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ScienceDirect
Carbohydrate Polymers 71 (2008) 300–309
Carbohydrate Polymers
www.elsevier.com/locate/carbpol

Demethylation of a model homogalacturonan with the salt-independent pectin methylesterase from citrus: Part II. Structure–function analysis

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Received 15 February 2007; received in revised form 25 May 2007; accepted 30 May 2007
Available online 10 June 2007

Abstract

Non-calcium sensitive pectin (NCSP), with a 94% degree of methylation (DM) was demethylated at pH 4.5 and 7.5 using monocomponent citrus salt-independent pectin methylesterase (PME). In a companion publication [Cameron, R. G., Luzio, G. A., Goodner, K., & Williams, M. A. K. (2008). Demethylation of a model homogalacturonan with the salt-independent pectin methylesterase from citrus: I. Effect of pH on demethylated block size and distribution. *Carbohydrate Polymers*], average demethylated blocks size ($\overline{BS}$), average number of demethylated blocks per molecule ($\overline{BN}$) and enzyme mode of action were characterized for a series of pectins using a *limited* digest with endo-polygalacturonase (EPG) to release demethylated blocks (DMBs) from each demethylated pectin. Rheology and calcium sensitivity measurements, on the same series of pectins, are presented herein to relate $\overline{BS}$ and $\overline{BN}$ values to functional properties. For the pH 4.5 – 50% DM sample with an excised $\overline{BS} = 8.6$ and $\overline{BN} = 3.9$, greater than 95% of the molecules appeared to be crosslinked with calcium ion and a storage modulus (G') value of 7.31 Pa was observed. For the pH 4.5 – 70% DM. The data indicated that for the pH 7.5 series, block number could be a limiting factor. The excised block size for the pH 7.5–60% DM sample was comparatively large at 12.2 compared to the pH 4.5 series. In contrast, block number of at least 2.0 appeared to be required before a significant level of calcium sensitivity was observed in the pH 7.5 series. Mixing the pH 4.5 – 70% DM sample with narrow-range size-classes of oligogalacturonic acids (OGalA) showed that small galacturonic acid oligomers with a degree of polymerization (DP) less than 13 could lower yield point (YP) and affect gelation, suggesting that even small excised DMBs need to be considered when relating pectin block size to functional properties. Published by Elsevier Ltd.

Keywords: Endo-polygalacturonase; Polysaccharide; Homogalacturonan; Rheology; Yield point; Storage modulus; G'; Loss modulus: G''; Mapping; Block size; Block number

1. Introduction

Citrus pectin, a complex polysaccharide, is composed of at least five different sugar moieties but 80–90% of its dry weight is galacturonic acid (GalA). The majority of the GalA is found in homogalacturonan (HG) regions of pectin, unbranched polymers of GalA in which a variable proportion of the GalA residues contain a methyl ester at their C6 position (Ridley, O'Neill, & Mohnen, 2001; Vincken et al., 2003). Pectin's functional properties and reactivity toward calcium and other cations is largely dependent on the amount of methylated GalAs and their distribution pattern within the HG stretches (Powell, Morris, Gidley, & Rees, 1982; Willats et al., 2001). Two general patterns of methyl ester distribution are recognized, random or ordered (Willats et al., 2001). Demethylation of pectin can be accomplished by enzymatic (PME) or chemical (alkaline demethylation) means. For enzymatic demethylation three different modes of action have been hypothesized (Denes, Baron, Renard, Pean, & Drilleau, 2000), two of which lead to blockwise (ordered) removal of esters.

Previous studies have demonstrated that plant PMEs can demethylate pectin in an apparent ordered process,

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0144-8617/$ - see front matter Published by Elsevier Ltd.
doi:10.1016/j.carbpol.2007.05.038

described as processive hydrolysis of the methyl esters. The details of this catalytic process are not well understood. Catalysis by plant PMEs creates DMBs (Daas, Meyer-Hansen, Schols, De Ruiter, & Voragen, 1999; Denes et al., 2000; Duvetter et al., 2006; Hotchkiss et al., 2002; Kim, Teng, & Wicker, 2005; Limberg et al., 2000a; Limberg et al., 2000b; Savary, Hotchkiss, & Cameron, 2002). The presence or absence of these blocks and how these blocks are produced is important since it affects the reactivity of pectin with cations such as calcium. The presence of blocks and the nature of calcium ion crosslinks of blocks is believed to follow an egg box type interaction, or junction zone, in which two HG chains containing a sequence (or DMB) of adjacent non-esterified GalA units are coordinated to a middle layer of calcium ions (Grant, Morris, Rees, Smith, & Thom, 1973; Kohn, 1975; Limberg et al., 2000a; Rees, 1972; Rees, 1981). A minimum number of 9 consecutive non-esterified units was determined for an interaction to occur (Liners, Thibault, & Van Cutsem, 1992). Other determinations have been made where the minimum number was estimated to be 8 to 12 GalA units (Powell et al., 1982). In separate work threshold values of 15–20 residues were estimated for cooperative binding of Ca^{2+} by OGalA in an egg box-like system (Kohn & Furda, 1967; Kohn & Luknar, 1977) and in modeling for junction zones interactions (Braccini & Perez, 2001).

