach pH 5. The slurry was then concentrated to 2 L. Precipitation of demethylated pectin was performed by adding 3 volumes of ethanol to the slurry. After 17 h at 4 °C, the suspension was filtered through Nylon (150 μm). The precipitate was washed twice with ethanol 70%, then twice with ethanol 96% and finally twice with acetone before being dried in a ventilated oven at 40 °C. The recovered deesterified pectin was dissolved in 2.5 L water to produce a concentration of ~10 g/L. 0.25 N HCl was added to the pectin solution to reach a final concentration of 0.1 N HCl. Hydrolysis was performed at 80 °C for 72 h. After cooling, the acid-soluble and acid-insoluble fractions were separated by centrifugation of the mixture at 15,000 × g for 20 min. The acid-insoluble fraction, corresponding to HG, was washed twice by 0.1 M HCl, then distilled water, and resuspended in distilled water (unmethylated HG in H^+ form). An aliquot of the suspension was taken in which 1 N NaOH was added drop by drop under magnetic stirring to reach pH 6. The solution obtained thereby was freeze-dried to yield unmethylated HG (quoted HG0) in Na^+ form.

2.1.2. Preparation of highly methylated HG

Highly methylated HG was prepared according to Renard and Jarvis (1999). Tetrabutyl ammonium hydroxide (TBA) was added to the rest of HG0 in H^+ form to reach pH7 and the resulting solution (HG-TBA) was extensively dialyzed against distilled water before freeze-drying. HG-TBA (40 g) was dissolved in dimethyl sulfoxide (900 mL) in a capped Erlenmeyer flask by overnight magnetic stirring at room temperature. Methyl iodide was then added (HG-TBA/CH_3I, 1/1, mol/mol) (3 times 2 mL at 30 min intervals). The solution was kept in the dark under continuous gentle stirring for 24 h. Residual CH_3I was removed through a nitrogen flux (1 h at room temperature). The solution was dialyzed twice against 0.2 M NaCl, then extensively against distilled water before freeze-drying.

2.1.3. Demethylation of highly methylated HG with NaOH

Aliquots (1 g) of highly methylated HG were demethylesterified by adequate amounts of 0.2 M NaOH (Table 1) to yield samples of given DM. At the end of the reaction, samples were freeze-dried. The samples thereby obtained are quoted **HGx-By** (with B = basic deesterification, x = DM of "mother" HG and y = final DM achieved).

2.1.4. Demethylation of highly methylated HG with p-PME

Aliquots of highly methylated HG (1 g) were deesterified by *p*-PME (Sigma 5400; 194 U/mg) to yield a series of *p*-PME-deesterified

Table 1
Chemical and enzymatic demethylesterification conditions applied and nomenclature of the different homogalacturonan samples.

Nomenclature	Chemical and enzymatic conditions	DM_i[a]	DM_f[b]
B-series			
HG96-B82	0.82 mol NaOH/g of HG, 18 h, 4 °C	96	82
HG96-B69	1.54 mol NaOH/g of HG, 18 h, 4 °C	96	69
HG96-B56	2.26 mol NaOH/g of HG, 18 h, 4 °C	96	56
HG96-B40	2.98 mol NaOH/g of HG, 18 h, 4 °C	96	40
HG96-B20	3.70 mol NaOH/g of HG, 18 h, 4 °C	96	20
P-series			
HG96-P75	353 units *p*-PME/g of HG, 25 min, 30 °C	96	75
HG96-P64	353 units *p*-PME/g of HG, 70 min, 30 °C	96	64
HG96-P56	1765 units *p*-PME/g of HG, 40 min, 30 °C	96	56
HG96-P36	1765 units *p*-PME/g of HG, 120 min, 30 °C	96	36
HG96-P14	1765 units *p*-PME/g of HG, 480 min, 30 °C	96	14
BP-series			
HG96-B77-P63	5.5 units *p*-PME/g of HG, 5 min, 30 °C	77	63
HG96-B76-P56	27 units *p*-PME/g of HG, 5 min, 30 °C	76	56
HG96-B76-P49	109 units *p*-PME/g of HG, 15 min, 30 °C	76	49
HG96-B82-P48	109 units *p*-PME/g of HG, 15 min, 30 °C	82	48

[a] DM of the initial "mother" homogalacturonan.
[b] Final DM reached.

HG samples of varying DM. Highly methylated HG aliquots were dissolved in phosphate buffer 100 mM pH 7.6 for 30 min at 30 °C. Adequate amounts of *p*-PME were added and solutions were kept under gentle stirring at 30 °C for various times (Table 1). At the end of the reaction, enzymes were inactivated: solutions were cooled in an ice bath and pH was brought to 5 with 2 N phosphoric acid. Solutions were then extensively dialyzed, pH was brought to 6 with NaOH and solutions were finally freeze-dried. Various samples were recovered; they are quoted **HGx-Py** (with P = PME deesterification, x = DM of "mother" HG and y = final DM achieved).

2.1.5. Demethylation of selected NaOH-demethylated HG with p-PME

Aliquots of selected samples from HG-B series (1 g) were deesterified by *p*-PME (Sigma 5400; 194 U/mg) to yield a series of HG-B-P samples of varying DM. The selected HG-B aliquots were dissolved in phosphate buffer 100 mM, pH 7.6 for 30 min at 30 °C. Adequate amounts of *p*-PME were added and solutions were kept under gentle stirring at 30 °C for various times (Table 1). At the end of the reaction, solutions were treated as described above. Various samples were recovered; they are quoted **HGx-By-Pz** (with B = basic deesterified HG, P = PME deesterification, x = DM of "mother" HG, y = DM achieved by basic deesterification of HGx, and z = final DM achieved).

