A REFEREED PAPER

CHARACTERIZATION OF ESTERIFIED BLOCKS IN PECTIN HOMOGALACTURONAN REGIONS AFTER DE-ESTERIFICATION WITH THE THERMALLY TOLERANT PECTIN METHYLESTERASE FROM CITRUS FRUIT

RANDALL G. CAMERON[1], GARY LUZIO, STEVEN W. KAUFFMAN
AND KAREL GROHMANN[2]
U.S. Department of Agriculture, Agricultural Research Service
South Atlantic Area Citrus and Subtropical Products Laboratory
600 Avenue S, N.W.
Winter Haven, FL 33881

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Abstract. **A non-calcium sensitive pectin, with a degree of esterification (DE) of 73%, was demethylated at pH 7.5 with a mono-component preparation of a thermally tolerant pectin methylesterase (TT-PME, EC 3.1.1.11) isolated from citrus fruit tissue. The DE of the parent pectin (73%) was lowered to 66.5% and 59%. Endo-polygalacturonase (EPG) was used to digest the pectin and estimate the maximum length of methyl-protected blocks in the homogalacturonan (HG). Following enzymatic demethylation and EPG digestion, the pectin was exhaustively demethylated with 0.1 M LiOH. Methyl-protected block size was estimated by high performance anion exchange chroma-**

tography coupled to an evaporative light scattering detector. The largest EPG protected block for the 66.5% DE pectin was a 19-mer although GA oligomers up to 44 residues were observed for the 59% DE pectin.

Citrus fruit peel contains 10-15% pectin on a fresh weight basis (Grohmann and Baldwin, 1992; Grohmann et al., 1995). Pectin, a complex polysaccharide, is composed of at least five different sugar moieties but 80-90% of its dry weight is galacturonic acid (GA). The vast majority of the GA is found in homogalacturonan (HG) regions of pectin, which are unbranched polymers of GA in which a variable proportion of the GA residues may contain a methyl ester at their C6 position. The functional properties of pectin are thought to be dependent on the fraction of these GA residues that are methylated and their distribution along the HG stretches (Taylor, 1982; Willats et al., 2001). Two patterns of methyl ester distribution are generally recognized (Willats et al., 2001), either random or ordered (blockwise). Analyzing these patterns of demethylation is key to understanding their relationship to function. Demethylation of pectin can be accomplished either by enzymatic (pectin methylesterase; PME) or chemical (alkaline demethylation) means. For enzymatic demethylation three different modes of action have been hypothesized (Denes et al., 2000). These were based on the mode of action of starch degrading enzymes (Greenwood and Milne, 1968). Starch degrading enzymes are depolymerizing enzymes while pectin methylesterases are methyl ester hydrolases so the comparison may be an over simplification. The three postulated modes of action are: (a) single chain mechanism—processive demethylation of all adjacent methyl esters from an initial binding site; (b) multiple chain mechanism—only a single methyl ester is hydrolyzed for each enzyme binding event; and (c) multiple attack mechanism—a limited number of adjacent methyl esters are hydrolyzed for each binding event.

Previous studies have demonstrated that plant PMEs can demethylate pectin in an ordered fashion, producing blocks of demethylated GA, at least at neutral pH (Cameron et al., 2003a; Daas et al., 1999; Denes et al., 2000; Hotchkiss et al., 2002; Limberg et al., 2000a). Lengths of the demethylated blocks have been estimated with indirect statistical methods on oligomers of galacturonic acid with varying DEs (Catoire et al., 1998; Denes et al., 2000; Grasdalen et al., 1996). Demethylated block length also has been estimated by enzymatic methods using exo- and endo-polygalacturonase (Limberg et al., 2000a). Lengths of partially esterified blocks were estimated by Limberg et al. (2000b) using pectin lyase which cleaves the HG in highly methylated regions, although these fragments would represent a portion of a methylated fragment since the enzyme cleaves within a fully methylated stretch.

In this study we utilized both enzymatic (endo-polygalacturonase, EPG) and chemical (alkaline demethylation) means to probe the changes in partially methylated (EPG-protected) block size resulting from decreasing the DE by demethylation with the thermally tolerant PME (TT-PME) isolated form Valencia orange fruit tissue (Cameron et al., 1998) and are able to directly visualize their degree of polymerization (DP) with evaporative light scattering detection following high performance anion exchange chromatography (Cameron et al., 2003b, c).