Extensive analyses for the presence of DMBs in pectin have been published. Lengths of the DMBs have been estimated with indirect statistical methods on GalA oligomers with varying DMs (Catoire, Pierron, Morvan, du Penhoat, & Goldberg, 1998; Denes et al., 2000). DMB length also has been estimated by enzymatic methods using exo-polygalacturonase (exo-PG) and EPG (Limberg et al., 2000a). Lengths of partially esterified blocks were estimated by (Limberg et al., 2000b) using pectin lyase (cleaves the HG in highly methylated regions), although since the enzyme cleaves within a fully methylated stretch, these fragments would contain a portion of a methylated fragment. Proton NMR resonances for H-4 and H-5 protons of $GalA_1$ have different chemical shifts depending on whether the GalA unit and its next neighbors are methylated (Andersen, Larsen, & Grasdalen, 1995; Grasdalen, Einar Bakoy, & Larsen, 1988), have been used to determine the ratio of an unmethylated GalA triad sequence relative to mixed or fully methylated triads. Enzymatic fingerprinting using endopectin lyase and EPG II (Limberg et al., 2000b) and characterization using pectin-specific antibodies (Willats et al., 2000) revealed discernible differences between the methyl-esterification patterns on the model pectins produced by the action of plant PME (producing blockwise demethylation) and fungal PME or base catalysis (producing random demethylation). EPG has been used for analyzing methylation patterns with special focus on the presence of DMBs in the analyzed pectins (Daas et al., 1999). Another approach (Limberg et al., 2000a) was used with a combination of EPG and exo-PG to quantify the amount of GalA units located in DMBs by measuring the amount of liberated GalA after combined digestion.

PME action patterns and the presence of DMBs have been shown to have significant effects on the rheological properties of pectin (Luzio, 2003; Powell et al., 1982; Schmelter, Wientjes, Vreeker, & Klaffke, 2002; Willats et al., 2001). Yield stress measurements for pectins with a blockwise distribution of unmethylated GalAs, indicate that interchain associations in the presence of calcium ion are stronger than those involving pectins with random distributions for DM values greater than 45% (Powell et al., 1982). Rheological properties of pectins have been related to measurements of degree of blockiness (DB), calculated by measuring $GalA_1$, $GalA_2$ and $GalA_3$ released from pectins treated with EPG (Lofgren, Guillotin, Evenbratt, Schols, & Hermansson, 2005). A high DB value can be related to the blockwise distribution of unmethylated GalA residues in pectin (Daas, Voragen, & Schols, 2000; Daas et al., 1999).

Since DM and DMB size are related to pectin functionality, detailed knowledge of pectin fine-structure, block sizes and their numbers could aid in understanding functional properties obtained from rheological measurements. Chromatography has allowed for separation and detection of GalA oligomers (Cameron, Luzio, Kauffman, & Grohmann, 2004; Hotchkiss, Lecrinier, & Hicks, 2001). Now it is also possible to quantify individual GalA oligomer fractions by HPLC using an evaporative light scattering detector (Cameron & Grohmann, 2005; Cameron, Luzio, Goodner, & Williams, 2008). As reported in a companion publication (Cameron et al., 2008), a demethylated pectin series was produced by a salt-independent citrus PME from high DM pectin that originally contained no DMBs and was not calcium sensitive. For this demethylated pectin series, average demethylated blocks size ($\overline{BS}$), average number of demethylated blocks per molecule ($\overline{BN}$) and enzyme mode of action were characterized using a *limited* digest with endo-polygalacturonase (EPG) to release demethylated blocks (DMBs) from each demethylated pectin.

The work reported herein focuses on determining the functional properties of the demethylated pectin series prepared and structurally analyzed in the companion study. The functional properties measured were: the relative amount of calcium sensitive pectin as measured by the calcium sensitive pectin ratio (CSPR), yield point (YP), storage modulus (G') and loss modulus (G'') values in the presence of calcium ion. The purpose is to relate structural determinations with functional properties. Resulting rheological and calcium sensitivity properties are discussed with regards to results from block size and number characterization of the demethylated pectin series.

2. Materials and methods

2.1. Materials

Endo-polygalacturonase (EPG-M2, Lot 00801) was purchased from Megazyme International Ireland Limited (Bray, Ireland). Esterified pectin (94% DM, 97% anhydro-GalA, P9561, Lot 114K0940), polygalacturonic acid (PGalA) (P3889, lot 044K0700) and all other chemicals were purchased from Sigma–Aldrich (St. Louis, MO, USA). Salt-independent PME was isolated as previously described (Cameron, Baker, & Grohmann, 1998; Cameron et al., 2008).

2.2. Pectin demethylation, demethylation analysis and block determination

Methods for pectin demethylation and demethylation analysis were described in our companion manuscript (Cameron et al., 2008), but details as to the computation of substrate to enzyme molar ratio was not provided as required for discussion herein. Estimation of substrate to enzyme mole ratio in these reactions was computed from specific activity value (salt-independent PME) of 350 U mg protein^{-1}, the amount of enzyme in each reaction mixture of 350 U and the molecular weight of the enzyme of 34,000 μg μmol^{-1} which yields 2.9×10^{-2} μmols of enzyme used per reaction (Cameron et al., 2008). Each reaction contained 20 g of pectin with a wt. ave. molecular weight of 43,280 g mol^{-1} or 462 μmols of pectin. The ratio, μmols of pectin to μmols of enzyme, yields a value of 1.6×10^4.

Analysis and determination of block size $\overline{BS}$ together with block number $\overline{BN}$ was performed by HPAEC – ELSD (high performance anion exchange chromatography – evaporative light scattering detector) as described in our companion manuscript (Cameron et al., 2008) for the pectin samples tested herein.

