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Utilisation of model pectins reveals the effect of demethylated block size frequency on calcium gel formation

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ABSTRACT

Calcium-mediated gelation of LMP is thought to arise from formation of a dense network of Ca^{2+}-cross-linked DMB meeting a required minimum average length along pectin chains. The use of MP containing specific average DMB size ($\overline{BS}$) types, in the range of 3–100 and in varying proportion (0–100%), has afforded further insights into the gelling behaviour of pectins with a certain DM in the presence of Ca^{2+} ions. It clearly appeared that a required minimum $\overline{BS}$ and a required minimum average frequency ($\overline{BSF}$) of the required minimum $\overline{BS}$ are conditions that must be satisfied by a pectin for formation of a highly dense Ca^{2+}-cross-linked DMB network equaling an elastically stable, strong, and cohesive gel. Furthermore, there is a clear contribution of the pectin branched domains to gelation and formation of a firmer and more cohesive gel. The results suggest that this pectin portion may function, not only as a "maintainer" of the pectin molecular weight to a sufficiently high level which fosters good gelation regarding the gelling rate and the strength and nature of the gel formed, but also as junction-zone-terminating structural elements that limit the appearance of undesirable phenomena, notably turbidity, syneresis, and precipitation.

1. Introduction

Pectic substances are a large family of at least eight blocks co-biopolysaccharides from plant origin, of which unbranched HG and neutral sugar-branched RG-I are the most frequently reported (Yapo, 2011). The well-known functional property of pectins, since their first discovery and crude characterisation in 1790–1825, is gel-formation under specified conditions. This functional property is (almost exclusively) conferred by the pectin HG regions, which are made of (1,4)-linked α-GalA*p* residues, partially methylesterified at *C*-6 position and sometimes partly acetyl-esterified at O-2/O-3 positions. The amount and $\overline{DP}$ of the pectin HG domains influence its gelling characteristics and the strength of the gel formed (Yapo, 2009). It is widely believed that the DM of pectin governs its mechanism of gelation. However, it should be kept in mind that extracted (crude) pectins are typically heterogeneous, with respect to individual polymer chains size, charge distribution, and charge density, so that the overall DM is always a mean value.

Depending on DM, pectins are distinguished as low methoxy pectins (LMP; DM < 50) and high methoxy pectins (HMP; DM ≥ 50). In general, LMP are produced from HMP using four kinds of deesterification, viz. by alkali (e.g., NaOH) treatment at cold temperature (LMP with random distribution of deesterified GalA units), by ammonia treatment (amidated LMP with random distribution of deesterified GalA units and amide groups partially substituted for methoxy groups), by fungal PME treatment (LMP with non-blockwise distribution of deesterified GalA residues), and by plant PME (LMP with blockwise distribution of deesterified GalA residues). HMP are believed to form gels at high sugar

Abbreviations: ADLP, alkali-deesterified lemon pectin; BME, beta-mercaptoethanol; BS, demethylated galacturonic acid block size; BSF, demethylated galacturonic acid block size frequency; CCHMP, commercial citrus highly-methylesterified pectin; DB, degree of blockiness; DB_{abs}, absolute degree of blockiness; DE, degree of esterification; DM, degree of methylesterification; DMB, demethylated galacturonic acid block; DP, degree of polymerisation; EndoPG, endopolygalacturonases; GalA, galacturonic acid; GFC, gel filtration chromatography; HG, homogalacturonans; HGA, homogalacturonic acids; HMP, high methoxy pectins; HPAEC-PAD, high performance anion exchange chromatography-pulsed amperometric detection; IEC, ion exchange chromatography; LMP, low methoxy pectins; LP, lemon pectin; MP, model pectins; MRGI, modified type one rhamnogalacturonans; Mw, molecular weight; MWCO, nominal molecular weight cut-off; MWD, molecular weight distribution; OGA, oligogalacturonides; PAE, pectin acetylesterases; PGA, polygalacturonic acids; PME, pectin methylesterases; PME-PDP, partially deesterified pectin with pectin methylesterase; RGI, type one rhamnogalacturonans; SAOS, small amplitude oscillatory shear; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; UMPS, ultra-methylated pectin strands sample.

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(e.g., sucrose) concentration (>55 wt%) and acid (low pH 2.2–2.8) conditions, thereby yielding the so-called "high methoxy pectin-sugar-acid-gel (HMP-SAG)". The mechanism of gelation of HMP is usually explained by formation of acid-and-sugar-promoted junction zones, between the pectin methylesterified HG domains, which are then stabilised by intermolecular hydrophobic interactions between methylester groups and by intermolecular hydrogen bonds between carboxyl groups of unesterified GalA residues and secondary alcohols (Oakenfull & Scott, 1984). This pectin functionality is currently exploited in the food industry for manufacturing jams, jellies, and marmalades.

Gelation of LMP, in contrast, does not require sugar and acid (low pH) conditions, but does need multivalent cations (especially Ca^{2+}), thereby yielding the so-called "calcium gel". Domains of utilisation of calcium gel are, for instance, formulation of low-calorie jellies and stabilisation of acidic yoghourts. A widely accepted mechanism of gelation for LMP is based on the so-called "egg-box junction zone model", in which deprotonated carboxyl groups of DMB of the pectin HG domains are believed to be cross-linked by Ca^{2+} ion bridges, thus forming intermolecular junction zones, which are then stabilised by van der Waals interactions, hydrogen bonds, and electrostatic interactions (Grant, Morris, Rees, Smith, & Thom, 1973; Kohn, 1975, 1987; Morris, Powell, Gidley, & Rees, 1982). However, the ability of LMP to form egg-box-type junction zones does not only depend on its degree of de-methylation (100-DM), but also on inter- and intra-molecular distribution patterns of unesterified GalA residues of the individual polyelectrolyte chains (Fraeye, Duvetter, Doungla, van Loey, & Hendrickx, 2010; Kohn & Luknar, 1977; Voragen, Pilnik, Thibault, Axelos, & Renard, 1995).

Introduction of two "blocky" parameters, namely DB which represents the ratio of the amount of unesterified (mono-, di- and tri-) GalA residues, released by EndoPG, to the total amount of unesterified GalA residues in pectin, and DB_{abs} which accounts for the ratio of the amount of unesterified (mono, di-, and tri-) GalA residues, liberated by EndoPG, to the total amount of (esterified and unesterified) GalA residues within pectin (Guillotin et al., 2005), have allowed to posit that the more blockwise the distribution of unesterified GalA residues on pectin chains is, the higher the probability of these pectin strands to be cross-linked by Ca^{2+} ions and to form stable egg-box junction zones, which results in stable, strong, and cohesive calcium gels. Thus, strong correlation between the DB_{abs} of pectin and gel strength (or stiffness) has recently been reported by alkali- or plant PME-demethylation of partially methylesterified pectins (e.g., 79% methylesterified commercial apple pectin), thereby confirming the importance of the (de-)methylesterification pattern for gel forming abilities (Fraeye et al., 2010). Nevertheless, acid-extracted (commercial citrus and apple) pectins are a mixture of non-homogenous pectin strands with varying size, DM, DB, and DB_{abs}, and therefore it would not be one study too many to use an ultra (chemically) methylated, size-homogenous, pectin HG as the starting sample to unequivocally substantiate existence of such a strong correlation. This original idea, which has been advanced for the very first time a year earlier (B.M. Yapo, unpublished), is being investigated with amply interesting results.

Furthermore, the minimum DMB required for formation of stable egg-box junction structures has variably been reported to be 6–13, 7, 9, 12, 14, and 15–20 GalA residues as succinctly summarised elsewhere (Fraeye et al., 2010; Taylor, 1982; Vincent & Williams, 2009). The present study aims at reporting on the gelling behaviour of partially demethylated MP, with different specific $\overline{BS}$ types, in the presence of Ca^{2+} ions. By mixing, in the presence of Ca^{2+} ions, a fixed amount of CCHMP (95% DM), with varying amounts of commercial triGalA or laboratory-produced HGA, having a narrow MWD, we have surprisingly observed that good gelation occurred with the HGA/CCHMP mixture under certain conditions, whereas no gelation occurred with the triGalA/CCHMP mixture over the whole range (from 0 to 100% of triGalA) or with CCHMP alone (100% CCHMP). This revealed that the required minimum $\overline{BS}$ and $\overline{BSF}$ in pectins are the key determinants for calcium-promoted gelation of partially demethylated pectins.

