Demethylation of a model homogalacturonan with a salt-independent pectin methylesterase from citrus: I. Effect of pH on demethylated block size, block number and enzyme mode of action

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Abstract

A model homogalacturonan (HG) was used to produce a demethylated series by reacting it with a citrus salt-independent pectin methylesterase (PME) at pH 4.5 and 7.5. HGs with a predicted degree of methylesterification (DM) of 90%, 80%, 70%, 60% and 50% were produced. HG structural properties and PME mode of action were probed by performing a *limited* digest with an endo-polygalacturonase. Liberated demethylated blocks (DMB) were separated and quantified by HPAEC coupled to an evaporative light scattering detector. A significant increase in maximum and average size of released DMBs was observed between 80% and 70% DM at pH 7.5 and between 70% and 60% at pH 4.5. The observed distribution of fragment lengths was compared to theoretical modeled distributions. A multiple-attack mechanism with a degree of processivity (p) of $p = 1$ at pH 4.5 and $p = 10$ at pH 7.5 modeled the observed shorter fragments well, while in all cases the amount of larger fragments detected was more successfully described by a single-chain mechanism. Published by Elsevier Ltd.

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1. Introduction

In-vitro and in-vivo pectin functionality depends on the amount of ion-binding groups attached to the polymer and their distribution along the backbone. Therefore, the development of methodologies to predictably generate novel, non-random patterns of methylesterification, and efficiently characterize their distributions, is of great interest.

Pectin, composed mostly of galacturonic acid (GalA) and smaller amounts of several different neutral sugars, is generally recognized to contain three structural domains (Ridley, O'Neill, & Mohnen, 2001; Vincken et al., 2003) . The dominant domain is the homogalacturonan region (HG), a linear polymer of GalA (Hotchkiss, Bahtimy, & Fishman, 1996; Zhan, Janssen, & Mort, 1998). Each domain has functional properties related to its structure.

Functional properties of the HG region are dependent on the amount and distribution of the methyl and acetyl esters. Methyl esters are the dominant form in most species but acetyl esters are abundant in sugar beets (Ralet et al., 2005; Ridley et al., 2001). Degree of methylation (DM) has a very strong influence on pectin functionality, being a major basis for differentiation of pectins used in the food industry (May, 1990). Studies have shown that the distribution of methyl esters in the HG region of pectin influences its functional properties (Guillotin et al., 2005; Luzio, 2003; Schmelter, Wientjes, Vreeker, & Klaffke, 2002; Willats et al., 2001). Willats et al. (2001) demonstrated that for water holding capacity of gels made from pectins with differing methyl ester distribution patterns in their HG region, the distribution pattern was more important than the DM.

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Spatial distribution patterns of methyl esters within HG regions are demonstrably related to the mechanism of demethylation. Demethylation occurs either by enzymatic action or alkaline demethylation, resulting in either ordered or random patterns. Enzymatic tools potentially can be used both to modify the amount and pattern of methylesterification and to characterize the chain. Plant PMEs, with a basic pI, have been shown to produce ordered distributions of demethylated stretches within HG regions while fungal PMEs, with an acidic pI, and base catalyzed demethylations produce random distributions (Grasdalen, Andersen, & Larsen, 1996; Limberg et al., 2000a, 2000b; Neiss, Cheng, Daas, & Schols, 1998; Ralet & Thibault, 2002; Verlent, Smout, Duvetter, & Van Loey, 2005). Using EPG digested pectins, and based on amounts of monomer, dimer and trimer of GalA liberated from pectic substrates, Daas, Meyer-Hansen, Schols, De Ruiter, and Voragen (1999) coined the term "Degree of Blockiness" (percentage of non-esterified GA residues released during the EPG digest) to differentiate pectins with numerous small unesterified sequential GA residues from those with larger unesterified blocks. Differences in distribution patterns of de-esterified blocks produced by ordered or random mechanisms have also been demonstrated (Duvetter et al., 2006; Williams, Foster, & Schols, 2003).

The resulting differences in distribution patterns and size of DMBs due to the action of plant or fungal PMEs are believed to be related to the mode of action of the different enzymes. PME modes of action have been proposed based on starch degrading enzymes (Greenwood & Milne, 1968; Robyt & French, 1967, 1970) and enzymatic modification of other polymeric substrates (Breyer & Matthews, 2001; Campa et al., 2004). The two extreme types of enzyme action are the single-chain (enzyme–substrate complex is formed and the enzyme does not dissociate from the polymer until it reaches the end of the molecule or a blocking residue) and the multi-chain (the enzyme randomly forms an enzyme–substrate complex and dissociates after each reaction) mechanisms. A variant of the single-chain mechanism, and intermediate between it and the multi-chain mechanism, is the multiple-attack mechanism where some numbers of reactions are catalyzed by the enzyme between formation of the enzyme–substrate complex and dissociation from the polymer. These three models describe a continuum of enzyme processivity, from dissociation following a single catalytic event to dissociation following tens, hundreds or thousands of catalytic events.

Fungal PMEs are generally described to possess the multi-chain mode of action leading to a random pattern of demethylation (Limberg et al., 2000a; Ralet & Thibault, 2002; Willats et al., 2001). Plant PMEs appear to be processive enzymes, introducing DMBs into the HG region (Catoire, Pierron, Morvan, Herve du Penhoat, & Goldberg, 1998; Daas et al., 1999; Limberg et al., 2000a, 2000b; Ralet & Thibault, 2002; Willats et al., 2001). Variability in the degree of processivity of plant PME isozymes has been reported for mung bean (Catoire et al., 1998;

Goldberg et al., 2001) and citrus (Kim, Teng, & Wicker, 2005). Reaction conditions have been reported to affect the mode of action of apple PME, (Denes, Baron, Renard, Pean, & Drilleau, 2000) suggesting that a single-chain mechanism occurred at pH 7.0 shifting to a multi-chain mechanism at pH 4.5.

The possibility of designing the functional properties of pectin by manipulating the size and distribution of DMBs in the HG region via reaction with various PMEs under controlled conditions could create the potential to engineer properties required for novel food or non-food applications. Such new applications could greatly increase the value of residues generated during processing of many pectin-rich crops (Willats, Knox, & Mikkelsen, 2006). Four chromatographic forms of PME have been identified from citrus and three of them have been characterized at the biochemical level (Cameron & Grohmann, 1995, 1996; Cameron, Savary, Hotchkiss, & Fishman, 2005b, 2003b; Savary, Hotchkiss, & Cameron, 2002). The three characterized forms each have unique properties that would enable them to be managed for selective demethylation of pectins. One is thermally tolerant (Cameron et al., 2005b) allowing for the selection of reaction conditions that would enable it to be the only active PME in a reaction cocktail. Another is salt-dependent, a characteristic that also allows for activity control (Cameron et al., 2003b; Christensen, Nielsen, Kreiberg, Rasmussen, & Mikkelsen, 1998). The different citrus PMEs have been shown to have a differential affect on the stability of citrus juice-cloud (Cameron, Baker, & Grohmann, 1998) suggesting their modes of action on the pectin present in juice-cloud are distinctively different from each other. Demethylation of pectin in the juice-cloud is recognized as the causative agent for juice-cloud destabilization (Guyer, Miller, Bissett, & Veldhuis, 1956; Stevens, Pritchett, & Baier, 1950; Versteeg, Rombouts, Spaansen, & Pilnik, 1980; Wicker, Ackerley, & Hunter, 2003).

Here we report on the action pattern of the salt-independent PME, the dominant PME form present in citrus peel, (Savary et al., 2002) and the size and distribution of DMBs in the HG following controlled demethylations at pH 4.5 and 7.5. We generated ten pectin samples from a highly-esterified starting material using this little-studied salt-independent citrus PME. More specifically these samples have post-PME treatment degrees of methylesterification (DM) of 50%, 60%, 70%, 80% and 90%; each sample generated at pH 4.5 or 7.5. As these samples were anticipated to contain relatively blocky structures we endeavored to use an alternative method to the complete enzyme digestion method utilized by Daas et al. (1999; Daas, Voragen, & Schols, 2000, 2001). These complete digests described by Daas and coworkers clearly show that the pectins did not have a random intramolecular distribution but for very blocky structures they tend simply to yield monomer, dimer and trimer, thus are relatively uninformative for making a minimum estimate of the largest DMB length, average block size or average number of blocks per pectin molecule. The PME generated samples studied here were

treated with EPG for a limited time, the value of which was assessed with preliminary experiments, so it could be assumed that no further processing of GalA fragments occurred once they were released from the chain. The EPG used is commercially available; hence the complex, costly and time consuming procedures for purifying a monocomponent enzyme preparation are avoided. Additionally, it is a research tool available to all. This methodology allowed us to estimate the minimum size of the largest DMB, average block size, average number of blocks per pectin molecule and to mathematically model the enzyme mode of action. The expected distributions of released demethylated blocks were simulated in-silico using different PME processivities and subsequently, the amount of each of the GalA oligomers contained in the simulated digest was calculated. These simulations were compared with experimental data obtained by the *limited* EPG digest and analysis by high performance anion exchange chromatography (HPAEC) coupled to an evaporative light scattering detector (ELSD) (Cameron & Grohmann, 2005; Cameron, Hotchkiss, Kauffman, & Grohmann, 2003a).