Materials and Methods

Apparatus. Controlled pectin demethylation was accomplished using a Radiometer PHM 290 pH STAT Controller (Hach Company, Loveland, Colo.) at pH 7.5. The HPLC system was composed of a Perkin Elmer Binary LC 250 Pump (Shelton, Conn.) and a Perkin Elmer Series 200 Autosampler connected to a CarboPac PA1 (4 × 250 mm; Dionex Corporation, Sunnyvale, Calif.) anion exchange column. Detection of analytes was accomplished with an ESA, Inc., Model 301 Evaporative Light Scattering Detector (Chelmsford, Mass.). Data collection was accomplished with an A/D converter connected to a Hewlett Packard (Palo Alto, Calif.) personal computer using EZ Chrome Elite software (Scientific Software, Pleasanton, Calif.).

Monocomponent TT-PME Preparation. A monocomponent preparation of the thermally-tolerant PME (TT-PME) from Valencia orange fruit peel was prepared as previously described (Cameron et al., 1998).

Pectin. A non-calcium sensitive pectin was obtained from Danisco (Grinsted URS 1200 lime pectin; Copenhagen, Denmark) that had a DE of 73%. A 2% solution of the pectin was precipitated with two volumes of isopropanol and centrifuged at 7500 rpm for 30 min at 4 °C in a Beckman J2-21 centrifuge. The pellets were washed with 50 mL of deionized water and then mixed with two more volumes of isopropanol, recentrifuged and the pellets were dried. The washed pectin was made to a stock concentration of 1% and contained 0.02% sodium azide. Aliquots of 1% pectin (preheated to 30 °C) were pipetted into a water-jacketed reaction vessel for demethylation with the TT-PME. Sodium chloride was added to give a final concentration of 0.2 M. The pH was adjusted to pH 7.5 and deionized water was added to make a final concentration of 0.5% pectin after the addition of TT-PME. Only minor adjustments to pH were required prior to starting the enzymatic demethylation. A recirculating water bath was used to maintain a temperature of 30 °C in the water jacket surrounding the reaction vessel. The TT-PME was added to the pectin in aliquots of 20 to 50 μL depending on the desired endpoint DE (66.5%, 59% or 49%). A 10 mM NaOH titrant was used to maintain the set pH. The reaction was stopped by adding two volumes of 37 °C ethanol to the pectin mixture. The pectin was centrifuged and washed as described above. After washing the pellets were solubilized in 10 mL of deionized water and lyophilized.

EPG Treatment. Endo polygalacturonase (EC 3.2.1.15, Megazyme International Ireland Limited, Bray, Ireland) digestion of the demethylated pectins was accomplished by solubilizing the demethylated pectins in 50 mM sodium acetate, pH 4.7, 0.02% sodium azide to a final concentration of 1% (w/v). Endo polygalacturonase (EPG) was added to a final concentration of 0.05 U/mL. Aliquots were removed after five minutes and placed in a boiling water bath for 10 min to inactivate the EPG. The remainder of the mixtures were allowed to continue reacting overnight to obtain an endpoint EPG digest. The EPG digested pectin was then refrigerated at 4 °C. A 2% sample of sodium polygalacturonic acid (PGA) also was digested with EPG for 3 h at room temperature to serve as a reference for identifying the degree of polymerization of galacturonic acid oligomers present in the demethylated samples after EPG treatment and alkaline demethylation.

Alkaline Demethylation. Aliquots of the EPG digested pectin were brought to 0.1 M LiOH with a 2 M solution of LiOH (w/v) and held at 4 °C overnight. Formic acid was then added to lower the pH to between 4.0 and 5.0.

High Performance Anion Exchange Chromatography. An ammonium formate gradient (50 mM for 5 min, 50 to 600 mM-

2 convex gradient over 55 min, 600 to 800 mM linear gradient over 120 min, 800-850 mM linear gradient over 15 min, followed by 50 mM for 15 min, total run duration of 210 min) at pH 4.4 was used to separate the oligogalacturonide fragments. The nebulizer and the evaporation chamber temperatures of the evaporative light scattering detector (ELSD) were set at 60 °C and 152 °C respectively. The photomultiplier sensitivity was set at 700 V. Nitrogen was used as detector carrier gas at a pressure of 138 kPa.