2. Materials and methods

2.1. Materials

CCHMP (95% DM, 85% GalA), citrus PGA (95% purity, $\overline{M_w}$ 25–50 kDa, lot 81325), triGalA, diGalA, and orange peel PME (P5400, lyophilised powder, ≥150 U/mg protein; 25–50% protein) were purchased from Sigma–Aldrich Co. (St. Louis, MO). MonoGalA was bought from Fluka (Buchs, Switzerland). Commercial polygalacturonase preparation (EPG-M2), produced by *Aspergillus aculeatus*, was purchased from Megazyme International Ireland Ldt. (Bray, Co., Wicklow, Ireland). Protein molecular weight makers in the range of 6–200 kDa, viz. aprotinin (6.5 kDa), α-lactalbumin (14.4 kDa), soybean trypsin inhibitor (21.5 kDa), carbonic anhydrase (31 kDa), ovalbumin (45 kDa), bovin serum albumin (66.2 kDa), phosphorylase b (97.4 kDa), β-galactosidase (116.3 kDa), and myosin (200 kDa) were from Biorad Laboratories, Inc. (Hercules, CA). A pullulan kit with a narrow MWD ($\overline{M_w}$~6.0, 10.0, 21.7, 48.8, 113.0, 210.0, 393.0, and 805.0 kDa) was from American Polymer Standards Corp. (Mentor, OH).

LP64 (64% DM, 85% GalA) was produced by dilute citric acid treatment of lemon peel, followed by dialysis and alcohol-precipitation (Yapo, 2009). Two homogenous HGA samples, referred to as HGA60 ($\overline{DP}$ 60) and HGA100 ($\overline{DP}$ 100), were purified from citric-acid-extracted alkali-deesterified pineapple flesh and lemon peel (ADLP) pectins, respectively (Yapo, 2009). The acid-generated RGI oligomers product (7.0%, w/w), obtained concomitantly with HGA100 (90.0%, w/w) from ADLP, is referred to as modified RGI (MRGI). UMPS (98% DM, 82% GalA) was produced from CCHMP as a soluble fraction in cupric sulfate solution containing excess of Cu^{2+} ions as follows. Solution (1%) of CCHMP (95% DM) was gradually added to 7% $CuSO_4 \cdot 5H_2O$ (Yapo, 2010). The mixture was stored at 4 °C to allow complete formation of insoluble Cu-pectinate complexes and was centrifuged to separate the precipitate formed from the supernatant. The precipitate was then purified to give a final pectinate product accounting for ~28% (w/w) and having an average DM of ~90%. This fraction was not further examined in this study. The pectin in supernatant was purified by precipitation-and-washing with ethanol, thereby yielding ~70% (w/w) final pectin product with a slightly increased DM (~98%) compared to the "parent" pectin. This product is referred to as an ultra-methylated pectin strands sample (UMPS). A partially de-methylesterified pectin (to a desired DM) from UMPS by the PME and then purified as Al-pectinate precipitate is referred to as PME-PDP. Table 1 shows some chemical and macromolecular characteristics of the initial pectic samples used.

2.2. Analysis of homogeneity and action patterns of enzyme preparations

2.2.1. Homogeneity

The two commercial enzyme preparations, viz. *A. aculeatus* EPG-M2 and orange peel P5400 were analysed for homogeneity in "native state" by GFC and in denatured state by SDS-PAGE. GFC was performed with a high resolution Superdex-200 HR 10/30 column (Amersham Biosciences Corp., NJ, USA). Elution of proteins was monitored spectrophotometrically at a scanning UV wavelength range of 215–280 nm. MWD of protein eluates was monitored with BioRad protein molecular weight standards as specified above and apparent $\overline{M_w}$ was estimated as described elsewhere (Andrews,

Table 1
Chemical and macromolecular characteristics of the initial pectic samples used for the preparation of model pectins.

	CCHMP	UMPS	PME-PDP	LP64	HGA60	HGA100	PGA	MRGI
GalA (%, w/w)	84.5 (3.1)	82.4 (3.7)	89.6 (2.1)	84.9 (3.1)	94.2 (2.3)	93.1 (1.2)	95.4 (1.5)	25.7 (1.5)
NS (%, w/w)	7.9 (2.5)	11.6 (1.8)	4.6 (1.8)	5.3 (0.5)	Tr	Tr	Tr	68.4 (3.2)
DM (mol%)	95.2 (1.5)	97.8 (1.2)	19.8 (2.6)	64.0 (3.0)	<5	<5	nd	nd
[η] (mL/g)	392.4 (3.6)	307.1 (2.8)	294.8 (3.7)	435.7 (3.9)	40.6 (0.5)	87.5 (1.2)	128.1 (3.2)	22.7 (1.4)
M_v (kDa)	90.5 (5.6)	81.4 (7.3)	77.2 (6.4)	120.7 (4.1)	10.4 (0.6)	18.1 (0.9)	25–50	ND
I_p (M_w/M_n)	ND	ND	ND	ND	1.04 (0.3)	1.09 (0.2)	ND	ND
DP	ND	ND	ND	ND	59 (4)	102 (5)	142–284	ND

Values are means ± SD (n = 3).
nd, not detected.
ND, not determined.
NS, total neutral sugar.
Tr, traces.

1964). Experiments were performed in three independent runs and mean ± SD values were used. Denaturating SDS-PAGE was carried out according to a Laemmlli gel system as described by Hempelmann (2008). Reducing SDS-PAGE was performed by incorporating a BME reducing agent in the sample (buffer) preparation. Stacking and resolving (or separating) gel solutions contained 4% and 12% (poly)acrylamide, respectively. Electrophoresis was performed in a BioRad Protean II slab cell apparatus. Staining was done with Coomassie brilliant blue R-250. Destaining solution was made of 45% methanol, 10% glacial acetic acid, and 45% double distilled water. The $\overline{M_w}$s of resolved proteins were estimated after Weber and Osborn (1969) using BioRad protein molecular weight markers as specified above.

2.2.2. Action patterns

The action pattern of EPG-M2 was investigated by a combination of reducing (end) groups and viscometric measurements using PGA (or HGA60) as substrate. The reaction was carried out by appropriately adding diluted amounts of EPG-M2 (in 50 mM acetate buffer/50 mM NaCl at pH 5.5) to 1.0% PGA (or HGA60) solution and incubating the mixture at 40 °C (optimum pH and temperature conditions specified by the manufacturer) for reaction time varying from 0 to 24 h. The reaction was then quenched by heating in boiling water for 5 min. The increase of reducing groups was quantified using monoGalA as standard. One unit was defined as the amount of enzyme that catalysed the release of 1 μmol of reducing groups from the substrate used per min under the conditions specified above. The accompanying changes in (specific) viscosity of substrate solutions were determined as previously described (Yapo & Koffi, 2006). The ratio of EPG-M2-generated monoGalA to higher-size OGA, from the substrate, was roughly estimated by extensively dialysing the enzymatic digest in tubing of 1,000 MWCO (Spectra Por) against double distilled water. As checked by production of reducing groups and decrease of sample solution (specific) viscosity, EPG-M2 was inactive on CCHMP and UMPS, but was partially active against LP64.

The PME action pattern was studied using CCHMP or UMPS as substrate. Since action of PME on the substrate resulted in acidity increase (protonation of carboxyl groups at *C*-6 of deesterified GalA residues) and methanol formation, the activity of the enzyme could be followed by quantifying the acidity or the methanol formed. However, only the increase in acidity was here measured by continuous automatic titration with CO_2-free 0.1 N NaOH at pH 7.5 and at 30 °C (optimum pH and temperature conditions specified by the producer) or by following changes in pH of the reaction medium of 0.5% water-solution of the substrate containing 50 mM NaCl at pH 7.5, according to an adaptation of the potentiometric method of Gonzalez and Rosso (2011). One unit was defined to be the amount of orange peel PME that catalysed production of 1 μmol of carboxyl groups per min at pH 7.5 and at 30 °C. The action pattern of the orange peel PME was determined by a differential precipitation method of PME-treated pectins using two (Cu^{2+} and Al^{3+}) or three (Cu^{2+}, Ca^{2+}, and Al^{3+}) multivalent metal cations with different binding affinities and capacities for pectinates. Therefore, solutions (0.5%) of UMPS were treated with three different quantities (U) of orange peel PME solution at pH 7.5 and at 30 °C to achieve extensive de-methylation within 5 min, 10 min, and 30 min, and then tested for differential precipitation with cupric sulfate, calcium chloride, or aluminum chloride containing excess of Cu^{2+}, Ca^{2+}, or Al^{3+} ions, respectively. Briefly, the enzymatic reaction was quenched by decreasing the pH to 3.9 (with dilute HCl) and the PME-treated mixtures were gradually added to solutions of cupric sulfate (containing 0.28 N Cu^{2+} ions), aluminum chloride (containing 0.33 N Al^{3+} ions) at pH 3.9, or calcium chloride (containing 0.28 N Ca^{2+} ions), and were allowed to stand for full formation of insoluble complexes, and centrifuged, and proceeded as above to yield fractions of cation-precipitated pectinates and cation-soluble pectins (in supernatants). Part of the Cu-pectinates was re-dissolved in water and was further tested for precipitation with aluminum chloride or calcium chloride. Experiments were carried out in three independent runs.