2.2. Analytical

2.2.1. Galacturonic acid and neutral sugars

The galacturonic acid content was quantified, after saponification of the samples (0.2 M NaOH, 30 min, room temperature) and neutralization (0.2 M HCl), according to the automated meta-hydroxybiphenyl method (Thibault, 1979).

Individual neutral sugars were analysed as their alditol acetate derivatives (Blakeney, Harris, Henry, & Stone, 1983) by gas chromatography after a hydrolysis in 2 M trifluoroacetic acid at 121 °C for 2.5 h. *Myo*-inositol was used as an internal standard. The quantification was performed on a DB-225 fused-silica capillary column (30 mL × 0.32 mm i.d., J&W Scientific, Courtabœuf, France).

2.2.2. Degree of methylation

DM was determined by titrimetry. All samples were recovered in acidic form by passing through a strong H$^+$-exchanger (Rohm and Hass Amberlite IR 120) (25 cm^3). Free carboxylic acid functions were quantified at the neutralization point by conductimetric titration with NaOH of known molarity and total carboxylic function were determined by colorimetry on the same solutions after saponification (Ralet & Thibault, 2002). DM was calculated by:

$$\mathrm{DM} = 100 \times \frac{(\text{total carboxylic functions} - \text{free carboxylic functions})}{\text{total carboxylic functions}}$$

2.3. Macromolecular characteristics

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, Shodex, Showa Denko KK, Miniato, Japan) eluted with 0.05 M NaNO$_3$ buffer containing 0.02% NaN$_3$ as preservative at a constant flow rate of 42 mL/h. A differential refractometer (RI) (ERC 7517A) and a multiple-angle laser light-scattering device (MALLS) (Mini Dawn, Wyatt, Santa Barbara, CA, USA) operating at three angles (41°, 90° and 138°), a differential viscometer (T-50A, Viscotek, Malvern Instruments Ltd., Worcestershire, UK) were used as detectors. Samples were solubilised in 0.05 M NaNO$_3$ buffer containing 0.02% NaN$_3$, left overnight with tail-over-head continuous mixing, heated at 40 °C for 15 min, cooled and filtered (PVDF filter 13 mm diameter, 0.45 μm pore size) (Whatman Inc., Sandford, ME, USA) prior to analysis. Samples were injected automatically through a 50 μL loop. Data for molar mass determinations were analysed using Astra 1.4 software (Wyatt, Santa Barbara, CA, USA) taking dn/dc 0.146. Unsmoothed M_w and M_n values were obtained from raw data. Intrinsic viscosity values were obtained using Tri SEC Software (Version 3.0, Viscotek, Malvern Instruments Ltd., Worcestershire, UK).

2.4. Calcium activity

HGs were dissolved in ultra-pure water (∼7 mequiv/L) by overnight magnetic stirring at room temperature. Percolating the samples through a strong H$^+$-exchanger (Rohm and Hass Amberlite IR 120) allowed the recovery of HG samples in H$^+$ form at a concentration of ∼1 mequiv/L. Conductimetric measurements were carried out at 25 ± 0.2 °C with a CDM83 conductimeter (Radiometer Analytical) equipped with a double platinum electrode CDC 241U (Radiometer Analytical). The cell constant was determined with 0.05% (w/w) NaCl before each set of measurements. The titrations were performed with freshly prepared Ca(OH)$_2$ (10 mequiv/L). The experimental calcium activity coefficient at the neutralization points (γCa^{2+}exp) was determined by means of a dual-wavelength spectrophotometric method (Φ = A530/A493 nm) using tetramethylmurexide as an activity probe for calcium ions (Dronnet, Renard, Axelos, & Thibault, 1996; Kohn & Furda, 1967; Ralet et al., 2001; Ralet, Crépeau, Buchholt, & Thibault, 2003). Values reported correspond to the ratio of the activity of calcium ions in the presence of HG to the activity of calcium ions in ideal CaCl$_2$ solutions at the same ionic concentration. Analyses were performed in duplicate. Standard deviation was ≤2%.

Calcium activity coefficients were compared to theoretical predictions from Manning's model (γCa^{2+}theo) (Manning, 1969, 1975):

$$\gamma \mathrm{Ca}^{2+}\mathrm{theo} = e^{-|z|\bar{\xi}/2} \quad \text{when} \quad z\bar{\xi} < 1$$

$$\gamma \mathrm{Ca}^{2+}\mathrm{theo} = \frac{e^{-1/2}}{|z|\,\bar{\xi}} \quad \text{when} \quad z\bar{\xi} \geq 1$$

where $\bar{\xi}$ is the structural charge density $\bar{\xi} = 1.61 \times (100 - \mathrm{DM})/100$ and z is the charge of the counterion.

2.5. Degree of blockiness

Six microliters of endo-PG (endo-PGII, 36 nkat/mL) purified as described by Bonnin et al. (2002) were added to 3 mL of HG solution (∼1 mg/mL) in 50 mM Na-acetate buffer pH 4. Hydrolysis was performed at 40 °C for 3 days, 6 μL of fresh enzyme being added at *t* = 24 h and *t* = 48 h. Hydrolysates were filtered (0.45 μm Millipore) and analysed by HPAEC. Analyses were performed in triplicate.

A Waters system equipped with a Carbopac PA1 column (2 mm) with pulsed amperometric detection was used. Flow rate of the eluant was constant at 0.25 mL/min. The elution was carried out with four linear gradient phases of (i) 250–500 mM Na-acetate in 100 mM NaOH (0–20 min), (ii) 500–600 mM Na-acetate in 100 mM NaOH (20–40 min), (iii) 600–700 mM Na-acetate in 100 mM NaOH (40–60 min), and (iv) 700–800 mM Na-acetate in 100 mM NaOH (60–65 min). The column was reconditioned by washing with 800 mM Na-acetate containing 100 mM NaOH for one minute and then re-equilibrated with the starting buffer. Borwin Software (JMBS Developments, Grenoble, France) was used for data acquisition and processing. Monomer, dimer and trimer of GalA were used as standard (Bonnin et al., 2002).