Mono-, di- and tri-galacturonic acid oligomers were purchased (Sigma-Aldrich, St. Louis, Mo.) and tetra-, penta-, hexa-, 19-, and 20-mer GA oligomers were kindly provided by Dr. Arland Hotchkiss (U.S. Department of Agriculture, Agricultural Research Service, Eastern Regional Research Center, Philadelphia, Pa.). These standards were used to identify the retention times of oligomers produced by EPG digestion of polygalacturonic acid (PGA) and the demethylated pectins.

Molecular weight determination. The HPSEC-multi-angle light scattering (MALS) system consisted of a pump (P500 Pharmacia, Amersham Biosciences, Piscataway, N.J.) with an in-line degasser and in-line filters (0.1 mm pore size, Millipore, Bedford, Mass.). The mobile phase was 50 mM lithium citrate (pH 4.7) (62484, tri-Lithium citrate tetrahydrate, Fluka BioChemika, Steinheim, Switzerland) was prefiltered through a 0.1 mm filter and continuously purged with helium. Flow rate was 0.6 mL/min. Due to the limited sample size, the eluants were centrifuged (model 5415 C, Eppendorf AG, Hamburg, Germany) for 4 min at 14,000 rpm to remove particles. Samples were injected with an 80 μL sample loop directly on line to limit the sample needed for analysis. A set of three linear (8 mm pore size, PL-aquagel-OH 40, 50 and 60) SEC columns with an operating range of 100-10,000,000 MW, determined by the manufacturer with polyethylene oxide and polyethylene glycol, (Polymer Laboratories, Inc., Amherst, Mass.) was employed for the separation. The columns were connected in series (largest pore size first), enclosed an oven and kept at 34 °C. Two detectors were present in line, a multi-angle light scattering detector and a refractive index (RI) detector (Wyatt Technologies, Santa Barbara, Calif.). The multi-angle, digital signal processing light scattering detector (DAWN® EOS) was equipped with a K5 flow cell and a He-Ne laser-light source (L = 633 nm). Prior to the measurements, the DAWN® EOS was calibrated using 0.2 mm-filtered HPLC quality toluene. The refractive index detector was the Optilab® DSP interferometric refractometer (Wyatt Technologies, Santa Barbara, Calif.) with a P10D cell operating at 633 nm and a constant temperature of 40 °C. RI instrument calibration was done with a syringe pump (SP100I, World Precision Instruments, Sarasota, Fla.). Results were processed using the software provided by the manufacturer (DNDC for Windows vs. 5.90.03, and Astra for Windows vs. 4.90.07, Wyatt Technologies). Elution data, molecular weight as weight average (MW) and peak molecular weight (Mp), and molecular radius (Rw) were processed or calculated using the Astra software. Previously published values of specific refractive index increments (dn/dc) were used for MW determination and mass recovery (Fishman et al., 2000). Debye type plots of $R_i(\theta) = Kc_1$ vs. $\sin^2\theta/2$, where $R_i(\theta)$ is the excess Rayleigh ratio at an angle θ and concentration c, were used to extrapolate to 0° angle for each slice of the elution chromatogram. The extrapolation was carried out according to a fitting of first order regression.