2. Materials and methods

2.1. Materials

All chemicals were purchased from Sigma–Aldrich (St. Louis, MO, USA) unless otherwise indicated. Endo-polygalacturonase (EPG-M2) was purchased from Megazyme International Limited (Bray, Ireland; Lot 00801). Salt-independent PME was isolated as previously described (Cameron et al., 1998; Savary et al., 2002).

2.2. Pectin demethylation

Pectin (97% AGA, 94% DM) was made to a final concentration of 1% (w/v) in 0.2 M LiCl at pH 7.5 (pH adjusted with LiOH). Lithium was used as a counter ion due to the increased pectin solubility associated with it (Fishman, Chau, Kolpak, & Brady, 2001). Two liters of the pectin solution were added to a water-jacketed 5 L stirred bioreactor and allowed to equilibrate to 30 °C. One milliliters of enzyme solution containing approximately 350 U (as estimated at pH 7.5 by pH-stat titration) (Cameron et al., 2005b) was added to the pectin solution and pH was maintained at either pH 4.5 or 7.5 with a Radiometer PHM290 pH-stat controller using 1 M LiOH as the titrant. The reaction was allowed to continue until sufficient titrant was added to obtain the desired DM at which time the entire contents of the bioreactor were allowed to flow into two volumes of warm (37 °C) ethanol. Evacuation of the bioreactor contents into the ethanol was completed in less than 30 s. The precipitated demethylated pectin was stored at 4 °C. Subsequent testing by pH stat titration of the precipitated pectin for residual PME activity indicated no activity remained. The majority of the liquid associated with the precipitated pectin was removed by filtration over

Whatman Qualitative #3 filter paper (32 cm diameter) using a vacuum Buchner Funnel connected to water aspirator. A nylon support was placed under the filter paper. The filtered pectin was removed with a spatula and placed into liquid nitrogen. The frozen pectin was then lyophilized. The lyophilized pectin was weighed, placed into plastic containers and then into a larger container containing drierite and stored at −80 °C.

2.3. EPG digests

Solutions containing 0.25% of the individual demethylated pectins were made in 50 mM lithium acetate (pH 5.5) and 0.02% lithium azide. Residual PME activity in the precipitated pectins was tested by pH-Stat titration and none was observed. Samples were equilibrated to 30 °C in benchtop incubator with stirring. EPG M2 was added at 5×10^{-4} U mL^{-1}. Aliquots (10 mL) were collected at 5 min (preliminary trials demonstrated that no detectable fragments were released at 1 min and that large fragments were being hydrolyzed with longer digestions). The aliquot was pipetted into 160 μL of concentrated HCl to drop the pH to ~2, quenching the enzyme activity. The aliquot was then heated to boiling in a microwave oven (~10 to 12 s) and then placed into a boiling water bath for 10 min.

2.4. Chromatography

2.4.1. SEC–MALLS

The SEC–MALLS (size exclusion-multiangle laser light scattering) system consisted of a pump (Model 1000D syringe pump, ISCO, Lincoln, Nebraska) and an in-line filter (0.02 mm pore size), Anodisc 25, Whatman, Maidstone, UK; (Cameron, Luzio, Kauffman, & Grohmann, 2004). Mobile phase (100 mM ammonium formate) was degassed under vacuum prior to loading into the syringe pump. Mobile phase flow rate was 0.60 mL/min. Pectin samples were injected onto a set of three linear (PL-aquagel-OH 50 and 60, 8 μm pore size, 300×5.7 mm, Polymer Laboratories Inc., Amherst, MA and a TSK-GEL, 10 μm pore size, 600×7.5 mm, TOSOHaas, Montgomeryville, PA) SEC columns with an operating range of 100–10,000,000 MW. The columns were connected in series (largest pore size first), enclosed in a column heater, and kept at 37.0 ± 0.2 °C. The detectors used were present in line in the following order: MALLS and RI. The MALLS (DAWN® EOS, Wyatt Technologies, Santa Barbara, CA) was equipped with a K5 flow cell and a He–Ne laser light source ($L = 633$ nm). Prior to the measurements, the MALLS was calibrated using 0.2 mm filtered HPLC quality toluene. The RI (Optilab DSP®, Wyatt Technologies, Santa Barbara, CA) had a P100 cell (10 mm path length) operating at 633 nm and a constant temperature of 42.0 °C. Results were processed to determine MW (DNDC for Windows ver. 5.90.03, and Astra for Windows ver. 4.90.07, Wyatt Technologies). A previously published value

of dn/dc for pectin (0.146 mL/g) was used for MW determination and mass recovery (Fishman, Chau, Hoagland, & Ayyad, 2000).

2.4.2. HPAEC

Partially digested samples were analyzed by HPAEC–ELSD (Cameron & Grohmann, 2005). The chromatography system was composed of a Perkin–Elmer Series 200 Pump (Shelton, CT, USA) and a Perkin–Elmer Series 200 Autosampler connected to a CarboPac PA1 (4 × 250 mm; Dionex Corporation, Sunnyvale, CA, USA) anion exchange column. Detection of analytes was accomplished with a Sedex 75S Evaporative Light Scattering Detector (Lawrenceville, NJ, USA). Data collection was accomplished with an A/D converter connected to a Dell (Round Rock, TX, USA) personal computer using EZ Chrome Elite software (version 3.1.6, Agilent Technologies, Inc., Palo Alto, CA, USA). Three to five replicate chromatography runs were performed for each pectin in the demethylation series. The DP of GalA oligomers was identified by comparison to elution times of previously run digests of polygalacturonic acid (PGA) and co-elution with known standards (Cameron & Grohmann, 2005; Cameron et al., 2003a, Cameron, Luzio, Baldwin, Narciso, & Plotto, 2005a, 2004). Masses for each oligomer (GalA$_n$; where n is the number of demethylated GalA residues in the oligomer) were estimated using a pooled calibration curve constructed using GalA$_2$, GalA$_3$, GalA$_6$ and GalA$_8$. Significant differences among the slopes and intersects for the individual calibration curves (each curve contained five concentration levels and each level was replicated three times) were tested using an ANCOVA test (performed using GraphPad Prism version 4.03 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com). Estimated mass concentrations for each GalA$_n$ were converted to molar concentration per mL, C_n. The molar concentration of pectin per mL (C_p) was estimated using the average molecular weight obtained from MALLS–SEC. The average number of DMBs released from a molecule of length n ($\overline{B}_n$) for each oligomer of GalA$_n$ was estimated with Eq. (1).

$$\overline{B}_n = \frac{C_n}{C_p} \tag{1}$$

The average number of blocks per molecule ($\overline{B}$) is the sum of the average number of DMBs of size n:

$$\overline{B} = \sum_{n=3}^{z} \overline{B}_n \tag{2}$$

The average DMB size released ($\overline{BS}$) was estimated according to Eq. (3)

$$\overline{BS} = \frac{\sum_{n=3}^{z} n\overline{B}_n}{\overline{B}} \tag{3}$$

Statistical analysis of the results were performed in Excel 2003 (Microsoft, Redmond, WA) and Statistica Version 7.1 (Statsoft, Tulas, OK). Data were inspected for outliers using Dixon's Q-test (Dixon, 1953), the ''huge'' test (Marascuilo, 1971) and the Grubbs' test (Grubbs, 1969). All statistical significance tests were performed at the 95% confidence level.