2.3. CSPR assay

This assay was modified from that published previously (Cameron, Savary, Hotchkiss, & Fishman, 2005; Gerrish, 2000; Hotchkiss et al., 2002). Pectin samples (0.1 g) were weighed into duplicate tubes to the nearest milligram and dissolved in 100 mL of deionized (DI) H_2O. The solutions were allowed to mix overnight at RT after heating to 70 °C for 15 min. A 10 mL aliquot of the pectin solution was pipetted into a centrifuge tube where 10 mL of cold (4 °C) 30 mM $CaCl_2$ in 8% isopropyl alcohol was added. The tubes were vortexed for 15 s and were placed into a refrigerator at 4 °C for 60 min. The tubes were centrifuged at 10,000*g* for 30 min at 4 °C. The supernatant was removed and discarded by siphoning off the top layer without disrupting the pellet. The pellet was resuspended in 20 mL of DI water by vortexing with 1 mm glass beads for 1 min. A control was performed by pipetting a 10 mL aliquot of the original 0.1% pectin solution into a centrifuge tube containing 10 mL of DI water. A 300 μL sample from the resuspension was removed and assayed colorimetrically for GalA (Luzio, 2004). Larger aliquots were used when a relatively small pellet was observed. Assays were performed in duplicate. The CSPR was determined by dividing the amount of pectin that precipitates in 30 mM calcium and 8% IPA (B) by the total amount of pectin in the control (A) and multiplying by 100. Therefore, CSPR values close to 100% indicates a CSP, whereas a non-calcium sensitive pectin (NCSP) has a low percentage value. To confirm the assay, a CSPR standard curve was generated from 10 different mixtures made up to known percentages of CSP and NCSP. The results produced a quantitative recovery of CSP with linear response having an R value = 0.991 (data not shown).

2.4. Rheological measurements

2.4.1. Oscillatory experiments

The viscoelasticity after the gel formation was characterized by oscillatory measurements using a stress controlled AR 2000 Rheometer (TA Analytical, Wilmington, DE) equipped with 60 mm acrylic parallel plate geometry. The rheological measurements were conducted in three replicates. Pectin solutions at 0.5% concentration were prepared by dissolving 0.1 g of pectin in 20 mL of DI water by stirring for 24 h. The pH was adjusted to 7.0 and 0.02 g of CaH_2PO_4 was added and mixed overnight, with no gel formation observed. The pH was then adjusted to 5.5 with dilute acid which results in a uniform release of calcium ion and is similar to a previously described method of calcium-pectin gel formation (Powell et al., 1982). After 2 h of standing, a 4 mL sample was placed under the parallel plates. After gap adjustment, 1000 μm, a manufacturer designed cover was used over the geometry to prevent evaporation. The measurements were performed at 20.0 °C at a frequency of range of 0.1 Hz to 600 rad s^{-1} in log mode to measure G' and G''. The storage and loss moduli, G' and G'', were monitored in the linear region (0.5% strain) at a constant frequency of 1 Hz. All rheological measurements were performed at 20 °C.

2.4.2. Rotational experiments

Yield point (the point on the stress–strain curve at which the increase in strain is no longer proportional to the increase in stress) was measured in the rotational mode using a flow down procedure. Instrument, geometry and sample preparation was the same as that used for oscillatory experiments. Following equilibration at a temperature of 20.0 °C the sample was pre-sheared at a shear rate of 10 s^{-1} and then the shear was ramped down from 10 to 0.001 s^{-1} in log mode. Yield point was computed from average stress values determined in the region of low shear where shear stress was relatively constant. Typically this occurred at a shear rate between 0.03 and 0.005 s^{-1}. All rheological measurements were conducted in duplicate.

Correlation of YP data with CSPR data was performed using SigmaPlot 9.0 from Systat Software (Richmond, CA).

2.5. Production of narrow-range size-classes

A 2% (w/v) solution of PGalA acid in acid form was prepared in 50 mM lithium acetate (LiOAc), pH 4.7 to yield the lithium salt solution of PGalA (LiPGalA) and digested with 0.05 U mL^{-1} EPG for 4.5 h at room temperature with constant stirring. The EPG digested LiPGalA was heated in a microwave until boiling to denature the enzyme. After cooling it was brought to pH 2.0 with concentrated HCl and then stored overnight at 4 °C. The precipitated material was pelleted by centrifugation at 23,500*g* for 30 min at 4 °C. The pelleted precipitate, representing the high degree of polymerization (DP) size-class (Cameron, Luzio, Baldwin, Narciso, & Plotto, 2005; Hotchkiss & Hicks, 1990; Hunt, Cameron, & Williams, 2006), was solubilized in 50 mM LiOAc and re-precipitated by adjusting to pH 2 with concentrated HCl and storing overnight at 4 °C followed by centrifugation as described above. The pelleted high DP (DP 13-42) material was solubilized in 50 mM LiOAc and stored at 4 °C.

The supernatant from the initial centrifugation of the pH 2 precipitation contained the low- and medium-DP fragments (Cameron, Luzio, et al., 2005; Hotchkiss & Hicks, 1990). It was brought to 50 mM sodium acetate (NaOAc) and 22.5% EtOH and then placed at 4 °C overnight to precipitate the medium-DP fragments (DP 7–21). The supernatant (low-DP fragments, DP 2–13) was pooled and stored at 4 °C. Following solubilization of the pelleted material in 50 mM LiOAc and re-precipitation with 50 mM NaOAc and 22.5% EtOH this material, representing the medium-DP fragments, was centrifuged again and the pellets were solubilized in 50 mM LiOAc and stored at 4 °C. The oligomers present in each size-class were characterized by high performance anion exchange chromatography coupled to an evaporative light scattering detector (Cameron & Grohmann, 2005).

2.6. Competition experiments

Three pectin solutions at 0.5% concentration were prepared by dissolving 0.1 g of a 70% DM sample of pectin which had been prepared from a 94% DM pectin by demethylation at pH 4.5 using the salt-independent PME isolated from citrus tissue in 20 mL of DI water by stirring for 24 h. To separate solutions 0.15 g of either the low-DP fragments, medium-DP fragments or high-DP fragments were added. The pH was adjusted to 7.0 and 0.02 g of CaH_2PO_4 was added. Each solution was mixed for 16 h and the pH was adjusted to 5.5 with dilute acid. After 24 h of standing, a 5 mL sample was placed under 60 mm parallel plates. Yield point was measured in the rotational mode using a flow down procedure as noted above.