Results and Discussion

Alkaline demethylation followed by an overnight digestion with EPG demonstrated that these treatments were successful in converting the parent pectin, which showed no significant level of GA oligomers, to oligomers of GA (Fig. 1) and would be capable of allowing us to investigate the mode of action of the TT-PME. The parent pectin was reduced to primarily mono-, di-, tri- and tetra-GA. Larger DP oligomers were identified from co-chromatography with oligomers of DP 4, 5, 6, 19 and 20 (data not shown) and a partial EPG di-
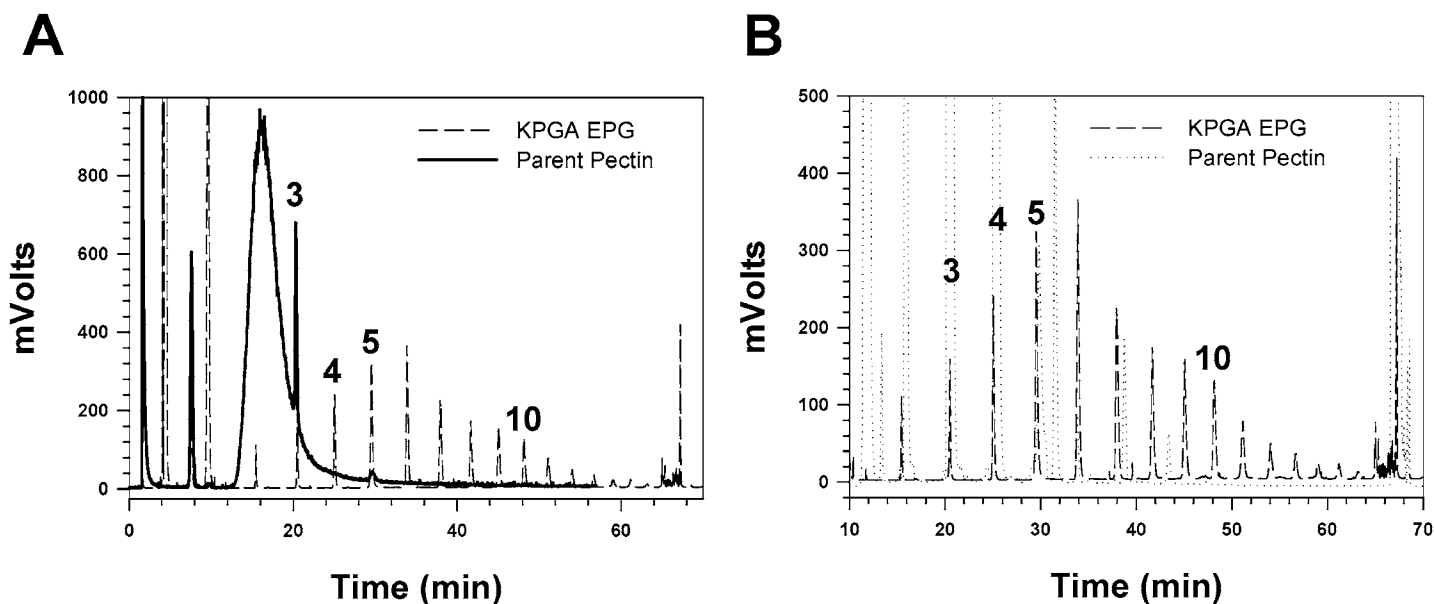

Fig. 1. High Performance Anion Exchange Chromatography (HPAEC) of Parent Pectin. A: Parent pectin (73% DE) before demethylation or EPG digestion B: Parent pectin after 1 hr room temperature alkaline demethylation with NaOH followed by overnight EPG digestion. Dashed line is EPG digest of K+ PGA showing reference GA oligomers.
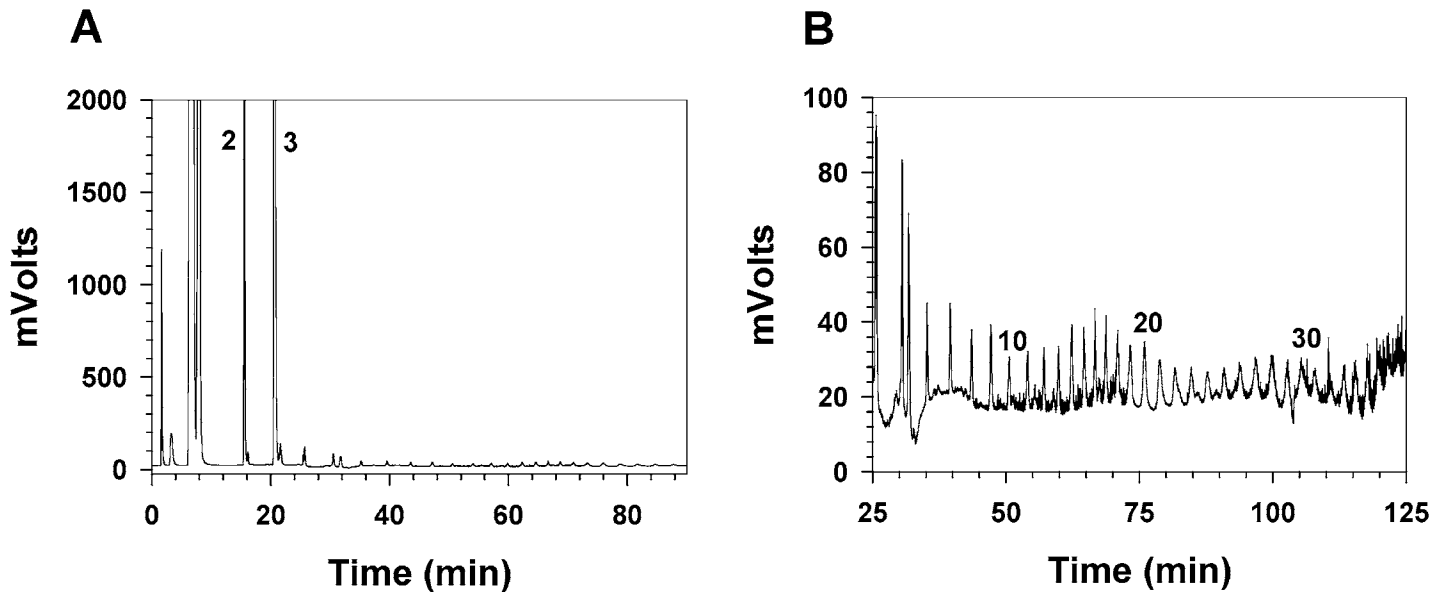
Fig. 2. A: HPAEC of parent pectin following overnight EPG digestion and LiOH demethylation. B: Zoom of the region from DP 5-35.