Degree of blockiness was calculated by measuring the amount of monomers, dimers and trimers of GalA released divided by the amount of free GalA present in the sample (Daas et al., 1999, 2000; Löfgren et al., 2005). Once the amount of released oligomers is expressed as a percentage of total GalA, it is called absolute degree of blockiness (DB_{abs}) (Guillotin et al., 2005; Ström et al., 2007).

$$DB = \frac{[(1 \times dp1) + (2 \times dp2) + (3 \times dp3)]M_w^{GalA}}{(1 - DM/100)m_{HG}(m_{GalA}/m_{HG})} \times 100$$

$$DB_{abs} = \frac{[(1 \times dp1) + (2 \times dp2) + (3 \times dp3)]M_w^{GalA}}{m_{HG}(m_{GalA}/m_{HG})} \times 100$$

3. Results and discussion

3.1. HG isolation and tailoring

Lime pectin was deesterified before isolating HG in order to avoid differences in susceptibilities of acid hydrolysis of glycosidic bonds due to the presence of ester groups. The deesterification was performed under cold alkaline conditions to minimize β-elimination of the GalA chains. The recovered HG0 after 72 h of acid hydrolysis of deesterified lime pectin contained >99 sugar mol% of GalA with traces of Rha, Xyl and Gal. HG0 exhibited a weight-average molar mass of 17,430 g/mol (±10%) (~100 GalA units) with a low dispersity index (1.1) and an intrinsic viscosity of 70.7 (±3%) mL/g. Those results are in good agreement with values reported in the literature for HG obtained from citrus pectin using the same procedure (Thibault et al., 1993; Yapo, Lerouge, Thibault, & Ralet, 2007).

Methylation of HG0 was carried out with some modifications of the procedure developed by Matricardi, Dentini, Crescenzi, and Ross-Murphy (1995) and Renard and Jarvis (1999). A DM of 96 was reached. Such a high DM made HG96 hardly soluble in water at room temperature but visual solubilisation could be achieved by heating the sample solutions for 15 min at 40 °C. HG96 exhibited a weight-average molar mass of 23,070 (±10%) (~121 GalA units) and a polydispersity index of 1.25. It is noteworthy that, due to the presence of remaining aggregates, the weight-average molar mass value is most probably overestimated. An intrinsic viscosity value of 78.6 (±3%) mL/g was observed. Degradation of HG stretches did not occur during the methylation process, contrary to what was reported by Renard and Jarvis (1999).

HG96 has been modified by enzymatic and chemical methods to generate two series of model HGs. Whatever their final DM (DM_f), HGs from B- and P-series exhibited homogenous molar mass distributions (polydispersity index 1.18–1.21) with no major degradation compared to the "mother" HG. Average degrees of polymerization of 80–117 were calculated from the weight-average molar mass values.

In order to potentially generate another type of methylesterification pattern, selected NaOH-demethylated HG samples of DM ~ 80 (either already available, either newly generated) were treated with *p*-PME. Again, no major degradation compared to the "mother" HG has been noticed.

3.2. Methylester distribution of the generated HG model samples

The distribution pattern of methyl esters over the different HG model samples was quantified using the degree of blockiness (DB) and the absolute degree of blockiness (DB_{abs}) (Table 2).

A polynomial relationship between DB and DM_f ($DB = 97.228 - 2.0075DM_f + 0.010486DM_f^2$) was observed for HG samples from the B-series, DB values decreasing rapidly when DM_f increases (Fig. 1A). On the opposite, DB values observed for samples from the P-series were high and did not depend upon DM_f in the range observed, in agreement with Ström et al. (2007) findings. HG96-B20 exhibited a DB value close to DB values observed for HG samples from the P-series. Although NaOH is known to induce demethylation in a random fashion, sequences of non-methylesterified GalA residues long enough to enable degradation by endo-PG are statistically generated when DM_f decreases below a value of 35–40 (Ralet & Thibault, 2002). Two samples from the BP-series (HG96-B77-P63 and HG96-B76-P56) could not be differentiated from samples from the B-series. For those samples, the difference between the DM of the "mother HG" (initial DM, DM_i = 76 or 77) and the final DM (DM_f) was small. For the other samples from the BP-series, the DB values observed were intermediate between the B- and P-series trends. Those samples are the ones for which the difference between the DM_i and DM_f is important.

Table 2
Degrees of blockiness (DB), absolute degree of blockiness (DB_{abs}), experimental calcium activity coefficient (γCa^{2+}exp) and ratio of experimental to theoretical values of calcium activity coefficient (γCa^{2+}exp/theo) of the different homogalacturonan samples.

HG samples	DB	DB_{abs}	γCa^{2+}exp	γCa^{2+}exp/theo
B-series				
HG96-B82	4	1	0.82	1.10
HG96-B69	9	3	0.65	1.07
HG96-B56	21	9	0.56	1.31
HG96-B40	33	20	0.43	1.37
HG96-B20	56	44	0.08	0.34
P-series				
HG96-P75	61	15	0.23	0.34
HG96-P64	81	29	0.14	0.27
HG96-P56	73	32	0.12	0.28
HG96-P36	71	46	0.08	0.27
HG96-P14	66	57	0.06	0.27
BP-series				
HG96-B77-P63	5	2	0.62	1.22
HG96-B76-P56	20	9	0.56	1.31
HG96-B76-P49	43	22	0.29	0.79
HG96-B82-P48	53	28	0.34	0.94

According to some authors, DB_{abs} is more informative than DB and provides further valuable information on HG methylation patterns, especially for extremely blocky pectins (Guillotin et al., 2005; Ström et al., 2007). As shown in Fig. 1B, plotting DB_{abs} values versus DM_f did however not give more information on methylation patterns of HG model series than plotting DB values versus DM_f (Fig. 1A).