3. Results and discussion

3.1. Development of calcium sensitivity

The introduction of calcium sensitivity into pectin, the ability of pectin molecules to bind to each other through a calcium bridge forming an extended network, can be quantified using the CSPR assay (Gerrish, 2000; Hotchkiss et al., 2002). As reported, randomly demethylated pectins having low DM values of less than 50% have been shown to be reactive to calcium, whereas calcium reactivity at DM values greater than 50% required the presence of enzymatically produced DMBs of GalA based on yield stress measurements (Powell et al., 1982). All samples tested herein had DM values greater than 50% (Cameron et al., 2008), thus the CSPR assay (in this experimental system) can be used as an indicator for the enzymatic formation of blocks when starting with high DM pectin (DM = 94, wt. ave. MW = 43 kDa) initially free of blocks. Previous data (Cameron et al., 2003; Hotchkiss et al., 2002) provided evidence that orange PMEs demethylate pectin in a block-wise manner. Increases in CSPR have been shown to be concomitant with changes in NMR chemical shifts as shown for both the salt-dependent and salt-independent PMEs (Hotchkiss et al., 2002). These chemical shifts agree with reported values for H5 $GalA_3$ blocks (4.75 ppm) (Kim et al., 2005; Neiss, Cheng, Daas, & Schols, 1998). In the CSPR assay calcium sensitive pectins (CSP) can be separated from non-calcium sensitive pectins (NCSP) by centrifugation in a 8% IPA solution containing 30 mM calcium ion (Hotchkiss et al., 2002). Quantification of precipitated pectin, as reported herein, was achieved by resuspending the pellet and performing a GalA colorimetric assay on the resulting solution (see Section 2).

Calcium sensitivity (CS) was not rapidly introduced into the parent pectin (94% DM, wt. ave. MW = 43.3 kDa). As shown in Table 1, at pH 4.5 the introduction of CS required a reduction in DM from 94% to 67.6%, where 11.4% of the pectin was observed to be CS as shown in the pH 4.5 – 70% DM pectin series. The CSPR value

Table 1
Calcium sensitive pectin ratio (CSPR) and measured DM for pH 4.5 and 7.5 demethylations with salt-independent orange PME, error values (in parenthesis) for DM and CSPR determined by propagation of error

Sample	DM %	CSPR %
pH 4.5		
90	89.5 (4.6)	0.6 (17.6)
80	77.4 (1.5)	2.0 (8.2)
70	67.6 (1.9)	11.4 (4.5)
60	61.0 (2.4)	38.6 (8.8)
50	55.0 (2.5)	95.3 (5.0)
pH 7.5		
90	90.2 (2.6)	0.4 (11.7)
80	78.0 (3.4)	0.3 (23.5)
70	70.1 (2.1)	0.8 (7.6)
60	61.1 (1.5)	45.6 (10.5)

increased to 38.6% for the pH 4.5 – 60% series and to 95.3% for the pH 4.5 – 50% series. With the pH 7.5 – 70% series the CSPR value was 0.8% and increased to 45.6% for the pH 7.5 – 60% series. These data can be compared to previous shifts in CS with a salt-independent PME and parent pectin with a higher molecular weight pectin (wt. ave. MW = 120 kDa) with lower initial DE of 76% that showed an increase in CSPR from 4% to 82% with a small change in DE of 6% (Hotchkiss et al., 2002). The differences between previously published and current results are likely due to the different substrates used in the separate experiments, since reaction conditions and enzyme used were the same (Cameron et al., 2008; Hotchkiss et al., 2002). The one sample that showed a CSPR greater than 90% in the present work was the pH 4.5 – 50% series with a DM that was reduced to a value of 55%. This data indicates the effect the substrate and/or extent of demethylation can have on the introduction of CS by a block demethylating enzyme and the potential value of being able to assay for block size and block number in understanding such differences.

3.2. Rheological observations

The same series of samples used in the CSPR assay was examined for yield point, and storage and loss moduli, G' and G'' which are important properties measured for gels such as those made by crosslinking pectin with calcium ions. The storage modulus G', corresponding to the stress in phase with the strain, is a measure of the energy stored in the material; for a gel, it is directly related to the crosslink density of the network and is the elastic component of a gel. The loss modulus G'', corresponding to the 90° out-of-phase stress amplitude, is a measure of the energy dissipated as heat (Axelos, Lefebvre, Qiu, & Rao, 1991) and is the apparent viscous component in a gel. These values are typically obtained by doing an oscillatory frequency scan at low levels of stress (applied force per unit area of the gel sample) or strain (ratio of the displacement caused by the stress relative to the thickness of the gel sample) to minimize disruption of the gel. By scanning the frequency, the mechanical response of the material can be explored and the gel characterized. Strong gels typically have G' values which an order of magnitude or greater than G'' whereas with weak gels the difference between modulus values is much smaller. The yield point or YP, is the stress at which a material begins to plastically deform. Prior to the YP the material will deform elastically and can return to its original shape when the applied stress is removed. Once the yield point is passed some fraction of the deformation will be permanent and non-reversible. YP is also the lowest stress at which strain increases without increase in stress. YP is another important physical property of many pectin-calcium gels which are made under dilute conditions.