gest of polygalacturonic acid (Fig. 1). EPG has been shown to have very little activity against highly methylated pectin although lower levels of methylation are tolerated (Benen et al., 1999; Daas et al., 2000). For Type 1 EPG relative activity against partially methylated pectin increased from 3% (relative to PGA) for a DE of 75% to 18% at DE 60 and 43% at DE 45 (Benen et al., 1999). An overnight EPG digest of the parent pectin followed by alkaline demethylation revealed no large accumulation of oligomers greater than a DP of 3 (Fig. 2). The presence of smaller peaks in a DP range of 4-45 suggests that the methyl-protected stretches were large and that their length was evenly distributed over a wide DP range. Large methyl-protected fragments are expected in a randomly methylated pectin with a DP of 73% since the probability of two adjacent unmethylated GAs would be $0.27 \times 0.27 = 0.0189$ (with a DE 0f 73% only 27% of the GAs are unmethylated so the probability that a given GA would be unmethylated is 0.27). Daas et al. (2000) suggests that a minimum unmethylated block size of 4 is required for EPG to cleave the HG chain, at least in a highly methylated pectin. The probability of this occurring in a 73% DE pectin is $0.27^4 = 0.0053$. However analysis of 5 min, 30 min, 60 min and overnight EPG digests by MALS indicates that the average molecular weight was reduced (Table 1) and that EPG cleavage sites are present in the parent pectin.

The molecular weight MW determinations by MALS require the measurement of the specific dn/dc of the polymer. Previously published values of dn/dc were used for MW de-

termination and mass recovery (Fishman et al., 2000). Results are given in Table 1 for mass recovery (mass of entire chromatogram peak), MW (average molecular weight over entire chromatogram peak), Mp (peak molecular weight from MW value at the maximum concentration value), and Rw (molecular radius) for each time point.

With no EPG digestion, the mass recovery was 852 μg and this was set as the 100 percent value. The MW with no digestion was 151 kDa, the Mp was 81.1 kDa, and the Rw was 29 nm. After only 5 min of EPG digestion the mass recovered decreased to 816 μg, the MW decreased to 91.2 kDa, the Mp decreased to 60.7 kDa and the Rw decreased to 26 nm. These values would be expected if approximately one glycosidic bond per pectin molecule were hydrolyzed in the first 5 min of EPG digestion. The loss in MW continued for all time points. At the 1400-minute digestion, the mass recovered decreased to 705 μg, the MW decreased to 22.3 kDa, the Mp decreased to 14.2 kDa and the Rw decreased to 18.3 nm. These data demonstrate a significant loss in MW and indicate that near complete EPG digestion was achieved with the 1400-minute digestion.

These values may not represent an accurate determination of MW since there is a significant loss in recovered mass relative to the undigested sample. With the overnight digestion the mass recovery was just 82.7 percent. It is likely that a low molecular weight fraction (<3 kDa) co-elutes with the salt peak at the end, and this low molecular weight material is not included in the final MW or mass balance values. If this were

Table 1. MW (average molecular weight over entire chromatogram peak), Mp (peak molecular weight from MW value at the maximum concentration value), mass recovery (mass of entire chromatogram peak), and Rw, (molecular radius) for EPG digestion at different time intervals for 73% DE pectin. Mass recovery based for 0 time value set as the 100 percent value.