2.4.3. Methanol determination

Degree of methylation was determined using a modified procedure (Levigne, Thomas, Ralet, Quemener, & Thibault, 2002). Pectin sample (6.75 mg) in 675 μL of a solution was mixed with 75 μL of a 100 mM CuSO$_4$ solution. To achieve saponification, 750 μL of 1 M NaOH was added and the mixture was left at 4 °C for 1.5 h in sealed vessels. Reaction mixtures were centrifuged for 12 min at 11,000g at room temperature. Supernatants were neutralized through a 1 mL syringe equipped with a Maxiclean IC-H device (Alltech) prior to injection. Neutralized supernatants, 100 μL sample size, were injected onto a C l8 column (Superspher 100 RP-18 end capped, Merk KGaA, 250 × 4) with a guard column (LiChroCART, 4 × 4). Elution was carried out with 4 mM sulfuric acid at 0.5 mL min^{-1} at 25 °C coupled to an RI detector (Optilab DSP®, Wyatt Technologies, Santa Barbara, CA) equipped with a P100 cell (10 mm path length) operating at 633 nm and a constant temperature of 42 °C. Data were collected and processed using EZ Chrome Elite. The concentration of methanol was determined using a standard curve obtained from known concentrations of methanol. Separately, the GalA content was determined colorimetrically by a microtiter plate colorimetric method (Luzio, 2004) using the same sample solutions of pectin as prepared for methanol determination. Degree of methylation was computed from the ratio moles of methanol released by saponification to the moles of GalA from the microtiter plate assay.

2.5. Capillary electrophoresis

Experiments were carried out using an automated CE system (HP 3D), equipped with a diode array detector. Electrophoresis was carried out in a fused silica capillary of internal diameter 50 μm and a total length of 46.5 cm (40 cm from inlet to detector). The capillary incorporated an extended light-path detection window (150 μm) and was thermostatted at 25 °C. Phosphate buffer at pH 7.0 was used as a CE background electrolyte (BGE) and was prepared by mixing 0.2 M Na$_2$HPO$_4$ and 0.2 M NaH$_2$PO$_4$ in appropriate ratios and subsequently reducing the ionic strength to 90 mM. All new capillaries were conditioned by rinsing for 30 min with 1 M NaOH, 30 min with a 0.1 M NaOH solution, 15 min with water and 30 min with BGE. Between runs it was found that similar harsh washing conditions were required. Detection was carried out using UV absorbance at 191 nm with a bandwidth of 2 nm. Samples were loaded hydrodynamically (various injection times at 5000 Pa, typically giving injection vol-

umes of the order of 10 nL), and typically electrophoresed across a potential difference of 20 kV. All experiments were carried out at normal polarity (inlet anodic) unless otherwise stated.

2.6. Mathematical modeling

2.6.1. Starting substrate generation

The starting substrate was modeled as a set of pectic chains, each simulated by two one-dimensional arrays, with each array element representing one residue. One array tracks the residue type (GalA, esterified GalA), while the other monitors the residue position with regard to its fragment (non-reducing end, middle, reducing end). All substrates are initialized as PGA and subsequently attain their required DM via an esterification algorithm. For random distributions Bernoulian statistics are used. Distributions in the DP of the chains can be accounted for by generating a set of chains, with the relevant number modeled by arrays of different length. Intermolecular DM distributions are simulated in a similar way with relevant numbers of chains being assigned slightly different DM values in order to build up a selected DM distribution for the simulation set. Here distributions in the DP and DM have been modeled simply by Gaussians with user-defined average values and widths at half height (FWHH), with each chain in the simulation having its DM and DP assigned at random, in order that the properties of the ensemble match those selected for the substrate replica.

For all simulations carried out here the starting substrate was assigned a number average degree of methylesterification of 94 (FWHH = 5) and a degree of polymerization of 500 residues (FWHH = 100). Up to 10^5 chains were used in any one simulation. The randomness of the generated starting intramolecular DM distribution was checked by plotting log(frequency) versus blocklength and ensuring that this resulted in a linear plot with a gradient of $\log((100 - DM)/100)$.

2.6.2. Generation and measurement of GalA blocklength distribution

Once the starting substrate was generated a de-esterification algorithm was run until the substrate set had achieved its specified endpoint (i.e. the degree of methylesterification had been reduced to the corresponding experimental condition). This algorithm removed methyl groups according to a simple model of PME with three salient features: (i) each iteration selected a chain and a residue at random, (ii) if the residue encountered was unesterified (this assumes that one unesterified GalA residue is required for successful substrate binding) and the residue to its right was methylesterified then the methyl group was removed (iii) subsequent methylester groups existing were also removed until either, an unesterified group or the end of a chain was encountered, or the user-defined degree of multiple attack was reached. By selecting the degree of multiple attack to be 1 a multi-chain mechanism can be simulated, and

additionally, by setting this multiple degree to a large value single-chain models also were investigated. Further modifications of these criteria are trivial and there effects will be investigated in further work.

After the substrate had been modified accordingly its newly generated intramolecular methylester distribution was interrogated. Fig. 1(a) shows the relative number (N) of the different GalA blocklengths E-$(G)_n$-E found in pectins de-esterified according to the described PME model to 60%, 70% or 80%, assuming a very large degree of multiple attack (i.e. a single-chain mechanism). It is observed that the clearly linear section of the log plot for DM 80 fits to give: the relative probability of modifying the blocklength by one residue is 0.94. This likely has its origins in the relative probability of contiguous methylesterified blocks in the original starting material and suggests that at least where the total demethylation is limited
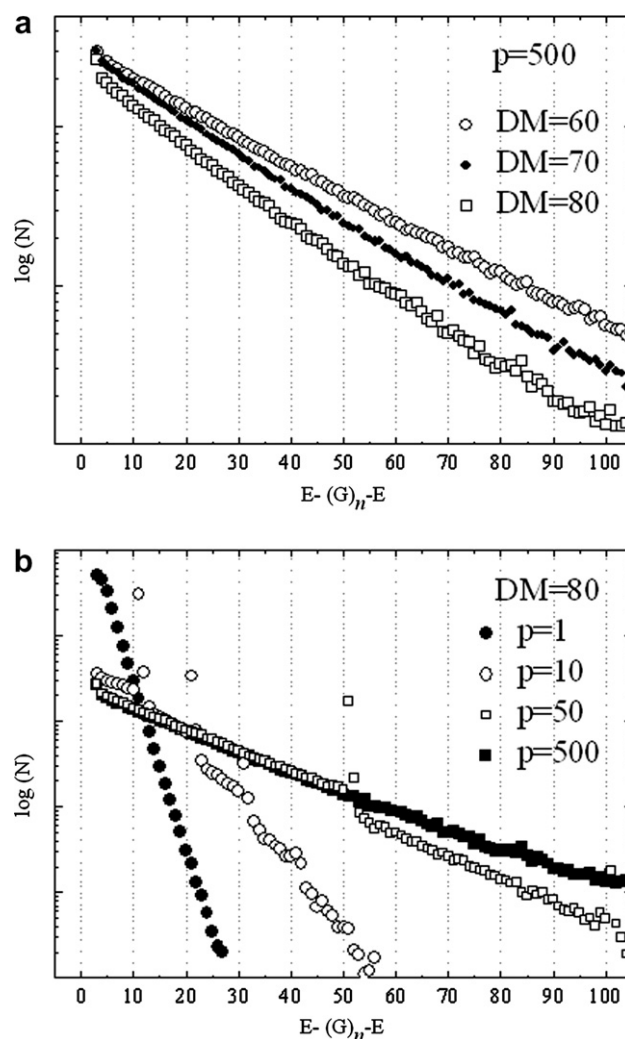

Fig. 1. (a) The relative number (N) of the different GA blocklengths E-$(G)_n$-E found in pectins de-esterified according to the described PME model assuming a very large degree of multiple attack ($p = 500$). (b) How this relative number (N) of the different GalA blocklengths, E-$(G)_n$-E, existing in the 80% PME demethylesterified substrate varies according to the degree of multiple attack.

(94 → 80%) this methylester distribution is translated into the GalA distribution without significant amalgamation of stretches that seem to be visible once a more significant demethylesterification is achieved.