With the pH 4.5 series no significant gel formation was observed (based on storage modulus values) in the 90% and 80% DM samples (Table 2) which is consistent with CSPR values being 2% or less. The first appearance of weak gel formation, based on storage modulus, was observed in the 70% DM sample where values for G', G'' and YP of 1.06, 0.502 and 0.171 Pa, respectively, were observed. These data correspond with an increase in CS as observed with the CSPR assay where this sample had a value of 11.4% (Table 1). The maximum values were observed in the pH 4.5 – 50% DM series with G', G'' and YP of 7.31, 1.99 and 0.353 Pa, respectively, where the CSPR also reached the highest observed value of 95.3% (Table 1). Linear regression analysis of CSPR data against YP exhibited an R value of 0.93 (slope $= 3.6 \times 10^{-3}$, $\sigma = 7 \times 10^{-4}$; intercept $= 0.047$, $\sigma = 0.03$). Increasing CSPR values also correlated with an increase in G' (data not shown) values, which indicates that with an increase in the number of molecules containing calcium sensitive blocks (based on CSPR) there is a corresponding increase in gel strength based on the storage modulus of G'.

In the pH 7.5 – 70% series demethylation series very weak gel formation was observed (Table 2) with G', G'' and YP values of 0.017, 0.033 and 0.0.042 Pa and a CSPR value of 0.8% (Table 1). In the pH 7.5 – 60% series the G', G'' and YP were 1.65, 0.465 and 0.260 Pa, respectively,

Table 2
Yield point, storage modulus (G''), loss modulus (G'), $\overline{BS}$ and $\overline{B}_n$ for pectins with calcium added that were demethylated at pH 4.5 and 7.5

Sample	YP (Pa)	G' (Pa)	G'' (Pa)	$\overline{BS}$	$\overline{B}_n$
pH 4.5					
90	<0.01 (n.d.)	<0.01 (n.d.)	<0.01 (n.d.)	n.d.	n.d.
80	<0.01 (n.d.)	<0.01 (n.d.)	<0.01 (n.d.)	4.1	3.0
70	0.171 (0.01)	1.06 (0.07)	0.502 (0.11)	5.3	3.6
60	0.2 (0.039)	0.94 (0.03)	1.65 (0.208)	7.9	3.1
50	0.353 (0.078)	7.31 (4.5)	1.99 (0.349)	8.6	3.9
pH 7.5					
90	<0.01 (n.d.)	<0.01 (n.d.)	<0.01 (n.d.)	n.d.	n.d.
80	<0.01 (n.d.)	<0.01 (n.d.)	<0.01 (n.d.)	5.3	1.7
70	0.042 (0.002)	0.017 (0.01)	0.033 (0.008)	10.2	2.0
60	0.26 (0.019)	1.65 (0.56)	0.465 (0.283)	12.2	2.0

Averages for YP shear stress were obtained from values collected in shear rate range 0.01–0.10 s^{-1}. $\overline{BS}$ and $\overline{B}_n$ obtained from (Cameron et al., 2008), σ values in parenthesis. n.d., not determined.

indicating a stronger gel formation (than the 70% DM) which corresponded with the increase in CSPR to a value of 45.6%. Values for the 60% DM are somewhat similar to previously published results (Powell et al., 1982) where a 92% DM pectin with a MW of 43,000 was demethylated by an orange PME mixture (not monocomponent) at pH 7.0 where yield stress (approximately 0.3 Pa at 1.5% pectin concentration) was observed at a DE of 60%. A randomly demethylated pectin, at this same DM value, showed no yield stress behavior (Powell et al., 1982). As with the pH 4.5 samples, the data for CSPR and YP correspond with the appearance of gel formation in the pH 7.5 series. The data for both pH series is consistent with the concept that with the formation of an increased concentration of molecules containing functional blocks, as shown by CSPR, leads to stronger gel formation as indicated by increases in YP, G' and G''.

3.3. Block size involved in crosslinking

It was important to estimate the smallest block size that could crosslink, so only blocks of sufficient size for crosslinking were considered for comparison to functional data. In a series of competition experiments, OGalA fragments prepared by controlled EPG hydrolysis of PGalA were purified by selective precipitation, producing three narrow-range size-classes of GalA oligomers see Fig. 1 and (Cameron, Luzio, et al., 2005). The low DP size-class was dominated by fragments of DP 2–13 with a median DP of 7. The medium DP size-class was dominated by fragments of DP 7–21 with a median DP of 14 and the high DP size-class had a DP of 13–42 units with a median DP of 28. In separate experiments, each size-class at 0.15% (based on GalA concentration), was mixed with pH 4.5 – 70% sample at a concentration of 0.5%, then reacted with calcium ion and YP was measured. As shown in Table 3, the YP was measured in each of the three size-classes, including a control sample with no added fragments. With the addition of the low DP size-class the measured YP decreased from the control value of 0.163–0.0959 Pa, suggesting that these OGalAs were interacting with enzyme demethylated blocks and inhibiting crosslinking. This is represented in Fig. 2 with GalA oligomer molecule F binding to molecule E and acting as a chain termination point. Chain termination should weaken gel formation and decrease the YP. The addition of the medium DP size-class produced a larger decrease in YP, to a value of 0.0478 Pa (control = 0.163 Pa) suggesting stronger competitive binding to DMBs than the small DP size-class. This stronger interaction is consistent with data for the sudden anomalous decrease in γ^{2+}_{Ca} when the OGalAs had DP values between 11 and 16, indicating intermolecular binding of Ca^{2+} in aggregates of the OGalAs (Kohn & Furda, 1967; Kohn & Luknar, 1977), and also with computations involving interaction energies (Braccini & Perez, 2001). For the high DP size-class a large increase in YP, to a value of 0.584 Pa, was observed. This YP value was 3.6 times greater (from Table 3 data) than the control and 4 replicates were performed to ensure against possible artifact. Representative experimental runs are shown in Fig. 3, shear stress (Pa) versus shear rate (s^{-1}), in the low shear rate region, for samples that containing different fragments size ranges together with a control sample. The increase in YP with the high DP size-class suggested that these OGalAs are participating in, and facilitating, crosslinking of the pectin molecules and are being incorporated into the gel structure. It is feasible that high DP lengths are of sufficient size to create a bridge between two adjacent molecules and that a single oligogalacturonide is bridging two separate pectin molecules and facilitating network formation. If it is assumed that a block size of between 6 and 10 on one of two adjacent molecules is sufficient for Ca^{2+} mediated linking, and represents the smallest functional block, then a mixture of