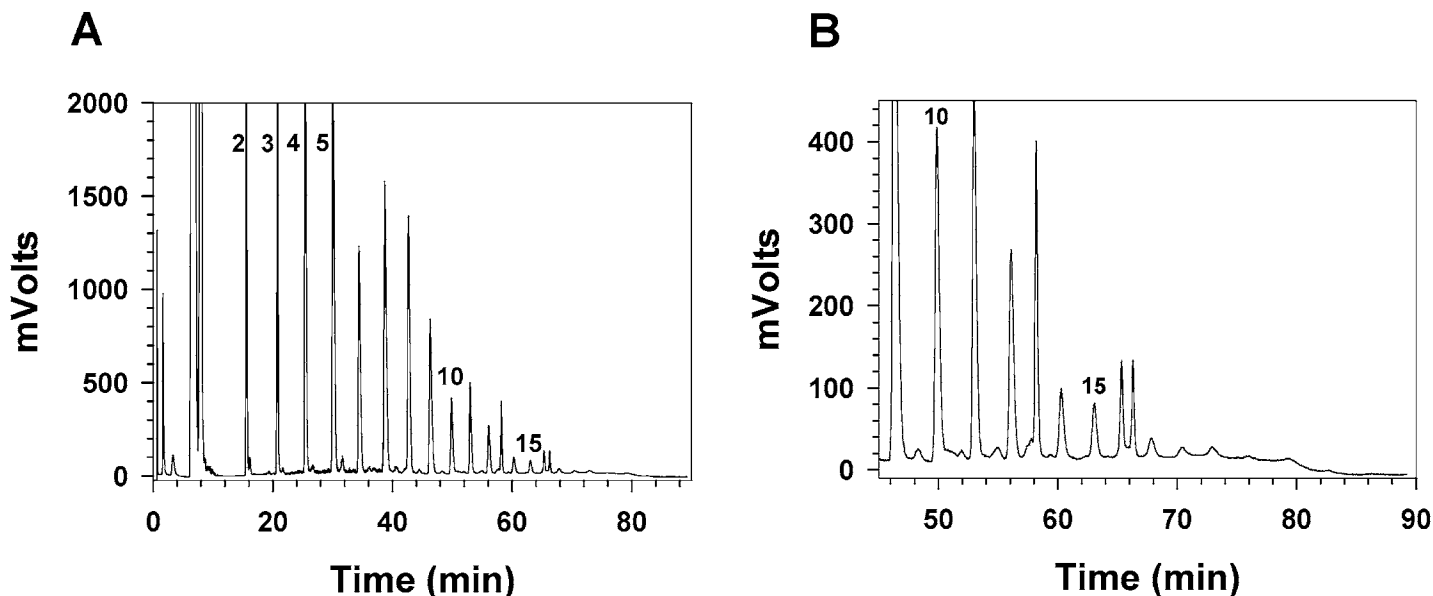| EPG digest time (min)	Mass recovered μg	Mass recovery %	MW kDa	Mp kDa	Rw (nm)
0	852	100.0	151.0 ± 0.9	81.1	29.0
5	816	95.8	91.2 ± 0.5	60.7	26.0
30	803	94.2	84.5 ± 0.5	57.8	24.3
60	809	95.0	80.7 ± 0.5	51.6	24.7
1400	705	82.7	22.3 ± 0.3	14.2	18.3

Fig. 3. A: HPAEC of parent pectin demethylated to DE 66.5% with TT-PME at pH 7.5 followed by an overnight EPG treatment and LiOH demethylation. B: Zoom of the region from DP 9-19.

the case, the MW should have lower values and would correspond closer to an average of the peaks observed with the ELSD chromatograms. Nevertheless, these data do demonstrate a significant loss in MW due to EPG digest and indicate that the digestion appears to be complete with the 1400-minute EPG digest sample. This data, coupled to the apparent increase in di- and tri-GA in the overnight EPG digest, indicates that although of low occurrence, some EPG cleavage sites are present in the parent pectin.

Reducing the DE to 66.5% with the TT-PME followed by an overnight EPG digest and alkaline demethylation resulted in the production of large peaks representing a DP of 4-16 and smaller peaks up to a DP of 17-19 (Fig. 3). The occurrence of these peaks demonstrate that the TT-PME had pro-

duced EPG cleavage sites which left methyl-protected fragments with an apparent DP between 4 and 19.