Fig. 1(b) shows how this relative number (N) of the different GalA blocklengths, E-(G)$_n$-E, existing in the 80% PME demethylated substrate varies according to the degree of multiple attack. It is clear that as expected this distribution depends crucially on the assumed degree of processivity of the enzyme. For stretches smaller than the modeled degree of multiple attack the relative amount of GalA runs is determined once again by the distribution of the original methylesterified blocks that are introduced by the methyl removing enzyme before it has to leave the chain. It is also interesting to note that large peaks appear at the number of the degree of multiple attack plus one, as clearly seen for the processivity, $p = 10$ and 50, arising from the production of contiguous methylesterified blocks longer than the degree of multiple attack. There is also some observed effect apparently related to multiples of this degree of attack for similar reasons.

2.6.3. Calculation of liberated fragments

Having generated different distributions of GalA blocklengths assuming that the PME had various degrees of processivity, the question now arises as to how these differences would be reflected in the DPs of the GalA oligomers released by a *limited* digest of the substrate with EPG. It should be remembered that the aim of the experimental work was to use conditions such that a limited digest was performed; specifically that once a GalA oligomer was removed from the polymer backbone it was not cut again. The problem was approached in two ways. Firstly, the digestion process itself was modeled in-silico using a method recently reported, that uses postulated subsite energies to calculate the success, or otherwise, of interactions between enzyme and substrate. The subsite energies used had not been measured specifically for this enzyme and best values available for EPG II from *Aspergillus niger* were used (Hunt, Cameron, & Williams, 2006) simply to allow exploratory investigations of the method to be performed, while it is clear that the relative amount of fragments smaller than the postulated subsite architecture (∼7) are likely to be incorrectly predicted.

Secondly, it was simply assumed that the enzyme acts at random within the unesterified blocks, in which case the relative probability of removing a k-mer from a block n long is given by:

$$\frac{(n-2)-k+1}{((n-2)^2+(n-2))/2} \tag{4}$$

Here the $n-2$ arises from assuming that the EPG needs two unmethylated residues in its active site so whatever scissions it makes it always leaves an unesterified residue on either side (i.e. the largest GalA oligomer extractable from a run of 10 unesterified residues would be eight). This expression simply says there is much more chance of obtaining a monomer than longer oligomers because there are many ways it can be generated (in the given example there are eight ways of doing this but only one way of removing an octomer, two ways of removing a heptamer etc). Now we are in a position to calculate (subject to the approximations made) the relative probabilities (and hence relative numbers of molecules) of the different DP fragments one would predict to obtain for PME models of varying multiple-attack degrees. The simulation described in the previous section gives the relative amounts, N, of different starting GalA runs of different lengths, E-(G)$_n$-E, in the polymer and a way of calculating, for each of those, the relative amount of each oligomer generated for a random chop-out from those regions.

Combining the approaches by summing the amount of each oligomer generated from each available length weighted by the probability of finding each starting contiguous length in the backbone we find the relative total number of GalA k-mers liberated is given by:

$$\sum_{n=k+2}^{n=\text{chainlength}} \frac{N(E-(G)_n-E)[(n-2)-k+1]}{\{[(n-2)^2+(n-2)]/2\}} \tag{5}$$

Fig. 2 shows the comparison of the two methodologies described above in calculating the predicted distribution of galacturonic acid oligomers that would be released from a DM 60% substrate that was de-esterified from 94% DM by a PME with a degree of multiple attack (processivity) given by 10. When the number of iterations performed in the explicit enzyme model was relatively low (10^4), so that no significant post-oligomer-removal processing occurred, the basic form of the predicted distribution is indeed in line with the second summation approach. This is taken as support for the second approach and it is favored owing to its
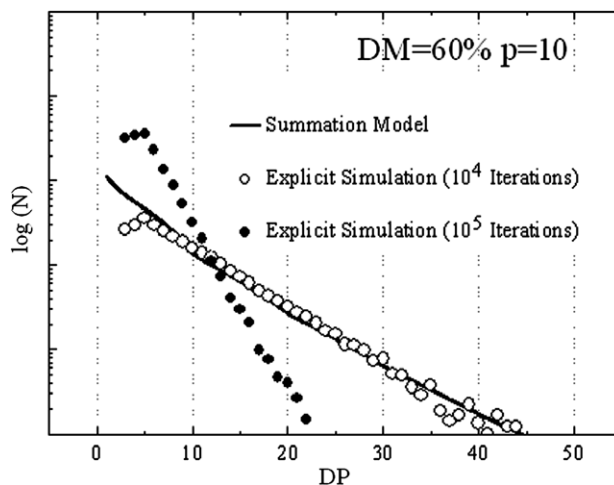

Fig. 2. Comparison of the two calculation methodologies described in the text for predicting the distribution of galacturonic acid oligomers that would be released from a DM 60% substrate that was de-esterified from 94% DM by a PME with a degree of processivity given by 10. When the number of iterations performed in the explicit enzyme model was relatively low (10^4), no significant post-oligomer-removal processing occurred.
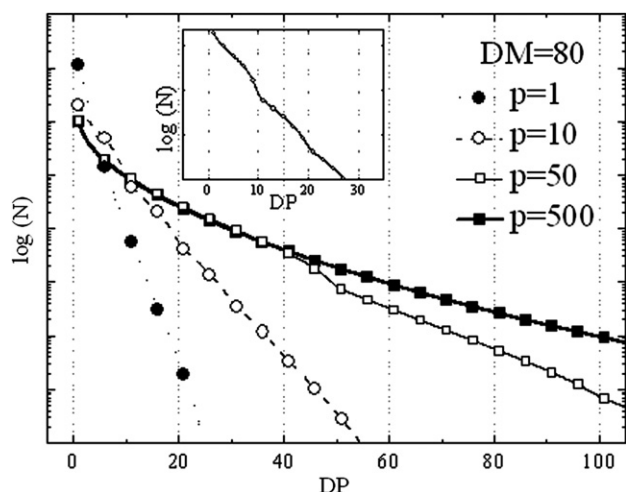
Fig. 3. Distributions of liberated GalA oligomers calculated from the GalA blocklength distributions shown in Fig. 1(b) calculated as described in the text.

simplicity of calculation compared with the full-blown enzyme degradation simulation. The first approach does have its benefits however, as it allows the effects of additional fragmentation to be investigated. The figure also shows that if the experimental assumption of limited digestion is incorrect (which naturally occurs in the algorithm as the number of iterations is increased as shown for 10^5) then the effect would be reflected in a general shortening of all oligomers as might be expected.

Fig. 3 shows how the GalA blocklength distributions shown in Fig. 1(b) translate into distributions of liberated GalA oligomers using the summation approach as given in Eq. (5). It is interesting to note that these oligomer distributions show points of inflection around the degree of multiple attack (see for example the inset plot of DP up to 30 for $p = 10$) that clearly originate from the way this feature generates peaks in the E-$(G)_n$-E distribution. The fact that this parameter is reflected in the liberated oligomers distribution raises hope that it might be possible with the methodology described here to determine the degree of multiple attacks experimentally.

3. Results and discussion

GalA (94–97%) and galactose (3–6%) were the only sugars detected in the parent pectin (data not shown). The lack of detectable rhamnose suggests that the parent pectin is a homogalacturonan. The Wt. Ave. Molecular Wt. of the parent pectin was estimated to be 43,280 daltons by SEC–MALLS, approximately equivalent to an HG stretch with a $GalA_n$ of 246. The DM of the parent pectin was reported by the manufacturer to be 94%, consequently the probability that a demethylated block of four GalAs, the minimum size estimated to be needed for relatively rapid EPG cleavage (Benen, Kester, & Visser, 1999; Chen & Mort, 1996), would be present is approximately 0.001. Although EPG II will hydrolyze trimers the rate is

extremely slow (Benen et al., 1999). Release of fragments by the five minute EPG digest of either the parent or 90% DM pectins was not detected so the 90% DM pectin was not used for further analyses and the 50% DM sample generated at pH 7.5 was lost during processing.

GalA oligomers were released from all pectins with a DM of ≤80% (Table 1; see Figs. 4 and 5 for representative chromatograms) following the 5 min EPG digest. Partially methylated fragments eluted as a large peak between 14 and 20 min. Unmethylated GalAs present in this partially methylated material are likely to be distributed either randomly, occur in very small blocks that could not be released by the EPG or as remnant fragment ends left behind following EPG cleavage of an interior region of a DMB. Peaks representing GalA oligomers were identified based on co-elution on the HPAEC–ELSD with purchased or purified standards (Cameron & Grohmann, 2005), comparison to elution of $GalA_n$ 3–17 oligomers with capillary electrophoresis (Hunt et al., 2006) and by HPLC–MS or SEC–MALLS of selected peaks. Further evidence that the individual peaks on chromatograms of EPG digests represent GalA oligomers is that no peaks were observed if the EPG digest was for only one minute and the larger peaks decreased, and eventually disappeared, if the digest was conducted for 15–30 min.