2.3. Preparation of oligogalacturonides with varying average size

Three categories of OGA, with regard to $\overline{DP}$, were prepared by controlled EPG-M2 digestion of PGA, followed by differential precipitations with acids and alcohols as described elsewhere (Cameron, Luzio, Baldwin, Narciso, & Plotto, 2005; Pressey & Avants, 1977) with further modification. Briefly, 2% PGA solution was digested with appropriately diluted EPG-M2 at pH 5.5, at 40 °C, and for 3 h. The reaction was stopped by lowering the pH of the medium to ~2 using dilute HCl, thereby simultaneously inactivating enzyme and rendering insoluble the fraction of OGA with higher $\overline{DP}$. The precipitate formed (fraction I) was collected by centrifugation (10,000 × *g*, 10 min, 20 °C), re-dissolved in water upon raising the pH to ~5 (with dilute NaOH), extensively dialysed against water, and freeze-dried. The supernatant was brought to pH 5.5 and was further treated at 40 °C for 2 h after fresh addition of appropriately diluted EPG-M2. After quenching the reaction by decreasing the pH to 2, 3 volumes of 95% ethanol were added and the mixture was stored at 5 °C for 48 h. The precipitate formed (fraction II) was collected and was treated as above. The supernatant, viz. the cold acid–alcohol–soluble material (fraction III), was brought to pH 5.0, concentrated by rotary evaporation at 40 °C, extensively dialysed, and freeze-dried. Fractions I, II, and III resolved well-enough in three distinct peaks (with some overlap) on GFC equipped with Sephadex G-50 F column as higher, intermediate, and lower sized OGA. Fractions I, II, and III are OGA with ca. $\overline{DP}34 \pm 2$, $\overline{DP}19 \pm 5$, and

$\overline{DP}7 \pm 3$, respectively, and were referred to as OGA34, OGA19, and OGA7, respectively. Experiments were carried out in three independent runs.

2.4. Model pectin-calcium gels

MP having specific $\overline{BS}$ types were first produced by binary mixtures of UMPS with different amounts of OGA3 (tri-GalA), OGA7, OGA19, OGA34, HGA60, or HGA100 so that the proportion of the specific $\overline{BS}$ varied from 0 (UMPS alone) to 100% (OGA or HGA alone) within the mixture. They are referred to as MP3, MP7, MP19, MP34, MP60, and MP100 series with respect to the specific $\overline{BS}$ type. Table 3 shows the chemical characteristics of the MP60 and MP100 series. Other MP were prepared by binary mixtures of UMPS with PME-PDP (MP-B) and UMPS with ADLP (MP-C) (Table 4). Ternary mixtures were also prepared (Table 4).

Calcium gel systems containing 1.0 wt% pectin material were prepared with the parent pectin (CCHMP) and with various MP at pH 5.5 as previously described (Yapo & Koffi, 2006), except that no sugar (sucrose) was added to the preparations. Calcium effect was excluded by using the stoichiometric ratio of binding ($R = 2\ [Ca^{2+}]/[-COO^-]$), which describes the relationship between the molar concentrations of Ca^{2+} ions and ionisable carboxyl groups of polygalacturonate on the basis of the pectin demethylesterification degree (100 − DM). The behaviour of gelling systems was monitored by dynamic SAOS tests, performed in a Bohlin CVO stress-controlled Rheometer (Bohlin Instruments Ltd., Cirencester, UK) using a cone-plate geometry (40 mm plate diameter, 4° cone angle, and 150 μm gap).

The viscoelastic parameters measured were the storage (or elastic) modulus (G'), which is directly related to the amount (or density) of elastically effective cross-links formed as well as to the strength (or firmness) of the resulting gel; the loss (or viscous) modulus (G''), which is indicative for relaxation of cross-links or the energy dissipated as heat; and the phase angle ($\tan\delta = G''/G'$), which is a parameter used to describe the nature of a gel (Luzio & Cameron, 2008; Oosterveld, Beldman, Searle-van Leeuwen, & Voragen, 2000; Yapo & Koffi, 2006). An amplitude sweep was carried out to ensure that the selected strain amplitude (1%) was within the linear viscoelastic region of Ca-pectin gels. A preliminary frequency sweep within the 0.1–100 Hz range allowed us to make the choice to perform all the measurements at 1 Hz and at 20 °C with insignificant frequency-dependence on gel batches cooled to room temperature for 3 h. G' was plotted against $\overline{BSF}$ of MP to possibly correlate the latter parameter with the pectin gelling ability. Experiments were done in three independent runs.

2.5. Molecular weight distribution of pectic samples

The MWD of produced OGA and pectin samples was analysed by GFC on a Sephadex G-50 F column (G5080; Sigma–Aldrich Co.) and a high resolution Superdex-200 HR 10/30 column (Amersham Biosciences Corp., NJ), respectively. MWD was determined using pullulan, monoGalA, diGalA, triGalA, PGA, and HGA standards with known or measured intrinsic viscosities under specified conditions as previously described (Yapo & Koffi, 2006). To better estimate unknown $\overline{M_w}$, the so-called universal calibration technique (UCT) was used by plotting $\log([\eta] \times \overline{M_w})$ versus the elution volume (V_e) of standards. Experiments were performed in three independent runs.

2.6. Analytical

The GalA content of pectic samples was quantified by the modified colorimetric sulfamate-meta-hydroxydiphenyl assay using monoGalA standard (Yapo, 2010). The DE of pectic samples was determined by potentiometry (Yapo, 2009). The acetyl content was assayed by the colorimetric hydroxamic acid reaction using glucose pentaacetate standard (McComb & McCready, 1957). The neutral sugar (NS) content was more accurately measured by an adaptation of the tri-reagent colorimetric assay of Kunerth and Youngs (1984) by replacing the carbazole assay by the metahydroxydiphenyl one. Reducing (end) groups were estimated by a manual (nanomole range-sensitive) colorimetric 2,2′-bicinchoninate assay using monoGalA standard (Waffenschmidt & Jaenicke, 1987). The protein content of samples was determined colorimetrically by a Folin-phenol reagent assay (Lowry, Rosebrough, Farr, & Randall, 1951) using bovin serum albumin standard. All the measurements were performed in triplicates.

3. Results and discussion

3.1. Homogeneity and patterns of action of enzyme preparations

Two commercial enzyme preparations, viz. EPG-M2 and P5400 were to be used for the production and/or characterisation of partially demethylated MP calcium gel preparations. The results of such experiments could be well-interpreted only if the enzyme preparations used were homogenously pure. Therefore, homogeneity, activity, and action pattern analyses were first carried out.

3.1.1. EPG-M2 preparation

Two methods, viz. reducing groups and viscometric measurements, were used to appraise the activity and action pattern of EPG-M2. Treatment of PGA (or HGA) by EPG-M2 slowly released reducing groups, which hardly exceeded 1% of the substrate GalA residues after 1.5 h of reaction and reached ∼14% after 24 h. To confirm that the enzyme acted preferentially in an endo-manner, the 24 h-EPG-M2-digested PGA was extensively dialysed in 1000 MWCO tubing and the ratio of monoGalA to longer OGA ($DPn \geq 2$) was estimated, assuming that the dialysate (retained in the 1000 MWCO tubing) was monoGalA-free and that no OGA as great as diGalA were removed. Previous work has indeed shown that extensive dialysis in 1000 MWCO tubing of EndoPG-degraded PGA caused only slight removal of OGA as small as DP2 (Mort, Moerschbacher, Pierce, & Maness, 1991). It appeared that the GalA content of the dialysate accounted for >82% of the total GalA, suggesting that less than 18% monoGalA residues were likely generated by the enzyme from substrate, which is in reasonable agreement with the determined amount of reducing sugar after 24 h. The difference might be due to removal of a part of EPG-M2-generated OGA with $DPn \geq 2$, especially diGalAs, concomitantly with monoGalAs by dialysis.