Lowering the DE to 59% with subsequent overnight EPG treatment and alkaline demethylation produced a completely different chromatogram (Fig. 4). The larger DP methyl-protected oligomers in the range of 4-15 are not present in the 59% DE pectin after alkaline demethylation. No oligomers larger than a dimer are present in abundance. The lack of these larger oligomers suggests that the TT-PME mode of action may change as the charge density on the HG chain increases (resulting from a lower degree of methylation). Apparent changes in PME mode of action also have been observed in other studies (Catoire et al., 1998; Goldberg et al., 2001) suggesting an opposite situation to what is reported
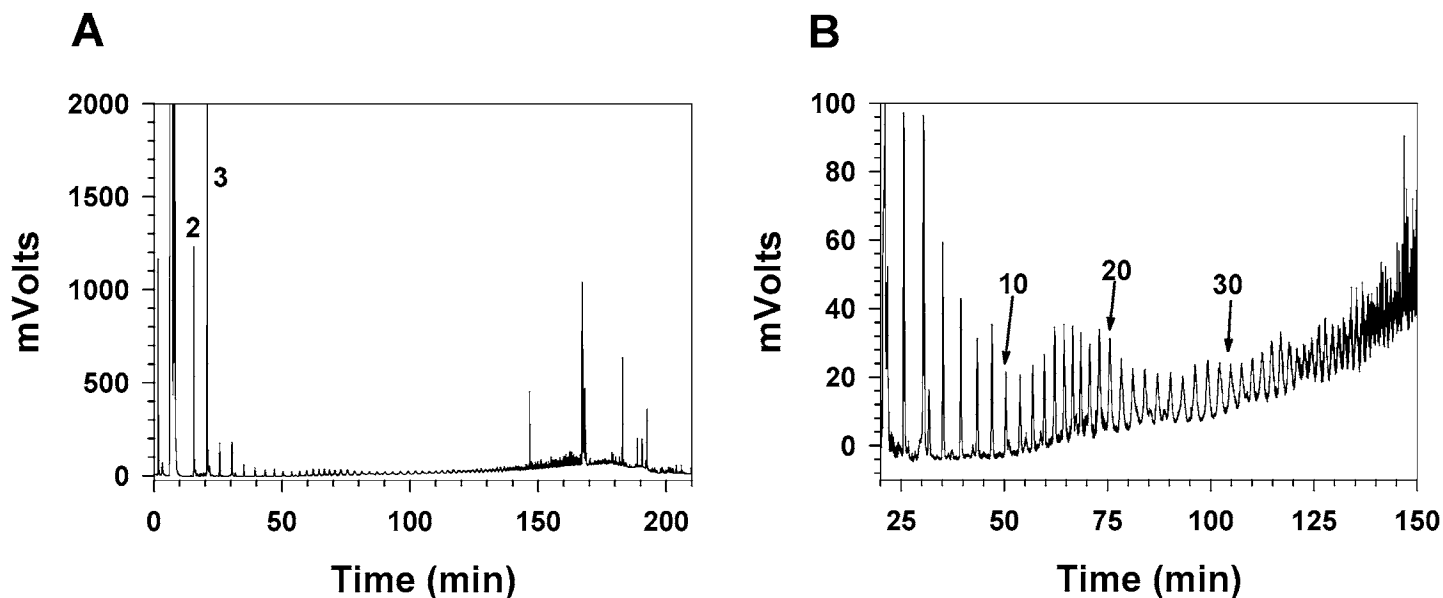

Fig. 4. A: HPAEC of parent pectin demethylated to DE 59% with TT-PME at pH 7.5 followed by an overnight EPG treatment and LiOH demethylation. B: Zoom of the region from DP 4-40.

here. In their work on mung bean PME Catoire et al. (1998) reported a switch from a multiple attack mechanism at pH 7.6 to a single chain mechanism at pH 5.6. Denes et al. (2000) also observed a pH dependent mode of action for apple PME. They reported a switch from single chain mechanism at pH 7.0 to a multiple attack mechanism at pH 4.5. A similar switch in mode of action for the TT-PME might also help explain the results we observed. If the TT-PME is producing shorter demethylated blocks (multiple attack mechanism) or is demethylating in a more random fashion (multiple chain mechanism), stretches in the HG with a relatively low DE (lower than the population average) would be produced and could reduce the requirement for consecutive demethylated GA residues for EPG activity as observed by Benen et al. (1999) and Daas et al. (2000). Further work is required to elucidate the mode of action of the TT-PME and the effect of demethylation on this EPG's mode of action.

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