Table 1
Effect of demethylation pH and degree of methylation on the average length of the longest demethylated block released by EPG and observed by HPAEC–ELSD

Pectin series (%)	Oligomer DP	
	pH 7.5	pH 4.5
90	0 (90.2%[a], 90.1%[b])	0 (89.5%[a], 90.0%[b])
80	14 (78.0%[a], 80.2%[b])	8 (77.4%[a], 80.2%[b])
70	42 (70.1%[a], 70.3%[b])	19 (67.6%[a], 69.8%[b])
60	39 (61.1%[a], 60.5%[b])	43 (61.0%[a], 61.5%[b])
50	–	>50 (48.9%[b])

[a] DM estimated by HPLC methanol determination of demethylated pectin.
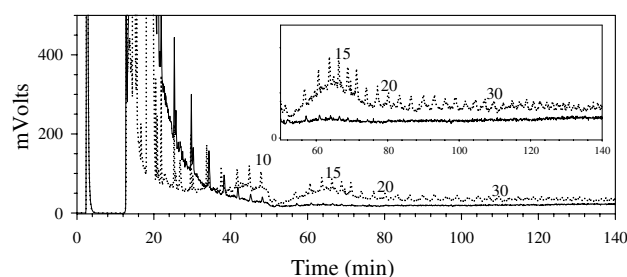[b] DM estimated from amount of base added during initial pH-stat demethylation.

Fig. 4. Representative composite HPAEC–ELSD chromatogram of GalA oligomers present after a 5 min EPG digest of the pH 7.5 80% (solid line) and 70% (dashed line) DM pectins. Numbers indicate oligomer DP of the corresponding peak. The inset is a zoom of the chromatogram for the indicated region.
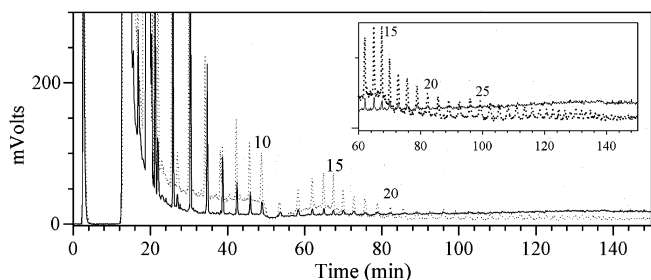
Fig. 5. Representative composite HPAEC–ELSD chromatogram of GalA oligomers present after a 5 min EPG digest of the pH 4.5 70% (solid line) and 60% (dashed line) DM pectins. Numbers indicate oligomer DP of the corresponding peak. The inset is a zoom of the chromatogram for the indicated region.

The mode of action of EPGs from *A. niger* have been well characterized (Benen et al., 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 always require one residue to the right and left of the active site to be unesterified for cleavage (Benen et al., 1999), consequently the oligomer size observed by HPAEC–ELSD is a minimum estimate of the blocklengths in the polymer. However, a connection between these two distributions is provided by the extensive modeling. Blocklengths in the backbone may be even larger than suspected if the EPG is acting on demethylated stretches already released from the HG. However, we saw no evidence of an increased accumulation of small oligomers in low DM samples where larger oligomers dominated the fragments observed. The *Aspergillus aculeatus* EPG used in this study has not been as well characterized

as the *A. niger* enzymes but preliminary studies (unpublished data) demonstrated that its behavior on GalA dimer and trimers was similar to *A. niger* EPG II. Additionally, unlike the EPGs from *A. niger*, this enzyme is commercially available so that it is not necessary to undertake the laborious and time consuming procedures of first purifying a monocomponent or homogenous enzyme preparation.

The lengths of EPG-released oligomers were a continuum although small oligomers ($GalA_n < 9$) were present in greater amounts in terms of number of molecules (Fig. 6), even with an average DM of 50% (pH 4.5 series). A large increase in the size of the longest released oligomer was observed between DM 80% and 70% for the pH 7.5 pectins and between DM 70% and 60% for the pH 4.5 pectins (Figs. 4 and 5). The largest oligomers released were in the pH 4.5–50% DM pectin samples (Table 1) where oligomers with a GalA > 50 were observed. For the 80% and 70% DM pectins longer oligomers were observed in the pH 7.5 series than in the pH 4.5 series. Very similar curves for oligomer length vs. nMol mL^{-1} (Fig. 6) were obtained for each pectin series and the general shape of the curve did not seem dependent on the DM of the pectin although the number of small oligomers was greater in the pH 4.5 series.

The most obvious difference within a pH series was the $GalA_n$ of the longest observed oligomer, although the number of fragments in a range of $GalA_7$–$GalA_{18}$ appeared to increase as the average DM decreased, while the amount of fragments with a $GalA_n > 18$ seemed to remain relatively constant although statistically significant differences were present between treatments (pH 7.5 vs. 4.5; ANOVA or *t*-tests). Additionally, in the pH 4.5 demethylated pectin the number of oligomers with a $GalA_n \leqslant 5$ consistently
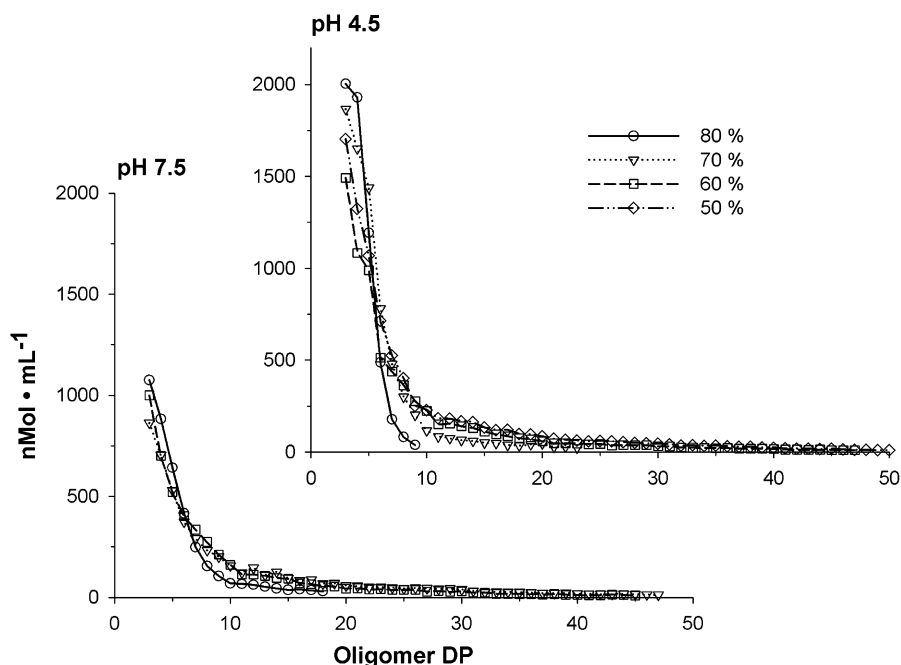

Fig. 6. Distribution of average amount of oligomers released after a 5 min EPG digest.

was significantly higher than the pH 7.5 demethylated pectin (Table 2).

Using pooled slope (1.48136) and intersect (6.15777) values for calibration curves constructed with GalA$_2$, GalA$_3$, GalA$_6$ and GalA$_8$ oligomers, that were tested (ANCOVA) and shown not to have statistically different slopes ($F = 1.7882$, DFn = 3, DFd = 91, $P = 0.155$) and intersects ($F = 1.60367$, DFn = 3, DFd = 94, $P = 0.1938$), the C_n for each oligomer peak could be estimated. This number and the C_p of substrate molecules, calculated from the concentration of the parent pectin (based on the Wt. Ave. Mol. Wt. estimated from MALLS–SEC), could be used to estimate the average GalA$_n$ of a DMB ($\overline{BS}$) and the average number of DMBs per molecule ($\overline{B}_n$, Table 3). The results suggest that the $\overline{BS}$ increases as the DM decreased within a pH series. An increase for $\overline{B}_n$ was observed in the pH 7.5 series between 80% and 70% DM but no consistent trend was observed for the pH 4.5 series. A large increase in $\overline{BS}$ was observed between 80% and 70% DM for the pH 7.5 series while a similar increase, thought not as large in magnitude, in the pH 4.5 series was not observed until the transition from 70% to 60% DM.