Activity determination by viscometric measurements showed that the substrate residual (specific) viscosity was less than 50% after only 15 min of reaction, <20% after 1.5 h and <3% after 3 h, from which it remained almost unchanged up to 24 h-hydrolysis, indicating that the enzyme preferentially hydrolysed its substrate in an endo-manner and by a multi-chain attack mode. The latter method is therefore better than the former, which is in fact an “exclusion method”, to investigate the endo-enzyme action pattern. MWD pattern of EPG-M2, as analysed by GFC, revealed the presence of a sole major peak with an estimated $\overline{M_w}$ of 43.0 ± 3.0 kDa and SDS-PAGE with and without BME showed a single band at $\overline{M_w}$ of ca. 39.0 kDa (see Table 2 for data summary). These results, taken altogether, indicate that EPG-M2 is a homogenously pure (monocomponent) Endo-PG preparation (in agreement with the manufacturer specification), which randomly cleaves inside the substrate chains to produce mainly OGA with a $DPn > 1$, in a multi-chain attack action pattern.

Table 2
Macromolecular characteristics and action patterns of the commercial enzyme preparations used.

	EPG-M2	P5400
Enzymes features		
Supplied form optimum conditions	Liquid pH 5.5 and 40 °C	Lyophilised powder pH 7.5 and 30 °C
Activity	2291 U/mg protein	≥153 U/mg powder
Protein	27.0 ± 5 mg% (w/v)	25–50 g% (w/w)
Enzyme activity characteristics		
Reducing sugar (%)	14.2 ± 1.6.	nd
MeOH production (%)	nd	80.0–92.0
Residual viscosity (%)	<3	>95.0
Molecular characteristics		
MWD-GFC	One major peak	One major peak
Average M_w (kDa)	43.0 ± 3.0	37.0 ± 4.0
SDS-PAGE	Single band	Single band
Average M_w (kDa)	39.0	35.0
SDS-PAGE + BME	Single band	Two bands
Average M_w (kDa)	39.0	28.0 11.0
Macrostructure	Monomeric	Heterodimeric
Composition	Monocomponent	Monocomponent
Purity level	Highly purified	Highly purified
Homogeneity level	Homogenous	Homogenous
Action patterns	Cleaves inside substrate by a multi-chain attack mode	De-methylesterifies pectins by a preferentially processive and single chain attack mode

Values are means ± SD (n = 3)
nd, not detected

3.1.2. P5400 preparation

3.1.2.1. Enzymatic activity. The PME preparation was able to deesterify CCHMP (95% DM), UMPS (98% DM), and LP64 (64% DM), indicating that this orange peel PME, like most plant PME, did not require long enough DMB, as binding site, to be active on highly and almost fully methyl-esterified pectin strands. This is consistent with previous work as summarised elsewhere (Taylor, 1982). At the end of reaction, the yields of methanol produced did not attain 100% (~80–92% (Table 2), depending on initial substrate), which was confirmed by the quantification of 5–20% residual DM (depending on the initial substrate) for the partially deesterified final pectin products. This indicated that the orange peel PME was unable to totally deesterify its substrate, in accord with previous reports on plant (orange and tomato) PME (Evans & McHale, 1968; Hills, Ogg, & Speiser, 1945; Yoo et al., 2009).

To date, it is not clearly known what may cause incomplete deesterification of pectins by most PME. However, it appeared that the residual DM (20%) of partially deesterified product from UMPS was higher than the residual DM (16%) of product from CCHMP, which in turn was higher than the residual DM (5%) of product from LP64, suggesting that the lower the homogeneity of charge distribution and density over initial pectin chains, the higher the deesterification extent of pectin caused by the orange peel PME used. Moreover, when varying amounts of OGA and HGA (DP1-60) were added to reaction mixtures, the enzyme activity was found to be abolished when the added OGA concentration exceeded a threshold value, which decreased with the OGA size (data not shown). Lin, Feng, Chung, and Lan (1990) also observed, by testing miscellaneous monosaccharides, that GalA at a concentration ≥0.1 M, totally inhibited citrus and tomato PME which were used to deesterify 0.02% solutions of pectin having ~60% DM, though these workers, in contrast, reported that complete deesterification was achieved with each PME under normal conditions.

Our results suggest that 100% deesterification is difficult to attain with the orange peel PME, probably because of increased amounts of relatively short or long DMB, which might progressively hinder its activity, even when the accompanying increasing acidity is consistently neutralised to pH 7.0–7.5 by (automatic) addition of a base (NaOH) solution. PME inhibition may gradually occur whenever the catalytic site (but not the binding site) of the enzyme is confronted by large amounts of relatively long DMB at the reducing end of (or inside) pectin chains. The residual specific viscosity of the substrate was >95%, suggesting that only little depolymerisation of pectin occurred during deesterification, which corroborates the early report (MacDonnell, Jansen, & Lineweaver, 1945) that orange peel contains no pectin-depolymerising enzymes, especially (endo- and/or exo-)polygalacturonases.

3.1.2.2. Action pattern. In this study, the action pattern of the orange peel PME used was investigated using an unusual differential precipitation method with two or three multivalent metal cations, viz. cupric, aluminum and/or calcium ions. Cupric ion has long since been shown to have a strong selectivity and binding capacity to pectinates (Wunsch, 1952) and is therefore a "powerful tool", as effective as IEC, to purify (crude) pectin extracts (Hwang, Roshdy, Kontominas, & Kokini, 1992; Yapo, 2010). Treatment of CCHMP (95% DM) with Cu^{2+} ions yielded ~28% (w/w) of a precipitated pectinate product (with an average DM of 89.5 ± 3.2%, based on three measurements), whereas no pectinate precipitates were formed with Ca^{2+} or Al^{3+} ions, thus confirming the strong affinity of Cu^{2+} ions for pectinate chains, even up to such a high average esterification level. In the presence of Cu^{2+} ions, ~70% (w/w) of CCHMP remained soluble and appeared to be made of extremely methylesterified pectin strands (UMPS, 98% DM) that did not allow any strong "sheet-like" binding by Cu^{2+} ions to cause formation of insoluble complexes (precipitate). Deesterification of UMPS with an excess of enzyme (as calculated on the basis of the number of mole of methoxy groups releasable from the pectin), followed by precipitation tests with Cu^{2+} ions showed that ~90% (w/w) of the PME-treated sample could be recovered as Cu-pectinate precipitate. This suggests that ~10% (w/w) of UMPS were either not at all attacked (or not sufficiently deesterified) by the enzyme to give a $\overline{BS}$ which promotes tight sheet-like binding leading to pectinate precipitation. A possible non-attack by the enzyme of Cu-soluble pectin fraction was corroborated by the finding for this product of an average DM that was almost identical to that of untreated UMPS. Furthermore, only ~57% of the PME-treated UMPS formed Al-pectinate in the presence of excess of Al^{3+} ions, thus leaving more than 40% soluble. Also, only the two-third of pectinate purified by precipitation with Cu^{2+} ions (i.e., Cu-pectinate) was found capable of forming Al-pectinate precipitate. This suggests that precipitation of pectin by Al^{3+} ions required the presence of larger DMB on pectin chains, compared to Cu^{2+} ions. The observation of differential precipitation of pectinates supports the work of Heri, Neukom, and Deuel (1961) in which IEC-fractionation, on diethylaminoethyl-cellulose columns, of an orange peel PME-produced LMP (56% DE), from an extremely esterified pectin (97% DE), gave several subfractions varying from faintly deesterified (92% DE) to extensively deesterified (24% DE) pectin materials. No precipitate was obtained by mixing the Cu-precipitated pectinate with an excess of Ca^{2+} ions, though a turbid mixture was observed. Hence, the pectinate precipitating capability of the three multivalent cations was as follows; $Cu^{2+} \gg Al^{3+} \gg Ca^{2+}$. This is in accord with the previous reports that below 50% DM, pectins are almost quantitatively recovered by precipitation with Al^{3+} ions, but only partially with Ca^{2+} ions, whereas above 50% DM, Al-pectinate and Ca-pectinate precipitates decreased gradually and drastically, respectively, when DM increased, approaching zero % at very high DM (Joslyn & De Luca, 1957; Yapo, 2010). GFC analysis of the orange peel PME showed one major elution peak with an estimated $\overline{M_w}$ of 37.0 ± 4.0 kDa and SDS-PAGE without BME also revealed a single band at a $\overline{M_w}$ of ca. 35.0 kDa (see Table 2 for results summary), in agreement with previous work (Kim, Teng, & Wicker, 2005). With BME, in contrast,