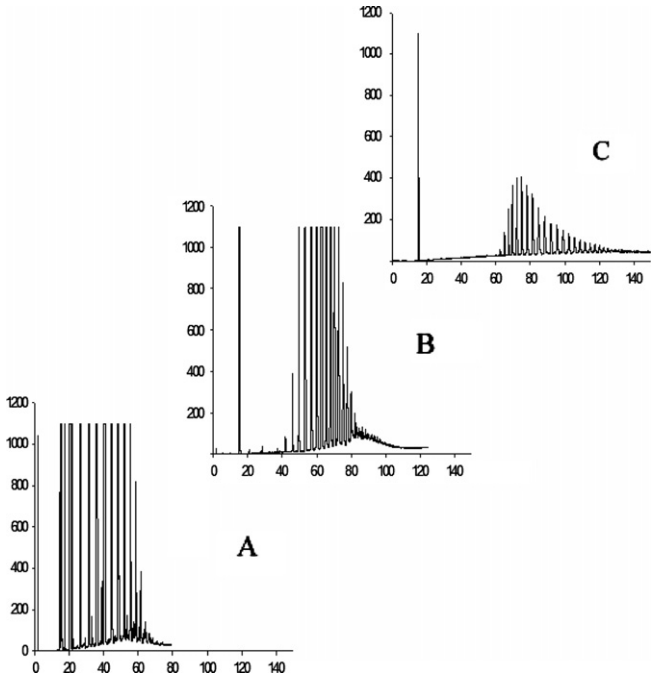

Fig. 1. Narrow-range size-classes obtained as noted in Section 2.5 and analyzed by HPAEC using an evaporative light scattering detector. Representative chromatograms are (A) low DP size-class dominated by DP 2–13 OGalAs; (B) medium DP size-class dominated by DP 7–21 OGalAs; (C) high DP size-class dominated by DP 13–42 OGalAs.

Table 3
Yield point value (Pa) dependency of sample pectin (pH 4.5 – 70% DM) when reacted with calcium and either small, medium or large DP of GA oligomers are added as compared to control with no added oligomers

Fragments added	Control	Small	Medium	Large
Size range	–	2–13	7–21	13–42
Median DP		7	14	28
Average (Pa)	0.163 (2)	0.0959 (2)	0.0478 (3)	0.584 (4)
σ	0.009	0.010	0.006	0.029

Shear stress values measured in shear rate range 0.005–0.03 s^{-1} were averaged, number of repeats shown in parenthesis.

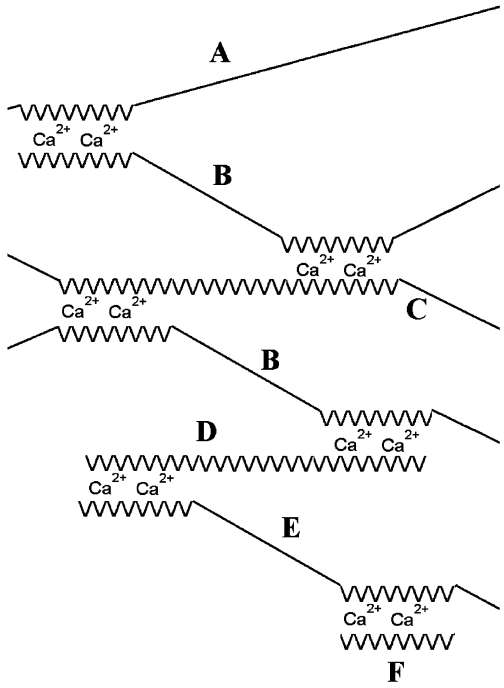

Fig. 2. Representative diagram of single calcium crosslinks (saw tooth lines) in partially esterified GalA polymers or unesterified GalA oligomers molecules that contain functional blocks. Molecule A with a single block sufficient for a calcium crosslink and acting as chain termination point. Molecule B with two crosslink blocks acting as chain extension. Molecule C with a single block of sufficient size to crosslink to separate molecules each containing blocks. Molecule D which is a long GalA oligomer that is linking to two separate molecules each containing a functional block. Molecule E with two blocks but interacting with an GalA oligomer, molecule F, which is acting as a chain termination point. Straight lines indicate esterified regions, which may also contain unesterified GalAs, but do not contain functional blocks. Block and nonblock regions are not drawn in proportion and number of calcium ions shown for each linkage is not intended to be an accurate representation. Trifunctional molecules are not depicted but are important for gelation.

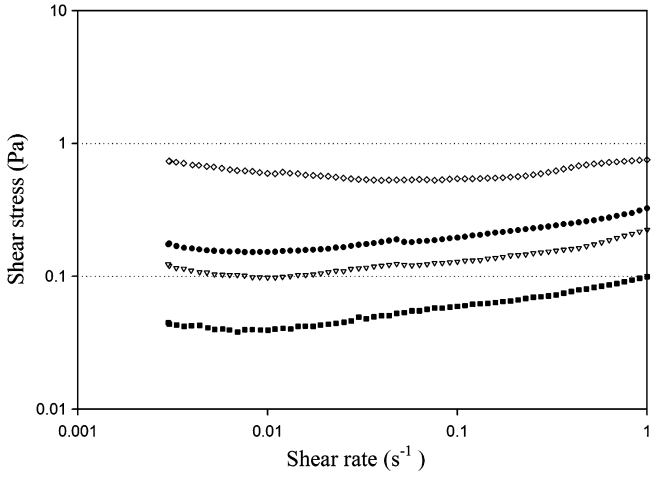

Fig. 3. Yield point measured using a flow down procedure in a rotational mode for sample pH 4.5 – 70% DM with added galacturonic acid oligomers showing single representative runs. Data shown is in low shear region (<1 s^{-1}) control (●) no added oligomers, small oligomers (▽) DP 2–13, medium oligomer (■) DP 7–21 and large oligomers (◇) DP 13–42 added.

large fragments from the high DP range with a median DP of 28 could be sufficient to bridge between adjacent molecules and thus be incorporated into a gel structure. As represented in Fig. 2, this is illustrated by molecule D, which represents a large GalA oligomer, crosslinking two separate pectin molecules each containing two functional blocks, where a functional block is any block of sufficient size to crosslink with calcium ion.