The intermolecular DM distribution of the samples has also been investigated by performing CE measurements on the 70% and 60% DM samples (Fig. 7). Discrimination of species with differing degrees of methyl esterification is clearly evidenced in the CE results (Fig. 7), based on the grounds of differential electrophoretic mobilities (Strom, Ralet, Thibault, & Williams, 2005; Zhong, Williams, Goodall, & Hansen, 1998, 1997). It can be seen that, at both pH
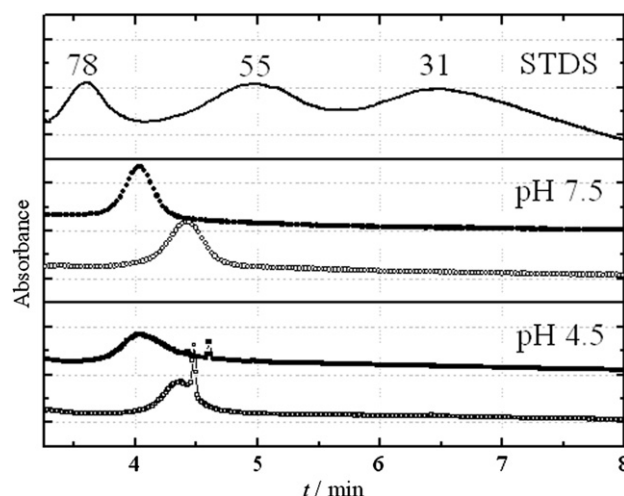

Fig. 7. Typical electropherograms obtained from standard pectin samples of known average degrees of methyl esterification compared with those made by de-esterification with PME, as described herein, at pH 7.5 and 4.5, respectively. ■ = 70% DM; □ = 60% DM.

values, samples with high and low degrees of methyl esterification have been generated. Owing to its inherent separation quality the width of the CE peaks offers some insight into the intermolecular distribution of the degree of methyl esterification that exists within the sample. While low DM pectins generated with plant PMEs are typically found to have broader distributions than those de-esterified with alkali or fungal enzymes (Williams et al., 2003), these samples in which limited methyl de-esterification has occurred do not appear to have significantly broader distributions, with a typical full width at half height of ∼10% DM units.. It also seems possible that for the sample modified at pH 4.5 a small amount of low molecular weight sample may have been generated manifest in the small sharper peak observed.

Fig. 8 shows the predictions for liberated GalA oligomers, as calculated by the summation method described in 2.6.3, for DM 80% pectin samples demethylated assuming PME has a multiple-attack degree given by 1, 5, 10 or 500 compared with experimental data obtained at (a) pH 4.5 and (b) pH 7.5. Figs. 9 and 10 show these predictions and data for pectins with DM 70% and 60%, respectively.

A number of features are worthy of comment. In the majority of cases, particularly for samples modified at pH 7.5, the experimental data for DP < 20 seems qualitatively to best fit the form of those curves simulated assuming that the degree of multiple attack of the PME used is 10. Also noteworthy in that context is the fact that a number of the experimental curves do show some kind of change of slope or inflection at around DP 10 that could be indicative of the processivity as described at the end of the mathematical modeling section. A degree of multiple attack of around 10 is certainly not unprecedented. However, it is also clear from experiments where the data extends well above DP 20 that the relative frequencies of these longer length fragments are much better predicted by a single-

Table 2
Comparison of C_n for pH 7.5 and 4.5 demethylated pectins

Oligomer DP	Degree of methylation (%)		
	80	70	60
3	***	****	*
4	****	***	****
5	****	***	****
6	NS	**	NS

Means were compared using Student's t-test (one-tailed) assuming unequal variances.

NS, not significant.
* $P < 0.05$.
** $P < 0.01$.
*** $P < 0.005$.
**** $P < 0.001$.

Table 3
Average demethylated block size ($\overline{BS}$) and number of blocks ($\overline{B}_n$) per molecule of the average block size for each pectin series

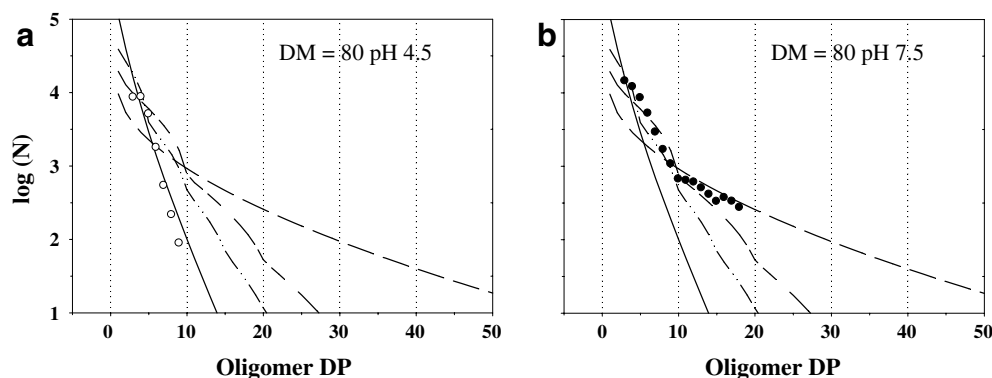| DM	pH 7.5		pH 4.5	
	$\overline{BS}$	$\overline{B}_n$	$\overline{BS}$	$\overline{B}_n$
80	5.3	1.7	4.1	3.0
70	10.2	2.0	5.3	3.6
60	12.2	2.0	7.9	3.1
50			8.6	3.9

Fig. 8. Predictions for liberated GalA oligomers for DM 80% pectin samples demethylated assuming PME has a multiple-attack degree given by 1, 5, 10 or 500 compared with experimental data obtained at (a) pH 4.5 and (b) pH 7.5. ○ Experimental data at pH 4.5; ● experimental data at pH 7.5; — $p = 1$; — ·· — $p = 5$; — – – $p = 10$; — — $p = 500$.
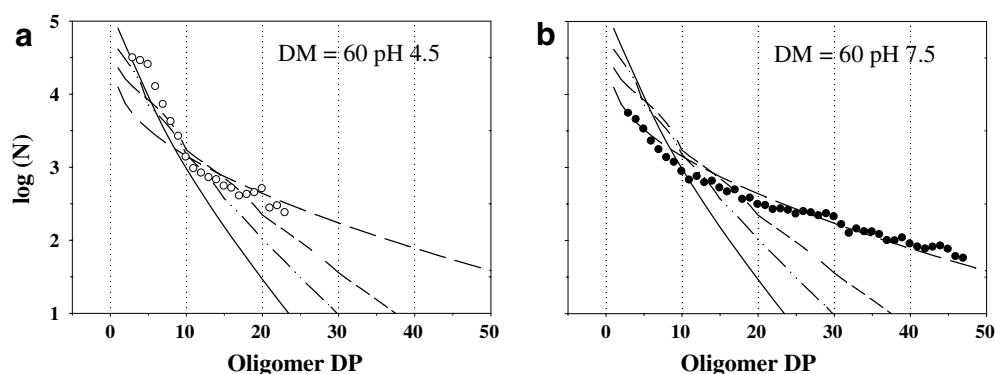

Fig. 9. Predictions for liberated GalA oligomers for DM 70% pectin samples demethylated assuming PME has a multiple-attack degree given by 1, 5, 10 or 500 compared with experimental data obtained at (a) pH 4.5 and (b) pH 7.5. ○ Experimental data at pH 4.5; ● experimental data at pH 7.5; — $p = 1$; — ·· — $p = 5$; — – – $p = 10$; — — $p = 500$.
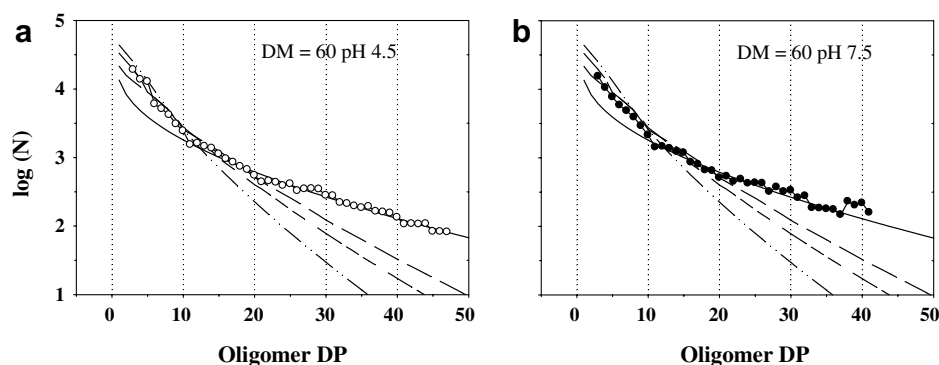

Fig. 10. Predictions for liberated GalA oligomers for DM 60% pectin samples demethylated assuming PME has a multiple-attack degree given by 1, 5, 10 or 500 compared with experimental data obtained at (a) pH 4.5 and (b) pH 7.5.

chain mechanism. That is, there are considerably higher amounts of these longer oligomers than would be predicted with a multiple-attack degree of 10. It is clear from Fig. 2 that the presence of multiple fragmentation of released fragments would only serve to exacerbate the situation.