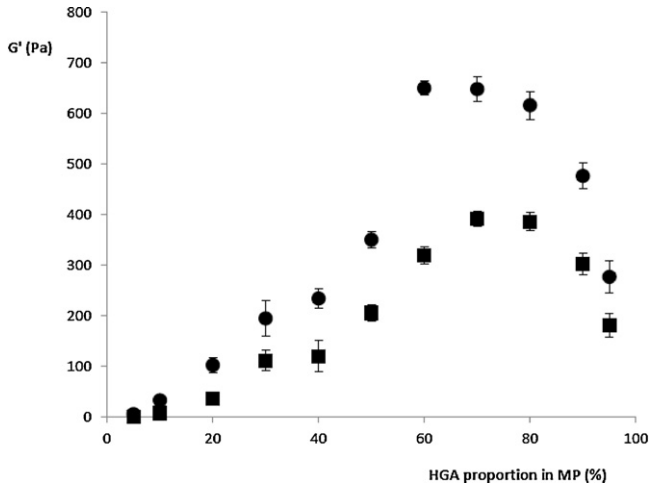

Fig. 1. Evolution of the storage modulus (G') of MP gels as a function of the proportion and size of HGA; (●) MP100 and (■) MP60.

SDS-PAGE showed two bands having lower $\overline{M_w}$ values (28.0 and 11.0 kDa), suggesting that the enzyme might consist of two oligomeric subunits. All these results led to the conclusions that P5400 is a homogenously pure PME preparation (as has been specified by the supplier), with a possibly heterodimer structure, that catalyses the release of methoxy groups from partially as well as from extremely esterified pectin strands in a preferentially processive and single chain-like attack mode to generate relatively long DMB on a part (and not on all) of the pectin chains at full reaction stage. To our knowledge, this is the first time that the action pattern of a plant PME has been determined, using ultra-methylesterified pectin as the starting substrate, by a relatively simple and rapid method of differential precipitation with two or three multivalent cations possessing different precipitating capacities instead of by the tedious and time-consuming (though more delineating) IEC-fractionation of the PME-treated pectin sample or by HPAEC–PAD following digestion of the PME-treated pectin by highly-purified EndoPG preparations. It is worth underlining that the initial DM of pectin should be no less than ~95% for this differential precipitation method to give accurate and reproducible results.

3.2. Effect of $\overline{BS}$ and $\overline{BSF}$ of pectin on its gelling behaviour

3.2.1. Model pectins

Gelation of LMP in the presence of Ca^{2+} ions is currently believed to occur by intermolecular bridges via Ca^{2+}-cross-linked DMB from different pectin chains with a required minimum $\overline{BS}$, which might vary from 6 to 20 as mentioned above. The parent pectin CCHMP (95% DM) as well as UMPS (98% DM) obtained from it did not form calcium gels, suggesting that both contain no pectin strands with a required minimum $\overline{BS}$ or very few of them, which did not enable a dense Ca^{2+}-cross-linked network to be formed. This is in line with the obtained zero% recovery of pectinate-precipitate with Ca^{2+} ions as pointed out earlier. The model pectins MP3 and MP7 (Section 2.4) were unable to form calcium gels ($G' < G''$) over the whole proportion range (0–100%). MP19 and MP34, above 50% proportion of OGA, formed a relatively weak gel-like structure ($G' > G''$; $G' < 50$ Pa) which strength remained almost unchanged as the proportion of OGA was increased up to 90%, after which undesirable phenomena (macroscopic separation and/or precipitation) appeared (data not shown).

The gelling behaviours of the MP60 and MP100 series are illustrated in Fig. 1. MP60 showed a gelling behaviour that was completely different from that of any of the above-mentioned MP with a lower $\overline{BS}$ type. Until 30% proportion of HGA60, MP60 was also incapable of forming "true" calcium gels ($G' > G''$; $G' < 100$ Pa). By contrast, above this proportion, stronger gelation occurred ($G' > G''$; $G' \geq 100$ Pa) and G' increased with increasing proportion of HGA60 within MP60, which might be explained by immobilisation, in stronger Ca^{2+}-cross-linked DMB networks, of the "nonfunctional" pectin strands, that is to say, the pectin strands which were not involved in formation of calcium-mediated junction zones. Since UMPS alone was non-gelling and remained in the form of a dispersed material, and pure dispersions of HGA60 were turbidity- or precipitation-prone, in the presence of the amount of Ca^{2+} ions needed for gelation, it can be hypothesised that the observed enhancements in the elastic modulus, from a certain proportion of HGA60 in the gelling mixture, resulted from the formation of a biphasic network in which UMPS was present predominantly in a dispersed phase that was surrounded by a continuous calcium pectinate gel network formed by the HGA component at a local concentration higher than its overall, nominal, concentration within the mixture. Therefore, the occurrence of gelation from a certain proportion of HGA60 in MP60 indicates that calcium-induced gelation of pectin is probably governed mainly by the pectin $\overline{BS}$ and $\overline{BSF}$ rather than by its DM, inasmuch as at 30% proportion of HGA60, the pectin element of the MP60 series appeared to be a HMP with an estimated 65.0% DM (see Table 3). This observation supports the reports that "weak gel" of tenuous network structure has been formed, in the presence of 10 mM calcium chloride, with (1 wt% solution of) a 68% esterified commercial citrus pectin at the start of incubation with fungal PME (O'Brien, Philp, & Morris, 2009), and that plant PME and fungal PME-deesterified commercial pectins with ~60–70% residual DM were able to form calcium gels with different strengths and elastic characters (Ngouémazong et al., 2012; Vincent & Williams, 2009).

G' increased with increasing proportion of HGA60 in MP60 up to 70% (G'_{60max}), with a steeper slope over 50% proportion, after which it remained nearly constant up to around 80% HGA60, and then sharply decreased as the proportion of HGA60, within the binary mixture, tended towards 100%. As regards the gelling behaviour of MP100, the onset of "true" gelation ($G' > G''$; $G' \geq 100$ Pa) and maximum gel strength ($G'_{100max} > G'_{60max}$) were observed much earlier (20% and 60% HGA100 proportion, respectively), with also a steeper slope above 50% HGA100 proportion, whereas the following sharper decrease of G' appeared later (around 90% HGA100 proportion), compared with the gelling trend of MP60. These differences in the gelling behaviour and capability among MPs clearly stemmed principally from differences in $\overline{BS}$ and $\overline{BSF}$, considering that these were the two influential parameters which varied from one sample to another. It has previously been hypothesized, on the basis of the Flory's theory, that if a large proportion of (pectin) molecules have only one DMB, then the ability to form an extended network among the molecules would be limited and little or no gel formation would occur (Luzio & Cameron, 2008). However, the present results show that formation of a stable and relatively strong gel is possible provided that the single DMB is amply functional, that is to say, great enough in size and in quantity to allow a highly dense Ca^{2+}-cross-linked DMB network, which is capable of immobilising all the "non-functional" strands, to be formed. Furthermore, the gelling behaviour of the tricombination (UMPS + HGA100 + HGA60) showed an intermediate trend between that of MP60 and MP100, considering that G' was closer to that of MP60 when the ratio of HGA60 to HGA100 was >1 and closer to that of MP100 in the reverse case (data not shown).

In contrast, the gelling behaviour of the tricombinations (UMPS + HGA100 + OGA7) and (UMPS + HGA100 + OGA3) showed a severe decrease of G' as the increasing ratio of OGA to HGA100 was >20–30% (see Table 4 for examples), indicating gradual inhibition of formation Ca^{2+}-cross-linked DMB networks by these OGA which might be more reactive than HGA100 with Ca^{2+} ions. Luzio and

Table 3
Chemical characteristics of model pectins of the MP60 and MP100 series.