In order to get a better insight into the DB and DB_{abs} values and potentially better differentiate the HG series, DM_i was tentatively taken into account by plotting DB and DB_{abs} versus $(DM_i - DM_f)/DM_i$. As shown in Fig. 1C and D, only HG96-B77-P63 was still not (DB) or hardly (DB_{abs}) differentiable from the B-series trend, while HG96-B76-P49 and HG96-B82-P48 were shown to follow the P-series trend.

3.3. Calcium binding properties of the generated HG model samples

The reactivity toward calcium ions is another approach used to determine the distribution pattern of methyl groups over HG domains (Kohn et al., 1983; Löfgren et al., 2005; Ralet et al., 2001; Slavov et al., 2009; Ström et al., 2007; Thibault & Rinaudo, 1985). Since the length of HG is similar for all samples, the only intrinsic parameter that can control calcium-binding properties is DM and its distribution pattern (Lutz, Aserin, Wicker, & Garti, 2009).

The experimental calcium activity values (γCa^{2+}exp) observed for HG samples from the B-series (Table 2) were in the range reported by Ralet et al. (2001) for pectin samples deesterified with alkali, while values obtained for HG samples from the P-series were

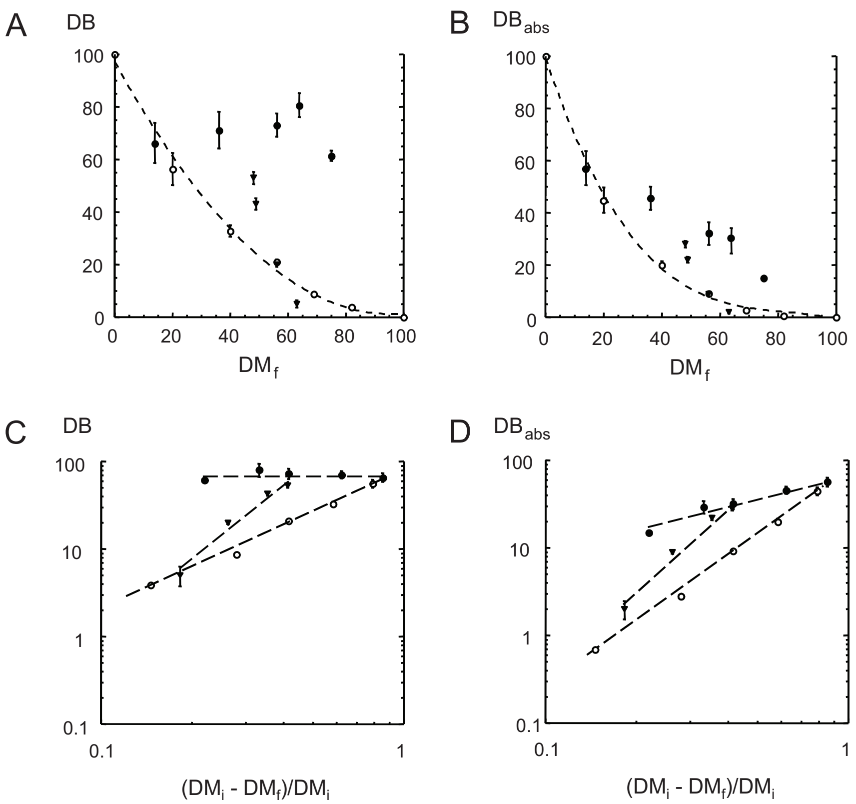

Fig. 1. Degree of blockiness (DB) and absolute degree of blockiness (DB$_{abs}$) as a function of the degree of methylesterification (DM) for the three series of homogalacturonans. DM$_i$, DM of the initial "mother" HG; DM$_f$, final DM reached. ○, B-series; ●, P-series; ▼, BP-series.

much lower than those reported by the same authors for pectin samples deesterified by the same *p*-PME. This indicates that *p*-PME treated-HGs generated in the present work can bind calcium ions more tightly than the previously studied *p*-PME treated-pectins. This difference could be due to the DM of the initial mother sample, i.e. DM$_i$ = 81 in Ralet et al. (2001) work versus DM$_i$ = 96 in the present study. This emphasizes again the importance of DM$_i$, the average length of demethylated blocks generated by *p*-PME increasing with a rise of DM of the mother sample.

For the different HG series, the dependency of γCa^{2+}exp upon DM$_f$ is presented in Fig. 2A. For HG samples from the B-series, a linear relationship between DM$_f$ and γCa^{2+}exp was observed in the DM range 40–80 ($\gamma Ca^{2+}\text{exp} = 9.03 \times 10^{-3}\text{DM}_f + 0.058$). A drop in γCa^{2+}exp values was then noticed below DM$_f$ 40, in agreement with previous reports (Kohn, 1975; Kohn & Luknar, 1975; Ralet et al., 2001; Thibault & Rinaudo, 1985). Indeed, sequences of non-methylesterified GalA residues long enough to enable dimerisation through calcium ions are statistically generated when DM$_f$ decreases below a value of 35–40 (Ralet & Thibault, 2002). The increase in γCa^{2+}exp values when increasing DM$_f$ is very limited for HG samples from the P-series. Cross-linking *via* calcium bridges seems to exist for all HG samples from the P-series, whatever their DM$_f$. The formation of demethylated blocks long enough to induce full calcium bridging of HG molecules is also obvious for HG-B20, in agreement with previous findings (Ralet et al., 2001). For HG samples from the BP-series, two samples (HG96-B77-P63 and HG96-B76-P56) matched with the B-series trend, while the two other samples (HG96-B76-P49 and HG96-B82-P48) exhibited γCa^{2+}exp values in between B-series and P-series trends. These results are in total accordance with what was observed using the "degree of blockiness" approach.