The interaction data experiment with the low DP size-class fragments indicated blocks as small as 6–13 may be involved in crosslinking, which is consistent with previous data (Liners & Cutsem, 1992; Powell et al., 1982). DMBs of this size in the demethylated pectin sample could be attacked by EPG, releasing a GalA fragment smaller than the actual DMB size. The mode of action of EPGs from *Aspergillus niger* have been well characterized (Benen, Kester, & Visser, 1999; Chen & Mort, 1996; Parenicova, Benen, Kester, & Visser, 1998) and shown to be unable to hydrolyze GalA dimers and only very slowly hydrolyze GalA trimers (Benen et al., 1999). Additionally, they cleave one residue left of the reducing end (Benen et al., 1999), consequently excision by EPG followed by HPLC analysis should yield a minimum value for block size. There is also a mathematical basis for this since there is a greater probability of excising a small oligomer than a larger oligomer from a large block (Cameron et al., 2008) which could decrease the value for average block size. An analysis of EPG excised block size and block number was performed by HPAEC – ELSD (high performance anion exchange chromatography – evaporative light scattering detector) and discussed in our companion manuscript (Cameron et al., 2008) on the pectin samples tested herein.

As discussed in this companion manuscript, additional degradation of released oligomers by EPG could further reduce the oligomer size and increase block number, however there was no chromatographic evidence of increased accumulation of small oligomers in low DM samples where larger oligomers dominated the fragments observed with a 5 min EPG digest (Cameron et al., 2008). Longer incubation times with EPG, 15 min or greater, did show a limited amount of degradation of long oligomers, and thus these conditions were avoided. In addition, mathematical modeling data has been shown to be consistent with the concept that released oligomers are not being further digested during excision under these circumstances (Cameron et al., 2008). Thus both block size and number should be largely unaffected by post excision attack of released oligomers by EPG under these experimental conditions. Attacking a $GalA_8$ DMB in an intact pectin molecule by EPG could result in a released GalA fragment as small as $GalA_3$ or $GalA_4$ if the middle of the block is excised, thus excised blocks of $GalA_3$ or $GalA_4$ and larger were counted to relate block counts to functional properties, since these oligomers could result from a block of sufficient size for calcium interaction to occur.

3.4. Comparison of block values with physical properties

Following EPG digestion and DMB analysis, the data for excised block size and number (Cameron et al., 2008) is shown in Table 2 for the different samples. For a network structure to form as shown in Fig. 2, it is reasonable to assume that each molecule should contain two blocks or more (as depicted by molecule B) or a single block of sufficient size (as depicted by molecule C) so that interaction could occur with at least two separate pectin molecules that also contain blocks large enough for linking with calcium ion. As indicated by theory the average number of blocks required per molecule must be >2 and that some fraction of molecules in a given population must be trifunctional or more (contain 3 blocks or more) for a gel to form (Flory, 1941). Trifunctional molecules are not depicted in Fig. 2. Some molecules could contain a single smaller block and yet be incorporated into the structure but could represent a terminating molecule (as depicted by molecule A). If a large proportion of molecules have only one block then the ability to form an extended network among the molecules would be limited and little or no gel formation would occur. This representative model should also be valid if more than two molecules make up a single junction zone (Powell et al., 1982) or as suggested by modeling data (Braccini & Perez, 2001) where these types of dimer–dimer interactions are thought to be weaker than the interactions occurring in a dimer interaction (Powell et al., 1982). In addition, work reported here and elsewhere indicates that a minimum block size is approximately 6–13 for calcium interaction to occur, keeping in mind that the excised block size being determined could be smaller based on mode of action of EPG.

One sample of interest is the pH 7.5 – 70% series that had a CSPR and YP value of 0.8% and 0.0.042 Pa, respectively. This sample had a mean excised block size ($\overline{BS}$) of 10.2 with a mean block number per molecule ($\overline{BN}$) of 2.0. The $\overline{BS} = 10.2$ for the pH 7.5 – 70% DM sample appears to be sufficient for crosslinking if the minimum block size required for crosslinking is from 6 to 13 based on the data from the competition experiments and by comparison to the pH 4.5 – 50% sample which had a smaller $\overline{BS}$ but formed a relative strong gel. $\overline{BN}$ could be over estimated if additional degradation of excised blocks is occurring, although as noted, evidence suggests that no additional degradation is observed (Cameron et al., 2008). If a block number of >2 for each molecule represents a minimum number for a network to form (Flory, 1941), then the pH 7.5 – 70% DM series may have insufficient blocks on all molecules in the population (assuming an uneven distribution) for effective crosslinking of the entire population of molecules and this could result in the low or limited CSPR values that were observed.