Proportions (%, w/w)	HGA60	UMPS	MP60 series		HGA100	UMPS	MP100 series	
			DM (mol%)	GalA (%, w/w)			DM (mol%)	GalA (%, w/w)
P_0	0.00	100.00	97.84 (1.24)	82.43 (3.72)	0.00	100.00	97.84 (1.24)	82.43 (3.72)
P_1	5.01 (0.02)	94.99 (0.08)	93.06 (1.13)	83.02 (1.25)	4.97 (0.05)	95.03 (0.07)	93.57 (1.45)	82.92 (1.19)
P_2	10.03 (0.05)	89.97 (0.05)	87.10 (0.85)	83.48 (1.04)	9.98 (0.01)	90.02 (0.11)	89.24 (2.16)	83.56 (1.09)
P_3	19.98 (0.09)	80.02 (0.14)	78.54 (2.01)	84.71 (1.76)	20.03 (0.02)	79.97 (0.04)	79.21 (1.97)	84.47 (2.41)
P_4	29.97 (0.01)	70.03 (0.09)	65.21 (3.08)	86.05 (2.01)	30.08 (0.08)	69.92 (0.01)	68.07 (2.07)	85.61 (1.57)
P_5	40.07 (0.03)	59.93 (0.05)	59.47 (1.97)	87.23 (1.39)	39.97 (0.06)	60.03 (0.09)	56.87 (1.26)	86.67 (1.09)
P_6	49.92 (0.07)	50.08 (0.07)	48.79 (1.07)	88.51 (1.12)	50.06 (0.04)	49.94 (0.16)	49.25 (2.03)	87.58 (1.79)
P_7	60.04 (0.03)	39.96 (0.04)	40.23 (2.05)	89.35 (2.47)	59.93 (0.07)	40.07 (0.08)	39.7 (0.91)	88.41 (2.32)
P_8	70.03 (0.08)	29.97 (0.13)	29.86 (1.75)	90.56 (1.08)	70.08 (0.02)	29.92 (0.07)	31.24 (1.23)	89.75 (2.21)
P_9	79.92 (0.11)	20.08 (0.05)	19.89 (1.64)	91.60 (3.02)	80.04 (0.17)	19.96 (0.21)	22.87 (2.41)	90.84 (1.17)
P_{10}	90.02 (0.07)	9.98 (0.02)	9.96 (1.83)	92.89 (1.24)	89.93 (0.05)	10.07 (0.05)	11.32 (1.28)	91.78 (1.25)
P_{11}	94.96 (0.01)	5.04 (0.08)	6.52 (3.02)	93.54 (1.09)	95.02 (0.08)	4.98 (0.10)	7.08 (2.45)	92.53 (1.63)
P_{12}	100.00	0.00	<5	94.21 (2.34)	100.00	0.00	<5	93.14 (1.21)

Data are means ± SD (n = 3).
P_i, series of model pectin preparations containing different proportions of HGA and UMPS.

Cameron (2008), from the observation that addition of OGA with $\overline{DP}$7–14 to PME-deesterified pectin to 70% DM-pH 4.5 substantially decreased the yield point of the Ca-pectin gels prepared, compared to control sample with no added OGA and to samples with added OGA of higher average DP ($\overline{DP}$28), suggested that short OGAs were interacting with DMB and competitively inhibiting cross-linking.

3.2.2. *Gelling behaviour of MP in the presence of Endo-PG: evidence for correlation with the "blocky parameter" DB_{abs}*

To establish a possible relation between the gelling behaviour of MP and the two common "blocky parameters" DB and DB_{abs} in addition to $\overline{BS}$ (Luzio & Cameron, 2008) and $\overline{BSF}$ (in the present study), the gelling conditions of the MP100 series yielding the highest gel strength (that is to say, the MP100 element, which comprises 40% UMPS and 60% HGA100 (see Table 3)) was used to prepare gels in the presence of added EPG-M2 so that the HGA-depolymerising enzyme gradually hydrolysed its substrate within the gelling system. As expected, G' decreased with decreasing proportion of residual HGA100 (and hence with increasing amounts of EndoPG-generated products, viz. OGA of shorter sizes) in the gelling system. Stable gelation no longer occurred ($G' \ll 100$ Pa) when the HGA100 residual proportion fell below ~30% (Fig. 2), indicating extensive impairment of formation of (dense) Ca^{2+}-cross-linked DMB networks. However, the lower limit of formation of a relatively strong and stable gel with MP100 was found to be around 20% under normal (EndoPG-free) conditions (see Fig. 1), suggesting that the inability of the partially degraded MP100 (to ~30% residual HGA100) to yield a stable gel might be caused by the great amount of EndoPG-generated OGA with shorter sizes within the gelling system. This was substantiated by purifying the partially degraded MP100 samples by precipitation-and-washing with ethanol and preparing gelling systems with the purified samples containing almost no more short EndoPG-generated OGA. It was indeed observed that the decrease of G' with decreasing residual HGA100 proportion was slower (Fig. 2) and steady gelation ceased to occur near 20% residual HGA100 ($G' < 20\%\ G'_{100max}$). This confirmed that OGA of relatively short sizes, which were within the gelling system, effectively hampered formation of dense Ca^{2+}-cross-linked DMB networks. It has been suggested that maximum gel strength, at a certain DM, is obtained when the length of DMB equals the minimum number of residues required to form a stable egg-box junction zone, since such distribution maximises the number of junction zones that can possibly be formed at that DM, resulting in maximum cross-link density (Fraeye et al., 2010). Our results show that maximum cross-link density (equaling maximum gel strength) is obtained when the DMB equals in size and in quantity the required minimum $\overline{BS}$ and $\overline{BSF}$, and hence these would actually be the two key parameters that determine Ca^{2+}-promoted gelation and the gel strength of PDP with a certain DM. The GalA recovery of alcohol-precipitated partially degraded MP100 was ~55% at full reaction which lasted ~5 h (longer treatments did not

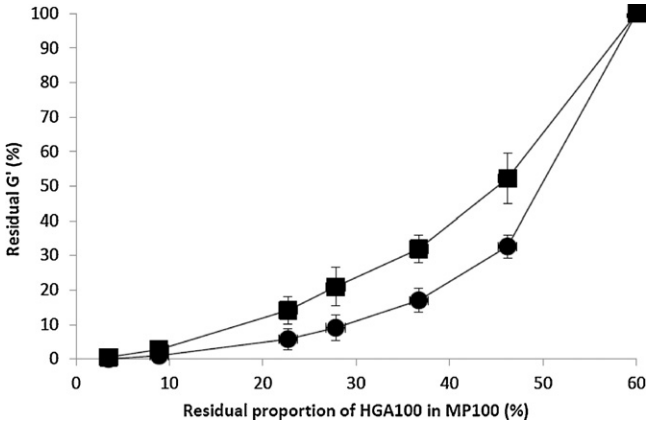

Fig. 2. Changes in the storage modulus (G') of gels prepared with EndoPG-degraded MP100 before (●) and after (■) purification by alcohol-precipitation-and-washing.

Table 4
Comparison of the strength (G') and the elastic character ($\tan\delta$) of gels prepared from diverse model pectins.

	MP-A	MP-B	MP-C	MP-D	MP-E	MP-F
Pectin mixtures	40% UMPS 60% HGA100	25% UMPS 75% PME-PDP	40% UMPS 60% ADLP	40% UMPS 45% HGA100 15% OGA7	40% UMPS 50% HGA100 10% OGA3	40% UMPS 54% HGA100 6% MRG-I
GalA (%, w/w)	88.4 ± 2.3	86.2 ± 1.7	84.9 ± 2.1	89.2 ± 1.6	91.3 ± 1.2	84.5 ± 2.6
DM (mol%)	39.7 ± 0.9	43.1 ± 2.5	41.2 ± 1.5	38.7 ± 1.4	39.2 ± 1.1	40.6 ± 1.8
G' (Pa)	649.0 ± 21.0	1297.0 ± 10.0	1036.0 ± 14.0	318.0 ± 12.0	232.0 ± 15.0	415.0 ± 25.0
Tan δ (G''/G')	0.09 ± 0.02	0.03 ± 0.01	0.07 ± 0.01	0.21 ± 0.08	0.48 ± 0.03	0.32 ± 0.05

Values are means ± SD (n = 3).