The physicochemical properties of polyelectrolytes in aqueous diluted solutions in absence of external salt depend on the charge parameter value and on the valency of the counterions (Rinaudo, 2009). Manning's theory (Manning, 1969, 1975) does not take into account the intermolecular trapping of calcium ions to carboxyl groups of two or more HG molecules. Hence, the ratio of experimental to theoretical calcium activity values [γCa^{2+}exp/theo] was shown to remain roughly constant at 1–1.4 in the absence of HG bridging through calcium ions and to drop to 0.3–0.5 for complete calcium bridging (Ralet et al., 2001, 2003; Thibault & Rinaudo, 1985).

γCa^{2+}exp/theo values were ≥ 1 for HG samples from the B-series of DM $\geq$ 40 (Table 2, Fig. 2B). For HG samples of the P-series and for HG96-B20, γCa^{2+}exp/theo values appeared very low (0.26–0.34), highlighting the formation of HG dimers/multimers through calcium ions, and hence the presence of long demethylated GalA stretches onto HG molecules (Fig. 2B). As already pointed out, for HG samples from the BP-series, two samples (HG96-B77-P63 and HG96-B76-P56) matched with B-series trend, while the two other samples (HG96-B76-P49 and HG96-B82-P48) exhibited γCa^{2+}exp/theo values in between B-series and P-series trends.

Taking into account DM$_i$ by plotting γCa^{2+}exp and γCa^{2+}exp/theo versus (DM$_i$ – DM$_f$)/DM$_i$ (Fig. 2C and D, respectively) did not lead to further differentiation of B-, P- and BP-series.

3.4. *Comparison of degradability by endo-polygalacturonase and calcium activity*

Ström et al. (2007) reported that plotting the storage modulus of calcium–pectin gels as a function of DB$_{abs}$ made the data collapse reasonably well onto a single master line ($r^2 = 0.86$). In the present work, a similarly good linear correlation between DB$_{abs}$ and 1/γCa^{2+}exp values was observed ($r^2 = 0.86$). However, some HG samples (HG96-B40, HG96-B76-P49, and HG96-B82-P48)

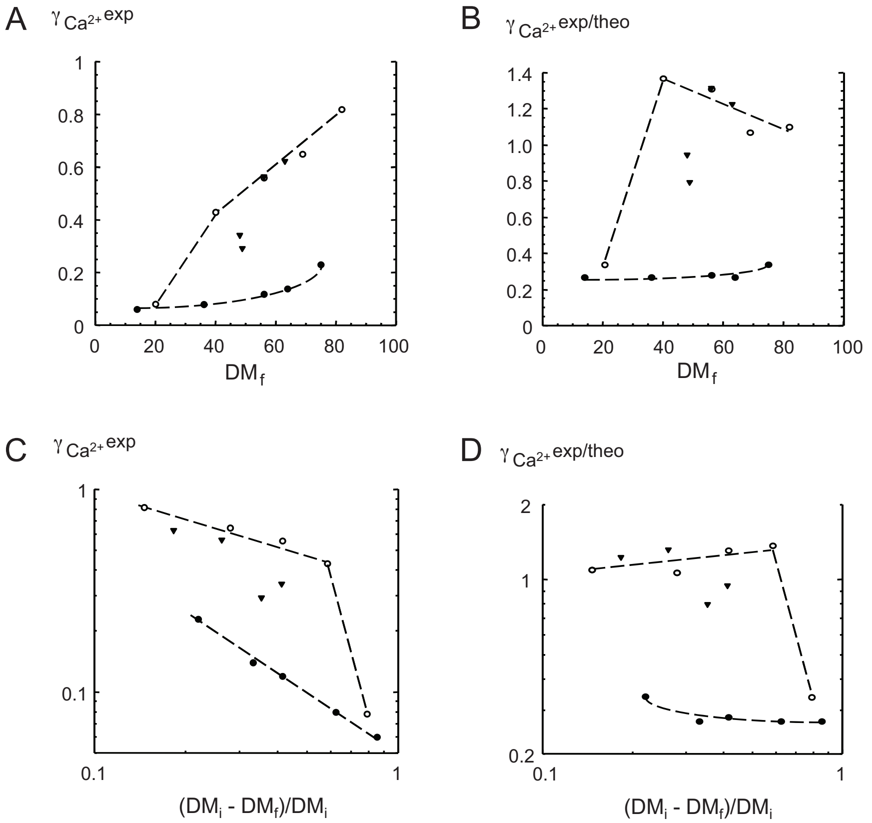

Fig. 2. Calcium activity coefficient (γCa^{2+}) as a function of the degree of methylesterification (DM) for the three series of homogalacturonans exp, experimental values; theo, theoretical values (Manning, 1969, 1975); DM_i, DM of the initial "mother" homogalacturonan; DM_f, final DM reached. ○, B-series; ●, P-series; ▼, BP-series.

did not follow this master line, exhibiting high DB_{abs} values for low $1/\gamma Ca^{2+}$exp values (Fig. 3). It can be hypothesised that those samples exhibit demethylated GalA stretches long enough to be degradable by endo-PGII but not long enough to promote the formation of HG dimers/multimers through calcium ions. On the basis of previously published data (Benen, Kester, & Visser, 1999; Chen & Mort, 1996; Luzio & Cameron, 2008), an average demethylated GalA stretches' degree of polymerization of 5–8 can be assumed for those samples.