There does also seem to be some indication (with the exception of DM = 60) that the DP < 10 data obtained from the sample modified at pH 4.5 has a steeper slope

than that observed from the sample processed at 7.5; suggesting that perhaps even a pure multiple-attack mechanism ($p = 1$) is more appropriate at describing this data range. However, once again, where data exists on longer fragments it looks more like a single-chain mechanism.

In summary, the data on the amount of different low DP GalA oligomers released can be reasonably described if the methylester distribution of the substrate was generated by a
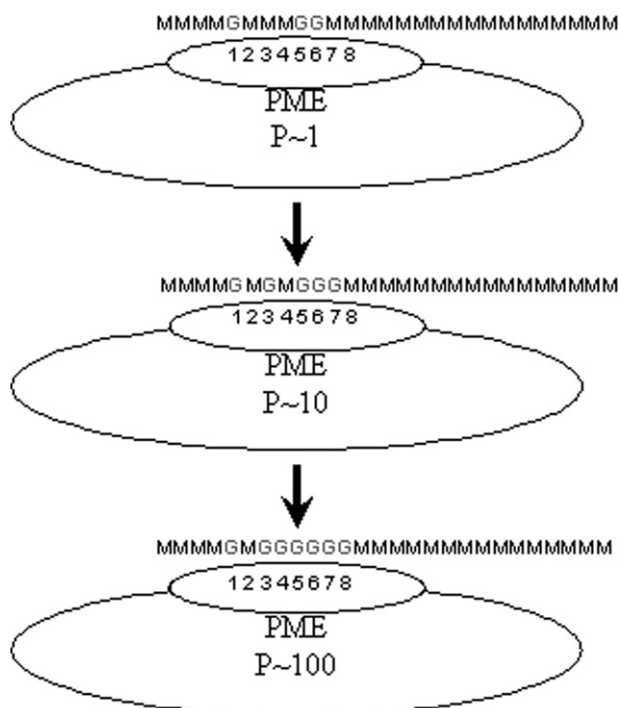
Fig. 11. Model of the proposed variably processive mode of action for the citrus salt-independent PME illustrating a change in degree of processivity (p), from ~1 to ~100, dependent on the methylation state of GalA units of pectin contained within, or surrounding, the enzyme active site. M, methylated GalA; G, GalA; numbers indicate hypothetical subsites within the active site cleft.

multiple-attack mechanism with $p = 1$ at pH 4.5 and $p = 10$ at pH 7.5. However, the relative amount of longer DP fragments is, in all cases, more successfully modeled by a single-chain mechanism. This transition is not understood in detail but it is suggested that it may arise from a variable degree of processivity (multiple attack; Fig. 11), which will be investigated in the future. In this proposed model for PME mode of action re-association of PME to previously minimally unmethylated regions with a relatively small block size is more likely to lead to an increase in the degree of processivity for demethylations resulting from subsequent binding events. That is, if the enzyme binding site is occupied by a region containing relatively few methylated GalAs, or the surrounding HG region outside of the binding cleft is relatively unmethylated, a higher degree of processivity may be induced. Versteeg (1979) reported a decrease in K_m for pectins with decreasing DM (95.6–32.3%) for two citrus PMEs he studied. An increase in the affinity of the PMEs for their substrate associated with a reduction in DM supports the variably processive mode of action we have presented here. Although the active cleft, and possible subsite number or binding energies, of processive PMEs has not been fully described crystallographic data has been presented (Johansson et al., 2002) suggesting that as many as seven GalA residues may reside within the binding cleft. It has been clearly shown that the transition between a low degree of processivity to a high degree of processivity is not a consequence of multiple fragmenta-

tion. While better agreement of a single model with the data over the entire range will involve further modeling, for example, using different rules of enzyme engagement with a more realistic variable degree of processivity, or including multiple isoforms, we believe that this work serves to demonstrate the promise of this technique. The possibility of detecting GalA oligomers up to DPs of 50 has permitted *limited fragmentation* in combination with mathematical modeling to be used as an additional useful tool in the study of fine structure.

Acknowledgements

References

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

Breyer, W. A., & Matthews, B. W. (2001). A structural basis for processivity. *Protein Science, 10*, 1699–1711.

Cameron, R. G., Baker, R. A., & Grohmann, K. (1998). Multiple forms of pectinmethylesterase from citrus peel and their effects on juice cloud stability. *Journal of Food Science, 63*, 253–256.

Cameron, R. G., & Grohmann, K. (1995). Partial purification and thermal characterization of pectinmethylesterase from red grapefruit finisher pulp. *Journal of Food Science, 60*, 821–882.

Cameron, R. G., & Grohmann, K. (1996). Purification and characterization of a thermally tolerant pectinmethylesterase from a commercial Valencia 'Fresh Frozen Orange Juice'. *Journal of Agricultural and Food Chemistry, 44*, 458–462.

Cameron, R. G., & Grohmann, K. (2005). Separation, detection and quantification of galacturonic acid oligomers with a degree of polymerization greater than 50. *Journal of Liquid Chromatography & Related Technologies, 28*, 559–570.

Cameron, R. G., Hotchkiss, A. T., Kauffman, S. W., & Grohmann, K. (2003a). Utilization of an evaporative light scattering detector for high-performance size-exclusion chromatography of galacturonic acid oligomers. *Journal of Chromatography A, 1011*, 227–231.

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

Cameron, R. G., Luzio, G., Kauffman, S. W., & Grohmann, K. (2004). Characterization of esterified blocks in pectin homogalacturonan regions after de-esterification with the thermally tolerant pectin methylesterase from citrus fruit. *Proceedings of the Florida State Horticultural Society, 117*, 410–415.

Cameron, R. G., Savary, B. J., Hotchkiss, A. T., & Fishman, M. L. (2005b). Isolation, characterization, and pectin-modifying properties of a thermally tolerant pectin methylesterase from *Citrus sinensis* var Valencia. *Journal of Agricultural and Food Chemistry, 6*, 2255–2260.

Cameron, R. G., Savary, B. J., Hotchkiss, A. T., Fishman, M. L., Chau, H. K., Baker, R. A., et al. (2003b). Separation and characterization of a salt-dependent pectin methylesterase from *Citrus sinensis* var. Valencia fruit tissue. *Journal of Agricultural and Food Chemistry, 7*, 2070–2075.

Campa, C., Holtan, S., Nilsen, N., Bjerkan, T., Stokke, B., & Skjåk-Bræk, G. (2004). Biochemical analysis of the processive mechanism for epimerization of alginate by mannuronan C-5 epimerase AlgE4. *Biochemical Journal, 381*, 155–164.

Catoire, L., Pierron, M., Morvan, C., Herve du Penhoat, C., & Goldberg, R. (1998). Investigation of the action patterns of pectinmethylesterase isoforms through kinetic analyses and NMR spectroscopy. *Journal of Biological Chemistry, 273*, 33150–33156.

Chen, E. M., & Mort, A. J. (1996). Nature of sites hydrolyzable by endopolygalacturonase in partially-esterified homogalacturonans. *Carbohydrate Polymers, 39*, 129–136.

Christensen, T. M., Nielsen, J. E., Kreiberg, J. D., Rasmussen, P., & Mikkelsen, J. D. (1998). Pectin methyl esterase from orange fruit: Characterization and localization by in-situ hybridization and immunohistochemistry. *Planta, 4*, 493–503.