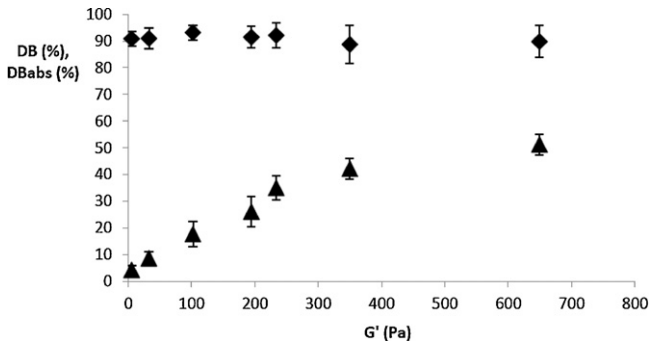

Fig. 3. Changes in the storage modulus (G') of MP100 gels as a function of approximate DB (♦) and DB_{abs} (▲).

result in any further degradation), indicating that ~45% of GalA was removed as ethanol-soluble materials, probably essentially OGA with DP1–3, which are known to be the main end-products of HGA digestion by EndoPG. Moreover, the GalA content of the purified partially degraded MP100 was found to be 84.0% and its DM increased up to 96.0, in line with the chemical characteristics of UMPS. Also, by GFC analysis, the purified material appeared to be composed of one major peak, nearly identical to the single peak of (untreated) UMPS, and another two peaks of higher elution volumes, corresponding to ethanol-insoluble OGA with much higher DP (data not shown). This indicated that the portion of the MP100 element that was extensively degraded, by EndoPG, as ethanol-soluble products stemmed from its sub-fraction HGA100 of which it accounted for ~90%. This result was expected, since the EndoPG used was highly active on HGA, but was totally inactive against CCHMP and UMPS as mentioned above. The method was therefore found to be useful to approximate the DB and DB_{abs} of the MP100 series under typical gelling conditions so as to correlate each of the two "blocky parameters" with the strength of the gel formed. Thus, approximate DB of the pectin elements of the MP100 series (with increasing proportions of HGA100) was calculated as the ratio of the amount of ethanol-soluble EndoPG-generated OGA to the amount of (GalA of) HGA100 and approximate DB_{abs} was calculated as the ratio of the amount of ethanol-soluble EndoPG-generated OGA to the total GalA amount of the pectin element. As so calculated, DB varied within a narrow range (Fig. 3), indicating that the gel strength was quasi-independent of the DB of MP. By contrast, G' increased with increasing DB_{abs} up to a certain threshold value (DB_{abs} ~ 60%), above which G' decreased as DB_{abs} further increased (not shown in Fig. 3). This suggests, under normal gelling conditions, that there exists a strong correlation between the DB_{abs} of MP and the strength of the resulting gel. The latter blocky parameter is therefore the best of the three commonly used parameters (DM, DB, and DB_{abs}) to relate the pectin de-methylesterification extent (or pattern) to its ability to form calcium gel, in good accord with previous work as summarised elsewhere (Fraeye et al., 2010).

3.2.3. Comparison of the gelling abilities of various model pectins: role of pectin branched domains

The gelling abilities of different MP, referred to as MP-A, MP-B, MP-C, and MP-F, were compared to one another with the aim of drawing information regarding the possible role played by the number of DMB and the pectin "hairy" (or branched RGI) regions in the formation of calcium-pectin gels (Table 4). As described in Section 2.1, to produce PME-PDP, UMPS was first de-methylesterified with three different quantities (U) of PME (and hence for three different times) to the same residual DM of ~25% and then submitted to precipitation tests with Al^{3+} ions. It was observed that the higher the quantity of enzyme used to achieve deesterification (for a shorter reaction time), the higher the amount of Al-precipitated pectinate, suggesting the presence of higher proportion of longer PME-DMB in the resulting partially deesterified pectin product. This also indicated that the enzyme acted in a more processive way when pectin was demethylated by a higher amount of it for a shorter reaction time than by a lower quantity of it for longer reaction duration. The Al-purified pectinate (PME-PDP; 20% DM) with larger DMB (obtained from the PDP with a higher quantity of enzyme for a shorter reaction time) was added to UMPS to obtain a model pectin (MP-B) with ~40% DM. All the MP had similar DM in order to exclude its effect on the gelling ability. As can be seen in Table 4, MP-B exhibited the highest gel strength and also the best gel nature (elastic character, as indicated by the lowest tan δ value), followed by MP-C, MP-A, and MP-F. The fact that MP-B displayed the highest gelling ability and the best elastic character might be explained by the size-homogeneity of this MP sample, considering that UMPS and PME-PDP were practically of the same $\overline{M_w}$. It might also come from the presence of more than one DMB on some of the PME-PDP strands, and from variability in $\overline{BS}$ and $\overline{BSF}$, which probably promoted formation of various highly dense Ca^{2+}-cross-linked DMB networks. The gel strength of MP-C was higher than the gel strength of MP-F and this might here again be accounted for by difference in $\overline{M_w}$, considering that MP-F was made of appropriate proportions of the two acid-degradation products of ADLP, namely ~90% (w/w) HGA100 and ~10% (w/w) of MRGI. By contrast, the fact that MP-F showed a lower gelling ability than MP-A, which contained only one (HGA100) of the two acid-degradation products of ADLP, suggested that the added MRGI was likely to impair gelation. This is all the more probable as the gel prepared with MP-F was 3–4 times less elastic than the gel obtained with MP-A, as appraised by tan δ values. To date, the role of the neutral sugar-branched RG-I regions of pectin on (calcium-mediated) gel formation has not clearly been elucidated. In HMP-SAG, they are thought to function as junction-zone-terminating structural elements, via rhamnose inserts into the galacturonan chain that give rise to rhamnose kinks and to "hairy" regions, thereby avoiding micelle formation with ensuing undesirable phenomena, viz. turbidity, syneresis, and eventually precipitation (Voragen et al., 1995). In LMP calcium gel preparations, they may also serve as junction-zone-terminating structural elements. On the other hand, high proportion of neutral sugar-branched RGI regions within pectin is believed to damage its gelling properties due to steric hindrance. It has previously been reported that (limited enzymatic) de-branching of the neutral sugar-branches of pectin RGI domains did not significantly affect the stiffness (G' value) of calcium gels, compared with untreated pectins (Oosterveld et al., 2000; Schmelter, Wientjes, Vreeker, & Klaffke, 2002). By contrast, a pectin fraction representing the "smooth" HG regions of (an acid-extracted) sugar beet pectin, which was purified by treatment of the parent pectin with rhamnogalacturonase, followed by size-exclusion chromatography fractionation, was found to be capable of forming gel, in the presence of calcium and deesterifying enzymes (fungal PME in association or not with fungal PAE from *A. niger*) at a slower rate in addition to yielding a gel with lower stiffness, compared with the parental pectin (Oosterveld et al., 2000). According to these workers, the difference in gelling ability resulted from difference in apparent $\overline{M_w}$ between the two pectin materials. Their findings are corroborated by the data obtained in the present study. Hence, on the one hand, high proportion of neutral sugar-branched RGI domains of pectin may substantially reduce its ability to form a cohesive and strong calcium gel because of steric hindrance. On the other hand, extensive or complete degradation of the core of the branched RGI regions of pectin is likely to give a final pectin product with a relatively low gelling capability (because of lower apparent $\overline{M_w}$) in addition to augmenting the risk of shortening the limit of occurrence of undesirable phenomena as specified

above, owing to shortage of junction-zone-terminating structural elements.

4. Conclusions

Gelation of low methoxy pectins in the presence of calcium is believed to result from formation of a dense network of calcium-cross-linked demethylated galacturonic acid blocks, with an elusively known required minimum average length on the pectin chains. In an extension of this postulate, the utilisation, as starting pectin materials, of model pectins with specific average demethylated block sizes, in varying proportions, has clearly proven to be amply interesting by affording new insights into the calcium-induced gelation of partially de-methylesterified pectin with a certain degree of esterification. The study indeed highlighted, for the first time, that a *quantitatively sufficient and functional demethylated block* is a prerequisite to be fulfilled by a pectin sample for formation of a highly dense calcium-cross-linked network giving an elastically stable, strong, and cohesive gel.

Of the two currently used "blocky parameters" for describing structure-function relationship, the absolute degree of blockiness is the one which appears to effectively explain the relationship between the pectin pattern of de-methylesterification and the resulting gel strength. This will further be shown in specific forthcoming work whereby naturally (hydrogen fluoride-hydrolysis-produced) or chemically (tetrabutylammonium fluoride-iodomethane)-produced highly methylated homogalacturonans with homogenous size, charge distribution, and density were utilised as the starting pectin materials. Furthermore, the pectin rhamnogalacturonan-I domains appear to be involved in formation of a stable, strong, and cohesive gel, most likely as an "enhancer" of the pectin molecular weight, which boosts the gelling properties in regard to the gelation rate and firmness of the resulting gel and as junction-zone-terminating structural elements, which extend the limit of appearance of undesirable phenomena, such as turbidity, syneresis, and precipitation.