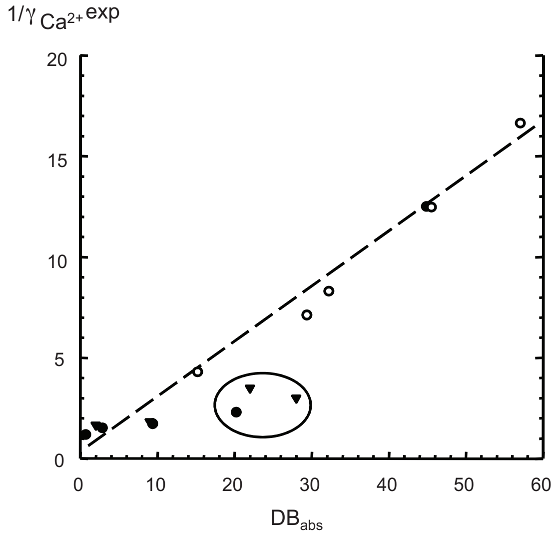

Fig. 3. Calcium sensitivity ($1/\gamma Ca^{2+}$exp) as a function of absolute degree of blockiness (DB_{abs}) for the three series of homogalacturonans. γCa^{2+}exp, experimental calcium activity coefficient values. ○, B-series; ●, P-series; ▼, BP-series.

Using the alternative graphical representation γCa^{2+}exp/theo = f(DB) (Fig. 4) evidenced the presence of three main families of HGs: (i) HGs with no or very short demethylated blocks exhibiting low DB values and high γCa^{2+}exp/theo values; (ii) HGs with long demethylated blocks exhibiting high DB values and low γCa^{2+}exp/theo values; and HGs with short demethylated blocks exhibiting medium DB and γCa^{2+}exp/theo values. HGs with no or very short demethylated blocks encompass all samples from the B-series with a DM ≥ 40 and two samples from the BP-series (HG96-B77-P63 and HG96-B76-P56) for which the difference between the DM of the "mother HG" (DM_i) and the final DM (DM_f) was small. HGs with long demethylated blocks encompass all

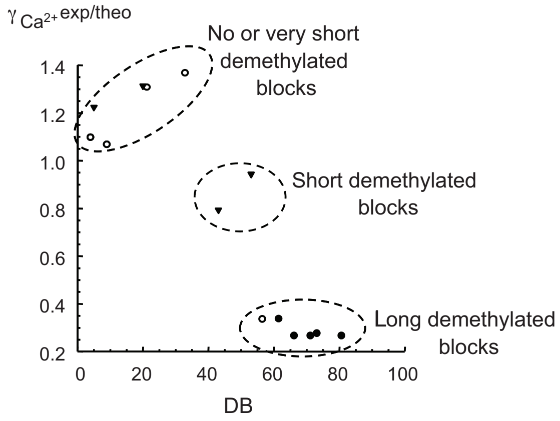

Fig. 4. Variations of the ratio of experimental to theoretical values of calcium activity coefficient (γCa^{2+}) with the degree of blockiness (DB) for the three series of homogalacturonans. ○, B-series; ●, P-series; ▼, BP-series.

samples from the P-series and the very lowly methylated HG96-B20 sample. HGs with short demethylated blocks encompass the two samples from the BP-series (HG96-B76-P49 and HG96-B82-P48) for which the difference between the DM_i and DM_f was important.

4. Conclusion

In the present work, purified HGs have been chemically and/or enzymatically tailored with the aim of mastering methyl groups' distribution in order to get a better insight into how to best analyse the methylester distribution in relation to reactivity toward calcium. Randomly demethylesterified HGs of various DM were classically obtained by alkali deesterification of a very highly methylated "mother" HG. Manipulating the initial DM (DM_i) of randomly methyl-esterified "mother" HGs prior to enzymatic blockwise demethylesterification allowed the recovery of two HG types. HGs with few long deesterified blocks of GalAs were generated from a very high DM_i "mother" HG, while HGs with more numerous but shorter deesterified blocks of GalAs could be produced from "mother" HGs of lower DM_i.

The mastered model HGs generated in this work will be useful as a basis for the study of pectins of unknown methylesterification patterns. However, HGs of high DM exhibiting subtle differences in methylesterification patterns – slightly blocky versus random – could not be differentiated one from the other on the basis of their DB, DB_{abs} or calcium sensitivity values. Alternative enzymatic degradation approaches, such as the use of pectin lyase, shall be considered. This will be described in a forthcoming paper.

References

Benen, J. A. E., Kester, H. C. M., & Visser, J. (1999). Kinetic characterization of *Aspergillus niger* N400 endopolygalacturonases I, II and C. *European Journal of Biochemistry*, *259*, 577–585.

Blakeney, A. B., Harris, P. J., Henry, R. J., & Stone, B. A. (1983). A simple and rapid preparation of alditol acetates for monosaccharide analysis. *Carbohydrate Research*, *113*, 291–299.

Bonnin, E., Le Goff, A., Körner, R., Vigouroux, J., Roepstorff, P., & Thibault, J.-F. (2002). Hydrolysis of pectins with different degrees and patterns of methylation by the endopolygalacturonase of *Fusarium moniliforme*. *Biochimica Biophysica Acta*, *1596*, 83–94.

Caffall, K. H., & Mohnen, D. (2009). The structure, function, and biosynthesis of plant cell wall pectic polysaccharides. *Carbohydrate Research*, *344*, 1879–1900.

Cameron, R. G., Luzio, G. A., Goodner, K., & Williams, M. A. K. (2008). Demethylation of a model homogalacturonan with a salt-independent pectin methyleterase from citrus. I. Effect of pH on the demethylated block size, block number and enzyme mode of action. *Carbohydrate Polymers*, *71*, 287–299.