Daas, P. J. H., Meyer-Hansen, K., Schols, H. A., De Ruiter, G. A., & Voragen, A. G. J. (1999). Investigation of the non-esterified galacturonic acid distribution in pectin with endopolygalacturonase. *Carbohydrate Research, 318*, 135–145.

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

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

Denes, J. M., Baron, A., Renard, C. M., Pean, C., & Drilleau, J. F. (2000). Different action patterns for apple pectin methylesterase at pH 7.0 and 4.5. *Carbohydrate Research, 4*, 385–393.

Dixon, W. J. (1953). Processing data for outliers. *Biometrics, 9*, 74–89.

Duvetter, T., Fraeye, I., Sila, D. N., Verlent, I., Smout, C., Hendrickx, M., et al. (2006). Mode of de-esterification of alkaline and acidic pectin methyl esterases at different pH conditions. *Journal of Agricultural and Food Chemistry, 20*, 7825–7831.

Fishman, M. L., Chau, H. K., Hoagland, P., & Ayyad, K. (2000). Characterization of pectin, flash-extracted from orange albedo by microwave heating, under pressure. *Carbohydrate Research, 323*, 126–138.

Fishman, M. L., Chau, H. K., Kolpak, F., & Brady, J. (2001). Solvent effects on the molecular properties of pectins. *Journal of Agricultural and Food Chemistry, 49*, 4494–4501.

Goldberg, R., Pierron, M., Bordenave, M., Breton, C., Morvan, C., & du Penhoat, C. H. (2001). Control of Mung bean pectinmethylesterase isoform activities. Influence of pH and carboxyl group distribution along the pectic chains. *Journal of Biological Chemistry, 276*, 8841–8847.

Grasdalen, H., Andersen, A. K., & Larsen, B. (1996). NMR spectroscopy of the action pattern of tomato pectinesterase: Generation of block structure in pectin by a multiple-attack mechanism. *Carbohydrate Research, 289*, 105–114.

Greenwood, C. T., & Milne, E. A. (1968). Starch degrading and synthesizing enzymes: A discussion of their properties and action pattern. *Advances in Carbohydrate Chemistry and Biochemistry, 23*, 281–366.

Grubbs, P. E. (1969). Procedures for detecting outlying observations in samples. *Technometrics, 11*, 1–21.

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.

Guyer, R. B., Miller, W. B., Bissett, O. W., & Veldhuis, M. K. (1956). Stability of frozen concentrated orange juice. I. The effect of heat treatment on enzyme inactivation and cloud stability of frozen concentrates made from pineapple and valencia oranges. *Food Technology, 10*, 10–11.

Hotchkiss, A. T., Bahtimy, K., & Fishman, M. L. (1996). Analysis of pectin structure by HPAEC–PAD. *Modern Methods of Plant Analysis, 17*, 129–146.

Hunt, J. J., Cameron, R., & Williams, M. A. K. (2006). On the simulation of enzymatic digest patterns: The fragmentation of oligomeric and polymeric galacturonides by endo-polygalacturonase II. *Biochimica et Biophysica Acta, 1760*, 1696–1703.

Johansson, K., El-Ahmad, M., Friemann, R., Jörnvall, H., Marković, O., & Eklund, H. (2002). Crystal structure of plant pectin methylesterase. *FEBS Letters, 514*, 243–249.

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.

Levigne, S., Thomas, M., Ralet, M. C., Quemener, B., & Thibault, J. F. (2002). Determination of the degrees of methylation and acetylation of pectins using a C18 column and internal standards. *Food Hydrocolloids, 16*, 547–550.

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

Limberg, G., Korner, R., Buchholt, H. C., Christensen, T. M., Roepstorff, P., & Mikkelsen, J. D. (2000b). Quantification of the amount of galacturonic acid residues in blocksequences in pectin homogalacturonan by enzymatic fingerprinting with exo- and endo-polygalacturonase II from *Aspergillus niger*. *Carbohydrate Research, 327*, 321–322.

Luzio, G. A. (2003). Effect of block deesterification on the psuedoplastic properties of pectin. *Proceedings of the Florida State Horticultural Society, 116*, 425–429.

Luzio, G. A. (2004). Determination of galacturonic acid content of pectin using a microtiter plate assay. *Proceedings of the Florida State Horticultural Society, 117*, 416–421.

Marascuilo, L. A. (1971). Statistical methods for behavior science research. *McGraw-Hill Series in Electrical and Computer Engineering* (578). New York: McGraw Hill.

May, C. D. (1990). Industrial pectins: Sources, production and applications. *Carbohydrate Polymers, 12*, 79–99.

Neiss, T. G., Cheng, H. N., Daas, P. J. H., & Schols, H. A. (1998). NMR and statistical analysis of the galacturonic acid and methyl ester distributions in pectic polysaccharides. *Polymer Preprint, 1*, 688–689.

Parenicova, L., Benen, J. A. E., Kester, H. C. M., & Visser, J. (1998). pgaE encodes a fourth member of the endopolygalacturonase gene family from *Aspergillus niger*. *European Journal of Biochemistry, 251*, 72–80.

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

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

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

Robyt, J. F., & French, D. (1967). Multiple attack hypothesis of α-amylase action: Action of porcine pancreatic, human salivary, and *Aspergillus oryzae* α-amylases. *Archives of Biochemistry and Biophysics, 122*, 8–16.

Robyt, J. F., & French, D. (1970). Multiple attack and polarity of action of porcine pancreatic α-amylase. *Archives of Biochemistry and Biophysics, 138*, 662–670.

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

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.

Stevens, J. W., Pritchett, D. E., & Baier, W. E. (1950). Control of enzymatic flocculation of cloud in citrus juices. *Food Technology, 4*, 469–473.

Strom, A., Ralet, M.-C., Thibault, J.-F., & Williams, M. A. K. (2005). Capillary electrophoresis of homogeneous pectin fractions. *Carbohydrate Polymers, 60*, 467–473.

Verlent, I., Smout, C., Duvetter, T. M. E. H., & Van Loey, A. (2005). Effect of temperature and pressure on the activity of purified tomato polygalacturonase in the presence of pectins with different patterns of methyl esterification. *Innovative Food Science and Emerging Technologies, 6*, 293–303.

Versteeg, C. (1979). Pectinesterases from the orange: their purification, general characteristics and juice cloud destabilizing properties, Agricultural University,Wageningen, The Netherlands., Ph.D.

Versteeg, C., Rombouts, F. M., Spaansen, C. H., & Pilnik, W. (1980). Thermostability and orange juice cloud destabilizing properties of multiple pectinesterases from orange. *Journal of Food Science, 45*, 969–971,99.

Vincken, J.-P., Schols, H. A., Oomen, R. J. F. J., McCann, M. C., Ulvskov, P., Voragen, A. G. J., et al. (2003). If homogalacturonan were a side chain of rhamnogalacturonan I. Implications for cell wall architecture. *Plant Physiology, 132*, 1781–1789.

Wicker, L., Ackerley, J. L., & Hunter, J. L. (2003). Modification of pectin by pectinmethylesterase and the role in stability of juice beverages. *Food Hydrocolloids, 17*, 809–814.

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

Willats, W. G., Orfila, C., Limberg, G., Buchholt, H. C., van Alebeek, G. J., Voragen, A. G., et al. (2001). Modulation of the degree and pattern of methyl-esterification of pectic homogalacturonan in plant cell walls. Implications for pectin methyl esterase action, matrix properties, and cell adhesion. *Journal of Biological Chemistry, 276*, 19404–19413.

Williams, M. A., Foster, T. J., & Schols, H. A. (2003). Elucidation of pectin methylester distributions by capillary electrophoresis. *Journal of Agricultural and Food Chemistry, 51*, 1777–1781.

Zhan, D., Janssen, P., & Mort, A. J. (1998). Scarcity or complete lack of single rhamnose residues interspersed within the homogalacturonan regions of citrus pectin. *Carbohydrate Research, 308*, 373–380.

Zhong, H.-J., Williams, M. A. K., Goodall, D. M., & Hansen, M. E. (1998). Capillary electrophoresis studies of pectins. *Carbohydrate Research, 1–2*, 1–8.

Zhong, H. J., Williams, M. A. K., Keenan, R. D., Goodall, D. M., & Rolin, C. (1997). Separation and quantification of pectins using capillary electrophoresis: a preliminary study. *Carbohydrate Polymers, 32*, 27–32.