The data is somewhat different for the pH 4.5 series. For the pH 4.5 – 70% DM sample the CSPR and YP values were 11.4% and 0.171 Pa, respectively. In this sample, $\overline{BN}$ of 3.6 may be sufficiently large to allow for network crosslinking (if >2 is a minimum) but excised $\overline{BS}$ was low with an average value of 5.3. For this sample, block size on many of the molecules in the entire population may be insufficient for crosslinking and the observed CSPR value of 11.4% could be reasoned. One sample that had a CSPR value of greater than 90% was the pH 4.5 – 50% DM series where the CSPR and YP were 95.3% and 0.353 Pa. This sample also had the highest values of G' and G'' of 7.31 and 1.99 Pa, respectively, indicating a relative strong gel formation. The relatively high storage modulus value of G' is consistent with a large proportion of molecules being incorporated into the gel structure which was indicated by the CSPR data. For this sample ($\overline{BS} = 8.6$ and $\overline{BN} = 3.9$) the excised block size and block number appear to be sufficient for crosslinking the majority of molecules in the population. The major difference between the 50% DM sample and the 70% DM sample for the pH 4.5 series was $\overline{BS}$ and not $\overline{BN}$, supporting the concept that a limitation in gelation for the 4.5–70% DM sample could be block size.

CSPR data indicates that for the pH 4.5 – 50% DM sample 95.3% of the molecules contains sufficient block size and number for crosslinking to occur, even though it's $\overline{BN}$ (≈4) is a relatively small number. Four represented the largest $\overline{BN}$ observed in all samples tested. A low value for $\overline{BN}$ is consistent with a processive characteristic for this PME. A processive characteristic is also suggested by the pH 7.5 – 60% DM series, which had a CSPR value of 45.6% and low $\overline{BN} = 2.0$. Substrate to enzyme molar ratio in the demethylation reactions was estimated to be 1.6×10^4 or higher depending on the specific activity value used. With PME molecules in the demethylation reaction being many orders of magnitude less than the total number of pectin molecules, enzyme migration from molecule to molecule must occur during the process for a high proportion of molecules to contain blocks as indicated by the reported results for the samples with DM values of 60% and less. One enzyme model consistent with a high proportion of pectin molecules participating in the calcium-induced crosslinking would be a variably processive attack, with release, relocation and rebinding occurring as discussed in our companion manuscript (Cameron et al., 2008). In this proposed model, rebinding to a previously formed unmethylated section of the molecule is more likely to lead to a productive block demethylation to extend the block and thereby maintain a low block number on each molecule. In this model, enzyme binding to larger blocks results in a concomitant increase in degree of processivity before release. This mode of action has been supported by mathematical modeling of the block data (Cameron et al., 2008), where it could be a mechanism in the formation of the DMBs of 10 or less over a large population of molecules. Blocks in this size range could participate in calcium linked network formation as suggested by the competition data discussed herein.

4. Conclusion

Pectin gelation in the presence of calcium ion has, in addition to biological implications, importance for both industrial and food applications and the gelation mechanisms affected by the presence of unesterified blocks. The ability to describe fine-structure (tallying the DMBs) and correlate it with functional properties such as modulus and yield point has benefits for understanding pectin's physical properties in the presence of calcium ion. Measuring rheology properties and other physical properties can be straightforward, but obtaining an accurate count of demethylated block size and number is more problematic. Progress in chromatography now allows for quantification of large DMBs (to a DP of 50 or greater) and is another step toward a direct structural analysis of pectin's HG regions. Recent methodology using *limited* EPG digestion combined with chromatographic analysis has allowed for the estimation of $\overline{BS}$ and $\overline{BN}$. A $\overline{BN}$ of between 2 and 4 appears to be conducive for calcium interactions and introduction of rheological properties together with an excised $\overline{BS}$ of approximately 8 or greater. Comparison of the extent of calcium reactivity by CSPR and modulus values with $\overline{BN}$ indicates that very few blocks need to be present on each molecule for a large proportion of molecules to be incorporated into the calcium sensitive gel structure.

If DMB architecture on all pectin molecules is biologically advantageous, the release, relocation and rebinding of enzyme molecules that accompanies limited processivity would be more productive for distributing a low concentration of enzyme molecules over a substrate population that typically is in much higher concentration. In contrast, an even distribution of DMBs would be restricted if the enzyme had absolute processivity and enzyme–substrate ratios were low. As suggested by the results reported here, with PME molecules being orders of magnitude less than the total pectin molecules, enzyme relocation must occur for most molecules to contain DMBs as indicated by CSPR for the samples with lower DM values. These observations are consistent with a mode of action described as variably processive attack. The mathematical modeling data presented in our companion paper (Cameron et al., 2008) suggested that variably processive attack, particularly at low pH and for small DMBs, was occurring. As indicated in the results presented here for rheological and calcium reactivity data, this new block size and block count methodology is one step closer toward the goal of relating absolute counts of block size and number to pectin functionality in order to provide full structure–function analysis that will allow for engineering pectin structure and tailoring functionality.

Acknowledgments

The authors acknowledge the technical assistance Steven W. Kauffman and Elena Stewart for technical expertise and to Dr. Karel Grohmann for his comments concerning this manuscript.

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Glossary

$\overline{BN}$: block number
$\overline{BS}$: block size
CS: calcium sensitivity
CSP: calcium sensitive pectin
CSPR: calcium sensitive pectin ratio
DI: deionized
DM: degree of methylation
DMB: demethylated block
DP: degree of polymerization
ELSD: evaporative light scattering detector
EPG: endo-polygalacturonase
EtOH: ethanol
Exo-PG: exo-polygalacturonase
G': storage modulus
G'': loss modulus
GalA: galacturonic acid
HG: homogalacturonan
HPAEC: high performance anion exchange chromatography
HPLC: high performance liquid chromatography
IPA: isopropyl alcohol
LiOAc: lithium acetate
MW: molecular weight
NaOAc: sodium acetate
NCSP: non- calcium sensitive pectin
OGalA: oligogalacturonic acid
Pa: pascals
PGalA: polygalacturonic acid
PME: pectin methylesterase
R: correlation coefficient
RT: room temperature
YP: yield point