Chen, E. M. W., & Mort, A. J. (1996). Nature of sites hydrolyzable by endopolygalacturonase in partially-esterified homogalacturonans. *Carbohydrate Polymers*, *29*, 129–136.

Coenen, G. J., Kabel, M. A., Schols, H. A., & Voragen, A. G. J. (2008). CE-MSn of complex pectin-derived oligomers. *Electrophoresis*, *29*, 2101–2111.

Daas, P. J. H., Arisz, P. W., Schols, H. A., De Ruiter, G. A., & Voragen, A. G. J. (1998). Analysis of partially methyl-esterified galacturonic acid oligomers by high-performance anion-exchange chromatography and matrix-assisted laser desorption/ionization time-of-flight spectrometry. *Analytical Biochemistry*, *257*, 195–202.

Daas, P. J. H., Meyer-Hansen, K., Schols, H. A., De Ruiter, G. A., & Voragen, A. G. J. (1999). Investigation of the non-esterified galacturonic acid distribution in pectin with endopolygalacturonase. *Carbohydrate Research*, *326*, 120–129.

Daas, P. J. H., Voragen, A. G. J., & Schols, H. A. (2000). Characterisation of non-esterified galacturonic acid sequences in pectin with endopolygalacturonase. *Carbohydrate Research*, *326*, 120–129.

Daas, P. J. H., Voragen, A. G. J., & Schols, H. A. (2001). Study of the methyl ester distribution in pectin with endo-polygalacturonase and high-performance size-exclusion chromatography. *Biopolymers*, *58*, 195–203.

Denès, J.-M., Baron, A., Renard, C. M. G. C., Péan, C., & Drilleau, J.-F. (2000). Different action patterns for apple pectin methylesterase at pH 7.0 and 4.5. *Carbohydrate Research*, *327*, 385–393.

Dronnet, V., Renard, C. M. G. C., Axelos, M. A. V., & Thibault, J.-F. (1996). Characterisation and selectivity of divalent metal ions binding by citrus and sugar-beet pectins. *Carbohydrate Polymers*, *30*, 253–263.

Fraeye, I., Doungla, E., Duvetter, T., Moldenaers, P., Van Loey, A., & Hendrickx, M. (2009). Influence of intrinsic and extrinsic factors on rheology of pectin–calcium gels. *Food Hydrocolloids*, *23*, 2069–2077.

Grasdalen, H., Barkoy, O. E., & Larsen, B. (1988). Determination of the degree of esterification and the distribution of methylated and free carboxyl groups in pectins by H-1-NMR spectroscopy. *Carbohydrate Research*, *184*, 183– 191.

Guillotin, S. E., Bakx, E. J., Boulenguer, P., Mazoyer, J., Schols, H. A., & Voragen, A. G. J. (2005). Populations having different GalA blocks characteristics are present in commercial pectins which are chemically similar but have different functionalities. *Carbohydrate Polymers*, *60*, 391–398.

Kohn, R. (1975). Ion binding on polyuronates—Alginate and pectin. *Pure and Applied Chemistry*, *42*, 371–397.

Kohn, R, & Furda, I. (1967). Calcium ion activity in solutions of calcium pectinate. *Collection of Czechoslovak Chemical Communications*, *32*, 1925–1937.

Kohn, R., & Luknar, O. (1975). Calcium and strontium ion activity in solutions of the corresponding pectinates and its dependence on their degree of esterification. *Collection of Czechoslovak Chemical Communications*, *40*, 959–970.

Kohn, R., Markovic, O., & Machova, E. (1983). Deesterification mode of pectin by pectin esterases of Aspergillus foetidus. *Collection of Czechoslovak Chemical Communications*, *48*, 790–797.

Körner, R., Limberg, G., Christensen, T. M. I. E., Mikkelsen, J. D., & Roepstorff, P. (1999). Sequencing of partially methyl-esterified oligogalacturonates by tandem mass spectrometry and its use to determine pectinase specificities. *Analytical Chemistry*, *71*, 1421–1427.

Limberg, G., Körner, R., Buchholt, H. C., Christensen, T. M. I. E., Roepstorff, P., & Mikkelsen, J. D. (2000a). Analysis of different de-esterification mechanisms for pectin by enzymatic fingerprinting using endopectin lyase and endopolygalacturonase II from *A. Niger*. *Carbohydrate Research*, *327*, 293–307.

Limberg, G., Körner, R., Buchholt, H. C., Christensen, T. M. I. E., Roepstorff, P., & Mikkelsen, J. D. (2000b). Quantification of the amount of galacturonic acid residues in block sequences in pectin homogalacturonan by enzymatic fingerprinting with exo- and endo-polygalacturonase II from *Aspergillus niger*. *Carbohydrate Research*, *327*, 321–332.

Löfgren, C., Guillotin, S., Evenbratt, H., Schols, H. A., & Hermansson, A.-M. (2005). Effects of calcium, pH and blockiness on kinetic rheological behavior and microstructure of HM pectin gels. *Biomacromolecules*, *6*, 646–652.

Lutz, R., Aserin, A., Wicker, L., & Garti, N. (2009). Structure and physical properties of pectins with block-wise distribution of carboxylic acid groups. *Food Hydrocolloids*, *23*, 786–794.

Luzio, G. A., & Cameron, R. G. (2008). Demethylation of a model homogalacturonan with a salt-independent pectin methyleterase from citrus. II. Structure–function analysis. *Carbohydrate Polymers*, *71*, 300–309.

Manning, G. S. (1969). Limiting laws and counterion condensation in polyelectrolyte solution. *Journal of Physical Chemistry*, *51*, 924–933.

Manning, G. S. (1975). A limiting law for the conductance of the rod model of a salt-free polyelectrolyte solution. *Journal of Physical Chemistry*, *79*, 262– 265.