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References

Andrews, P. (1964). Estimation of the molecular weights of proteins by Sephadex gel-filtration. *Biochemical Journal*, *91*, 222–233.

Cameron, R. G., Luzio, G., Baldwin, E., Narciso, J., & Plotto, A. (2005). Production of narrow-range size-classes of polygalacturonic acid oligomers. *Proceedings of the Florida State Horticultural Society*, *118*, 406–409.

Evans, R., & McHale, D. (1968). Multiple forms of pectinesterase in limes and oranges. *Phytochemistry*, *17*, 1073–1075.

Fraeye, I., Duvetter, T., Doungla, E., van Loey, A., & Hendrickx, M. (2010). Fine-tuning the properties of pectin-calcium gels by control of pectin fine structure, gel composition and environmental conditions. *Trends in Food Science & Technology*, *21*, 219–228.

Gonzalez, S. L., & Rosso, N. D. (2011). Determination of pectin methylesterase activity in commercial pectinases and study of the inactivation kinetics through two potentiometric procedures. *Ciência e Tecnologia Alimentos*, *31*, 412–417.

Grant, G. T., Morris, E. R., Rees, D. A., Smith, P. J. C., & Thom, D. (1973). Biological interactions between polysaccharides and divalent cations: The egg-box model. *FEBS Letters*, *32*, 195–198.

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.

Hempelmann, E. (2008). SDS-protein PAGE and protein detection by silver staining and immunoblotting of *Plasmodium falciparum* proteins. In K. Moll, I. Ljungström, H. Perlmann, A. Scherf, & M. Wahlgren (Eds.), *Methods in malaria research* (5th ed., pp. 263–266). Virginia: MR4-American Type Culture Collection.

Heri, W., Neukom, H., & Deuel, H. (1961). Chromatographie von pektinen mit verschiedener verteilung der methylester-gruppen auf den fadenmolekeln. 16. Mitteilung über Ionenaustauscher. *Helvetica Chimica Acta*, *44*, 1945–1949.

Hills, C. H., Clyde, L., Ogg, C. L., & Speiser, R. (1945). Determination of methyl ester content of pectin. *Industrial & Engineering Chemistry, Analytical Edition*, *17*, 507–510.

Hwang, J., Roshdy, T. H., Kontominas, M., & Kokini, J. L. (1992). Comparison of dialysis and metal precipitation effects on apple pectins. *Journal of Food Science*, *57*, 1180–1184.

Joslyn, M. A., & De Luca, G. (1957). The formation and properties of aluminum pectinates. *Journal of Colloid Science*, *12*, 108–130.

Kim, Y., Teng, Q., & Wicker, L. (2005). Action pattern of Valencia orange PME de-esterification of high methoxyl pectin and characterization of modified pectins. *Carbohydrate Research*, *340*, 2620–2629.

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

Kohn, R. (1987). Binding of divalent cations to oligomeric fragments of pectin. *Carbohydrate Research*, *160*, 343–353.

Kohn, R., & Luknar, O. (1977). Intermolecular calcium-ion binding on polyuronates—Polygalacturonate and polyguluronate. *Collection of Czechoslovak Chemical Communications*, *42*, 731–744.

Kunerth, W. H., & Youngs, V. L. (1984). Modification of the anthrone, carbazole, and orcinol reactions for quantitation of monosaccharides. *Cereal Chemistry*, *61*, 344–349.

Lin, T.-P., Feng, T.-Y., Chung, Y.-H., & Lan, C.-L. (1990). Quantification of methyl ester content of pectin by pectinesterase. *Botanical Bulletin of Academia Sinica*, *31*, 273–278.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. *The Journal of Biological Chemistry*, *193*, 265–275.

Luzio, G. A., & Cameron, R. G. (2008). Demethylation of a model homogalacturonan with the salt-dependent pectin methylesterase from citrus: Part II. Structure-Function analysis. *Carbohydrate Polymers*, *71*, 300–309.

MacDonnell, L. R., Jansen, E. F., & Lineweaver, H. (1945). The properties of orange pectinesterase. *Archives of Biochemistry*, *6*, 389–401.

McComb, E. A., & McCready, R. M. (1957). Determination of acetyl in pectin and in acetylated carbohydrate polymers. *Analytical Chemistry*, *29*, 819–821.

Morris, E. R., Powell, D. A., Gidley, M. J., & Rees, D. A. (1982). Conformations and interactions of pectins. I-Polymorphism between gel and solid states of calcium polygalacturonate. *Journal of Molecular Biology*, *155*, 507–516.

Mort, A. J., Moerschbacher, B. M., Pierce, M. L., & Maness, N. O. (1991). Problems encountered during the extraction, purification, and chromatography of pectin fragments and some solutions to them. *Carbohydrate Research*, *215*, 219–227.

Ngouémazong, D. E., Tengweh, F. F., Fraeye, I., Duvetter, T., Cardinaels, R., Loey, A. V., et al. (2012). Effect of de-methylesterification on network development and nature of Ca^{2+}-pectin gels: Towards understanding structure—Function relations of pectin. *Food Hydrocolloids*, *26*, 89–98.

Oakenfull, D., & Scott, A. (1984). Hydrophobic interaction in the gelation of high methoxyl pectins. *Journal of Food Science*, *49*, 1093–1098.

O'Brien, A. B., Philp, K., & Morris, E. R. (2009). Gelation of high-methoxy pectin by enzymic de-esterification in the presence of calcium ions: A preliminary evaluation. *Carbohydrate Research*, *344*, 1818–1823.

Oosterveld, A., Beldman, G., Searle-van Leeuwen, M. J. F., & Voragen, A. G. J. (2000). Effect of enzymatic deacetylation on gelation of sugar beet pectin in the presence of calcium. *Carbohydrate Polymers*, *43*, 249–256.

Pressey, R., & Avants, J. K. (1977). Occurrence and properties of polygalacturonase in *Avena* and other plants. *Plant Physiology*, *60*, 548–553.

Schmelter, T., Wientjes, R., Vreeker, R., & Klaffke, W. (2002). Enzymatic modifications of pectins and the impact on their rheological properties. *Carbohydrate Polymers*, *47*, 99–108.

Taylor, A. J. (1982). Intramolecular distribution of carboxyl groups in low methoxyl pectins—A review. *Carbohydrate Polymers*, *2*, 9–17.

Vincent, R. R., & Williams, M. A. K. (2009). Microrheological investigations give insights into the microstructure and functionality of pectin gels. *Carbohydrate Research*, *344*, 1863–1871.

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 applications* (pp. 287–339). New York: Marcel Dekker.

Waffenschmidt, S., & Jaenicke, L. (1987). Assay of reducing sugars in the nanomole range with 2,2′-bicinchoninate. *Analytical Biochemistry*, *165*, 337–340.

Weber, K., & Osborn, M. (1969). The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. *The Journal of Biological Chemistry*, *244*, 4406–4412.

Wunsch, G. (1952). Über schwermetallpektinate. *Die Makromolekulare Chemie*, *8*, 124–133.

Yapo, B. M., & Koffi, K. L. (2006). Yellow passion fruit rind—A potential source of low-methoxyl pectin. *Journal of Agriculture and Food Chemistry*, *54*(7), 2738–2744.

Yapo, B. M. (2009). Pineapple and banana pectins comprise fewer homogalacturonan building blocks with a smaller degree of polymerisation as compared

with yellow passion fruit and lemon pectins: Implication for gelling properties. *Biomacromolecules*, *10*(4), 717–721.

Yapo, B. M. (2010). Improvement of the compositional quality of monocot pectin extracts contaminated with glucuronic acid-containing components using a step-wise purification procedure. *Food and Bioproducts Processing*, *88*(2/3), 283–290.

Yapo, B. M. (2011). Pectic substances: From simple pectic polysaccharides to complex pectins—A new hypothetical model. *Carbohydrate Polymers*, *86*(2), 373–385.

Yoo, S.-H., Lee, B.-H., Savary, B. J., Lee, S., Lee, H. G., & Hotchkiss, A. T. (2009). Characteristics of enzymatically-deesterified pectin gels produced in the presence of monovalent ionic salts. *Food Hydrocolloids*, *23*, 1926–1929.