Matricardi, P., Dentini, M., Crescenzi, V., & Ross-Murphy, S. B. (1995). Gelation of chemically cross-linked polygalacturonic acid derivatives. *Carbohydrate Polymers*, *27*, 215–220.

Mohnen, D. (2008). Pectin structure and biosynthesis. *Current Opinion in Plant Biology*, *11*, 266–277.

Ralet, M.-C., Cabrera, J. C., Bonnin, E., Quéméner, B., Hellìn, P., & Thibault, J.-F. (2005). Mapping sugar beet pectin acetylation pattern. *Phytochemistry*, *66*, 1832– 1843.

Ralet, M.-C., Crépeau, M.-J., & Bonnin, E. (2008). Evidence for a blockwise distribution of acetyl groups onto homogalacturonans from a commercial sugar beet (*Beta vulgaris*) pectin. *Phytochemistry*, *69*, 1903–1909.

Ralet, M.-C., Crépeau, M.-J., Buchholt, H. C., & Thibault, J.-F. (2003). Polyelectrolyte behaviour and calcium binding properties of sugar beet pectins differing in their degrees of methylation and acetylation. *Biochemical Engineering Journal*, *16*, 191–201.

Ralet, M.-C., Dronnet, V., Buchholt, H. C., & Thibault, J.-F. (2001). Enzymatically and chemically de-esterified lime pectin: Characterization, polyelectrolyte behavior calcium binding properties. *Carbohydrate Research*, *336*, 117–125.

Ralet, M.-C., & Thibault, J.-F. (2002). Interchain heterogeneity of enzymatically deesterified lime pectins. *Biomacromolecules*, *3*, 917–925.

Renard, C. M. G. C., & Jarvis, M. C. (1999). Acetylation and methylation of homogalacturonans 1: Optimisation of the reaction and characterisation of the products. *Carbohydrate Polymers*, *39*, 201–207.

Ridley, B. L., O'Neil, M. A., & Mohnen, D. (2001). Pectins: Structure, biosynthesis and oligogalacturonide-related signalling. *Phytochemistry*, *57*, 929–967.

Rinaudo, M. (2009). Polyelectrolyte properties of a plant and animal polysaccharide. *Structural Chemistry*, *20*, 277–289.

Round, A. N., Rigby, N. M., MacDougall, A. J., & Morris, V. J. (2010). A new view of pectin structure revealed by acid hydrolysis and atomic force microscopy. *Carbohydrate Research*, *345*, 487–497.

Savary, B. J., Hotchkiss, A. T., & Cameron, R. G. (2002). Characterization of a salt-independent pectin methylesterase purified from Valencia orange peel. *Journal of Agricultural and Food Chemistry*, *50*, 3553–3558.

Slavov, A., Garnier, C., Crépeau, M.-J., Durand, S., Thibault, J.-F., & Bonnin, E. (2009). Gelation of high methoxy pectin in the presence of pectin methylesterases and calcium. *Carbohydrate Polymers*, *77*, 876–884.

Ström, A., Ribelles, P., Lundin, L., Norton, I., Morris, E. R., & Williams, M. A. K. (2007). Influence of pectin fine structure on the mechanical properties of calcium–pectin and acid–pectin gels. *Biomacromolecules*, *8*, 2668–2674.

Thibault, J.-F. (1979). Automated method for the determination of pectic substances. *Lebensmittel-Wissenschaft und Technologie*, *12*, 247–251.

Thibault, J.-F., Renard, C. M. G. C., Axelos, M. A. V., Roger, P., & Crépeau, M.-J. (1993). Studies of the length of homogalacturonic regions in pectins by acid hydrolysis. *Carbohydrate Research*, *238*, 271–286.

Thibault, J.-F., & Rinaudo, M. (1985). Interactions of mono- and divalent counter ions with alkali- and enzyme-deesterified pectins in salt-free solutions. *Biopolymers*, *24*, 2131–2144.

Vincent, R. R., Cucheval, A., Hemar, Y., & Williams, M. A. K. (2009). Bio-inspired network optimization in soft materials—Insights from the plant cell wall. *European Physical Journal E*, *28*, 79–87.

Voragen, A. G. J., Pilnik, W., Thibault, J.-F., Axelos, M. A. V., & Renard, C. M. G. C. (1995). Pectins. In A. M. Stephen (Ed.), *Food polysaccharides and their interactions* (pp. 287–340). New York: Marcel Dekker.

Willats, W. G. T., Knox, J. P., & Mikkelsen, J. D. (2006). Pectin: New insights into an old polymer are starting to gel. *Trends in Plant Science*, *17*, 97–104.

Willats, W. G. T., McCartney, L., Mackie, W., & Knox, J. P. (2001). Pectin: Cell biology and 516 prospects for functional analysis. *Plant Molecular Biology*, *47*, 9–27.

Willats, W. G. T., Orfila, C., Limberg, G., Buchholt, H. C., van Alebeek, G. J. W. M., Voragen, A. G. J., et al. (2001). Modulation of the degree and pattern of methyl-esterification of pectic homogalacturonan in plant cell walls—Implications for pectin methyl esterase action, matrix properties, and cell adhesion. *Journal of Biological Chemistry*, *76*, 19404–19413.

Williams, M. A. K., Cucheval, A., Ström, A., & Ralet, M.-C. (2009). Electrophoretic behavior of copolymeric galacturonans including comments on the information content of the intermolecular charge distribution. *Biomacromolecules*, *10*, 1523–1531.

Yapo, B. M., Lerouge, P., Thibault, J.-F., & Ralet, M.-C. (2007). Pectins from citrus peel cell walls contain homogalacturonans homogeneous with respect to molar mass, rhamnogalacturonan I and rhamnogalacturonan II. *Carbohydrate Polymers*, *69*, 426–435.